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REVIEW ARTICLE

Secondary transport of metal–citrate complexes: the CitMHS family

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Abstract
Primary and secondary transport of citrate has been extensively studied in pathogenic and non-pathogenic bacteria. Primary transporters of citrate complexed with metal ions, particularly Fe, have also garnered attention, with the *fec* system of *E. coli* being a classic example. In contrast, little is known about secondary transporters of metal–citrate complexes. Recently, a family of proteins responsible for secondary metal–citrate transport in bacteria was discovered and designated as the CitMHS transporter family. Several members have been functionally characterized to date and serve as the foundation for understanding this family. Three subfamilies have been categorized, depending on the main metal ion transported. These subfamilies are the Mg^{2+}–citrate transporter, the Ca^{2+}–citrate transporter, and the Fe^{3+}–citrate transporter. Each subfamily is believed to be substrate-selective due to the metal–citrate complexes being abundantly present in their environment and/or the ability of the complex to be metabolized by the organism. The implication of this family in the pathogenic access to Fe, information about transcriptional control, putative structure, predicted family members, members characterized to date and potential use in bioremediation are discussed.

Keywords: *Metal–citrate; secondary transport; iron; integral membrane; Streptomyces*

Introduction

While there has been extensive research conducted into citrate transport across membranes, there has been a relative dearth of research into membrane proteins that can transport metal-bound, complexed citrate (Sobczak and Lolkema, 2005). Most bacterial citrate transporters carry free citrate coupled to protons or Na and are inhibited by the addition of di- or tri-valent cations, since they do not recognize the metal–citrate complexes. Some transporters, however, have evolved in strains of species such as *Bacillus*, *Citrobacter*, *Neisseria*, *Klebsiella* and *Streptomyces*, that recognize citrate complexed with specific divalent and/or trivalent metal ions (Borrsma *et al.*, 1996; Korithoski *et al.*, 2005; Blancato *et al.*, 2008; Lensbouer *et al.*, 2008). It is believed that these organisms take up complexed citrate because it is predominantly available as such in their habitat. One particular family of secondary transporters is the CitMHS family, which transports metal–citrate complexes in symport with one/or two H^+ per metal–citrate complex. To date, the only functionally characterized systems for metal–citrate transport in this family are those of *Bacillus subtilis*, *Streptococcus mutans* and most recently *Enterococcus faecalis and Streptomyces coelicolor*. Lolkema's group demonstrated that Cit_{Bs1} from *B. subtilis* transported citrate complexed with Mg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} but not with Ca^{2+}, Ba^{2+} and Sr^{2+} (Krom *et al.*, 2000). Cit_{Bs2}, also from *B. subtilis*, transported citrate complexed to Ca^{2+}, Ba^{2+} and Sr^{2+} but not with Mg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}. The group of metal ions transported by Cit_{Bs1} includes the smaller cations, with a Pauling radius of less than ~0.80 Å, whereas the ions transported by Cit_{Bs2} of *B. subtilis* have radii larger than 0.98 Å. More recently, Cvitkovitch's group functionally characterized the Cit_{Bs1} homolog from *Streptococcus mutans* (Korithoski *et al.*, 2005). Citrate complexed to Fe^{3+} and Mn^{2+} was transported in this case, whereas the citrate complexes with Ca^{2+}, Mg^{2+} and Ni^{2+}

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were not. These authors in fact state that Fe^{3+} is the most efficient cofactor for citrate uptake in *S. mutans*. This suggests the intriguing possibility that, given that *S. mutans* is considered a major etiological agent of dental caries and oral cancer, it may be using the CitMHS system to access essential Fe and therefore may play a role in pathogenesis. Given members of the CitMHS family are postulated in bacteria such as *B. anthracis* and *Neiserria* spp., adding a further dimension to these systems. A fourth system characterized in native membranes is that of *E. faecalis* (Blancato *et al.*, 2006). The high amino acid sequence homology (73%) to the sequence of *S. mutans* led researchers to believe that it could be an Fe transporter, but the system was shown to be a Cit_{Bs2} (*B. subtilis*) homolog instead, transporting large ionic radii metals such as Ca. The transporter of *S. mutans* itself had been predicted to be a transporter for Mg^{2+}. This unpredictability clearly demonstrates our limited understanding of these systems. Sequence homology, while a good predictor of the presence of CitMHS family members, clearly does not yet allow us to predict metal co-factor preferences. Most recently, Fe^{3+}–citrate uptake was shown to occur in *Streptomyces coelicolor*, even in the presence of glucose (Lensbouer *et al.*, 2008), implying that this system is specifically targeting Fe. In a soil environment the use of citrate as a siderophore to allow access to limited Fe resources is certainly a viable alternate route to acquire the vital micronutrient.

