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# Functional Characterization of CitM, the $Mg^{2+}$-Citrate Transporter

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**Abstract.** The CitM transporter from *Bacillus subtilis* transports citrate as a complex with $Mg^{2+}$. In this study, CitM was functionally expressed and characterized in *E. coli* DH5α cells. In the presence of saturating $Mg^{2+}$ concentrations, the $K_m$ for citrate in CitM was 274 μM, similar to previous studies using whole cells of *B. subtilis*. CitM has a high substrate specificity for citrate. Other di- and tricarboxylic acids including succinate, isocitrate, *cis*-aconitate and tricarballylic acid did not significantly inhibit the uptake of citrate in the presence of $Mg^{2+}$. However, CitM accepts complexes of citrate with metal ions other than $Mg^{2+}$. The highest rate of citrate transport was seen in the presence of $Mg^{2+}$, followed in order of preference by $Mn^{2+}$, $Ba^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Ca^{2+}$. Citrate transport by CitM appears to be proton coupled. The transport was inhibited in transport buffers more alkaline than pH 7.5 and not affected by pH at acidic values. Transport was also inhibited by ionophores that affect the transmembrane proton gradient, including FCCP, TCC and nigericin. Valinomycin did not affect the uptake by CitM, suggesting that transport is electroneutral. In conclusion, the cloned CitM transporter from *B. subtilis* expressed in *E. coli* has properties similar to the transporter in intact *B. subtilis* cells. The results support a transport model with a coupling stoichiometry of one proton coupled to the uptake of one complex of $(Mg^{2+}\text{-citrate})^{1-}$.

**Key words:** Citrate — $Mg^{2+}$-citrate — Metal-citrate complex — *Bacillus subtilis* — Cations

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## Introduction

Citrate is an important source of energy for bacteria. Most bacteria contain concentrative citrate transporters located on the cytoplasmic membrane to allow the absorption of citrate from the environment. Many of the bacterial citrate transporters carry free citrate coupled to protons or sodium [15, 16, 21–24]. However, some unusual transporters exist in *Bacillus*, *Pseudomonas* and *Klebsiella* sp. that can absorb citrate as a complex with metal ions [5, 14, 25]. Citrate has three negatively charged carboxylic groups at neutral pH, two of which form stable complexes with a variety of divalent metal ions such as $Mg^{2+}$and $Ca^{2+}$ [7, 20].

The citrate transporter from *Bacillus subtilis*, called CitM, is a metal ion-citrate complex transporter that prefers $Mg^{2+}$-citrate as a substrate [2, 25]. The amino-acid sequence of CitM is approximately 60% identical to the sequence of the $Ca^{2+}$-citrate complex transporter, CitH, also from *B. subtilis* [4, 11]. Both CitM and CitH have similar predicted secondary structures containing 12 transmembrane domains. These transporters belong to a distinct family that is not related to the other families of citrate transporters.

Although the function of CitM has been characterized in native membranes, the results could have been complicated by the multiple pathways that exist for citrate transport in *B. subtilis* [11]. In this study, the cloned CitM from *B. subtilis* was expressed in *E. coli* DH5α cells, which do not have an endogenous citrate transporter. We find that CitM has a relatively low affinity for substrate and $Mg^{2+}$. Although the transporter is very specific for citrate, other divalent metal ions, including $Mn^{2+}$, $Ba^{2+}$, $Ni^{2+}$ and $Co^{2+}$, can substitute for $Mg^{2+}$. Transport appears to be driven by the proton electrochemical gradient. The results are similar to previous studies of CitM in native membranes and support an electroneutral

transport mechanism with a coupling stoichiometry of one $H^+$ and one $(Mg^{2+}\text{-citrate})^{-1}$ complex.

## Materials and Methods

### Construction of the CitM Expression Vector

The *citM* gene (Genbank accession number U62003) [4] was amplified from genomic DNA isolated from *B. subtilis* 168M (ATTC) using Polymerase Chain Reaction (PCR) with sequence-specific primers. The sense primer included a mutation that introduced an *NcoI* restriction site (underlined) at the start codon (in bold) to facilitate subcloning: 5′-CAG ACC **ATG** GTA GCA ATC TTA GGC TTT-3′. The introduction of the *NcoI* restriction site changed the first and second amino-acid residues from Val-Leu to Met-Val. The antisense primer was: 5′-AAT TGG ATC CTG AAT TA**T CA**T ACG GAA ATA G-3′, which included a stop codon (in bold type) and a *BamHI* restriction site (underlined) to facilitate subcloning. The PCR was done using the Clontech (Palo Alto, CA) Advantage PCR kit which contains a proofreading DNA polymerase, Klentaq, to minimize mutations. The PCR product was cloned into the pCR2.1 vector using the TopoTA cloning kit, according to manufacturer's directions (InVitrogen, San Diego, CA). The single internal *NcoI* site in the *citM* gene was removed by silent mutagenesis to facilitate subcloning (Quick change mutagenesis kit, Stratagen, La Jolla, CA). The *citM* DNA was then subcloned into the *NcoI*/*BamHI* sites of the pSE380 expression vector (InVitrogen). The recombinant plasmid containing *citM* DNA in pSE380 is called pSE380/CitM. The construct was sequenced at the Sealy Center for Molecular Science (University of Texas Medical Branch, Galveston, TX). Three separate clones were sequenced and all were identical to the published sequence except for a mutation at position 256, which changes the valine to alanine. The mutation may be due to a strain variation since we used strain 168M, whereas the published sequence is from *B. subtilis* strain 6GM.

