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Secondary Transporters for Citrate and the Mg^{2+}-Citrate Complex in *Bacillus subtilis* Are Homologous Proteins

ANDRÉ BOORSMA, MICHEL E. VAN DER REST, JUKE S. LOLKEMA,* AND WIL N. KONINGS

Department of Microbiology, Groningen Biotechnology and Biomolecular Sciences Institute, University of Groningen, 9751NN Haren, The Netherlands

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Citrate uptake in *Bacillus subtilis* is mediated by a secondary transporter that transports the complex of citrate and divalent metal ions. The gene coding for the transporter termed CitM was cloned, sequenced, and functionally expressed in *Escherichia coli*. Translation of the base sequence to the primary sequence revealed a transporter that is not homologous to any known secondary transporter. However, CitM shares 60% sequence identity with the gene product of open reading frame N15CR that is on the genome of *B. subtilis* and for which no function is known. The hydropathy profiles of the primary sequences of CitM and the unknown gene product are very similar, and secondary structure prediction algorithms predict 12 transmembrane-spanning segments for both proteins. Open reading frame N15CR was cloned and expressed in *E. coli* and was shown to be a citrate transporter as well. The transporter is termed CitH. A remarkable difference between the two transporters is that citrate uptake by CitM is stimulated by the presence of Mg^{2+} ions, while citrate uptake by CitH is inhibited by Mg^{2+}. It is concluded that the substrate of CitM is the Mg^{2+}-citrate complex and that CitH transports the free citrate anion. Uptake experiments in right-side-out membrane vesicles derived from *E. coli* cells expressing either CitM or CitH showed that both transporters catalyze electrogenic proton/substrate symport.

Citrate is very abundant in nature, and most bacteria have transport proteins in the cytoplasmic membrane that mediate the uptake of citrate. The carriers belong to the class of secondary transporters that use the free energy stored in transmembrane electrochemical gradients of ions to drive the uptake of the substrates (for a review, see reference 18). The citrate transporter CitH of *Klebsiella pneumoniae* is driven by the proton motive force (22), and the transporters CitS and CitC of *K. pneumoniae* and *Salmonella* serovars are driven by both the proton motive force and sodium ion motive force (9, 14, 19, 24). Mechanistically these transporters catalyze coupled translocation of citrate and H^+ and/or Na^+ (symport). A special case are the citrate carriers of lactic acid bacteria that take up citrate by an electrogenic uniport mechanism or by exchange with lactate, a product of citrate metabolism (citrolactic fermentation) (16, 17, 20). These citrate transporters are involved in secondary metabolic energy generation (12).

A number of structural genes coding for citrate transporters have been cloned, and the primary sequences have been deduced from the base sequences. The proton-dependent citrate carrier of *K. pneumoniae* CitH belongs to a large family of homologous proteins which also contains many sugar transporters (23). The Na^+-dependent citrate carriers CitS of *K. pneumoniae* and CitC of *Salmonella* serovars form, together with the citrate carriers of lactic acid bacteria CitPs, a distinct family termed the 2-hydroxy-carboxylate carriers (4, 15, 24). The malate transporter of *Lactococcus lactis*, MleP, that is involved in malolactic fermentation is also a member of this family (1).

Citrate is a chelator that forms stable complexes with various metal ions. The presence of divalent metal ions results in inhibition of citrate transport activity by the transporters mentioned above (16, 22, 24), showing that the metal ion-citrate complex is not a substrate of these citrate transporters. On the other hand, other bacteria including *Pseudomonas* and *Klebsiella* spp. and *Bacillus subtilis* are known to preferentially take up and degrade citrate in the metal ion-bound complex (2, 3, 10, 26). These microorganisms have been implicated in the prevention of mobilization of toxic metal wastes by chelators like citrate. Degradation of the metal ion-citrate complex would render the metal ion in an insoluble, immobilized state (7). A complication is that the nature of the metal ion in the complex determines whether the complex is degraded. Studies with *Pseudomonas fluorescens* have shown that at least for a number of metal ions the lack of degradation was limited by the lack of transport of the complex into the cells and not caused by the toxicity of the metal ion. The transporter seemed to recognize only the bidentate metal ion-citrate complexes that leave the hydroxyl group of citrate free and not tridentate complexes (10).

The citrate carrier of *B. subtilis* transports citrate in a complex with a wide variety of divalent metal ions. A study with membrane vesicles showed that the highest uptake rates were observed with Mn^{2+}, intermediate rates were observed with Zn^{2+}, Mg^{2+}, Be^{2+}, Ba^{2+}, Ca^{2+}, and Cu^{2+} and the lowest rates were observed with Co^{2+} and Ni^{2+} (2). Here, we report the cloning and sequencing of this transporter, termed CitM, and its functional expression in *Escherichia coli*. A search of the available databases of protein sequences revealed one homologous protein that was found to be a citrate transporter as well. However, the latter transports citrate in the uncomplexed state. The two genes that code for a new family of secondary transporters provide the starting point for engineering the substrate specificity of the transporters either to narrow or broaden the metal ion specificity in the metal ion-citrate complex that is recognized by the carrier.

* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751NN Haren, The Netherlands. Phone: 31-50-3632155. Fax: 31-50-3632154. Electronic mail address: j.s.lolkema@biol.rug.nl.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* JM101 harboring plasmid pWSKcitM coding for the divalent cation-dependent citrate carrier of *B. subtilis* and strain JM109(DE3) harboring plasmid pWSKcitH coding for the proton-

dependent citrate carrier of *B. subtilis* were grown in 6-liter flasks containing 1 liter of medium at 37°C and under vigorous shaking. JM101 was grown in Luria-Bertani (LB) medium, and JM109(DE3) was grown in LB medium or minimal medium containing citrate as the sole carbon source. Carbenicillin was added at a concentration of 100 μg/ml, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added when appropriate. The cells were harvested in the late exponential growth phase and used immediately for the uptake experiment or the preparation of membrane vesicles. *B. subtilis* 6GM was grown at 37°C with vigorous aeration in medium containing 0.8% tryptone (Difco), 0.5% NaCl, 25 mM KCl, and 10 mM sodium citrate.

Cloning and sequencing of CitM. Chromosomal and plasmid DNA isolation and all other genetic techniques were done by using the standard protocols described by Sambrook et al. (21) or the manufacturers' protocols. Chromosomal DNA isolated from *B. subtilis* 6GM was partially digested with the restriction enzyme *Hin*dIII, and the fragments were ligated in the multiple cloning site of vector pINIIIA (8) digested with the same enzyme. Two fragments of 0.9 and 1.8 kb were digested from clone pM5 by *Hin*dIII and ligated in the multiple cloning site of plasmid pBluescript II SK (Stratagene), yielding pSK0.9 and pSK1.8. Sets of nested deletions starting at both ends of the inserts were constructed from pSK0.9 and pSK1.8 with the Erase-a-base System (Promega). The plasmids were digested with *Kpn*I or *Sac*I to create protected 3′ overhangs and *Sal*I or *Bam*HI to allow digestion into the fragments. The subclones were sequenced on a Vistra 725 automated sequencer with Texas Red-labeled forward and reverse primers of the pBluescript vector (Fig. 1). The sequencing reactions were performed by using the Thermo Sequenase sequencing kit (Vistra Systems) with 7-deaza-dGTP according to the manufacturers' protocol.

Southern blotting. DNA probes were prepared by amplifying the regions on genomic DNA of *B. subtilis* that code for CitM and open reading frame (ORF) N15CR by PCR. The CitM probe is made up of approximately the first 487 nucleotides of *citM*, and the N15CR probe is made up of the last 463 nucleotides of N15CR. The probes were labeled with digoxygenin (DIG) by including DIG-dUTP in the PCR mixture. The reaction mixture contained the following (in a total volume of 30 μl): 3 μl of superTaq buffer; 10 ng of template DNA; 6 μl of a mixture of dATP, dCTP, and dGTP (0.65 mM each); 0.35 mM DIG-dUTP, 2.5 U of superTaq, and 1 μl of gelatin (5-mg/ml stock). The oligonucleotide primers were used at a concentration of 0.03 μg/μl. The forward and backward primers for the CitM probe were 5′-TTAAGGGGCCATGGATGTGTTAGC-3′ and 5′-CTCCCCAAGGAATCGTGTTC-3′, and for the N15CR probe they were 5′-GGTGGATGCAATGGCGCATTC-3′ and 5′-ATGAATCTTCTAGACACT CATAGGATCCTATTGATC-3′, respectively. The PCRs yielded fragments of the expected size, and control reactions in the absence of DIG-UTP yielded identical fragments. Restriction analysis confirmed that the correct regions had been amplified. The DIG-dUTP-labeled products were purified over a QiaQuick column (Qiagen), and approximately 1 μg of labeled probe was used per hybridization.

DNA was electrophoresed in agarose gels and blotted on Zeta-Probe Blotting Membrane (Bio-Rad) with the Vacuum Blotting Unit (LKB Bromma). Sample preparation and transfer were performed essentially as described by Sambrook et al. (21). The blots were incubated with the labeled probes overnight at 65°C. Subsequently, the membranes were washed twice with 300 mM NaCl–30 mM sodium citrate–0.1% sodium dodecyl sulfate (SDS) for 5 min and at room temperature and twice with 15 mM NaCl–1.5 mM sodium citrate–0.1% SDS for 15 min at 65°C. The membranes were used immediately for detection of hybridization.

