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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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***Bacillus subtilis* 168 was assayed for its growth on tricarboxylic acid (TCA) cycle intermediates and related compounds as the sole carbon sources. Growth of the organism was supported by citrate, D-isocitrate, succinate, fumarate and L-malate, whereas no growth was observed in the presence of *cis*-aconitate, 2-oxoglutarate, D-malate, oxaloacetate and tricarballylate. Growth of the organism on the tricarboxylates citrate and D-isocitrate required the presence of functional CitM, an Mg^{2+}–citrate transporter, whereas its growth on succinate, fumarate and L-malate appeared to be CitM-independent. Interestingly, the naturally occurring enantiomer D-isocitrate was favoured over L-isocitrate by the organism. Like citrate, D-isocitrate was shown to be an inducer of *citM* expression in *B. subtilis*. The addition of 1 mM Mg^{2+} to the growth medium improved growth of the organism 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 right-side-out membrane vesicles prepared from *E. coli* cells expressing *citM*. None of the other TCA cycle intermediates and related compounds tested were recognized by CitM. Uptake experiments using radioactive $^{63}Ni^{2+}$ provided direct evidence that D-isocitrate is transported in complex with divalent metal ions.**

Keywords: TCA cycle intermediate, promoter fusion, divalent metal ion–citrate complex, membrane vesicles, exchange

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. *Bacillus subtilis* runs a complete TCA cycle and is able to utilize almost all of the TCA cycle intermediates under aerobic conditions (Fortnagel & Freese, 1968; Wei *et al.*, 2000; Asai *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 C_4 dicarboxylates succinate and fumarate by *B. subtilis* during its growth on these substrates was shown to be mediated by the dicarboxylate transporter DctP (Asai *et al.*, 2000; Janausch *et al.*, 2002). Expression of *dctP* is positively regulated in response to external signals by a sensor kinase and regulator pair, DctS and DctR. The regulatory system is induced in the presence of succinate, fumarate and low concentrations of yeast extract and is repressed by malate (Asai *et al.*, 2000). Although it was not excluded that malate is also a substrate for the DctP transporter, several other transport systems have been shown to transport this compound. CimH (*yxkJ*) 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 (*yqkI*) 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 of *B. subtilis* on malate is unknown.

Older studies have reported the ability of the TCA cycle intermediates *cis*-aconitate and isocitrate to induce and

Abbreviations: FCCP, carbonylcyanide *p*-trifluormethoxy-phenylhydrazone; MSMYE, minimal salts medium/0·05 % yeast extract; RSO, right-side-out; TCA, tricarboxylic acid.

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competitively inhibit the uptake of citrate in wild-type cells of *B. subtilis*, suggesting the presence of a specific transport system in this organism (McKillen *et al.*, 1972). More-recent studies have identified a number of transporters for citrate in *B. subtilis*. CimH, mentioned above, catalyses 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 Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} or Zn^{2+}, and CitH recognizes citrate in complex with Ca^{2+}, Ba^{2+} or Sr^{2+} (Krom *et al.*, 2000). Expression of *citM* is strictly regulated: gene activation depends on the action of the two-component regulatory pair CitS–CitT, which senses the presence of citrate in the medium (Yamamoto *et al.*, 2000). Furthermore, gene expression is subject to catabolite repression (Warner *et al.*, 2000). The strict regulation of *citM* expression by medium components makes it likely that CitM is the main uptake system during growth of *B. subtilis* on citrate as the sole carbon source.

Here, we have studied the involvement of the Mg^{2+}–citrate transporter CitM in the growth of *B. subtilis* on TCA cycle intermediates. It follows that CitM is both necessary and sufficient for growth of the organism 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 demonstrated that CitM transports the complex of isocitrate and divalent metal ions.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *B. subtilis* 168 (*trpC2*), CITMd (*trpC2* Δ*citM ery*) (Yamamoto *et al.*, 2000) and CM002 [*trpC2 amyE*::(P_{citM}–*lacZ cat*)] (Warner *et al.*, 2000), and *E. coli* TOP10 (*φ*80Δ*lacZ*M15 Δ*lacX74 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^{-1}. CSE medium was C medium (Aymerich *et al.*, 1986) to which sodium succinate (6 g l^{-1}) and potassium glutamate (8 g l^{-1}) was added. LB-grown pre-cultures were diluted 200 times in minimal salts medium (2·72 g K_2HPO_4, 1 g KH_2PO_4, 1 g NH_4Cl, 0·284 g Na_2SO_4, 0·17 g $NaNO_3$, 0·15 g KCl, 25 mg $MgCl_2.6H_2O$, 22 mg $CaCl_2.6H_2O$, 15 mg $MnCl_2.4H_2O$ and 2·16 mg $FeCl_3.6H_2O$ per litre of medium) containing 0·05 % yeast extract (MSMYE medium) (Goel *et al.*, 1995; Asai *et al.*, 2000). TCA cycle intermediates were added to the medium at a final concentration of 5 mM after growth of the cells for 2·5 h (OD_{660} ~0·1). CSE-grown pre-cultures 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 r.p.m. Growth was monitored by measuring the optical density at 660 nm. When appropriate, antibiotics were added to the medium at the following concentrations: 5 μg chloramphenicol ml^{-1}; 0·3 μg erythromycin ml^{-1}; 50 μg ampicillin ml^{-1}. Qualitatively, *β*-galactosidase activity was measured by growing the cells on agar plates containing LB medium and 10 mM of the different carbon sources and 5 % of the chromogenic substrate X-Gal.