### Preparation of Cells

The pSE380/CitM construct was transformed into *E. coli* DH5α cells, which normally do not express a citrate transport system [10, 12]. Overnight cultures of *E. coli* DH5α cells harboring the recombinant plasmid pSE380/CitM or vector-only plasmid pSE380 were used to inoculate 100 ml TB medium containing 50 μg/ml carbenicillin. Cells were grown at 37°C until the $O.D._{650}$ reached ~0.8 to 0.9. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cell growth was continued for another 2 hours. The cells were harvested by centrifugation at 2000 × g (Beckman JA-25.50 rotor) for 10 minutes at 4°C. The cells were washed twice with HEPES-glucose buffer (100 mM HEPES, 0.1% D-(+)-glucose, pH 6.8 adjusted with 1M Tris) [25] and then resuspended in the same buffer to a final $O.D._{650}$ of ~10. For experiments using different pH values, cells were washed with HEPES-glucose buffer at pH 6.8 and then resuspended in HEPES-glucose buffer adjusted to the appropriate pH. In experiments using potassium-phosphate buffer, the cells were washed and resuspended in 50 mM K-phosphate, pH 7.0.

### Transport Assay

Cells were preincubated for 10 minutes at room temperature with $MgCl_2$ by combining 80 μl of cell suspension with 10 μl of 100 mM $MgCl_2$ stock solution prepared in HEPES-glucose buffer. Transport was initiated by adding 10 μl of substrate solution in which radioactive $^{14}$C-citrate (0.1 μCi/μl, Moravek Biochemicals, Brea, CA) was mixed with different concentrations of non-radioactive citrate. Transport was stopped with ice-cold HEPES-glucose buffer. In pilot experiments, we compared HEPES-glucose and 100 mM LiCl stop solutions (*results not shown*). The radioactivity retained by the filters was the same using both stop solutions, but there appeared to be leakage from the cells in LiCl (tested by waiting 30 sec before filtration). Therefore, uptakes were stopped by the addition of 1 ml of ice-cold HEPES-glucose buffer. For the measurement of uptake at time 0, the stop solution was added to the cell suspension before adding the radioactive substrate. The mixture was immediately filtered through a nitrocellulose filter (HAWP Millipore, 0.45 μm) using suction. The filter was washed twice, first with 1 ml and then with 4 ml of ice-cold HEPES-glucose buffer and immediately submerged in scintillation liquid. The radioactivity retained on the filter was counted. For experiments testing the inhibition of citrate transport by potential substrates such as isocitrate, cis-aconitate and tricarballylic acid, a final concentration of 1 mM of the test inhibitor was added together with the radiolabelled citrate. For experiments testing the effect of different metal ions, cells were preincubated for 10 minutes with a final concentration of 5 mM different divalent metal ion solutions prepared in HEPES-glucose buffer.

In experiments testing the effects of ionophores, the 80 μl of cell suspension was preincubated together with 10 μl $MgCl_2$ and 1 μl of ionophore stock solution dissolved in 100% ethanol. Control groups were preincubated in ethanol without ionophore. The final concentration of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was 25 μM. In experiments involving trichlorocarbanilide (TCC), a final concentration of 100 μM was used and the cells were suspended in 100 mM choline chloride, 10 mM HEPES, pH 6.8 to provide a source of anions for the TCC [1]. In experiments involving nigericin or valinomycin, the cells were first permeabilized with EDTA as described below and final concentrations of 50 μM for nigericin or 10 μM for valinomycin were used [l].

### Permeabilization of Cells

The outer membrane of *E. coli* prevents access of some large or hydrophobic molecules to the inner membrane where CitM is located. Therefore, in experiments involving the ionophores nigericin or valinomycin, the *E. coli* cell wall was first permeabilized using EDTA [1, 18]. Briefly, the cells were grown and washed as described above and resuspended in 1/30 the original culture volume. The cells were then equilibrated to 37°C and an equal volume of resuspension buffer containing 2 mM EDTA was added. The cells were then incubated exactly 3 minutes at 37°C before being diluted 100-fold with resuspension buffer containing 100 mM $MgSO_4$. The cells were then washed and resuspended in 100 mM HEPES buffer without glucose, since the cells can repair the permeabilization of the outer membrane in the presence of glucose [13]. For the experiments involving valinomycin, the cells were resuspended in 60 mM HEPES buffer containing 20 mM KCl or CholineCl.

### Protein Assay

The total membrane protein was measured using the *DC* Protein Assay kit (BioRad, Hercules, CA), according to the manufacturer's directions. The protein standard used was bovine plasma gamma globulin dissolved in HEPES-glucose buffer.

