Regulation and expression of the metal citrate transporter CitM of Bacillus subtilis

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Growth of *Bacillus subtilis* on citrate and isocitrate is supported by the Mg$^{2+}$-citrate transporter CitM

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**ABSTRACT**

*Bacillus subtilis* 168 was assayed for growth on TCA cycle intermediates and related compounds as sole carbon source. Growth was supported by citrate, D-isocitrate, succinate, fumarate and L-malate, whereas no growth was observed in the presence of cis-aconitate, α-ketoglutarate, D-malate, oxaloacetate and tricarboxylate. Growth on the tricarboxylates citrate and D-isocitrate required the presence of functional CitM, a Mg$^{2+}$-citrate transporter, while growth on succinate, fumarate and L-malate appeared to be CitM-independent. Interestingly, the naturally occurring D-isocitrate was favored over L-isocitrate. Like citrate, D-isocitrate was shown to be an inducer of *citM* expression. The addition of 1 mM Mg$^{2+}$ to the growth medium improved growth on both citrate and D-isocitrate suggesting that D-isocitrate was taken up by CitM in complex with divalent metal ions. Subsequently, the ability of CitM to transport D-isocitrate was demonstrated by competition experiments and by heterologous exchange in RSO membrane vesicles prepared of *E. coli* cells expressing the *citM* gene. None of the other TCA cycle intermediates and related compounds tested was recognized by CitM. Uptake experiments using radioactive $^{63}$Ni$^{2+}$ provided direct evidence that D-isocitrate is transported in complex with divalent metal ions.
INTRODUCTION

The two main roles of the tricarboxylic acid (TCA) cycle in cell metabolism are to generate metabolic energy by producing reducing equivalents and to provide the cell with intermediates for anabolism. 

*B. subtilis* runs a complete TCA cycle and is able to utilize almost all citric acid cycle intermediates under aerobic conditions (Fortnagel & Freese, 1968; Asai et al., 2000; Wei et al., 2000). Little is known about the identity and regulation of the transport systems responsible for the uptake of the growth substrates under these conditions. The uptake of the C4-dicarboxylates succinate and fumarate during growth on these substrates was shown to be mediated by the dicarboxylate transporter DctP (Asai et al., 2000; Janausch et al., 2002). Expression of the *dctP* gene is positively regulated in response to external signals by a sensor kinase and regulator pair, DctS and DctR. The system is induced in the presence of succinate, fumarate, and low concentrations of yeast extract and repressed by malate (Asai et al., 2000). Although it was not excluded that the latter is also a substrate for the DctP transporter, several other transport systems have been shown to transport malate. CimH mediates the uptake of malate (and citrate) in symport with protons (Krom et al., 2001), MeaN (yufR) transports malate in symport with Na⁺ ions (Wei et al., 2000), and MleN was shown to be a malate/lactate exchanger that couples the exchange reaction to proton uptake and Na⁺ efflux (Wei et al., 2000). The role of the individual transporters during growth on malate is unknown.

Older studies have reported the ability of the tricarboxylic TCA cycle intermediates cis-aconitate and isocitrate to induce and competitively inhibit the uptake of citrate in wild-type cells of *B. subtilis*, suggesting the presence of a specific transport system (McKillen et al., 1972). More recent studies have identified a number of transporters for citrate. CimH, mentioned above, catalyzes symport of citrate and protons (Krom et al., 2001). The secondary transporters CitM and CitH also transport citrate in symport with protons, but only in the presence of divalent metal ions (Boorsma et al., 1996; Krom et al., 2000). Uptake studies of the two transporters separately expressed in *E. coli* cells confirmed that the metal ion-citrate complex is the transported species and that the metal ion specificity of the two transporters is complementary, i.e. CitM transports citrate in complex with Mg²⁺, Mn²⁺, Ni²⁺, Co²⁺, Zn²⁺ and CitH recognizes citrate in complex with Ca²⁺, Ba²⁺ or Sr²⁺ (Krom et al., 2000). Expression of *citM* is strictly regulated: gene activation depends on the action of the two-component regulator pair, CitS-CitT, that senses the presence of citrate in the medium (Yamamoto et al., 2000). Furthermore, gene expression is subject to catabolite repression (this thesis). The strict regulation of *citM* expression by medium components makes it likely that CitM is the main uptake system during growth on citrate as the sole carbon source.
Growth on citrate and isocitrate is supported by CitM