E. coli TOP10 was transformed with plasmid pWSKCitM, which contains the gene encoding the Mg^{2+}–citrate transporter CitM under the control of the *lac* promoter (Krom *et al.*, 2000). Recombinant cells were grown in LB medium supplemented with 100 μg carbenicillin ml^{-1} and were induced with 0·1 mM IPTG at an OD_{660} value of 0·2, after which the cells were allowed to grow for an additional 2 h.

Preparation of membrane vesicles. *E. coli* TOP10 cells expressing CitM were harvested at an OD_{660} value of 0·8–1·0 and right-side-out (RSO) membrane vesicles were prepared by the osmotic shock lysis procedure described by Kaback (1983). The vesicles were resuspended in 50 mM potassium PIPES (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.

Transport assays.

(i) $^{63}Ni^{2+}$ uptake in whole cells. Cells of *B. subtilis* were harvested by centrifugation, washed once and resuspended in 50 mM potassium 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 a cell suspension with a final OD_{660} value 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)$^{-1}$, 468 MBq (mg Ni)$^{-1}$; Amersham] and citrate or DL-isocitrate was added to the cell suspension, 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 of $^{63}Ni^{2+}$ was stopped by the addition of 2 ml of ice-cold 0·1 M LiCl to the suspension, immediately followed by filtration of the suspension 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-^{14}C]citrate uptake in membrane vesicles of *E. coli*. RSO membrane vesicles of *E. coli* TOP10 containing CitM were diluted in 50 mM potassium PIPES (pH 6·5) supplemented with 10 mM $MgCl_2$ to a final membrane protein concentration of 50 or 100 μg ml^{-1} 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 potassium ascorbate and 100 μM phenazine methosulfate to the assay mix under a flow of water-saturated air with magnetic stirring, after which the uptake was initiated by the addition of [1,5-^{14}C]citrate (114 mCi $mmol^{-1}$, 4·218 GBq $mmol^{-1}$; Amersham) to the assay mix to a final concentration of 4·5 μM. The uptake of [1,5-^{14}C]citrate was quenched and the samples were treated as described above. Inhibitors were present at a concentration of 1 mM.

(iii) Exchange in membrane vesicles of *E. coli*. RSO membrane vesicles were allowed to accumulate [1,5-^{14}C]citrate or L-[U-^{14}C]proline (260 mCi $mmol^{-1}$, 9·62 GBq $mmol^{-1}$; Amersham) as described above for 1·5 min. Subsequently, in exchange experiments, various substrates were added to the assay mixes

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at the indicated concentrations and the internalized label was followed over time. In efflux experiments, 10 μM of the protonophore carbonylcyanide *p*-trifluormethoxy-phenylhydrazone (FCCP) was added to the assay mixes, which completely dissipates the proton motive force. Samples were taken between 10 s and 3 min and treated as described earlier.

β-Galactosidase assay. β-Galactosidase activity of the *B. subtilis* cells was determined at 28 °C by the method of Miller (1972) using ONPG as the substrate. Cells from 2 ml of a cell culture were harvested by centrifugation. The cell pellet was suspended in a buffer containing 100 mM Na-phosphate, 10 mM KCl, 1 mM $MgSO_4$ and 1 mM 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 min per cell density at 28 °C (Miller units). The values reported are means of two independent measurements. Background activities were measured in *B. subtilis* 168 and amounted to 0·3–0·5 Miller units.

RESULTS

Involvement of CitM in growth of *B. 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 *et al.* (2000). Growth of the cells was triggered in MSMYE medium. In the absence of an additional carbon source, growth of *B. subtilis* ceased at an OD_{660} value of ~0·3 (Fig. 1a), whereas upon the 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*, whereas the addition of *cis*-aconitate, 2-oxoglutarate and oxaloacetate to the culture medium did not result in significant growth of the organism (Fig. 1c, e, f). The naturally occurring enantiomer D-isocitrate supported growth of *B. subtilis* better than the mixture of D- and L-isocitrate added to the culture medium at the same total concentration, indicating that the former is the preferred substrate of *B. subtilis* (compare Fig. 1c, d). Tricarballylate, which is structurally related to citrate and isocitrate but lacks the hydroxyl group, did not support growth of *B. subtilis* (Fig. 1g). Also, D-malate, in contrast to L-malate, did not support growth of the organism.