Construction of the expression vectors. (i) pWSKcitM. The *citM* gene was amplified by PCR using the Vent polymerase (Biolabs) from chromosomal DNA isolated from *B. subtilis* 6GM. The forward primer 5′-TTA*AGGG*GCCATGG AT**GTG**TTAGC-3′ contained the putative ribosomal binding site (indicated in italic type) and the valine start codon (bold type) and two mutations that result in an *Nco*I restriction site (CCATGG) in front of the start codon. The *Nco*I site was engineered for future purposes. The backward primer 5′-GTCATTACGC CT*GAATTCC***TCA**TACG-3′ contained two mutations that create an *Eco*RI site (italic type) immediately behind the TGA stop codon (bold type). The *Eco*RI site at the 3′ end of the PCR product was cut, while the 5′ end was left blunt. The fragment was ligated into plasmid pWSK29 (25) digested with *Sma*I and *Eco*RI, yielding plasmid pWSKcitM. In pWSKcitM, the open reading frame coding for CitM is downstream of the *lac* promoter on the vector and the *B. subtilis* ribosomal binding site.

(ii) pWSKcitH. ORF N15CR coding for CitH was amplified from the same chromosomal DNA. The forward primer 5′-AAAA*AAGCTT*TTGAATAGGG GAGGTCATA*CCATGG*TTGCCATAC-3′ contained three mutations resulting in a *Hin*dIII site in front of the ribosomal binding site and a *Nco*I site around the start codon. The construction of the *Nco*I site results in the leucine-to-valine mutation in the second codon of the primary sequence. The backward primer 5′-ATGAATCT*TCTAGA*CACTCATA*GGATCC*TATTGATC-3′ was complementary to sequences downstream of the stop codon. Four base changes resulted in the introduction of *Bam*HI and *Xba*I sites (italic type) in the PCR product. The PCR product was digested with *Hin*dIII and *Bam*HI and ligated into plasmid pWSK29 (27) digested with the same two enzymes. In the resulting vector, pWSKcitH, the *citH* gene is downstream of the T7 promoter and the *B. subtilis* ribosomal binding site. The base sequences of the inserts in pWSKcitM and pWSKcitH were verified by sequencing the sense strand.

Transport assays. (i) Whole cells. Cells of *E. coli* harboring plasmids pWSKcitM and pWSKcitH were grown in LB broth as described above and washed twice in 50 mM potassium phosphate, pH 7. Uptake of [1,5-^{14}C]citrate was measured essentially as described by Lolkema et al. (14). The cells were resuspended in 95 μl of the same buffer with the additions indicated in the figure legend and incubated for 10 min at 37°C. At time zero, 5 μl of [1,5-^{14}C]citrate was added to give a final concentration of 4.5 μM. Uptake was stopped by adding 2 ml of an ice-cold 100 mM LiCl solution followed by immediate filtering through 0.45-μm-pore-size nitrocellulose filters. The filters were washed twice with the LiCl solution and immediately submerged in scintillation fluid to stop any further metabolic activity. The radioactivity retained on the filter was quantified in a liquid scintillation counter.

(ii) Membrane vesicles. Right-side-out membrane vesicles from the *E. coli* cells harboring plasmids pWSKcitM and pWSKcitH were prepared by the osmotic lysis procedure as described by Kaback (11). *E. coli* JM101/pWSKcitM was grown in LB medium, and JM109/pWSKcitH was grown in minimal medium supplemented with 20 mM sodium citrate. The membranes were resuspended in 50 mM potassium 1,4-piperazindiethansulfate (PIPES), pH 6.5, at a protein concentration of 15 mg/ml and stored in aliquots in liquid nitrogen. The membrane vesicles were energized by the potassium ascorbate-phenazine methosulfate (PMS) electron donor system. The membranes were diluted in 50 mM K-PIPES (pH 6.5) and 10 mM potassium ascorbate in a total volume of 100 μl and incubated for 5 min at 30°C under a constant flow of water-saturated air. PMS was added at a concentration of 100 μM, and the proton motive force was allowed to develop for 1 min, after which [1,5-^{14}C]citrate was added to a final concentration of 4.5 μM. Uptake was stopped, and the samples were processed as described above.

Materials. [1,5-^{14}C]citrate (111 mCi/mmol) was obtained from Amersham Radiochemical Center. Mono potassium phosphate and potassium hydroxide with low Na^+ content were obtained from Merck. All other chemicals were reagent grade and obtained from commercial sources.

Nucleotide sequence accession number. The complete base sequence of *citM* has been submitted to the GenBank database and is available under accession number U62003.