Here, we study the involvement of the Mg\(^{2+}\)-citrate transporter CitM in growth of *B. subtilis* on TCA cycle intermediates. It follows that CitM is both necessary and sufficient for growth on citrate as the sole carbon source. In addition, it is shown that CitM supports the growth of *B. subtilis* on isocitrate which, like citrate, is shown to be an inducer of *citM* expression. Subsequently, transport studies using membrane vesicles and resting cells demonstrate that CitM transports the complex of isocitrate and divalent metal ions.

**MATERIALS and METHODS**

**Bacterial strains and growth conditions**

Strains used in this study are *Bacillus subtilis* strains 168 (trpC2), CITMd (trpC2 ΔcitM ery; Yamamoto et al., 2000) and CM002 (trpC2 amyE::(P\(\text{citM}\)-lacZ cat); Chapter 2 of this thesis) and *E. coli* strain TOP10 (Φ80DlacZM15 ΔlacX74 recA; Invitrogen). Precultures of *B. subtilis* were grown overnight at 37 °C in Luria-Bertani (LB) or CSE medium, to which tryptophan was added to a final concentration of 20 µg/ml. CSE medium is C medium (Aymerich et al., 1986) to which sodium succinate (6 g/l) and potassium glutamate (8 g/l) was added. LB grown precultures were diluted 200 times in minimal salts medium (2.72 g of K\(_2\)HPO\(_4\), 1 g of KH\(_2\)PO\(_4\), 1 g of NH\(_4\)Cl, 0.284 g of Na\(_2\)SO\(_4\), 0.17 g of NaNO\(_3\), 0.15 g of KCl, 25 mg of MgCl\(_2\)•6H\(_2\)O, 22 mg of CaCl\(_2\)•6H\(_2\)O, 15 mg of MnCl\(_2\)•4H\(_2\)O, and 2.16 mg of FeCl\(_3\)•6H\(_2\)O per liter medium) containing 0.05% yeast extract (MSMYE medium; Goel et al., 1995; Asai et al., 2000). TCA cycle intermediates were added at a final concentration of 5 mM after growing the cells for 2.5 hrs (OD\(_{660}\) ~ 0.1). CSE grown precultures were diluted 25 times in CSE supplemented with 5 mM trisodium citrate, DL-isocitrate or cis-aconitate. The cells were grown in flasks under continuous shaking at 150 rpm. Growth was monitored by measuring the optical density at 660 nm (OD\(_{660}\)). When appropriate, antibiotics were added at the following concentrations: chloramphenicol, 5 µg/ml; erythromycin, 0.3 µg/ml; ampicillin 50 µg/ml. Qualitatively, β-galactosidase activity was measured by growing the cells on agar plates containing Luria-Bertani (LB) medium containing 10 mM of the different carbon sources and 5% of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

*E. coli* TOP10 was transformed with plasmid pWSKCitM which contains the gene coding for the Mg\(^{2+}\)-citrate transporter CitM under control of the lac promoter (Krom et al., 2000). Recombinant cells were grown in LB medium supplemented with 100 µg/ml carbenicillin and were induced with 0.1 mM isopropylthiogalactopyranoside (IPTG) at OD\(_{660}\) = 0.2, after which the cells were allowed to grow for an additional two hours.