### Data Analysis

Each data point represents the mean of 4 replicate samples. For most experiments, the counts in cells transformed with control

vector pSE380 have been subtracted from the counts in cells expressing CitM. For citrate kinetic calculations, the initial uptake rate of citrate was fit by nonlinear regression using Sigma Plot 2000 (SPSS) to the Michaelis-Menten equation: $v = V_{max} \cdot [S]/(K_m + [S])$, where $v$ is the initial uptake rate, $[S]$ is the concentration of the substrate, $V_{max}$ is the maximum uptake rate at saturating substrate concentrations, and $K_m$ is the substrate concentration producing one half of $V_{max}$ and is also called the Michaelis-Menten constant. In some experiments the data were fitted to the Hill equation: $v = (V_{max} \cdot [S])^n/(K_{0.5}^n + [S]^n)$ with an additional constant, $C$, which represents the uptake or binding of citrate in the absence of added $Mg^{2+}$. The $n$ represents the Hill coefficient.

In some of the experiments, the concentrations of free $Mg^{2+}$ and the $Mg^{2+}$-citrate complex were calculated using the MaxChelator program (Winmaxc v.2.05, http://www.stanford.edu/~cpatton/maxc.html) [3]. Although both divalent ($citrate^{2-}$) and trivalent ($citrate^{3-}$) forms of citrate are able to form complexes with $Mg^{2+}$, the complex with trivalent citrate ($Mg\text{-}citrate^{1-}$) is more abundant. For example, under the conditions used in many of our experiments (pH 6.8, 500 μM total citrate and 10 mM total $Mg^{2+}$), the concentration of the $Mg\text{-}citrate^0$ complex is only 1.2 μM, whereas the concentration of $Mg\text{-}citrate^{1-}$ is 468 μM.

## Results

### Time Course of Citrate Uptake

The time course of citrate uptake in the presence of saturating concentrations of $Mg^{2+}$ was measured in whole cells of *E. coli* DH5α transformed with the pSE380/CitM construct or with the pSE380 vector alone (Fig. l). Transport studies in bacteria often use potassium phosphate buffer to resuspend the cells and LiCl buffer to stop the reaction. However, previous studies of citrate transport in *B. subtilis* suggested that HEPES might be a better transport buffer than phosphate because the phosphate can form complexes with divalent metal ions [6, 8, 25]. Therefore, the time course of citrate transport was compared in potassium phosphate buffer and HEPES-glucose buffer.

In both buffers, the transport of citrate in *E. coli* cells transformed with the control plasmid, pSE380, was very low, verifying that *E. coli* DH5α do not express a native citrate transporter (Fig. 1*A*, *B*). The transport activity in cells expressing CitM was approximately tenfold higher when measured in HEPES-glucose buffer (Fig. 1*B*) than in potassium-phosphate buffer (Fig. 1*A*), in agreement with previous reports that phosphate may interfere with the transport assay. The linear range of transport measured in HEPES-glucose was in the first 30 seconds. There appeared to be some binding or rapid uptake in cells expressing CitM (Fig. 1*B*). In four experiments, the uptakes measured at time 0 were 10 ± 2% (mean ± SEM) of the uptakes measured at 30 seconds. The HEPES-glucose buffer was used for the following studies and 15- or 30-second time points were measured.

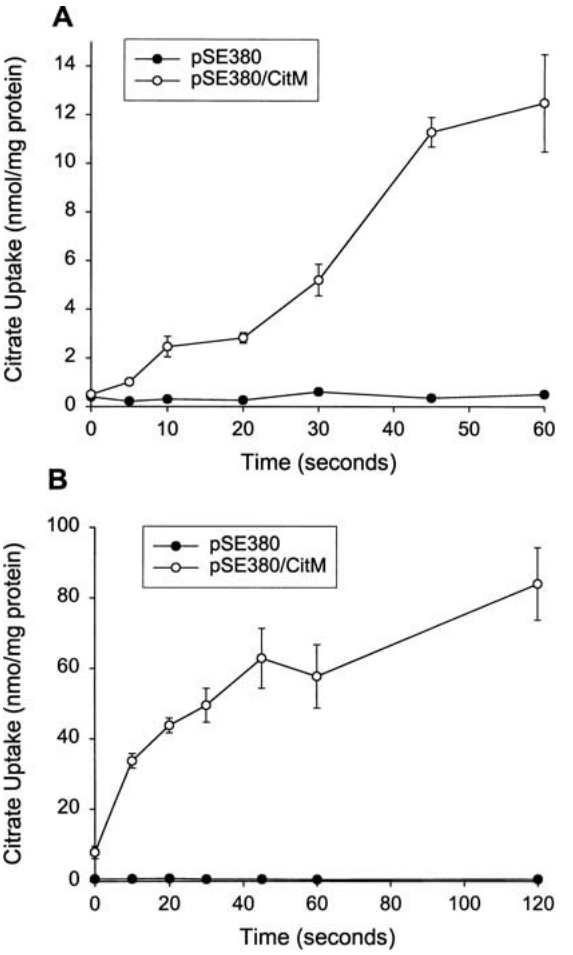


**Fig. 1.** Time course of the uptake of 1 mM citrate by CitM expressed in *E. coli*. Citrate uptakes were measured in whole cells of *E. coli* transformed with pSE380/CitM or with pSE380 vector. Cells were first preincubated with 10 mM $MgCl_2$ for 10 minutes and then $^{14}C$-citrate was added to activate the transport. (*A*) The uptakes were measured in 50 mM potassium phosphate buffer (pH = 7.0) and stopped with 100 mM LiCl buffer. (*B*) The uptakes were measured and stopped with 100 mM HEPES-glucose buffer (pH = 6.8). The data shown are means ± SEM of four replicate measurements.