RESULTS

Cloning and sequencing of CitM. The Mg^{2+}-dependent citrate carrier of *B. subtilis* that we will term CitM was cloned by conventional techniques. Chromosomal DNA of *B. subtilis* 6GM was partially digested with *Hin*dIII, and the fragments were cloned in the expression vector pINIIIA (8). *E. coli* is an ideal host for the cloning of citrate carriers, since it is not capable of taking up citrate but metabolizes it readily in the citric acid cycle. The expression vectors containing the chromosomal fragments were transformed to *E. coli* JM101, which was then grown on citrate indicator plates (Simmons agar). A number of blue colonies, indicative of citrate uptake and metabolism, were assayed for their ability to take up citrate in the presence and absence of 10 mM $MgCl_2$. A clone (plasmid pM5) that showed no uptake activity in the absence of Mg^{2+} and high uptake in the presence of Mg^{2+} was selected (not shown). Restriction analysis of pM5 revealed the presence of a 5.6-kb insert containing three additional *Hin*dIII sites (Fig. 1). A 2.8-kb *Eco*RI fragment of pM5 was subcloned into the *Eco*RI site of pINIIIA. Only one of the two orientations of the insert (plasmid pM6) showed Mg^{2+}-dependent citrate uptake activity, revealing the presence of the gene on the fragment and showing that the gene is expressed from the tandem promoter on pINIIIA. Further subcloning of pM6 resulted in the loss of the citrate-utilizing phenotype. pM6 was used to subclone the 0.9-kb *Eco*RI-*Hin*dIII fragment (pSK0.9) and the 1.8-kb *Hin*dIII-*Eco*RI fragment (pSK1.8) in pBluescript. The two subclones were used to make sets of nested deletions to determine the nucleotide sequence of the gene. Both subclones were sequenced in both directions as indicated in Fig. 1. Reconstruction of the base sequence of the insert on pM6 from the sequences of the two subclones revealed an ORF downstream of the promoter region on the vector with a length of 1,302 bp and starting with a GTG codon (Fig. 2). The length of the ORF conforms to the expected length of a gene coding for a bacterial secondary transporter. A putative ribosomal binding site is located upstream of the GTG start codon that shows

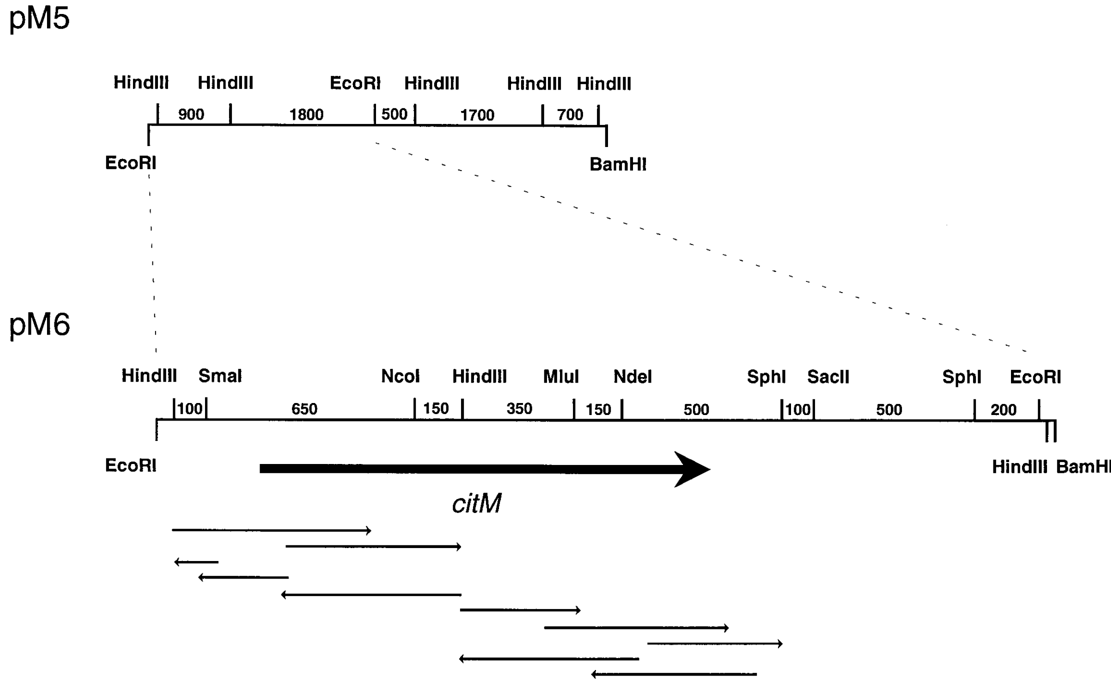

FIG. 1. Restriction map and sequencing strategy. (Top) The 5.6-kb fragment of *B. subtilis* chromosomal DNA in the *Eco*RI-*Hin*dIII-*Bam*HI multiple cloning site of the pINIIIA vector (pM5). (Bottom) The *Eco*RI fragment of pM5 in pINIIIA (pM6). The location and direction of the ORF coding for CitM are indicated by the large thick arrow. The two *Hin*dIII fragments of pM6 were subcloned in pBluescript, yielding pSK0.9 and pSK1.8, and sequenced in both directions. The small thin arrows indicate the regions and directions that were sequenced on these two fragments. The numbers indicate the lengths of the fragments in base pairs.

extensive similarity to the 3′ end of *B. subtilis* 16S rRNA. Neither a clear promoter sequence nor a terminator sequence were found upstream and downstream of the ORF, respectively.