**Preparation of membrane vesicles**

*E. coli* TOP10 cells expressing CitM were harvested at an OD\(_{660}\) of 0.8 - 1.0 and right-side-out membrane vesicles were prepared by the osmotic shock lysis procedure as described by Kaback (Kaback 1983). The vesicles were resuspended in 50 mM K-PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]) pH 6.5, aliquoted in 0.5 ml samples, rapidly frozen in liquid nitrogen and, subsequently, stored at –80 °C. The protein concentration was determined with the Bio-Rad DC Protein Assay kit (Bio-rad).
Transport assays

(i) $^{63}$Ni$^{2+}$ uptake in whole cells. Cells were harvested by centrifugation, washed once and resuspended in 50 mM K-PIPES, pH 6.5, and stored on ice until use. Transport activity was determined by the rapid-filtration method (Lolkema et al., 1994). Briefly, 98 µl of cell suspension with a final OD$_{660}$ of 1 was incubated for 5 min at 30 °C. At time point zero, 2 µl of a mixture of $^{63}$Ni$^{2+}$ (12.66 mCi/mg Ni, 468 MBq/mg Ni, Amersham) and citrate or DL-isocitrate was added, yielding a final concentration of 12.5 µM $^{63}$Ni$^{2+}$ and 0.125 - 2.5 mM citrate or 1 - 10 mM DL-isocitrate. Samples were taken at time points between 0 and 5 min. Uptake was stopped by the addition of 2 ml of ice-cold 0.1 M LiCl, immediately followed by filtration through a 0.45 µm pore-size nitrocellulose filter. The filters were washed once with the same LiCl solution and submerged in scintillation fluid. The retained radioactivity was counted in a liquid scintillation counter. Uptake rates were determined from the linear part of each uptake curve.

(ii) [1,5-14C]citrate uptake in membrane vesicles of E. coli. Right-side-out membrane vesicles of E. coli TOP10 containing CitM were diluted in 50 mM K-PIPES pH 6.5 supplemented with 10 mM MgCl$_2$ to a final membrane protein concentration of 50 or 100 µg/ml in a total assay volume of 100 µl. An electrochemical proton gradient was allowed to develop at 30 °C for 2 min after the addition of 10 mM K-ascorbate and 100 µM phenazine methosulfate (PMS) under a flow of water-saturated air with magnetic stirring after which the uptake was initiated by the addition of [1,5-14C]citrate (114 mCi/mmol, 4.218 GBq/mmol, Amersham) to a final concentration of 4.5 µM. The uptake was quenched and the samples treated as described above. Inhibitors were present at a concentration of 1 mM.

(iii) Exchange in membrane vesicles of E. coli. Right-side-out membrane vesicles were allowed to accumulate [1,5-14C]citrate or L-[U-14C]proline (260 mCi/mmol, 9.62 GBq/mmol, Amersham) as described above for 1.5 minute. Subsequently, in exchange experiments, various substrates were added at the indicated concentrations and the internalized label was followed in time. In efflux experiments, 10 µM of the protonophore carbonylcyanide p-trifluormethoxy-phenylhydrazone (FCCP) was added which completely dissipates the proton motive force. Samples were taken between 10 seconds and 3 minutes and treated as described earlier.

β-Galactosidase assay

β-Galactosidase activity of the cells was determined at 28 °C by the method of Miller (Miller 1972) using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. Cells from 2 ml of cell culture were harvested by centrifugation. The cell pellet was suspended in a buffer containing 100 mM NaP$_{i}$, 10 mM KCl, 1 mM MgSO$_4$, and 1 mM 1,4-dithiothreitol (DTT), pH 7.0 and the cells were lysed using the lysozyme treatment in the presence of 10 µM DNase. Specific β-galactosidase activities are expressed as the o-nitrophenol released per minute per cell density at 28 °C (Miller units). The values reported are averages of two independent measurements. Background activities were measured in B. subtilis strain 168 and amounted to 0.3-0.5 Miller units.
Growth on citrate and isocitrate is supported by CitM