To determine the involvement of CitM in the uptake of any of the substrates that supported growth of *B. subtilis*, the experiment was repeated with the CitM-deficient strain CITMd (kindly provided by Professor Sekiguchi, Shinshu University, Japan). Growth of the mutant strain in MSMYE medium was similar to that observed for the wild-type strain *B. subtilis* 168 (Fig. 1a). In contrast to the wild-type strain, 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 the growth conditions used here. Similarly, no growth of the mutant was observed upon the addition of DL-isocitrate and D-isocitrate to the medium (Fig. 1c, d), whereas 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 MSMYE medium contained 0·123 mM Mg^{2+}, apparently enough to support growth on citrate, but citrate was present in the medium in large excess in this study (5 mM). Accordingly, growth of *B. subtilis* on citrate was considerably improved when an additional concentration of 1 mM $MgCl_2$ was added to the medium (Fig. 1b). Higher concentrations of citrate, up to 10 mM, did not further improve growth of the organism (not shown). Similarly, growth of *B. subtilis* 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 enhancement of growth of the organism was observed on *cis*-aconitate (Fig. 1e; see Discussion). Apparently, growth of *B. subtilis* on these substrates had been limited by a low metal ion concentration in the medium. To exclude the possibility that the improved growth of *B. subtilis* in response to Mg^{2+} was due to the addition of $MgCl_2$ itself, growth of the organism was followed in the presence of the poor growth substrate tricarballylate and the good growth substrate L-malate, which is utilized independently of CitM. In both cases, the addition of 1 mM $MgCl_2$ to the medium did not affect the growth characteristics of *B. subtilis* (Fig. 1h).

In summary, 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 of the organism on succinate, fumarate and L-malate, while no growth of the organism was observed in the presence of *cis*-aconitate, 2-oxoglutarate, D-malate, oxaloacetate or tricarballylate.

Analysis of *citM* expression

Expression of *citM* by *B. subtilis* 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 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 in *B. subtilis* CM002 (not shown).

Induction of *citM* expression was followed over time during growth of *B. subtilis* CM002 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 their growth. The cultures were inoculated with uninduced cells. In the presence of citrate, β-galactosidase activity of the cultures increased

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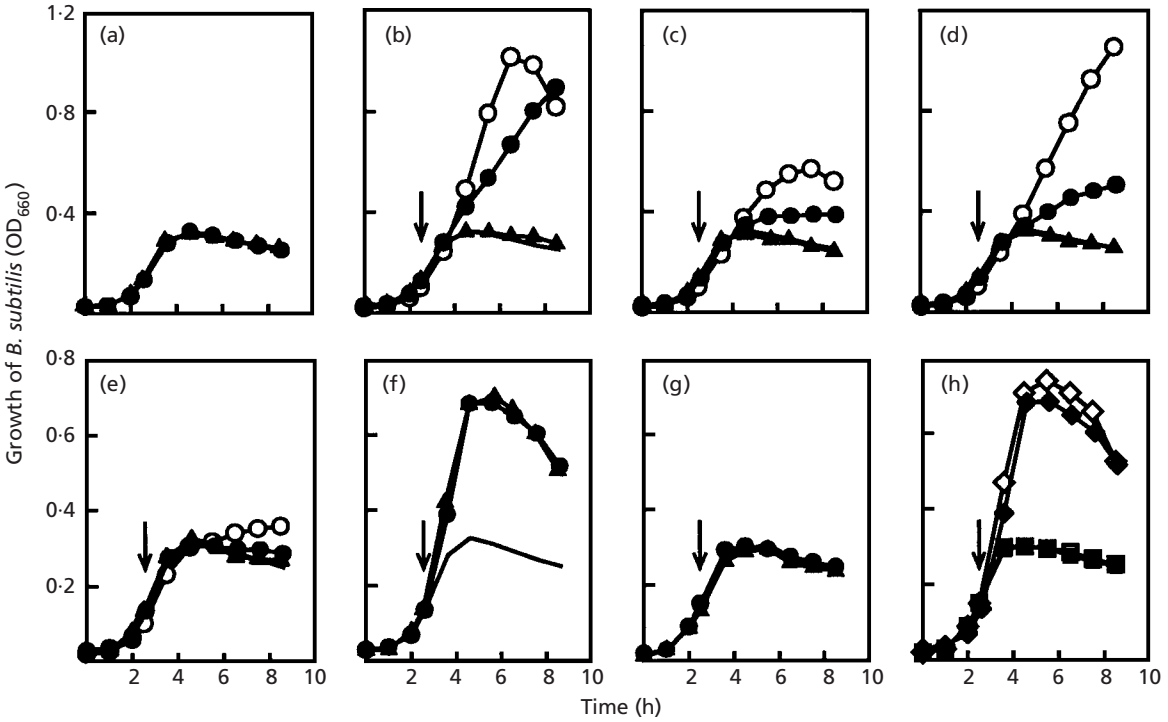