A nonredundant search of the available gene banks revealed an ORF of 1,278 bp with a base identity of 60% with the cloned gene. The ORF indicated as N15CR was detected in the *bglS-katB* intergenic region on the genome of *B. subtilis* 168. The ORF starts with an ATG codon and is preceded by a ribosomal binding site. No clear promoter region could be detected upstream. The alignment of the *citM* sequence with the N15CR ORF shows that the ATG codon that lies between the ribosomal binding site and the putative *citM* GTG start codon is unlikely to function as the initiator of translation (Fig. 2).

The presence of the *citM* gene and the N15CR ORF on the genome of *B. subtilis* 6GM was confirmed by PCR and Southern hybridization. DNA probes were prepared by amplifying the first 487 bp of *citM* and the last 463 bp of N15CR by PCR by using the chromosomal DNA of *B. subtilis* 6GM as the template. The probes were selected such that they contained no *Hin*dIII restriction sites. The PCR resulted in distinct DNA fragments of the expected length. The nucleotide analog DIG-dUTP was incorporated into the fragments for use as probes in Southern blotting (Fig. 3). The two probes detected unique but different fragments of *B. subtilis* 6GM genomic DNA digested with *Hin*dIII (Fig. 3, lanes 1 and 2). Both the fragments were of the expected length. Plasmids pWSKcitM and pWSKcitH that contain the *citM* gene and ORF N15CR (see below) hybridized exclusively with the *citM* and N15CR probes, respectively (lanes 5 and 6) in spite of the high sequence identity between the two genes. The lack of cross-reaction reflects the high stringency of the hybridization and washing conditions. Under these conditions, the two probes did not detect similar genes on the chromosome of *E. coli* and the thermophilic *Bacillus stearothermophilus* (lanes 3 and 4, respectively).

Primary sequence analysis. Translation of the base sequences of the cloned ORF and the homologous ORF from the *B. subtilis* gene bank results in two proteins with 60% amino acid sequence identity and an additional 18% similarity. The amino acid compositions of the two proteins are typical for integral membrane proteins, with average hydrophobicity values of 0.51 and 0.47 on the normalized scale of Kyte (5), respectively. The hydropathy profiles of the two sequences are remarkably similar. Figure 4 shows the average hydropathy profiles of the two aligned sequences (bold line) together with the two individual profiles (thin lines). Significant differences show up only in the region from positions 125 to 145 and to a lesser extent in the region from positions 310 to 330. In both regions, CitM is the more hydrophobic sequence. The high similarity of the two sequences suggests a similar folding in the membrane. Secondary structure prediction (6) results in 12 membrane-spanning, presumably α-helical, regions both for the CitM protein and the protein coded by the N15CR ORF. Assuming similar folds for the two proteins, merging of the two predictions results in the 12 transmembrane segments indicated by the bars in Fig. 4.

Substrate specificity. The *citM* gene and the N15CR ORF were amplified by PCR with *B. subtilis* chromosomal DNA as

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citM   TTAAGGGGGAATGGATGTGTTAGCAATCTTAGGCTTTCTCATGATGCTTGTGTTTATG
citH   TAGGGGAGGTCATACCATGCTTGCCATACTCGGTTTTGTGATGATGATTGTCTTTATG
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FIG. 2. Nucleotide sequences of the beginning of the *citM* gene and ORF N15CR on the *B. subtilis* 168 genome. Putative ribosomal binding sites are underlined, and the start codons are indicated in bold type. ORF N15CR is indicated citH. Vertical bars indicate identical bases.

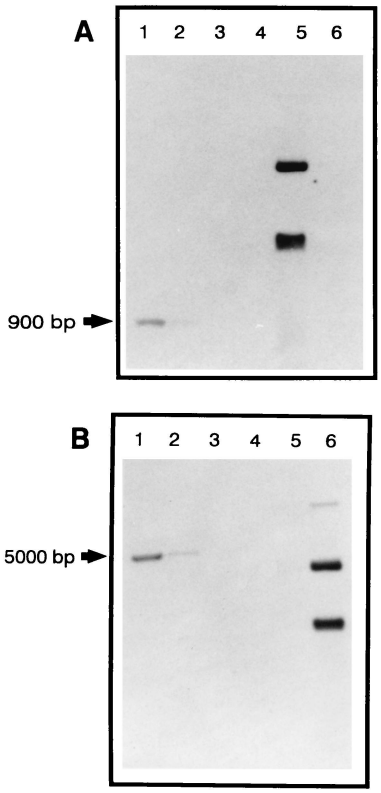

FIG. 3. Southern blot analysis of *B. subtilis*, *E. coli*, and *B. stearothermophilus* genomic DNAs. The blots were hybridized with the *citM* probe (A) and the N15CR probe (B). Lanes 1, 5 μg of genomic DNA from *B. subtilis* 6GM; lanes 2, 1 μg of *B. subtilis* 6GM genomic DNA; lanes 3, 5 μg of *B. stearothermophilus* genomic DNA; lanes 4, 5 μg of *E. coli* genomic DNA; lanes 5, 20 ng of plasmid pWSKcitM; lanes 6, 20 ng of plasmid pWSKcitH. Genomic DNA was digested to completion with *Hin*dIII. Plasmid DNA was not digested.

the template. The *citM* fragment was cloned downstream of the *lac* promoter region on plasmid pWSK29, a low-copy-number pBluescript derivative (25), yielding plasmid pSKcitM. The N15CR ORF was cloned behind the T7 promoter on the same plasmid, yielding pWSKcitH. The sequences of the cloned PCR fragments were verified in one direction and found to be identical to the base sequences of the original ORFs except for the second codon of *citH* which now codes for Val instead of Leu (see Materials and Methods for details).