RESULTS

Involvement of CitM in growth of *Bacillus subtilis* on TCA cycle intermediates

The involvement of the Mg\(^{2+}\)-citrate transporter CitM in growth of *B. subtilis* on TCA cycle intermediates and some related compounds was investigated using an assay developed by Asai (Asai *et al.*, 2000). Growth of the cells was triggered in a minimal medium containing 0.05% yeast extract (MSMYE medium). In the absence of an additional carbon source, growth ceased at an OD\(_{660}\) of ~0.3 (Fig. 1A), while upon addition of, for instance, citrate (arrow Fig. 1B) the cells were able to keep on growing for several hours, indicating that they took up citrate and used it for growth. Similarly, DL-isocitrate, L-malate, succinate, and fumarate supported growth of *B. subtilis*, while addition of *cis*-aconitate, \(\alpha\)-ketoglutarate, and oxaloacetate did not result in significant growth (Fig. 1C, E, F). The naturally occurring enantiomer D-isocitrate supported growth better than the mixture of D- and L-isocitrate added at the same total concentration, indicating that the former is the preferred substrate (compare Fig. 1C and D). Tricarballylate, which is structurally related to citrate and isocitrate, but lacks the hydroxyl group, did not support growth (Fig. 1G). Also, D-malate, in contrast to L-malate, did not support growth.

To determine the involvement of CitM in the uptake of any of the substrates that supported growth, the experiment was repeated with the CitM deficient strain CITMd (kindly provided by Prof. Sekiguchi, Shinshu University, Japan; Yamamoto *et al.*, 2000). Growth of the mutant in MSMYE medium was similar as observed for the wild-type strain (Fig. 1A). In contrast to the wild-type, the CITMd mutant did not reveal enhanced growth when citrate was added to the medium (Fig. 1B), suggesting that CitM is the only transporter in the wild-type cells that is used for citrate uptake under these growth conditions. Similarly, no growth of the mutant was observed on DL-isocitrate and D-isocitrate (Fig. 1C, D), while L-malate, succinate and fumarate supported growth of the wild-type and CITMd strains equally well (Fig. 1F and not shown).

CitM is known to transport exclusively citrate complexed to divalent metal ions (Krom *et al.*, 2000). The minimal MSMYE medium used in the studies contained 0.123 mM Mg\(^{2+}\), apparently enough to support growth on citrate, but citrate was present in large excess (5 mM). Accordingly, growth on citrate was considerably improved when an additional concentration of 1 mM MgCl\(_2\) was added to the medium (Fig. 1B). Higher concentrations up to 10 mM did not further improve growth (data not shown). Similarly, growth on isocitrate was significantly improved in the presence of 1 mM MgCl\(_2\), especially in the case of D-isocitrate (Fig. 1C, D). At higher Mg\(^{2+}\) concentrations, some growth enhancement was observed on *cis*-aconitate (Fig. 1E; see Discussion). Apparently, growth on these substrates had been limited by a low metal ion concentration. To exclude the possibility that the
improved growth in response to Mg$^{2+}$ was due to the addition of MgCl$_2$ itself growth was followed in the presence of the poor growth substrate tricarballylate and the good growth substrate L-malate which is independent of CitM. In both cases the addition of 1 mM MgCl$_2$ did not affect the growth characteristics (Fig. 1H).

**Figure 1.** Growth of *B. subtilis* on TCA cycle intermediates. Panels A-G. *B. subtilis* wild-type (●,○) and CITMd (▲) were grown in minimal salts medium containing 0.05% yeast extract (MSMYE) without further addition (A) or in the presence of 5 mM of the carbon sources citrate (B), DL-isocitrate (C), D-isocitrate (D), cis-aconitate (E), L-malate (F) or tricarballylate (G). The open symbols (○) represent growth of the wild-type strain in medium to which an additional concentration of 1 mM Mg$^{2+}$ was added. The solid line representing growth of the wild-type strain in the absence of any carbon source (A, ●) was copied to the other panels for easy reference. Panel (H), Growth of the wild-type strain on L-malate (●, ○) and tricarballylate (■, □) in the absence (●, ■) or presence (○, □) of 1 mM Mg$^{2+}$. The arrows indicate the time point of adding the carbon source.