Fig. 1. Growth of *B. subtilis* on TCA cycle intermediates. (a–g) *B. subtilis* wild-type (●, ○) and CITMd (▲) were grown in MSMYE without further addition (a) or in the presence of 5 mM of the carbon sources (b) citrate, (c) DL-isocitrate, (d) D-isocitrate, (e) *cis*-aconitate, (f) L-malate or (g) tricarballylate. 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. (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.

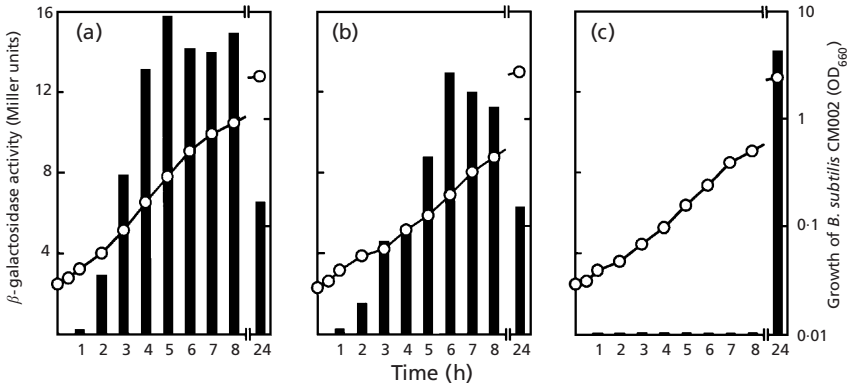

Fig. 2. Induction of *citM* expression. *B. subtilis* CM002 was grown in CSE minimal medium in the presence of 5 mM (a) citrate, (b) DL-isocitrate or (c) *cis*-aconitate. Growth was followed by measuring the OD_{660} (○). The β-galactosidase activity correlating with the *citM* promoter activity was indicated in Miller units (solid bars).

to reach a steady-state value in the late-exponential phase of growth (Fig. 2a). The pre-steady-state period represents the time required for the β-galactosidase expression level to equilibrate between the synthesis rate and the growth rate (Warner & Lolkema, 2002). A similar time dependence for β-galactosidase activity was observed when *B. subtilis* CM002 was grown in the presence of DL-isocitrate, but the level of expression appeared to be somewhat lower than that observed for citrate (Fig. 2b). No induction of expression 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 as high as that observed during growth of the organism on citrate or DL-isocitrate (Fig. 2c). The differences in β-galactosidase activity levels in the exponential growth phase between *B. subtilis* CM002 cultures grown on citrate or DL-isocitrate and on *cis*-aconitate could not be explained by different growth rates (Warner & Lolkema, 2002), since these were not affected much by the different substrates. To exclude the possibility that the observed induction of β-galactosidase activity by *cis*-aconitate

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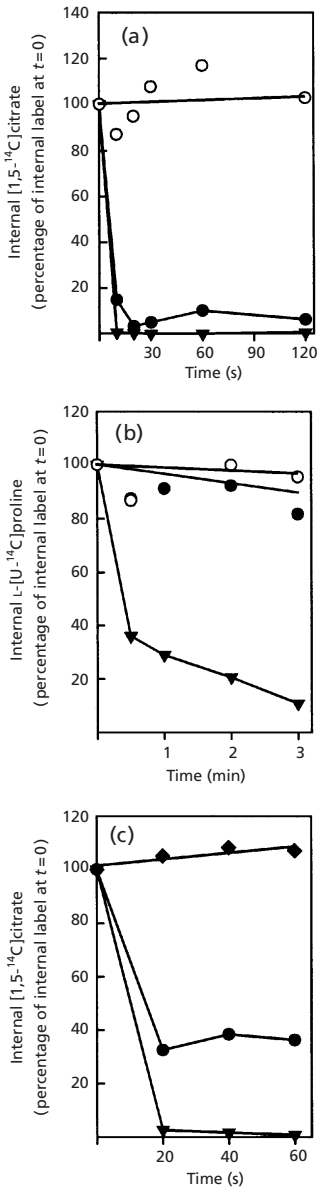