### Citrate Uptake as a Function of Citrate Concentrations

The kinetics of citrate uptake in *E. coli* expressing CitM is shown in Fig. 2. At saturating magnesium concentrations (10 mM), the $K_m$ for citrate was 339 μM and the $V_{max}$ was 252 nmol/mg · min (Fig. 2*A*). In three experiments, the mean $K_m$ was 274 ± 51 μM and the $V_{max}$ was 222 ± 47 nmol/mg · min (mean ± SEM). The native transporter expressed in *B. subtilis* has a $K_m$ of 550 μM [25]. The data were also analyzed by calculating the Mg-citrate concentrations instead of total citrate since the transported species is the Mg-citrate complex (Fig. 2*B*). Since the $Mg^{2+}$ concentration was 10 mM, the concentration of Mg-citrate was similar to the total citrate concentration and

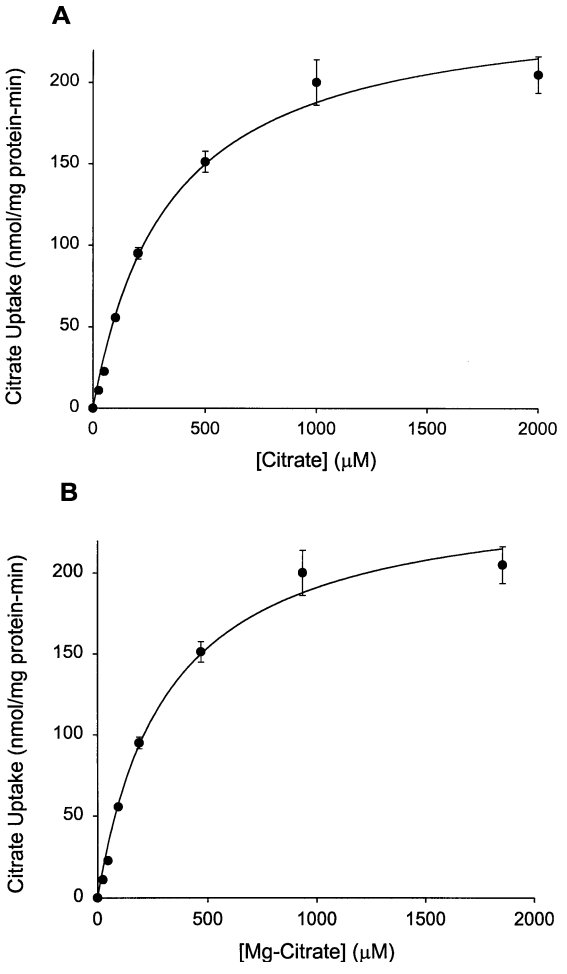


**Fig. 2.** (*A*) Citrate uptake kinetics of CitM expressed in *E. coli* DH5α cells. 30-second uptakes from different concentrations of citrate (50 μM to 2 mM) were measured in the presence of 10 mM $MgCl_2$ in HEPES-glucose buffer, pH = 6.8. The $K_m$ for citrate is 339 ± 46 μM and the $V_{max}$ is 252 ± 12 nmol/mg protein · min (the error represents SE of the regression). The data shown are means ± SEM of four replicate measurements. (*B*) The same uptake data (shown in *A*) were expressed as a function of the Mg-citrate concentration. The $K_m$ for citrate is 320 ± 44 μM and the $V_{max}$ is 252 ± 12 nmol/mg protein · min (the error represents SE of the regression).

there was very little difference in the kinetic values. For example, the $K_m$ for Mg-citrate in Fig. 2*B* is 320 μM.

## Substrate Specificity of CitM

The substrate specificity of CitM was tested by measuring the inhibition of uptake of radiolabelled citrate in the presence of saturating $Mg^{2+}$ concentrations. Four tricarboxylic acids (citrate, isocitrate, *cis*-aconitate and tricarballylic acid) and one dicarboxylic acid (succinate) were tested. Only citrate inhibited the transport of radiolabelled citrate, whereas there was no inhibitory effect of the other test substrates (Fig. 3). The uptake of $^3$H-succinate was also tested directly in DH5α cells expressing CitM but there was no difference from control cells transformed with pSE380 alone (*data not shown*). Therefore, CitM is highly specific for citrate.