Citric acid and metals: chemistry and biology

Citric acid is an important tricarboxylic acid that provides a source of carbon and energy in biological systems. Under aerobic conditions, citrate enters the Krebs cycle and is converted to cis-aconitate/iso-citrate via aconitase. Under anaerobic conditions, citrate is converted to oxaloacetate, which undergoes one of three differently described conversions to lactate, acetate, or succinate (Sobczak and Lolkema, 2005). Citrate is also important for chelating and transport. Plants use citric acid to solubilize inorganic Fe, which is the dominant ligand bound to Fe in xylem sap (Rellan-Alverez *et al.* 2010). Venomous snakes use citrate to chelate metals, inactivating metalloproteases in the snake venom. Following dilution, enzymes in the bitten host are activated (Marques-Porto *et al.*, 2008). Evidence even suggests that humans use citrate to facilitate Fe transport in the brain (Moos *et al.*, 2007). Binding of metal ions can occur at four sites: namely through the three carboxylate groups ($pK_1 = 3.13$, $pK_2 = 4.76$, $pK_3 = 6.40$) and hydroxyl group (pKa = 14.4; see Silva *et al.*, 2009). Two binding motifs have been described of citrate to divalent metal ions: bidentate and tridentate complexes (Figure 1; Francis and Dodge, 1993). Bidentate complexes bind through a dicarboxy motif and have been reported for Ni^{2+}, Ca^{2+}, and Ga^{3+}. Bidentate complexes involving the hydroxy group have been reported for Ti^{2+} and Al^{3+}. Tridentate complexes have been reported for Cu^{2+}, Cd^{2+}, Pb^{2+} and Fe^{3+}. A binuclear uranium complex, where the metal ions are bridged via the alkoxide groups, has also been observed.

Due to the bioavailability of metal–citrate complexes in nature and the constant ecological pressure for survival, it is not surprising that bacteria have evolved primary and secondary mechanisms for acquiring such complexes. The intriguing aspect remains with the specific recognition and uptake mechanisms. Primary citrate/metal–citrate transporters (e.g. *fec*A) use arginine to attract and bind to citrate (Yue *et al.*, 2003), but little is known about the residues that interact with the metal. The metal ion may not actually coordinate to the protein, but instead cause a conformation change in the citrate molecule

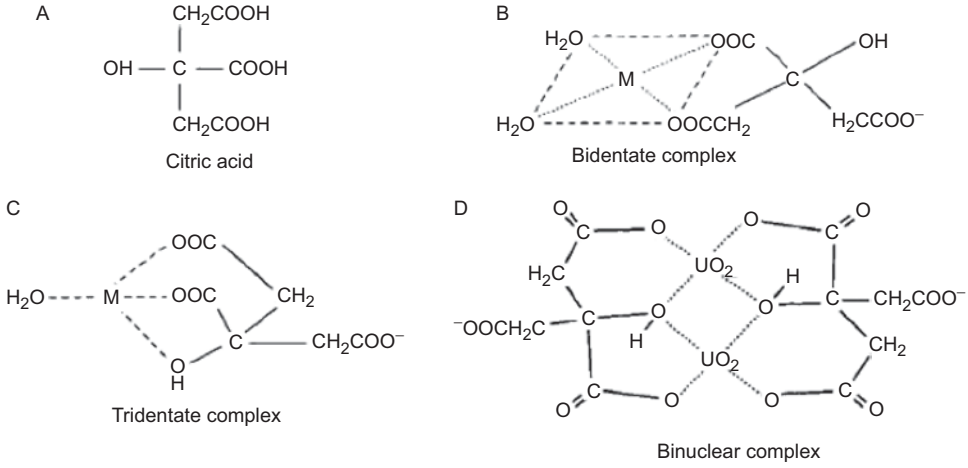

Figure 1. Types of metal–citrate complex. (A) citric acid; (B) a bidentate metal–citrate complex with two coordinating water molecules; (C) a tridentate metal–citrate complex with one water coordinating; and (D) a binuclear uranium complex. Figure is based on the work of Francis *et al.* (1992).

that is specific for the binding pocket. However, divalent metal transporters do interact with the metal ion, using aspartic acids and carbonyl groups for coordination (Eshaghi *et al.*, 2006). Therefore, substrate specificity and recognition remain ambiguous, although progress is being made.

While recognition and specificity are important, the ability to metabolize the metal–citrate complexes must also be considered. Francis *et al.* (1992) studied the ability of *Pseudomonas fluorescens* to metabolize metal–citrate complexes. They found that tridentate complexes (e.g. Cu^{2+}, Cd^{2+}, Pb^{2+}, and Fe^{2+}) were not metabolized, in opposition to what occurs with bidentate (Ca^{2+}, Ni^{2+}, and Fe^{3+}) complexes. Further investigation into why *P. fluorescens* was able to metabolize the Fe^{3+}–citrate bidentate complex and not the Fe^{2+}–citrate complex revealed that the hydroxyl group was important for metabolizing the citrate (Francis and Dodge, 1993). The same authors also showed that given enough time, the Fe^{2+}–citrate complex would oxidize and hydrolyze to the Fe^{3+}–citrate bidentate complex (Figure 2). The ability of *P. fluorescens* to metabolize bidentate complexes over tridentate ones reconfirms that the hydroxyl group needs to be uncoordinated, so that aconitase can recognize the citrate. *Clostridium sphenoides* was able to reduce di-U^{6+}-dicitrate to U^{4+}-dicitrate, which remained in solution (Francis and Dodge, 2008).

Discussion

Functionally characterized CitMHS members and substrate recognition

Five CitMHS members have been functionally characterized to date, with an empirically observed sixth member in *Pseudomonas fluorescens*. The metal–citrate complexes transported by each member are different, which makes prediction of the specific metal–citrate complexes transported by putative members difficult. Listed in table 1 are the characteristics of the CitMHS members studied to date.

Bacillus subtilis

B. subtilis is a Gram-positive soil dwelling bacterium. The complete genome of *B. subtilis* was sequenced in 1997 and was found to contain 4.2 million base pairs predicted to encode for 4100 genes (Wipat and Harwood, 1999). To fight starvation, the genome of *B. subtilis* encodes for many peptidases and polysaccharidases that allow it to metabolize a wide variety of carbon sources, including citrate. Interestingly, *B. subtilis* makes two proteins that transport metal–citrate complexes, which are the dominant forms of citrate in soil (Dessureault-Rompre *et al.*, 2008).