Fig. 3. Homologous and heterologous exchange catalysed by CitM. RSO membrane vesicles of *E. coli* expressing CitM were pre-loaded with (a, c) [1,5-^{14}C]citrate or (b) L-[U-^{14}C]proline as described in Methods. (a, b) After pre-loading, at $t=0$, either no further additions were made (○), or 1 mM citrate (a, b, ●), 10 μM FCCP (a, b, ▼), 1 mM tricarballylate (c, ◆), 1 mM *cis*-aconitate (c, ●) or 1 mM D-isocitrate (c, ▼) was added to the assay. The label retained by the membranes was indicated as the percentage of the internal label at $t=0$. The 100 % value corresponded to (a) 0·62 nmol (mg protein)$^{-1}$, (b) 0·59 nmol (mg protein)$^{-1}$ and (c) 1·16 nmol (mg protein)$^{-1}$.

might be the result of the slow conversion (hydration) of this TCA cycle intermediate into citrate or isocitrate during the course of bacterial growth, CSE minimal medium containing *cis*-aconitate was pre-incubated for 24 h at 37 °C under continuous shaking before inoculation. The induction pattern generated after this 'pre-treatment' was unchanged (not shown).

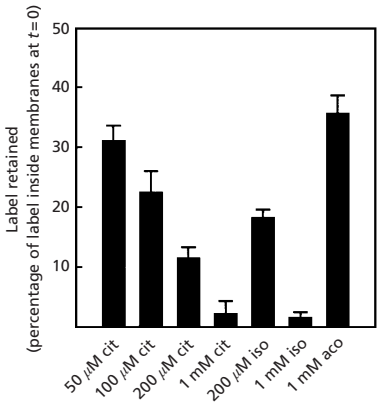

Fig. 4. Analysis of heterologous exchange with *cis*-aconitate. Label retained by RSO membrane vesicles of *E. coli* expressing CitM pre-loaded with [1,5-^{14}C]citrate upon the addition of the indicated concentrations of citrate (cit), D-isocitrate (iso) and *cis*-aconitate (aco). For details see the legend to Fig. 3. The bars and error bars give the mean + SD of the label retained at 20, 40 and 60 s 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)$^{-1}$.

Analysis of CitM substrate specificity

RSO membrane vesicles prepared from *E. coli* cells expressing the Mg^{2+}–citrate transporter CitM of *B. subtilis* catalysed the proton-motive-force-driven uptake of [1,5-^{14}C]citrate at a rate of 52·2 pmol min^{-1} (mg protein)$^{-1}$ at a [1,5-^{14}C]citrate concentration of 4·5 μM (not shown; Boorsma *et al.*, 1996). At a concentration of 1 mM, unlabelled citrate completely inhibited the uptake of labelled citrate, which is in agreement with the reported K_m for uptake (~50 μM; Krom *et al.*, 2000). DL-Isocitrate and D-isocitrate were also potent inhibitors of [1,5-^{14}C]citrate uptake (<10 % residual activity), while the presence of 1 mM of *cis*-aconitate in the growth medium resulted in 40 % residual activity. None of the TCA cycle intermediates (succinate, fumarate, L-malate, 2-oxoglutarate and oxaloacetate) nor the related compounds D-malate and tricarballylate resulted in significant inhibition of [1,5-^{14}C]citrate uptake when they were present at concentrations of 1 mM (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 labelled citrate and unlabelled 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 [1,5-^{14}C]citrate driven by the proton motive force until a plateau was reached. Addition of FCCP, a protonophore that dissipates the proton motive force, to the assay mix resulted in very rapid efflux of the label from the lumen of the vesicles. In fact, all of the label was released within 10 s of the addition of FCCP (Fig. 3a). When instead of FCCP

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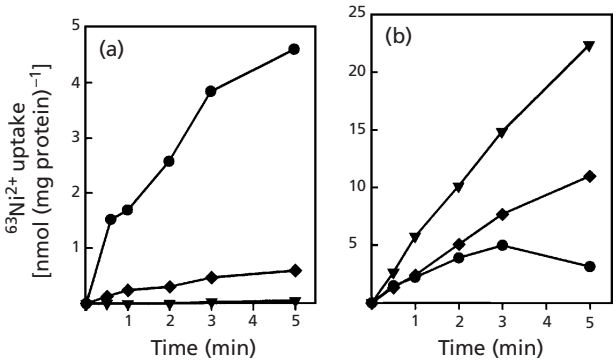

Fig. 5. $^{63}Ni^{2+}$ uptake in *E. coli* expressing CitM. The uptake of $^{63}Ni^{2+}$ in *E. coli* cells harbouring plasmid pWSK without an insert (a) and pWSKCitM carrying *citM* (b) was measured in the presence of no further additions (●), 5 mM citrate (▼) or 5 mM DL-isocitrate (◆).