Summarizing, CitM supports growth of *B. subtilis* on citrate and D-isocitrate in the presence of Mg$^{2+}$. No apparent involvement of CitM was observed during growth on succinate,
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fumarate and L-malate, whereas no growth was observed in the presence of cis-aconitate, α-ketoglutarate, D-malate, oxaloacetate or tricarballylate.

Analysis of citM gene expression

Expression of citM was determined qualitatively by growing B. subtilis CM002, a strain that contains the lacZ reporter gene fused behind the citM promoter region integrated into the chromosome (see Materials and Methods), on LB agar plates containing the chromogenic substrate X-Gal to monitor LacZ activity. Control plates scored negative. Supplementing the plates with different TCA cycle substrates revealed that besides citrate, DL-isocitrate, D-isocitrate and cis-aconitate were apparently able to induce citM expression (not shown).

Figure 2. Induction of citM expression. B. subtilis strain CM002 was grown in CSE minimal medium in the presence of 5 mM citrate (A), DL-isocitrate (B) or cis-aconitate (C). Growth was followed by measuring the OD660 (ê). The β-galactosidase activity correlating with the citM promoter activity was indicated in Miller units (bars). Bars and error bars represent the average of two independent measurements and standard deviation, respectively.

Induction of citM expression was followed in time during growth on CSE minimal medium in the presence of citrate, DL-isocitrate and cis-aconitate. The citM promoter activity was measured quantitatively by measuring the β-galactosidase activity of the cells during growth. The cultures were inoculated with uninduced cells. In the presence of citrate β-galactosidase activity increased to reach a steady-state value in the late-exponential growth phase (Fig. 2A). The pre-steady-state period represents the time required for the β-galactosidase expression level to equilibrate between synthesis rate and growth rate (this thesis). A similar time dependence was observed in the presence of DL-isocitrate, but the level of expression appeared to be somewhat lower (Fig. 2B). No induction by cis-aconitate
was observed during the exponential growth phase, but, surprisingly, after 24 h, long after the cells had entered the stationary phase, induction was equally high as observed during growth on citrate or DL-isocitrate (Fig. 2C). The differences in expression levels in the exponential growth phase between citrate and DL-isocitrate on the one hand and cis-aconitate on the other hand could not be explained by different growth rates (this thesis) since these were not much affected by the different substrates. To exclude the possibility that the observed induction by cis-aconitate might be the result of a slow conversion (hydration) into citrate or isocitrate during the course of growth, CSE minimal medium containing cis-aconitate was pre-incubated for 24 h at 37 °C under continuous shaking before inoculation. The induction pattern was unchanged (data not shown).

Analysis of CitM substrate specificity
Right-side-out (RSO) membrane vesicles, prepared from *E. coli* cells expressing the Mg²⁺-citrate transporter CitM of *B. subtilis* catalyzed pmf-driven uptake of ¹⁴C-citrate at a rate of 52.2 pmol/min-mg protein at a ¹⁴C-citrate concentration of 4.5 µM (not shown; Boorsma *et al.*, 1996). At a concentration of 1 mM, unlabeled citrate completely inhibited uptake of labeled citrate, which is in agreement with the reported Kₘ for uptake of ~50 µM (Krom *et al.*, 2000). Also DL-isocitrate and D-isocitrate were potent inhibitors (less than 10 % residual activity), while 1 mM cis-aconitate resulted in 40% residual activity. None of the TCA cycle intermediates, succinate, fumarate, L-malate, α-ketoglutarate, oxaloacetate, or the related compounds D-malate and tricarballylate resulted in significant inhibition at 1 mM concentrations (not shown).