The first CitMHS member was empirically identified in *B. subtilis* by Willecke *et al.* (1973). These authors studied citrate transport in *B. subtilis* cells and found that it was dependent on the presence of Mg^{2+} (Willecke *et al.*, 1973). Other divalent metal ions, Mn^{2+}, Zn^{2+}, and Co^{2+}, were also found to induce citrate transport. No metal–citrate uptake

Table 1. Bacteria containing the CitMHS members and corresponding metals transported with citrate. The p in $pCit_{Pf}$ indicates putative.

Bacteria	Protein	Metals transported	Reference
Bacillus subtilis	Cit_{Bs1}	Mg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}	Krom *et al.*, 2000
Bacillus subtilis	Cit_{Bs2}	Ca^{2+}, Ba^{2+}, Sr^{2+}	Krom *et al.*, 2000
Streptococcus mutans	Cit_{Sm}	Fe^{3+}, Mn^{2+}	Korithoski *et al*, 2005
Enterococcus faecalis	Cit_{Ef}	Ca^{2+}, Sr^{2+}, Mn^{2+}, Cd^{2+}, Pb^{2+}	Blancato *et al.* 2008
Streptomyces coelicolor	Cit_{Sc}	Fe^{3+}, Ca^{2+}, Pb^{2+}, Ba^{2+}, Mn^{2+}	Lensbouer *et al.*, 2008
Pseudomonas fluorescens	$pCit_{Pf}$	Ca^{2+}, Fe^{3+}, Ni^{2+}, Zn^{2+}	Francis *et al.*, 1992

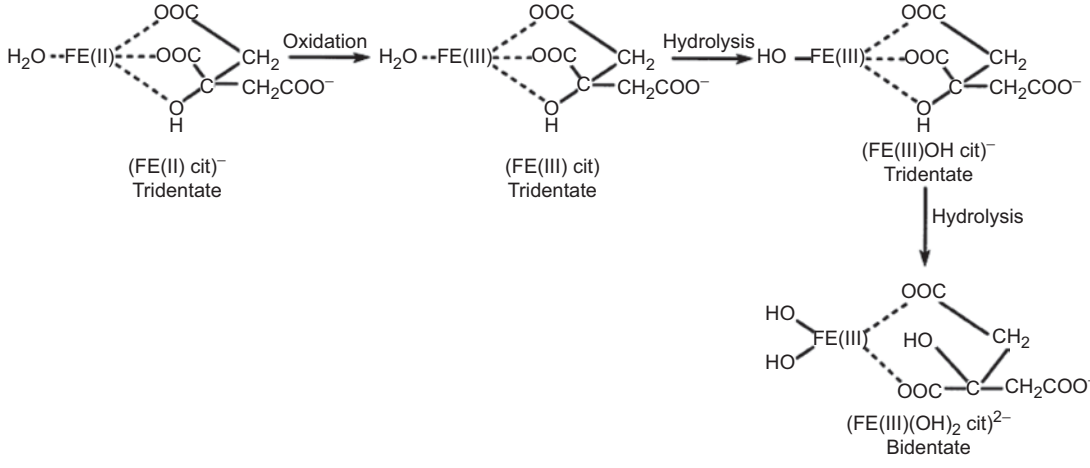

Figure 2. Oxidation and hydrolysis of tridentate ferrous–citrate to bidentate ferric–citrate. Figure was reproduced from Francis and Dodge (1993).

was observed when cells were grown in media that did not contain citrate as the sole carbon source.

In 1983, Bergsma and Konings took a more in-depth look at the metal–citrate transport of *B. subtilis* by using membrane vesicles. Their study indicated that the divalent metal ions Mg^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Be^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} were transported complexed to citrate. Using flow dialysis, it was found that one proton was symported with the metal–citrate complexes at pH 4.7, and two protons were symported at a pH of 8.0. Using the uncoupling agents *p*-trifluoromethoxyphenyl hydrazone, valinomycin, and nigericin it was demonstrated that the proton gradient was the driving force for metal–citrate transport.

In 1996 Borrsma *et al*. identified the gene that encoded for the Mg^{2+}–citrate transporter Cit_{Bs1} and a second gene that encoded for the free citrate transporter Cit_{Bs2}. Cit_{Bs1} and Cit_{Bs2} were identified as homologous proteins sharing 60% of the amino acid profile. The occurent of 12 membrane helices were predicted based on the hydropathy profile. The genes were cloned into expression vectors and overexpressed in *E. coli* JM109 (*DE3*) cells, and whole cell transport studies were consistent with the earlier results.

Krom *et al*. (2000) published a detailed look at the complementary uptake of Cit_{Bs1} and Cit_{Bs2} proteins and found that the Cit_{Bs1} protein transports Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} complexed to citrate where as the Cit_{Bs2} protein transports Ca^{2+}, Sr^{2+}, and Ba^{2+} complexed to citrate.