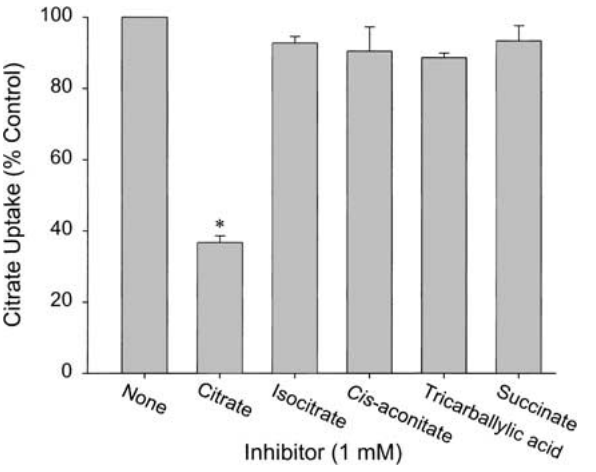


**Fig. 3.** Substrate specificity of CitM expressed in *E. coli*. 30-Second uptakes from 10 μM $^{14}$C-citrate were measured in the presence of 10 mM $MgCl_2$ and 1 mM test inhibitors or in the absence of test inhibitors. The results shown are the mean ± range of two separate experiments. The uptake of citrate in the absence of inhibitors was 1.0 ± 0.1 nmol/mg protein · min ($n$ = 2). (*, significantly different from uptake in absence of inhibitor, $p < 0.001$).

## Cation Effects on Citrate Uptake by CitM

Previous studies have shown that CitM transports citrate as a complex with $Mg^{2+}$ [4, 25]. In this study, we examined the effect of increasing total $Mg^{2+}$ concentrations while keeping total citrate concentrations constant. This would result in changing both Mg-citrate and free $Mg^{2+}$ concentrations. As shown in Fig. 4*A*, the uptake of radiotracer citrate appeared to be a sigmoidal function of the Mg-citrate concentrations in the medium. The apparent half-saturation constant ($K_{0.5}$) for Mg-citrate was 271 μM and the Hill coefficient was 2.6 (Fig. 4*A*). When the data were replotted as a function of free $Mg^{2+}$ concentrations, the curve fit was still sigmoidal but the Hill coefficient decreased to 1.6 (Fig. 4*B*). There was also measurable uptake of citrate in the absence of added $Mg^{2+}$ in *E. coli* expressing CitM compared with controls. The citrate uptake in the absence of added $Mg^{2+}$ was about 20 nmol/mg · min (Fig. 4).

CitM can carry complexes of citrate with divalent metal ions other than $Mg^{2+}$. As shown in Fig. 5, the substitution of $Mg^{2+}$ with $Mn^{2+}$ produced 76% of the citrate-transport rate. Substitution with $Ba^{2+}$, $Ni^{2+}$ and $Co^{2+}$ was less effective, producing citrate-uptake rates between 50% and 30% of that observed in $Mg^{2+}$. The uptake of citrate in the presence of $Ca^{2+}$

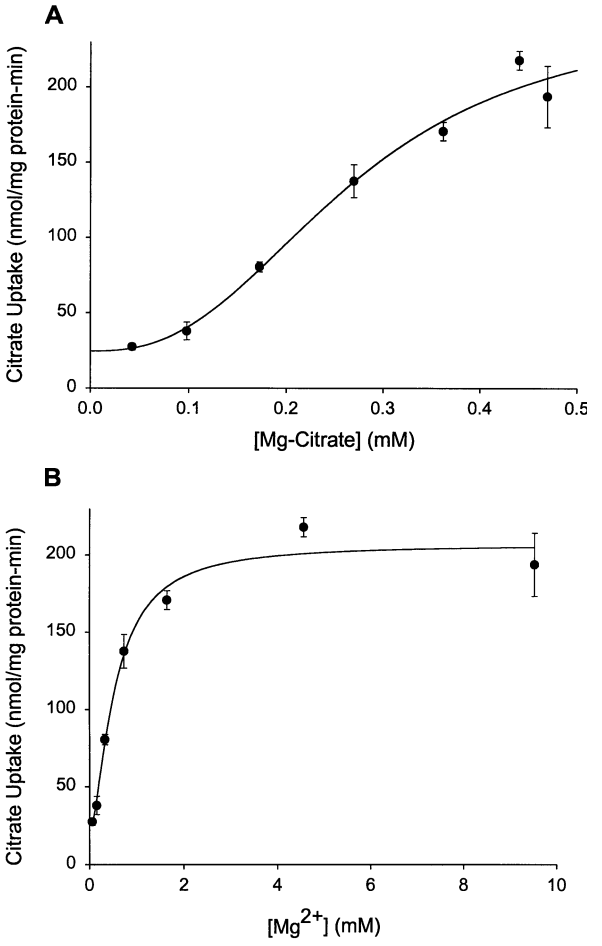


**Fig. 4.** Effects of varying $Mg^{2+}$ concentrations on citrate transport in CitM expressed in *E. coli* DH5α cells. 15-second uptakes from 500 μM $^{14}$C-citrate were measured in HEPES-glucose buffer, pH = 6.8 in the presence of different concentrations of total $MgCl_2$ (0–10 mM). (*A*) The uptake data expressed as a function of Mg-citrate concentration in the medium. The $K_{0.5}$ for Mg-citrate is 271 ± 68 μM, the $V_{max}$ is 226 ± 72 nmol/mg protein · min and the Hill coefficient *n* is 2.6. (*B*) The same uptake data as in *A*, expressed as a function of the free $Mg^{2+}$ concentration in the medium. The $K_{0.5}$ for $Mg^{2+}$ is 541 ± 113 μM, the $V_{max}$ is 187 ± 24 nmol /mg proteir · min, the Hill coefficient is 1.6. The uptake in the absence of added $Mg^{2+}$ is 20 ± 17 nmol/mg · min (the errors represent SE of regression). The data shown are the mean ± SEM of four replicate measurements.