Plasmid pWSKcitM was transformed to *E. coli* JM101 and plasmid pWSKcitH was transformed to *E. coli* JM109(DE3), a strain that contains a chromosomal copy of T7 polymerase, and the cells were plated on Simmons agar indicator plates. Surprisingly, both plasmids conferred the citrate-utilizing phenotype. Apparently, the N15CR ORF codes for a citrate transporter as well. We will term this transporter CitH. Figure 5 shows the uptake of citrate in cells harboring plasmids pWSKcitM (Fig. 5A) and pWSKcitH (Fig. 5B) in the presence of different concentrations of Mg^{2+}. Citrate uptake activity in cells expressing CitM is completely absent in the absence of Mg^{2+} ions. The uptake activity increases with increasing Mg^{2+} concentrations, which is consistent with the Mg^{2+}-citrate complex being the substrate of the carrier (2). In marked contrast, cells harboring plasmid pWSKCitH expressing the citrate carrier coded by ORF N15CR (*citH*) readily take up citrate in the absence of Mg^{2+}. Increasing concentrations of Mg^{2+} in the assay buffer result in decreasing uptake rates. Apparently, the substrate of CitH is free citrate, as is the case for the Na^+- and H^+-dependent citrate carriers of *K. pneumoniae* (22, 24) and the membrane potential-generating citrate carrier of *Leuconostoc mesenteroides* (16).

Coion specificity. The involvement of Na^+ ions in the uptake of citrate by CitM and CitH was investigated by measuring the uptake of citrate in *E. coli* JM101/pWSKcitM and JM109 (DE3)/pWSKcitH in the presence and absence of 10 mM NaCl. Prior to the experiments, the cells were washed three times in large volumes of potassium phosphate (pH 7) containing especially low levels of Na^+. The residual Na^+ ion concentration was at most a few micromolar. Furthermore, the uptake experiments were performed in plastic tubes to prevent Na^+ contamination from glassware. For both transporters, the uptake of citrate was not significantly different in the presence or absence of NaCl, indicating that Na^+ is not a coion for CitM or CitH (not shown).

A study with membrane vesicles prepared from *B. subtilis* cells has demonstrated that the Mg^{2+}-dependent citrate transporter CitM is a secondary transporter that is driven by the proton motive force (2). The high degree of similarity between CitM and CitH suggests that the same is true for CitH. The energy-coupling mechanism of both cloned transporters was investigated by preparing right-side-out membrane vesicles of *E. coli* cells expressing CitM or CitH (Fig. 6). The membranes were energized by the ascorbate-PMS electron donor system. In the presence of a proton motive force, both transporters accumulated citrate, indicating cotransport by CitM of the Mg^{2+}-citrate complex and protons and by CitH of citrate and protons (open circles in Fig. 6). In the presence of the K^+ ionophore valinomycin, which results in the dissipation of the membrane potential component of the proton motive force, the uptake activity was slightly less (squares). In the presence of nigericin, a K^+/H^+ antiporter, which dissipates the pH gradient across the membrane and results in a proton motive force that is composed solely of the membrane potential, significant uptake (triangles) above the background level (solid circles) is still observed. It is concluded that both CitM and CitH are electrogenic transporters that translocate net positive charge into the cells. The complex between citrate and Mg^{2+} is monovalent anionic ($MgCit^-$), and therefore, CitM cotransports at least two protons per Mg^{2+}-citrate complex. Electrogenic transport by CitH indicates cotransport of at least three or four protons, depending on translocation of $CitH^{2-}$ or Cit^{3-}, respectively.

DISCUSSION

The cloning and sequencing of the Mg^{2+}-dependent citrate carrier of *B. subtilis* CitM led to the surprising discovery of a second citrate carrier in *B. subtilis* CitH, and this gene was deposited in the databanks as ORF N15CR. The two transporters share common properties at different levels as follows. (i) The coding genes are homologous, and in the primary sequence alignment, about 60% residues are identical. (ii) The transporters function as electrogenic proton symporters. (iii) The genes coding for the transporters are present on the chromosome of *B. subtilis*, and identical genes were not found in *E. coli* and *B. stearothermophilus*. The most striking difference between the two transporters is that CitM transports the Mg^{2+}-citrate complex, while CitH transports free citrate. In *B. subtilis*, CitM is induced by citrate in the medium, and the absence of citrate uptake by membrane vesicle in the presence of EDTA indicates that CitM is the only transporter induced under these conditions (2). The present experiments with the cloned transporter in *E. coli* emphasize that CitM transports