Inhibition of uptake does not prove that the inhibitor is transported, i.e. the inhibitor may bind to the transporter without being translocated. Heterologous exchange of labeled citrate and unlabeled substrates provides an assay to demonstrate translocation of the latter (Bandell & Lolkema, 1999). Membrane vesicles prepared from *E. coli* cells expressing CitM were allowed to accumulate ¹⁴C-citrate driven by the proton motive force until a plateau was reached. Addition of FCCP, a protonophore that dissipates the proton motive force, resulted in very rapid efflux of the label from the lumen of the vesicles. In fact, all of the label was released within 10 seconds (Fig. 3A). When instead of FCCP, 1 mM of unlabeled citrate was added the release of label was also fast but 10-20% of the label was still inside the membranes at t = 10 sec. Release of label under these conditions is the result of homologous exchange during which the transporter transports unlabeled citrate into the vesicle in exchange for the exit of labeled citrate. To rule out the possibility that the addition of 1 mM citrate would dissipate the proton motive force, i.e. would mimic the effect of FCCP, the effect of 1 mM citrate on the accumulation level of ¹⁴C-proline was measured as a control.
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(Fig. 3B). No significant release of $^{14}$C-proline was observed, while FCCP resulted in the loss of label in 3 min, suggesting that the addition of 1 mM citrate did not affect the magnitude of the proton motive force significantly.

![Figure 3](image-url)

**Figure 3.** Homologous and heterologous exchange catalyzed by CitM. RSO membrane vesicles of *E. coli* expressing CitM were preloaded with $^{14}$C-citrate (A, C) or $^{14}$C-proline (B) as described in the Methods section. (A, B) After preloading, at $t = 0$, either no further additions were made (○), or 1 mM citrate (A, B, ●), 10 µM FCCP (A, B, ▼), 1 mM tricarballylate (C, ●), 1 mM cis-aconitate (C, ○), 1 mM D-isocitrate (C, ▼) was added. The label retained by the membranes was indicated as the percentage of the internal label at $t = 0$. The 100% value corresponded to 0.62 nmol/mg protein (A), 0.59 nmol/mg protein (B), and 1.16 nmol/mg protein (C).

Replacing unlabeled citrate in the assay with the same concentration of unlabeled D-isocitrate resulted in the same rapid release of label from the membranes, while the addition of tricarballylate was without effect (Fig. 3C). In agreement with the observed inhibition of $^{14}$C-citrate uptake by the two substrates, it follows that D-isocitrate is transported by CitM and tricarballylate is not. Addition of 1 mM *cis*-aconitate to the preloaded membranes also resulted in the release of label, however, the release was incomplete, leaving approximately 35% of the label inside (Fig. 3C). During homologous and heterologous exchange, the transporter effectively equilibrates the specific radioactivity of the internal and external pools of substrates, and, therefore, the extent of release of label from the membranes depends on the total pool of external exchangeable substrate (Bandell & Lolkema, 1999).

A titration with citrate as the exchangeable substrate showed that the addition of 50 µM citrate resulted in release of ~70% of the label and 200 µM in about 90% (Fig. 4). The same amount of D-isocitrate resulted in a similar release, showing that D-isocitrate is the exchangeable substrate. The release observed with 1 mM *cis*-aconitate corresponded to a
concentration of ~50 µM exchangeable substrate and, therefore, was most likely due to a contamination in \textit{cis-}aconitate, presumably citrate or isocitrate. A contamination of 50 µM citrate in a 1 mM \textit{cis-}aconitate solution would explain the observed inhibition of 14C-citrate uptake. In conclusion, citrate and D-isocitrate are substrates of CitM and \textit{cis-}aconitate is not.

\textbf{Figure 4.} Analysis of heterologous exchange with \textit{cis-}aconitate. Label retained by RSO membrane vesicles of \textit{E. coli} expressing CitM preloaded with 14C-citrate upon the addition of the indicated concentrations of citrate (cit), D-isocitrate (iso) and \textit{cis-}aconitate (aco). For details see the legend to Fig. 3. The bars and error bars give the average and standard deviation of the label retained at 20, 40, and 60 sec expressed as the percentage of the label inside the membranes at t = 0. The 100% value varied between 0.6 and 1.2 nmol/mg protein.