In 2001, Li and Pajor reinvestigated the Cit_{Bs1} protein, this time expressed in *E. coli* DH5α cells (Li and Pajor, 2002). It was shown that phosphate buffer interferes with transport due to the metal ions forming complexes with phosphate, and that HEPES caused a 10-fold increase in metal–citrate transport. The kinetic studies revealed that transport of citrate by Cit_{Bs1} in *E. coli* compared to *B. subtilis* was nearly identical, which indicates that using a Gram-negative organism for expression does not influence the ability of Cit_{Bs1} to transport citrate. Isocitrate, cis-aconitate and tricarballylic acid were also tested and CitBs1 was found to be specific for citrate (Warner and Lolkema, 2002), with uptake trends remaining the same in the presence of these other tricarboxylic acids. The uptake of Mg^{2+}–citrate by Cit_{Bs1} was tested in the range pH 5–8, and when pH was raised above the internal cell pH of 7.5, a 30% decrease in uptake was observed. Ionophores were tested to examine the effects of chemical and electrical gradients of ions on the transport of Mg^{2+}–citrate. When the electrogenic proton ionophore-like *p*-(trifluoromethoxy) phenylhydrazone (FCCP) was used, the transmembrane H^+ gradient was abolished, causing a reduction of 47% in Mg^{2+}–citrate transport. Trichlorocarbanilide, an anion/OH^- exchanger, changed the internal pH and reduced transport similarly as FCCP. Nigericin, an electroneutral K^+/H^+ exchanger, reduced uptake by 86%. The only siderophore that caused no change in uptake was valinomycin, which is an electrogenic K^+ ionophore that produces a K diffusion potential.

Streptococcus mutans

S. mutans is a Gram-positive bacterium and the principal cause of dental caries. In 2002, the genome of *S. mutans* UA15, was published (Ajkic *et al.*, 2002). The genome contains 2 million base pairs, encoding for 1963 putative genes. Of the putative genes 63% were assigned functions. *S. mutans* has the ability to metabolize a wide variety of sugars and sugar alcohols, and can transport and utilize primary metabolites such as citrate, which is commonly used as a preservative and bactericide for *Staphylococcus* bacteria. However, *S. mutans* is not able to survive solely on citrate as a carbon source.

Korithoski *et al.* (2005) functionally characterized the third CitMHS member, Cit_{Sm}. These authors were looking at the transport and metabolism of citrate by *S. mutans*, and they discovered a gene in the citrate gene cluster that was believed to be a homolog of the Cit_{Bs1} transporter. After doing functional transport assays in the native bacterium, it was found that *S. mutans* transports Fe^{3+}–citrate and Mn^{2+}–citrate. Mg^{2+}, Ni^{2+}, and Ca^{2+} were not transported by *S. mutans*. Expression studies were also conducted over the pH range 5–7 with and without citrate. As the pH decreased the expression of Cit_{Sm} increased. Expression levels increased, when citrate was added with the highest levels found at a pH 5. The end product of citrate uptake was found to be aspartic acid. The impact of Fe uptake by the CitMHS transporter was predicted to have pathogenic implication in *S. mutans*.

Enterococcus faecalis

E. faecalis is a Gram-positive bacterium that frequently inhabits the bowel. In 2003, the genome of *E. faecalis* V583 was sequenced, which revealed 3.2 million base pairs encoding for 3182 genes and three plasmids encoding for 155 genes (Paulsen *et al.*, 2003). *E. faecalis* is a known pathogen and causes urinary tract infections as well as endocarditis.

The functional characterization of the fourth CitMHS member was reported by Blancato *et al*. (2006) in *E. faecalis*. The putative CitMHS member in *E. faecalis* shares a high amino acid similarity to that of Cit_{Sm}, so these authors reasoned *E. faecalis* would transport Fe^{3+}–citrate. If fact, they found that the protein, designated Cit_{Ef}, was actually a homolog of Cit_{Bs2} from *Bacillus subtilis*. Cit_{Ef} transported Ca^{2+}, Sr^{2+}, Mn^{2+}, Cd^{2+}, and Pb^{2+}, but not Mg^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, or Fe^{3+}. It was concluded that the specificity appears to correlate with the size of the metal ion in the complex.

Streptomyces coelicolor

S. coelicolor is a common soil dwelling bacterium. In 2002 the genome was sequenced, which revealed 8.7 million base pairs encoding for 7825 putative genes (Bentley *et al.*, 2002). Within those putative genes, 20 secondary metabolite clusters were identified. These secondary metabolites include three antibiotics, siderophores, pigments, lipids, regulation factors, and growth inhibitors.

The fifth CitMHS member to be functionally characterized was Cit_{Sc} from *S. coelicolor* A3(2). When looking at the ability of CitMHS members to be used for bioremediation, a new Fe^{3+}–citrate transporter was discovered (Lensbouer *et al.*, 2008). Transport assays were conducted in the native bacterium as well as with JW4251 Δfec *E. coli* cells. The dominant ion transported was Fe^{3+}–citrate, followed by Ca^{2+}, Pb^{2+}, Ba^{2+} and Mn^{2+}. Cit_{Sc} appears to be a hybrid homolog of the Cit_{Bs2} transporter and the Cit_{Sm} transporter, which makes predicting the complexes transported very difficult.

In 2010 the first and, to date, only CitMHS mutation work was conducted to better understand the mechanism of metal–citrate transport (Lensbouer *et al.*, 2010). Using known permease structures (e.g. of LacY and CorA; see Abramson *et al.*, 2003; Eshaghi *et al.*, 2006) and several structure predicting programs (Gasteiger *et al.* 2003), some amino acids were targeted for mutation work (R161A, K452C, and R460C; Lensbouer *et al.*, 2010). These amino acids were predicted to attract the negatively charged metal–citrate complexes or be involved in salt-bridge formation. Results indicated that arginines are critical for metal–citrate uptake, whereas transport by the lysine mutant was still observed (Lensbouer *et al.*, 2010).