was similar to the uptake in the absence of added metal ions. It is likely that the transport solutions contain some residual divalent cations since the addition of the divalent cation chelator, EGTA, reduced the uptake further. There was no significant uptake of citrate in the presence of $Zn^{2+}$ when compared with the EGTA-containing solution. The uptake of radiotracer citrate in this experiment was not necessarily related to the concentration of the citrate-metal ion complex. For example, the concentration of the Ba-citrate complex was 63% of the total citrate compared with Mg-citrate (88% of total), which could account

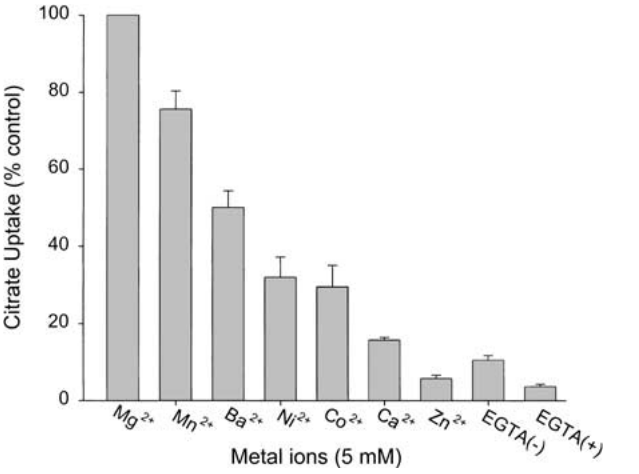


**Fig. 5.** Cation specifity of CitM expressed in *E. coli*. 30-second uptakes from 500 μM $^{14}$C-citrate were measured in the presence of 5 mM concentrations of different metal ions as shown in the figure. The uptakes were also measured in the absence of added metal ions, without EGTA (−) or with 1 mM EGTA (+). The data are expressed as a percentage of control uptake of citrate measured in $Mg^{2+}$ (82 ± 12 nmol/mg protein-min, mean ± SEM, $n = 4$). The data shown are the means ± SEM of 2–4 separate experiments.

for the decreased uptake activity in the presence of $Ba^{2+}$. However, $Zn^{2+}$ and $Ca^{2+}$, which produced the lowest transport activity, had the highest percentage of metal ion-citrate complex (Zn-citrate 99% and Ca-citrate 91% of total citrate).

## Effect of pH on Citrate Uptake by CitM

Previous studies have suggested that citrate transport by CitM is coupled to protons [2, 4]. Therefore, we examined the effect of pH on citrate uptake in *E. coli* expressing CitM. As shown in Fig. 6, there was little effect of pH on citrate transport between pH 5 and 6.8, whereas at pH 7.5 and 8.0 the transport was greatly reduced to only about 30% of the average uptake at pH 5 to 6.8. Since the intracellular pH of *E. coli* is normally between 7.5 to 8.0 [9], extracellular pH values above 7.5 should abolish the transmembrane pH gradient. The inset of Fig. 6 shows that the concentration of the Mg-citrate complex is not affected much by changes in pH. The decrease in concentration of Mg-citrate occurs at acidic pH, which is different from the decrease in transport activity. Therefore, the results suggest that citrate transport by CitM is dependent on an inwardly-directed pH gradient or an outwardly directed $OH^-$ gradient.

## Effect of Ionophores on Citrate Uptake in CitM

Ionophores were tested to examine the effect of chemical and electrical gradients of ions on the transport activity of CitM (Fig. 7). Two of the ionophores (FCCP and TCC) cross the cell wall readily

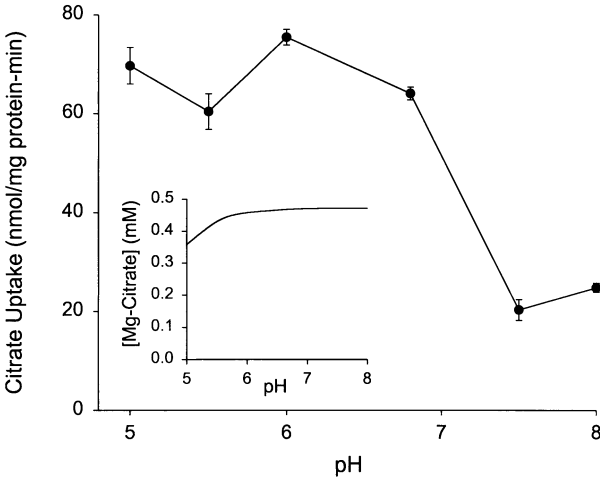


**Fig. 6.** Effect of external pH on uptake of citrate by CitM expressed in *E. coli*. Cells were resuspended in HEPES-glucose buffer at different pH values as shown in the figure and 30-second uptakes from 500 μM citrate were measured in the presence of 10 mM $MgCl_2$. Inset, the concentration of the Mg-citrate complex in the medium as a function of pH.