\textbf{Ni\textsuperscript{2+}-isocitrate uptake by CitM}

Resting cells of \textit{E. coli} TOP10 took up radioactive free \textit{63}Ni\textsuperscript{2+} at a rate of about 1.4 nmol/min-mg cell protein (Fig. 5A). In the presence of 5 mM of citrate, which renders the \textit{63}Ni\textsuperscript{2+} ions in the transport incompetent, complexed state, uptake was completely inhibited. The same concentration of isocitrate resulted in a residual uptake rate of ~10%, showing that isocitrate is somewhat less potent in complexing Ni\textsuperscript{2+}. Under the same conditions, \textit{E. coli} cells expressing the CitM protein, showed increased uptake of \textit{63}Ni\textsuperscript{2+} by the addition of the same concentration of both citrate and DL-isocitrate confirming that CitM transports Ni\textsuperscript{2+} complexed to citrate and DL-isocitrate (Fig. 5B).
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DISCUSSION

The *B. subtilis* genome codes for at least three different transporters for citrate, all of the secondary type. CimH, a member of the 2-hydroxycarboxylate transporter family, transports citrate and malate in symport with H⁺, and CitM and CitH, members of the MeCit transporter family, transport the complex of divalent metal ions and citrate (Krom et al., 2000, 2001). Moreover, open reading frame *yraO* codes for a third protein in the MeCit family and, potentially, is a citrate transporter as well. In this study we show that among these transporters the Mg²⁺-citrate transporter CitM is the only transporter involved in the uptake of citrate during growth on citrate as the sole carbon source. The conclusion is in line with the strict regulation of expression of the *citM* gene by components in the medium. The physiological function of the other citrate transporters is still elusive.

Citrate transporters are widely spread among bacteria, allowing them to utilize citrate by degradation via the tricarboxylic acid cycle or one of the citrate fermentation pathways (Bott 1997). In fact, *E. coli* is an exception to this rule; though it contains all the enzymes necessary for citrate metabolism, it cannot utilize citrate, because it lacks a functional citrate transport system (Bott 1997). In contrast to citrate, few reports are available on the utilization of isocitrate by bacteria and data on transporters with specificity for isocitrate are similarly scarce. It has been argued that transporters for citrate may transport isocitrate as well (Kay 1978), but this is definitely not generally true. For instance, the citrate transporters in the 2-hydroxycarboxylate transporter family, CimH of *B. subtilis*, CitP of

Figure 5. Ni²⁺ uptake in *E. coli* expressing CitM. The uptake of ⁶⁵Ni²⁺ in *E. coli* cells harboring plasmid pWSK without an insert (A) and pWSKCitM carrying the *citM* gene (B) was measured in the presence of no further additions (○), 5 mM citrate (▼), and 5 mM DL-isocitrate (○).
lactic acid bacteria, and CitS of *K. pneumoniae* do not recognize isocitrate (van der Rest *et al.*, 1992; Bandell *et al.*, 1997; Krom *et al.*, 2001). On the other hand, in *Pseudomonas fluorescens* a tricarboxylate transport system has been described that is induced by citrate and transports citrate and D-isocitrate. A second system in this organism is induced by tricarballylate and transports citrate, cis-aconitate and tricarballylate (Kay 1978). *Salmonella typhimurium* has been reported to be able to grow on citrate, cis-aconitate and isocitrate using a thus far unique uptake system, which involves a periplasmic binding protein that specifically binds citrate, isocitrate and L-erythro-2-fluorocitrate (Somers *et al.*, 1981; Sweet *et al.*, 1984; Widenhorn *et al.*, 1988). In this study we show that *B. subtilis* can grow on isocitrate as a sole carbon source and that the Mg\(^{2+}\)-citrate transporter CitM is responsible for the uptake from the medium by three criteria. (i) The *B. subtilis* CitM deficient strain CITMd lost the ability to grow on isocitrate completely (Fig. 1 C, D) as was also observed for growth on citrate. (ii) Like citrate, isocitrate appeared to be an inducer of citM gene expression, both on solid and on liquid media. (iii) Heterologous exchange experiments demonstrated that CitM transports isocitrate. Similarly to citrate, isocitrate is taken up in complex with a divalent metal ion.