Pseudomonas fluorescens (putative)

A putative sixth member of the CitMHS family is predicted to occur in the Gram-negative bacterium *P. fluorescens*. Francis *et al.* (1992) found that *P. fluorescens* has the ability to metabolize Ca^{2+}, Fe^{3+}, Zn^{2+} and Ni^{2+}, but not Fe^{2+}, Cu^{2+}, U^{6+}, Pb^{2+} or Cd^{2+} complexed to citrate. These authors isolated a strain of *P. fluorescens* from the low-level radioactive waste disposal site, West Valley, New York, and assessed metal–citrate metabolism by whole cell flux assays. However, the gene or genes responsible for the transport were never identified. Another strain, *P. fluorescens* SBW25, has been genomically sequenced, and a putative CitMHS transporter has been assigned with a locus tag of PFLU4350.

CitMHS members: known and predicted

As the number of sequenced genomes increases, more putative CitMHS members are being identified. Using the amino acid sequences for the functionally characterized CitMHS member Cit_{Bs1}, a BLAST search was conducted against the microbe proteomes on the NCBI server (Altschul *et al.*, 1997; 2005). Using the NCBI's scoring methodology, we excluded all scores that were below 200, although many putative members may exist below the 200 score. Based on the scoring methodology, no CitMHS members are predicted in the domain *Archae* or phylum *Spirochaetales*, but members are predicted in the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. To date, all five functionally characterized CitMHS members belong in the *Actinobacteria* or *Firmicutes* phyla. Two members belong to the order *Lactobacillales* and two members are in the order *Bacillales*. We identified a total of 278 predicted CitMHS members. As more genomes are sequenced, we predict that this number will grow significantly.

Known and predicted CitMHS members are found in environments that are high in citrate. The most common environments are soil, animals (including humans), and plants. Figure 3 is a phylogenetic tree of CitMHS members and 21 selected putative members listed in Table 2 (Felsenstein, 1989; 1993). Three main branches are seen (A, B, and C). Branch A represents the *Lactobacillales*, which are fermentable bacteria. Many of the members of this branch are pathogenic or opportunistic pathogens living in the digestive tract of animals, with the exception of *Lactobacillus*, which are known probiotics. Branch B contains members from *Xanthomonadaceae*, *Bacillales*, and *Pseudomonadaceae*. Many of these bacteria are pathogenic or opportunistic pathogens, with the exception of *Bacillus subtilis*. Branch C contains members from *Actinobacteria*, which are all considered as nonpathogenic.

The existence of three branches in the phylogenetic tree may suggest that three different evolutionary paths have occurred. Environmental factors significantly affect the expression of proteins and the functions that they perform. The lactic bacteria (A) may have uniquely developed metal–citrate transporters to facilitate the anaerobic degradation of citrate to oxaloacetate and subsequently to lactic acid. One factor that would have significantly affected the metal ions available would have been pH. The low pH of the mouth would allow for a favored Fe^{3+}–citrate formation compared to that of Mg^{2+} and Ca^{2+}, since as the pH increases Mg^{2+}–citrate and Ca^{2+}–citrate become more favorably complexed (Gustafsson, 2006). Cit_{Sm} is known to thrive in the mouth and was found to transport Fe^{3+}–citrate (Korithoski *et al.*, 2005). Plaque build-up in the mouth causes the release of acids, which lowers the pH. As the pH decreases, citric acid complexes to any available Fe^{3+} instead of Ca^{2+}. From the recent work in *S. mutans*, an increase in expression of Cit_{Sm} was observed as the pH decreased with the addition of citric acid (Korithoski *et al.*, 2005).