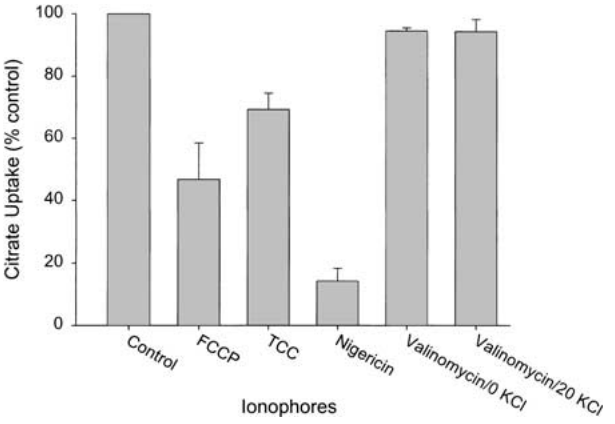


**Fig. 7.** Effect of ionophores on citrate uptake by CitM expressed in *E. coli*. 30-second uptakes from 500 μM citrate were measured in HEPES-glucose buffer in the presence of 10 mM $MgCl_2$ and ionophores: FCCP, TCC, nigericin and valinomycin. The valinomycin experiment was done in 20 mM extracellular KCl (20 KCl) or in 20 mM choline chloride (0 KCl). The data shown are the means ± range of two separate experiments.

and were used directly on the *E. coli* cells, whereas nigericin and valinomycin require prior permeabilization of the cell wall with EDTA. Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) is an electrogenic proton ionophore that can dissipate the transmembrane $H^+$ gradient [17]. Citrate uptakes in the presence of FCCP in CitM were reduced to about 47% of uptakes without FCCP (Fig. 7). Trichlorocarbanilide (TCC) is an anion/$OH^-$ exchanger that produces a change in intracellular pH without affecting membrane potential [1]. The addition of TCC also inhibited transport by CitM (Fig. 7). Nigericin is an electroneutral $K^+/H^+$ exchanger that also dissipates the transmembrane pH gradient without changing membrane potential [1, 17]. Nigericin reduced uptakes of citrate in CitM by 86%. Finally, valinomycin was used to test whether the membrane potential affects citrate transport by CitM. Valinomycin is an electrogenic $K^+$ ionophore that produces a diffusion potential of $K^+$. In bacteria the addition of valinomycin can also change intracellular pH depending on the concentration of extracellular $K^+$ [1]. However, extracellular $K^+$ concentrations below 25 mM affect the membrane potential without changing pH. Intracellular $K^+$ concentrations in bacteria are approximately 350 mM [9]. The combination of valinomycin and an outward gradient of $K^+$ should produce a hyperpolarization of the membrane potential, but there was no change in citrate transport under these conditions (Fig. 7). Therefore, CitM activity does not appear to be affected by membrane potential. Based on these results, the coupling stoichiometry is likely to be one proton coupled to one complex of $(Mg^{2+}\text{-citrate})^{1-}$, with no net movement of charge across the membrane.

## Discussion

The CitM transporter carries a complex of $Mg^{2+}$ and citrate across the cytoplasmic membrane of *B. subtilis*. The major findings of this study are that CitM expressed in *E. coli* DH5α cells is an electroneutral proton-coupled transporter for the $Mg^{2+}$-citrate complex. Furthermore, CitM is very specific for citrate as a substrate. Other di- or tricarboxylic acids, including succinate, isocitrate, cis-aconitate and tricarballylic acid are not substrates of CitM. However, the divalent metal ion carried by CitM is not limited to $Mg^{2+}$. The transporter also accepts $Mn^{2+}$, $Ba^{2+}$, $Ni^{2+}$ and $Co^{2+}$.

The apparent $K_m$ for citrate in CitM expressed in *E. coli* is around 270 μM. This agrees well with the $K_m$ of 550 μM reported in previous studies of whole cells of *B. subtilis*, also measured in HEPES-glucose buffer with saturating $Mg^{2+}$ concentrations [25]. However, there is some variability reported in the literature on the $K_m$ for citrate in CitM. Some of the variability is due to the use of phosphate buffer for assays, which typically results in a much larger apparent $K_m$. One exception is the study by Bergsma and Konings, which reported a $K_m$ of 40 μM in phosphate buffer using membrane vesicles from *B. subtilis* [2]. Vesicle experiments should provide a more accurate measurement of $K_m$ because they do not contain the cell wall found in whole cell studies, which could add complications of unstirred layers to the kinetic measurements. Interestingly, a recent study done with the cloned CitM expressed in whole cells of *E. coli* DH5α reported a $K_m$

for citrate of about 60 μM using PIPES buffer [11]. At the moment it is not clear why there are such large differences in measured $K_m$ between studies.