1 mM of unlabelled citrate was added to the assay mix, the release of the label was also fast, but 10–20 % of the label was still inside the membranes at $t = 10$ s. Release of the label under these conditions is the result of homologous exchange during which the transporter transports unlabelled citrate into the vesicle in exchange for the exit of labelled citrate. To rule out the possibility that the addition of 1 mM of citrate to the assay mix would dissipate the proton motive force, i.e. would mimic the effect of FCCP, the effect of 1 mM of citrate on the accumulation level of L-[U-^{14}C]proline in the RSO vesicles was measured as a control (Fig. 3b). No significant release of L-[U-^{14}C]proline was observed under these conditions, while treatment of the assay mix with FCCP resulted in the loss of the label in 3 min, suggesting that the addition of 1 mM of citrate to the assay mix did not affect the magnitude of the proton motive force significantly.

Replacing unlabelled citrate in the assay with the same concentration of unlabelled D-isocitrate resulted in the same rapid release of the label from the membranes, while the addition of tricarballylate to the assay had no effect on release of the label (Fig. 3c). In agreement with the observed inhibition of [1,5-^{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 of *cis*-aconitate to the pre-loaded membranes also resulted in the release of label; however, this release was incomplete, leaving approximately 35 % of the label inside the membranes (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 to the assay resulted in the release of ~70 % of the label; the addition of 200 μM citrate to the assay resulted in the release of ~90 % of the label (Fig. 4). The addition of the same amounts of D-isocitrate to the assay resulted in a similar release of label, showing that D-isocitrate is the exchangeable substrate. The release of label observed with 1 mM of *cis*-aconitate corresponded to a concentration of ~50 μM exchangeable substrate and, therefore, was most likely due to a contaminant in *cis*-aconitate, presumably citrate or isocitrate. Contamination of a 1 mM *cis*-aconitate solution with 50 μM citrate would explain the observed inhibition of [1,5-^{14}C]citrate uptake. In conclusion, citrate and D-isocitrate are substrates of CitM and *cis*-aconitate is not.

$^{63}Ni^{2+}$–isocitrate uptake by CitM

Resting cells of *E. coli* TOP10 took up radioactive free $^{63}Ni^{2+}$ at a rate of ~1·4 nmol min^{-1} (mg cell protein)$^{-1}$ (Fig. 5a). In the presence of 5 mM citrate, which renders the $^{63}Ni^{2+}$ ions in the complexed state, uptake of $^{63}Ni^{2+}$–citrate by *E. coli* TOP10 was completely inhibited. The same concentration of isocitrate resulted in a residual uptake rate of $^{63}Ni^{2+}$ of ~10 %, showing that isocitrate is somewhat less potent in complexing with Ni^{2+} than citrate. Under the same conditions as used for the above experiments, *E. coli* cells producing CitM showed increased uptake of $^{63}Ni^{2+}$ upon the addition of 5 mM of citrate or DL-isocitrate to the assay, confirming that CitM transports Ni^{2+} complexed to citrate or DL-isocitrate (Fig. 5b).

DISCUSSION

The *B. subtilis* genome encodes at least three different transporters for citrate, all of which belong to the secondary type of transporter. CimH, a member of the 2-hydroxycarboxylate transporter family, transports citrate and malate in symport with H^+; CitM and CitH, members of the MeCit transporter family, transport the complex of divalent metal ions and citrate (Krom *et al.*, 2000, 2001). Moreover, the ORF *yra*O encodes a third protein in the MeCit family which is, potentially, a citrate transporter as well. In this study, we have shown that among the citrate transporters the Mg^{2+}–citrate transporter CitM is the only transporter that is involved in the uptake of citrate during growth of *B. subtilis* on citrate as the sole carbon source. This conclusion is in line with the strict regulation of expression of *citM* by components in the medium. The physiological function of the other citrate transporters of *B. subtilis* is still elusive.

Citrate transporters are found in many bacterial species; these transporters allow bacteria to utilize citrate by its degradation via the TCA cycle or via one of the citrate-

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fermentation pathways (Bott, 1997). However, *E. coli* is an exception to this rule – although it contains all of 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 also transport isocitrate (Kay, 1978), but this is definitely not generally true. For instance, the citrate transporters of the 2-hydroxy-carboxylate transporter family (CimH of *B. subtilis*, CitP of lactic acid bacteria and CitS of *Klebsiella pneumoniae*) do not recognize isocitrate (van der Rest *et al*., 1992; Bandell *et al*., 1997; Krom *et al*., 2001). However, a tricarboxylate transport system has been described in *Pseudomonas fluorescens* 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 by 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 have shown 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. 1c, d), as was also observed for growth on citrate. (ii) Like citrate, isocitrate appeared to be an inducer of *citM* expression, on solid and in liquid media. (iii) Heterologous exchange experiments demonstrated that CitM transports isocitrate. Similar to citrate, isocitrate is taken up in complex with a divalent metal ion.