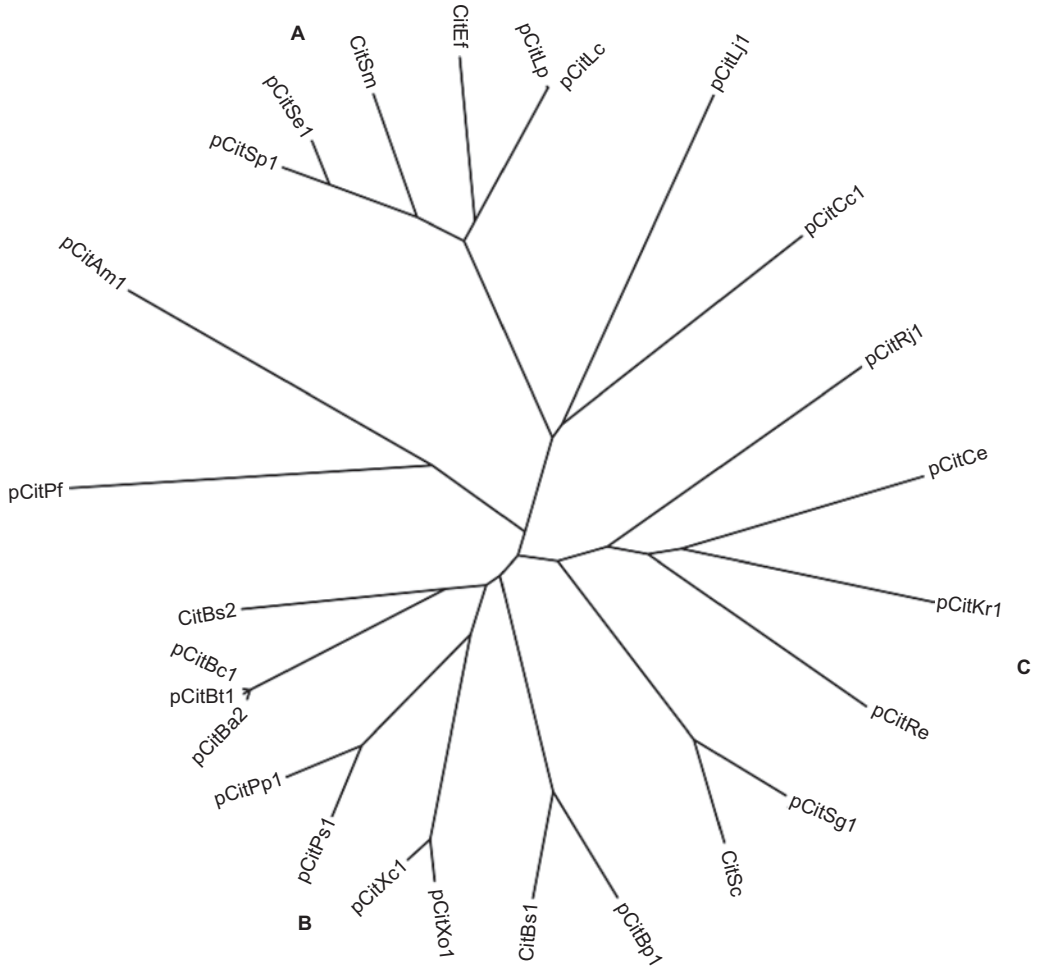

Figure 3. Unrooted phylogenetic tree of CitMHS members from Table 2. Three branches are highlighted as A, B, and C. PHYLIP was used to construct the tree (Felsenstein, 1989, 1993).

Table 2. Bacteria and their corresponding CitMHS member or putative member. The p in pCit refers to a putative CitMHS member.

Bacteria	CitMHS protein	Bacteria	CitMHS Protein
Lactobacillus casei	$pCit_{Lc}$	*Xanthomonas campestris*	$pCit_{Xc1}$
Lactobacillus paracasei	$pCit_{Lp}$	*Xanthomonas oryzae*	$pCit_{Xo1}$
Enterococcus faecalis	Cit_{Ef}	*Bacillus subtilus*	Cit_{Bs1}
Streptococcus mutans	Cit_{Sm}	*Bacillus pumilus*	$pCit_{Bp1}$
Streptococcus pyogenes	$pCit_{Sp1}$	*Streptomyces coelicolor*	Cit_{Sc}
Alkaliphilus metalliredigens	$pCit_{Am1}$	*Streptomyces griseoflavus*	$pCit_{Sg1}$
Pseudomonas fluorescens	$pCit_{Pf}$	*Ralstonia eutropha*	$pCit_{Re}$
Bacillus subtilis	Cit_{Bs2}	*Kocuria rhizophila*	$pCit_{Kr1}$
Bacillus cereus	$pCit_{Bc1}$	*Corynebacterium efficiens*	$pCit_{Ce}$
Bacillus thuringiensis	$pCit_{Bt1}$	*Rhodococcus jostii*	$pCit_{Rj1}$
Bacillus anthracis	$pCit_{Ba2}$	*Lactobacillus jensenii*	$pCit_{Lj1}$
Pseudomonas putida	$pCit_{Pp1}$	*Clostridium carboxidivorans*	$pCit_{Cc1}$
Pseudomonas syringae	$pCit_{Ps1}$	*Streptococcus equi*	$pCit_{Se1}$

Cit_{Ef} from *E. faecalis* is also in branch A. However, Cit_{Ef} was found to transport Ca^{2+}–citrate. A key difference between *S. mutans* and *E. faecalis* is that *E. faecalis* lives in the colon where the pH is closer to neutral. Therefore the dominant metal–citrate complexes would be expected to be Ca^{2+}–citrate. Furthermore, Fe is tightly regulated by the body, and very little Fe is present in fecal content compared to Ca (Nishimuta *et al.*, 2006).

Branches B and C contain mainly soil dwelling bacteria that grow in the rhizosphere. The rhizosphere contains micromolar concentrations of Fe and millimolar concentrations of Ca (Dessureault-Rompre *et al.*, 2008). Two characterized CitMHS members, Cit_{Sc} and Cit_{Bs2}, transport Ca^{2+}–citrate, as citrate and Ca are predominant in the rhizosphere. However, Cit_{Sc} transports Fe^{3+}–citrate, but Cit_{Bs2} does not. One explanation may be that Cit_{Bs2} has lost the ability to transport Fe^{3+}–citrate. It has been shown that Fe^{3+}–citrate was bioremediated by *P. fluorescens* (Francis and Dodge, 1993), and this organism contains a putative CitMHS member. If $pCit_{Pf}$ does

transport Fe^{3+}–citrate, than the divergent branching may represent Cit_{Bs2} losing the ability to transport Fe^{3+}–citrate. The evolution away from the Fe^{3+}–citrate transport may also explain Cit_{Bs1}. If Cit_{Bs2} evolved away from Fe^{3+}–citrate transport, it would be expected that the homologue Cit_{Bs1} would as well. It was demonstrated that Cit_{Bs1} takes up Mg^{2+}–citrate, which is vastly different than any of the other members. Currently, the methodology for predicting which metal–citrate complexes are transported is to look at the similarity of the amino acid sequences. However, a more accurate method may involve using the amino acid sequences as well as assessing environmental factors that occur where the organism is most commonly found.