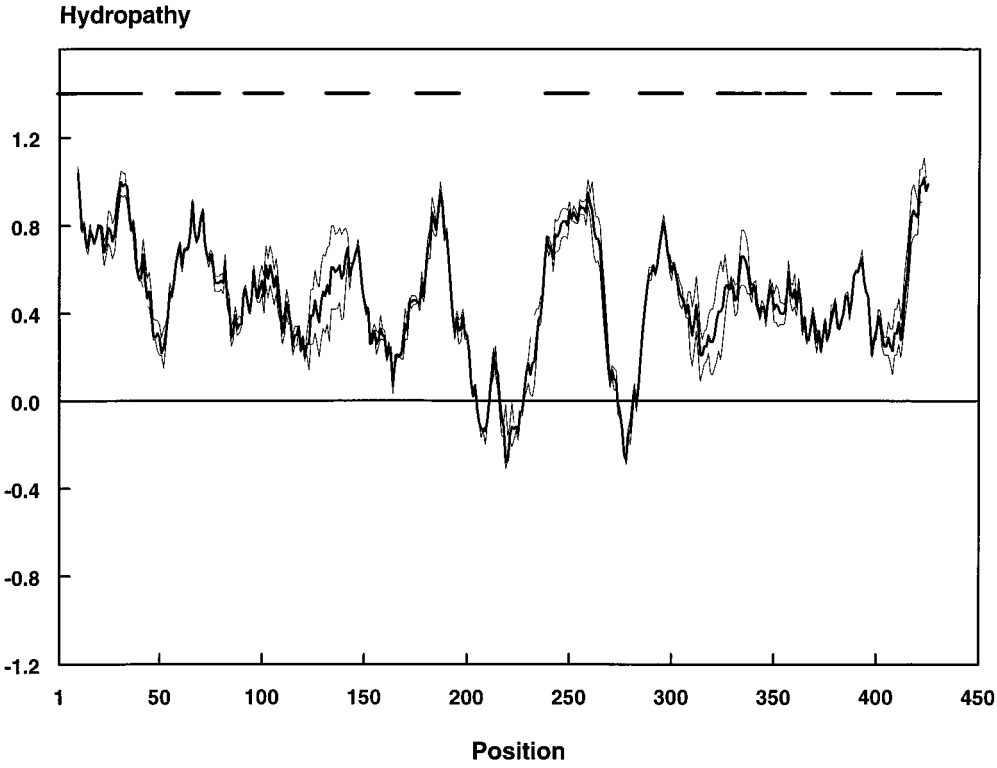

FIG. 4. Hydropathy profiles of the aligned primary sequences. Each position indicates the average hydrophobicity of the residues of both sequences in a window of 19 positions laid over the alignment shown in Fig. 3. Gaps in the window were ignored. The thin lines indicate the hydrophobicity profiles of the individual sequences. The profiles were computed, and then the gaps in the alignment were introduced in the profile. The scale is according to the normalized scale of Kyte (5). The bars on top indicate the 12 predicted transmembrane segments.

only citrate in the Mg^{2+}-complexed form (Fig. 5A, solid circles). Therefore, CitH is not induced under the same conditions in *B. subtilis*. ORF N15CR that codes for CitH lies between the genes *bglS* and *katB* on the *B. subtilis* genome. The ORF is coded in the direction opposite that of the two genes. The *bglS* gene codes for lichanase, an exported enzyme, that hydrolyzes mixed linked β-glucans (27), and *katB* codes for a catalase involved in sporulation (13). The *citH* gene is not