The results obtained with *cis*-aconitate are confusing; this confusion is likely to be caused by the presence of impurities in commercially available *cis*-aconitate preparations. Growth of *B. subtilis* in MSMYE medium resulted in complete lysis of the cells after 24 h incubation. When grown in the presence of *cis*-aconitate, we did not see additional growth of the organism, but the cells did not lyse and were still viable after 24 h incubation (not shown). Moreover, some *B. subtilis* growth enhancement was observed when 1 mM Mg^{2+} was added to the medium in addition to *cis*-aconitate, strongly suggesting the involvement of CitM in the uptake of this TCA cycle intermediate (Fig. 1e). The *citM*-expression studies showed induction of CitM by *cis*-aconitate, but only after prolonged incubation of *B. subtilis* in the stationary phase. Unfortunately, the exchange studies revealed the presence of a contaminant in *cis*-aconitate (~5 %) that was also a substrate of CitM and was, therefore, most likely to be citrate or isocitrate. No evidence was obtained that *cis*-aconitate itself is a substrate of CitM. We tentatively conclude that the growth effects and induction pattern observed when *B. subtilis* is grown in the presence of *cis*-aconitate, and previous claims made in the literature (McKillen *et al*., 1972), must be ascribed to impurities in the *cis*-aconitate used; hence, *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 *B. subtilis* growth on citrate and isocitrate is that optimal growth of the organism requires higher concentrations of Mg^{2+} to be present than normally required (compare Fig. 1b, d, h). No growth of *B. subtilis* was detected when *cis*-aconitate, 2-oxoglutarate, D-malate, oxaloacetate or tricarballylate was the sole carbon source. The lack of growth of *B. subtilis* on 2-oxoglutarate that we observed is in contradiction with a report claiming growth of this organism on 2-oxoglutarate mediated by a low-affinity, inducible transport system with a K_m of 6·7 mM (Fournier *et al*., 1972). Analysis of the *B. subtilis* genome (Kunst *et al*., 1997) provides no clue as to the identity of such a 2-oxoglutarate transporter.

CitM has been shown to take up citrate in complex with Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+}. The improved growth of *B. subtilis* on isocitrate upon the addition of extra Mg^{2+} (Fig. 1c, d) to the growth medium and the $^{63}Ni^{2+}$ uptake experiments (Fig. 5) indicated that isocitrate is also transported in complex with Mg^{2+} and Ni^{2+}. Further experiments showed that the uptake of [1,5-^{14}C]citrate in the presence of 10 mM of Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+} could, in all cases, be significantly inhibited by the presence of 1 mM of isocitrate in the medium (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 not substrates for CitM. Citrate appears to be a stronger chelator of divalent metal ions than isocitrate. For instance, the complex formation constants for Mn^{2+}–isocitrate and Mn^{2+}–citrate (2·55 and 3·54 mM, respectively) (Martell & Smith, 1977) indicate a 10-fold lower affinity of isocitrate for Mn^{2+} than citrate. In our experiments, about seven times more DL-isocitrate than citrate was required to inhibit the $^{63}Ni^{2+}$ uptake activity in whole cells of *B. subtilis* CITMd by 50 %. Four times less of the naturally occurring isomer D-isocitrate was needed in whole cells of *B. subtilis* than L-isocitrate, 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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REFERENCES

Asai, K., Baik, S. H., Kasahara, Y., Moriya, S. & Ogasawara, N. (2000). Regulation of the transport system for C_4-dicarboxylic acids in *Bacillus subtilis*. *Microbiology* **146**, 263–271.

Aymerich, S., Gonzy-Tréboul, G. & Steinmetz, M. (1986). 5′-Noncoding region *sacR* is the target of all identified regulation affecting the levansucrase gene in *Bacillus subtilis*. *J Bacteriol* **166**, 993–998.

Bandell, M. & Lolkema, J. S. (1999). Stereoselectivity of the membrane potential-generating citrate and malate transporters of lactic acid bacteria. *Biochemistry* **38**, 10352–10360.

Bandell, M., Ansanay, V., Rachidi, N., Dequin, S. & Lolkema, J. S. (1997). Membrane potential-generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins. Substrate specificity of the 2-hydroxycarboxylate transporter family. *J Biol Chem* **272**, 18140–18146.