Transcription regulation and induction of CitMHS transporters

Catabolite regulation in bacteria is diverse and complicated. To date, multiple mechanisms of control have been documented. In bacteria many catabolite genes are subject to catabolite control protein A (CcpA)-mediated catabolite repression (Kim *et al.*, 1998; Warner and Lolkema, 2003; Abranches *et al.*, 2008). CcpA is a bifunctional protein that acts as a repressor or inducer, depending on the presence of glucose and other primary metabolites (e.g. citrate). Genes that are subject to CcpA-mediated regulation contain carbon responsive elements upstream of the transcription start codon. Additionally, other corepressors are involved in CcpA regulation. The histidine-containing phosphocarrier protein (HPr), and phosphoenol-pyruvate-sugar phosphotransferase system (PTS) act as corepressors by forming a trans-acting complex with CcpA when phosphorylated (Pompeo *et al.*, 2007). This entire complex binds to carbon responsive elements (*cre*) or catabolite operators and inhibits expression.

Bacillus subtilis: transport and transcriptional regulation of the citrate gene cluster

In *B. subtilis*, expression of many genes is subject to CcpA-mediated catabolite repression, including the Cit_{Bs1} transporter and predicted Cit_{Bs2} transporter (Warner *et al.*, 2000; Repizo *et al.*, 2006). Two carbon responsive elements are found in the citrate gene cluster of *B. subtilis*. The first is found in the two component signal-transduction system, upstream of the Cit_{Bs1} transcriptional start codon (Figure 4; Repizo *et al.*, 2006; Fabret *et al.*, 1999). The transcription factor for the two-component system was found to match the consensus sequence for σ^A. In the coding sequence for CitS there is a site that matches the consensus *cre* sequence that represses the expression of the CitST system. When glucose concentrations are depleted, the CcpA is released from the *cre* site and allows for CitST to be transcribed. CitS is a sensor kinase that phosphorylates CitT when citrate is present. Phosphorylated CitT then binds to specific sequences located in the region between –62 and –113 upstream of Cit_{Bs1} and activates transcription. However, a second *cre* site exists between the CitT binding site and the Cit_{Bs1}

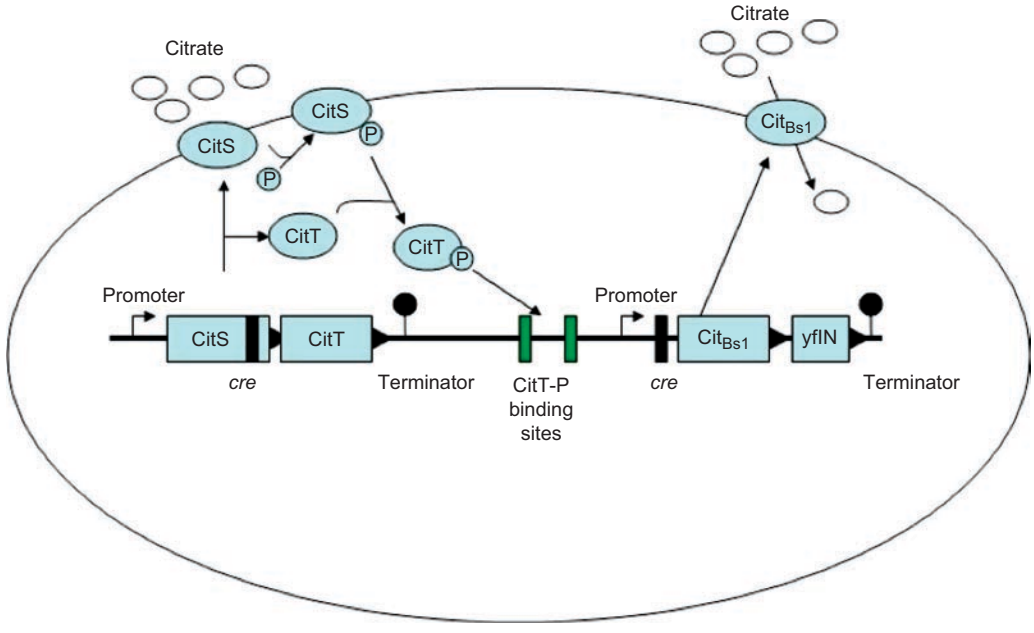

Figure 4. Transport and transcriptional regulation of the citrate gene cluster in *B. subtilis*. Cit_{St} is the two component signal and response regulator. Cit_{Bs1} is the Mg^{2+}–citrate transporter. Figure is based on work from Pompeo *et al.* (2007).

gene, which should not interfere with expression due to the already established low presence of glucose.

Streptococcus mutans and Enterococcus faecalis: transport and transcriptional regulation of the citrate gene cluster

S. mutans and *E. faecalis* do not use the two-component regulator system that is seen in *B. subtilis*. These two bacteria, which belong to the order *Lactobacillales*, regulate their citrate gene clusters with a GntR type of family regulator, CitO (Blancato *et al.*, 2008). GntR regulators are named after the *B. subtilis* GntR transcriptional regulator for the gluconate operon. They contain a conserved N-terminal helix-turn-helix domain that binds to DNA, while the C-terminus domain is involved in substrate binding. Because of the wide variety of N-terminus binding domains, eight subfamilies have been proposed (FadR, DevA, HutC, AraR, MocR, PlmA, DasR, and YtrA). CitO belongs to the FadR subfamily. Citrate binds to the C-terminus of CitO and induces the transcription of the citrate gene clusters (Figure 5). The *cre* sites may play a role in CitO induction. Blancato *et al.* (2008) studied the CitO regulator from *E. faecalis* and found that there were two binding domains in the intergenic region between Cit_{Ef} and oadH. When comparing the binding domains to the intergenic region between Cit_{Sm} and oadH of *S. mutans*, two regions align identically with those of *E. faecalis*. These regions are likely the binding domains for CitO for *S. mutans*, but no work has been conducted to elucidate the mechanism of expression for *S. mutans*.