The results with cis-aconitate are confusing which is likely caused by the presence of impurities in commercially available cis-aconitate. Growth of *B. subtilis* in MSMYE medium (0.05% yeast extract) resulted in complete lysis of the cells after 24 h of incubation. In the presence of cis-aconitate we did not see additional growth, but the cells did not lyse and were still viable after 24 h (not shown). Moreover, some growth enhancement was observed when in addition to cis-aconitate 1 mM Mg\(^{2+}\) was added to the medium, strongly suggesting the involvement of CitM (Fig. 1E). The expression studies showed induction of citM by cis-aconitate, but only after prolonged incubation in the stationary phase. Unfortunately, the exchange studies revealed the presence of a contamination in cis-aconitate (~5%) that is a substrate of CitM and, therefore, most likely citrate or isocitrate. No evidence was obtained that cis-aconitate itself is a substrate of CitM. We tentatively conclude that the observed growth effects and induction pattern in the presence of cis-aconitate, and previous claims made in the literature (McKellen *et al.*, 1972), must be ascribed to impurities and that cis-aconitate is not a growth substrate of *B. subtilis*.

In conclusion, *B. subtilis* is capable of growing on the TCA cycle intermediates citrate and isocitrate, mediated by CitM, on succinate and fumarate, mediated by DctP (Asai *et al.*, 2000), and on L-malate. A consequence of the involvement of CitM in growth on citrate and isocitrate is that optimal growth requires higher concentrations of Mg\(^{2+}\) than normally required (compare Fig. 1B, D, H). No growth was detected with cis-aconitate, α-ketoglutarate, D-malate, oxaloacetate and tricarballylate. The lack of growth on α-
Growth on citrate and isocitrate is supported by CitM

Ketoglutarate that we observed is in contradiction with a report claiming growth of *B. subtilis* on α-ketoglutarate mediated by a low-affinity, inducible transport system with a *Kₐ* of 6.7 mM (Fournier *et al.*, 1972). Analysis of *B. subtilis* genome (Kunst *et al.*, 1997) provides no clue about the identity of such a transporter.

CitM has been shown to take up citrate in complex with Mg²⁺, Zn²⁺, Mn²⁺, Co²⁺, and Ni²⁺. The improved growth on isocitrate upon addition of extra Mg²⁺ (Fig. 1C, D) and the ⁶³Ni²⁺ uptake experiments (Fig. 5) indicated that isocitrate is transported in complex with Mg²⁺ and Ni²⁺ as well. Further experimentation showed that the uptake ¹⁴C-citrate in the presence of 10 mM of Mg²⁺, Zn²⁺, Mn²⁺, Co²⁺, and Ni²⁺ could in all cases be significantly inhibited by 1 mM of isocitrate (not shown). These experiments suggest that the metal ion specificity in the complexes that are transported by CitM is the same for isocitrate and citrate, but it cannot be excluded that complexes with other divalent metal ions are substrates. Citrate appears to be a stronger chelator of divalent metal ions than isocitrate. For instance, the complex formation constants for Mn²⁺-isocitrate and Mn²⁺-citrate (2.55 and 3.54, respectively) (Martell & Smith, 1977) indicate a 10-fold lower affinity of isocitrate. In our experiments, about 7 times more DL-isocitrate than citrate was required to inhibit the ⁶³Ni²⁺ uptake activity in whole cells of *B. subtilis* CITMd by 50%. For the natural occurring isomer D-isocitrate 4 times more was needed, indicating that D-isocitrate is a better chelator than L-isocitrate (not shown). Consequently, optimal growth of *B. subtilis* on D-isocitrate requires relatively high concentrations of divalent metal ions in the medium.

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