Boorsma, A., van der Rest, M. E., Lolkema, J. S. & Konings, W. N. (1996). Secondary transporters for citrate and the Mg^{2+}–citrate complex in *Bacillus subtilis* are homologous proteins. *J Bacteriol* **178**, 6216–6222.

Bott, M. (1997). Anaerobic citrate metabolism and its regulation in enterobacteria. *Arch Microbiol* **167**, 78–88.

Fortnagel, P. & Freese, E. (1968). Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. *J Bacteriol* **95**, 1431–1438.

Fournier, R. E., McKillen, M. N., Pardee, A. B. & Willecke, K. (1972). Transport of dicarboxylic acids in *Bacillus subtilis*. Inducible uptake of L-malate. *J Biol Chem* **247**, 5587–5595.

Goel, A., Lee, J., Domach, M. M. & Ataai, M. M. (1995). Suppressed acid formation by cofeeding of glucose and citrate in *Bacillus* cultures: emergence of pyruvate kinase as a potential metabolic engineering site. *Biotechnol Prog* **11**, 380–385.

Janausch, I. G., Zientz, E., Tran, Q. H., Kröger, A. & Unden, G. (2002). C_4-dicarboxylate carriers and sensors in bacteria. *Biochim Biophys Acta* **1553**, 39–56.

Kaback, H. R. (1983). The *lac* carrier protein in *Escherichia coli*. *J Membr Biol* **76**, 95–112.

Kay, W. W. (1978). Transport of carboxylic acids. In *Bacterial Transport*, pp. 385–411. Edited by B. P. Rosen. New York: Marcel Dekker.

Krom, B. P., Warner, J. B., Konings, W. N. & Lolkema, J. S. (2000). Complementary metal ion specificity of the metal–citrate transporters CitM and CitH of *Bacillus subtilis*. *J Bacteriol* **182**, 6374–6381.

Krom, B. P., Aardema, R. & Lolkema, J. S. (2001). *Bacillus subtilis* YxkJ is a secondary transporter of the 2-hydroxycarboxylate transporter family that transports L-malate and citrate. *J Bacteriol* **183**, 5862–5869.

Kunst, F., Ogasawara, N., Moszer, I. & 48 other authors (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.

Lolkema, J. S., Enequist, H. & van der Rest, M. E. (1994). Transport of citrate catalyzed by the sodium-dependent citrate carrier of *Klebsiella pneumoniae* is obligatorily coupled to the transport of two sodium ions. *Eur J Biochem* **220**, 469–475.

Martell, A. E. & Smith, R. M. (1977). *Critical Stability Constants, vol. 3: Other Organic Ligands*. New York: Plenum.

McKillen, M. N., Willecke, K. & Pardee, A. B. (1972). Citrate transport by *Bacillus subtilis*. In *The Molecular Basis of Biological Transport*, pp. 249–70. Edited by J. F. Woessner & F. Huijung. New York: Academic Press.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Somers, J. M., Sweet, G. D. & Kay, W. W. (1981). Fluorocitrate resistant tricarboxylate transport mutants of *Salmonella typhimurium*. *Mol Gen Genet* **181**, 338–345.

Sweet, G. D., Kay, C. M. & Kay, W. W. (1984). Tricarboxylate-binding proteins of *Salmonella typhimurium*. Purification, crystallization, and physical properties. *J Biol Chem* **259**, 1586–1592.

van der Rest, M. E., Molenaar, D. & Konings, W. N. (1992). Mechanism of Na^+-dependent citrate transport in *Klebsiella pneumoniae*. *J Bacteriol* **174**, 4893–4898.

Warner, J. B. & Lolkema, J. S. (2002). LacZ-promoter fusions: the effect of growth. *Microbiology* **148**, 1241–1243.

Warner, J. B., Krom, B. P., Magni, C., Konings, W. N. & Lolkema, J. S. (2000). Catabolite repression and induction of the Mg^{2+}–citrate transporter CitM of *Bacillus subtilis*. *J Bacteriol* **182**, 6099–6105.

Wei, Y., Guffanti, A. A., Ito, M. & Krulwich, T. A. (2000). *Bacillus subtilis* YqkI is a novel malic/Na^+–lactate antiporter that enhances growth on malate at low protonmotive force. *J Biol Chem* **275**, 30287–30292.

Widenhorn, K. A., Somers, J. M. & Kay, W. W. (1988). Expression of the divergent tricarboxylate transport operon (*tctI*) of *Salmonella typhimurium*. *J Bacteriol* **170**, 3223–3227.

Yamamoto, H., Murata, M. & Sekiguchi, J. (2000). The CitST two-component system regulates the expression of the Mg–citrate transporter in *Bacillus subtilis*. *Mol Microbiol* **37**, 898–912.

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