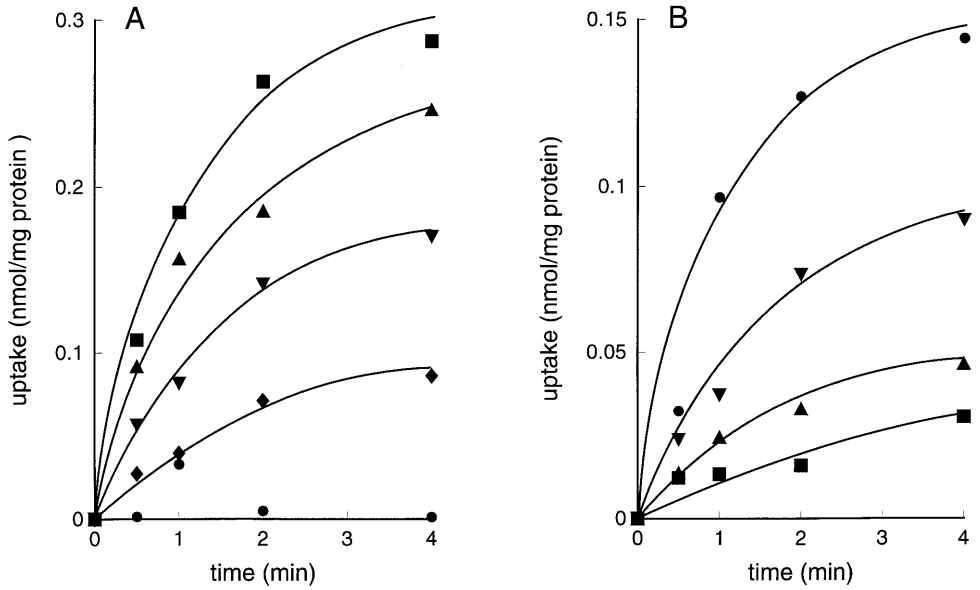

FIG. 5. Mg^{2+} ion dependence of the uptake activity of CitM (A) and CitH (B). [1,5-^{14}C]citrate uptake by *E. coli* JM101/pSKcitM (A) and *E. coli* JM109(DE3)/pSKcitH (B) was measured in 50 mM potassium phosphate (pH 7) supplemented with 0 (●), 0.5 (◆), 1 (▼), 5 (▲), or 10 (■) mM $MgCl_2$. The cell protein concentrations were 0.6 (A) and 1.2 (B) mg/ml.

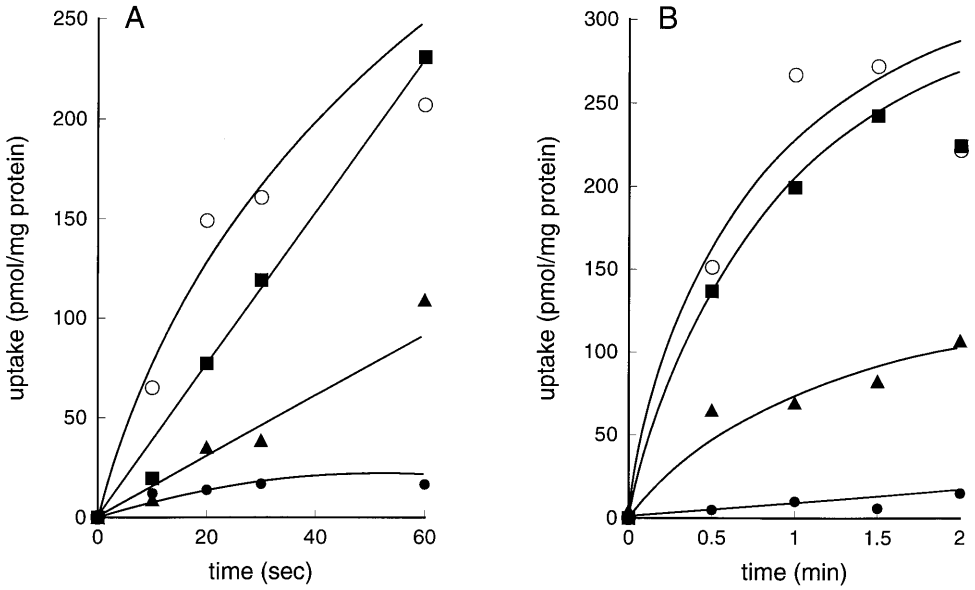

FIG. 6. Proton motive force-driven uptake by CitM (A) and CitH (B) in membrane vesicles. Uptake of [1,5-^{14}C]citrate in right-side-out membrane vesicles prepared from *E. coli* JM101/pSKcitM (A) and *E. coli* JM109(DE3)/pSKcitH (B) resuspended in K-Pipes (pH 6.5) with (A) and without (B) 10 mM $MgCl_2$ (○) and in the presence of 1 μM valinomycin (■) or 0.25 μM nigericin (▲). The membranes were energized with the ascorbate-PMS electron donor system. In the control experiment (●), the electron mediator PMS was omitted from the suspension.

preceded by a known promoter sequence and does not seem to be part of an operon structure, suggesting that the gene is silent. On the other hand, the gene is preceded by a ribosomal binding site and results in a functional transporter when expressed from a heterologous promoter. Either the gene has become silent only very recently on an evolutionary time scale, or more likely, the gene is expressed under special, unknown conditions using an unknown promoter sequence.

The high sequence identity of CitM and CitH suggests that the binding sites for the Mg^{2+}-citrate complex and citrate are not very different. The two transporters may be very suitable for the construction of chimeric proteins to localize the substrate binding site in the primary sequence. The successful construction of active chimeras can be tested on citrate indicator plates, and the dependence on Mg^{2+} provides an easy way to discriminate between the activities of the two transporters (Fig. 5). We constructed one chimera by making use of a conserved *Stu*I restriction site in the two genes around position 490. The hybrid protein consisted of the N-terminal CitM fragment and the C-terminal CitH fragment. Unfortunately, the resulting protein was inactive in citrate transport. We are now in the process of constructing a series of chimeras by introducing unique restriction sites at selected sites in the *citM* and *citH* genes. These chimeras will be discussed in a future publication.

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