The CitM transporter is very specific for the complex of citrate with a divalent cation. Citrate has three carboxyl groups that can form a stable complex with metal ions. In the case of magnesium, citrate forms a tridentate complex involving two of the carboxyl groups and the central hydroxyl group. The central hydroxyl group and carboxyl group are very important for the stability of the complex [7]. Although the tricarboxylic acids that were tested as potential substrates, isocitrate, *cis*-aconitate and tricarballylic acid, also form complexes with metal ions, they lack the central hydroxyl group necessary for the formation of the tridentate complex [7], which may result in structures that are not recognized by the CitM substrate binding site. The dicarboxylic acid succinate is not a substrate of CitM, presumably also because it does not form the appropriate complex with divalent metal ions. Alternately, the concentration of the metal ion-test substrate complex may be very low compared with the Mg-citrate concentration.

Although $Mg^{2+}$ is the preferred metal ion transported by CitM, other metal ions including $Mn^{2+}$, $Ba^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Ca^{2+}$ (in order of preference) can substitute for $Mg^{2+}$. The order of cation preference seen in our study is similar to that observed previously in *B. subtilis* cells [2]. However, as in the other measurements of CitM function, there have been reports of different cation preferences of CitM. The same order of cation preference was measured in whole cells of *B. subtilis* in a recent study [11]. Surprisingly, the same study reported a different order of cation preference for the cloned CitM expressed in *E. coli*, with the highest transport rate in $Ni^{2+}$, followed by $Co^{2+}$, $Mg^{2+}$, $Mn^{2+}$, and finally $Zn^{2+}$. It is not clear why such large differences between the cloned CitM and whole cells of *B. subtilis* were found in the same study. The highest transport rate for citrate was seen with $Mg^{2+}$, and *B. subtilis* has only two transporters for citrate-metal ion complexes, CitM and CitH. Since CitH does not transport $Mg^{2+}$ [11], one would expect that the activity of CitM would account for the high transport rate measured in $Mg^{2+}$.

In the experiments reported here, there was measurable uptake of citrate by CitM in the absence of added divalent metal ions. This is likely due to the presence of residual metal ions in the transport buffer since the transport in the presence of EGTA was lower than in its absence. Similar results were observed in recent whole-cell studies of CitH expressed in *E. coli*. The uptake of citrate in the absence of added metal ions was reduced to background levels by the addition of EGTA [11].

The transport activity of CitM in the $Mg^{2+}$-activation experiment appears to be a sigmoidal function of the Mg-citrate concentration. One explanation for this is that $Mg^{2+}$ may have two functions in CitM, much like the $Mg^{2+}$ in Mg-ATP requiring enzymes [19]. It is possible that the $Mg^{2+}$ is not only a transported substrate, as part of the Mg-citrate complex, but it may also act as an essential activator of transport by binding to the CitM transport protein at an allosteric site. However, the uptake rate at the same concentration of Mg-citrate was similar in the kinetic experiment shown in Fig. 2 (with free [$Mg^{2+}$] close to 10 mM) and in Fig. 4 (at low free [$Mg^{2+}$]). This raises the possibility that the residual divalent cations in the medium might have complicated the results by introducing uncertainty into the calculations of the Mg-citrate concentrations at low free [$Mg^{2+}$]. Therefore, more experiments are needed to distinguish these possibilities.

The mechanism of transport by CitM is likely to involve proton cotransport (or countertransport of $OH^-$). In our study, the transport of citrate by CitM was dependent on an inwardly-directed proton gradient. Transport was reduced when the extracellular pH was greater than 7.5 or in the presence of ionophores that disrupt the transmembrane pH gradient, including FCCP, nigericin and TCC. Valinomycin is an electrogenic $K^+$ ionophore, and under the conditions used in this study, the addition of valinomycin should change the membrane potential without affecting the pH [1]. However, valinomycin treatment had no effect on the transport of citrate by CitM in our studies. In previous studies of CitM activity in membrane vesicles of *B. subtilis*, the addition of valinomycin resulted in an inhibition of uptake, although the phosphate transport buffer used could complicate the results [2]. A second study reported little effect of valinomycin on the cloned CitM expressed in *E. coli,* but the experiments were not done using permeabilized cells, so it is unclear whether the valinomycin reached the inner membrane [4]. Furthermore, the experiments were done in the presence of 50 mM extracellular $K^+$, which should inhibit transport by the large decrease in the transmembrane pH gradient since valinomycin changes the pH in *E. coli* at extracellular $K^+$ concentrations above 25 mM [1]. The insensitivity of CitM to changes in membrane potential produced by valinomycin indicates that the transporter is electroneutral. Since the complex of $Mg^{2+}$ and citrate contains one net negative charge, it is likely that one proton is coupled to the movement of one $(Mg^{2+}\text{-citrate})^{1-}$ complex.

In conclusion, we have characterized the function of CitM expressed in *E. coli*. Although the endogenous CitM activity has been characterized in *B. subtilis*, the results could have been complicated by multiple transport pathways for citrate in those membranes. However, we find that the cloned CitM expressed in *E. coli* has very similar properties to the native transporter. CitM is a low-affinity transporter that carries the complex of citrate with metal ions

such as $Mg^{2+}$ or other divalent metal ions. The transporter is very specific for the citrate-metal ion complex and other tricarboxylic acids are not substrates. Finally, the transporter appears to be electroneutral with a likely coupling stoichiometry of 1 $H^+$: 1 $(Mg^{2+}\text{-citrate})^{1-}$.

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