Streptomyces coelicolor: Putative transcriptional regulation of CitSc

Three of the CitMHS transporters Cit_{Bs1}, Cit_{Sm}, and Cit_{Ef} are found in citrate gene clusters within the corresponding bacterial genome, but Cit_{Bs2} and Cit_{Sc} are not found in the citrate gene clusters. It is believed that Cit_{Bs2} is under the same transcriptional regulation as Cit_{Bs1}, but no work has been carried out to prove this. Cit_{Sc} is believed to have a dual transcription regulator. Metal–citrate uptake in *S. coelicolor* occurred when the organism was grown in *Streptomyces* minimal media with citrate being the only carbon source, but when glucose was added no metal–citrate was transported. However, when excess Fe and glucose were added, Fe–citrate was observed to be transported (Lensbouer *et al.*, 2008). These experiments suggest that Cit_{Sc} is under CcpA and Fe transcriptional regulation. Other systems such as the valine dehydrogenase (vdh) gene in *S. coelicolor* are under similar dual regulation (Figure 6; Tang and Hutchinson, 1995). Vdh is repressed by glucose and ammonia. When comparing the promoter regions of vdh, galactose operon, and glycerol operon to the putative Cit_{Sc} promoter region, a high similarity exists at the –35 and –10 regions, suggesting that partial transcription control is by CcpA. Fe boxes in *S. coelicolor* are sequences of DNA that bind the divalent

A

	-35	-10
$pCit_{Sc}$	GGTGGGATGTTCAAGGGCGAACGTTAGGT	
galP1	GGGGGGTGGTGGGTTGTGATGTGTTATGT	
gylRp	GGGGGGAGGTCGGCATGGACCGGTAGTGT	
vdh-P	GGGGGGCCGGTACACCCAGGCTCTAATCT	

B

Cit_{Sc}	GCTGCTTCGCGCCACCTAA
Con	TTAGGTTAGCTTCACCTAA
DesA	TTAGGTTAGGCTCACCTAA
Tox	TTAGGATAGGTTTACCTAA

Figure 6. Alignment of the putative promoter region upstream of Cit_{Sc} compared to known promoter regions for valine dehydrogenase, glactose operon, and glycerol operon (A). Alignment of the putative iron box upstream of Cit_{Sc} compared to iron boxes of the *Streptomyces* consensus sequence, *des*A of the desferrioxamine B biosynthesis from *Streptomyces pilosus*, and iron-regulated promoters *tox* (of the diphtheriae toxin) of *Corynebacterium diphtheriae* (B).

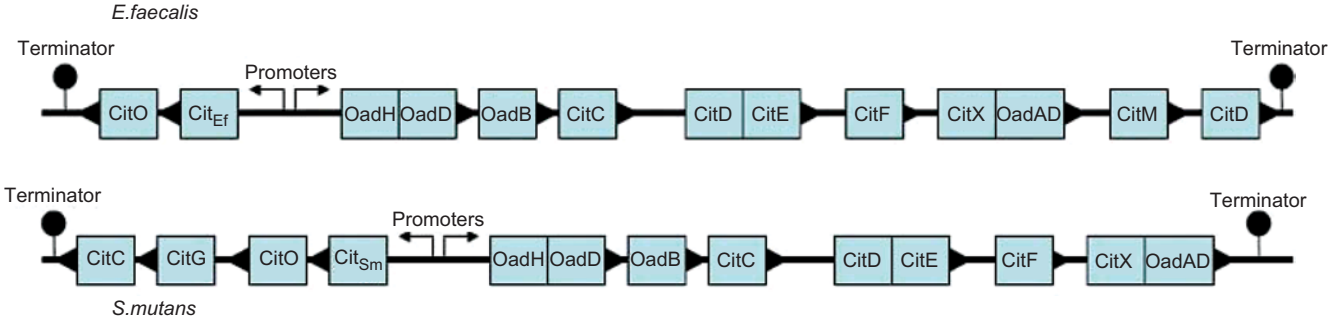

Figure 5. Citrate gene cluster of *E. faecalis* and *S. mutans*. CitO is the transcriptional activator. Cit_{Ef} and Cit_{Sm} are the metal–citrate transporters. OadHDBC encodes for the three subunits of oxaloacetate decarboxylase. CitCDEFG encodes the citrate lyase and auxiliary proteins. OadAD is believed to be a biotin carrier and CitX is an auxiliary protein. Figure is based on the work of Blancato *et al.* (2008).

metal-dependant regulators Dmd1 or Dmd2 (Flores *et al.*, 2004). Upstream of the Cit_{Sc} gene is a 19mer region that is very similar to the consensus sequence of Fe boxes in *S. coelicolor*. When comparing against the consensus sequence or other putative Fe boxes, the similarity ranges from 63.1% to 78.9%. The similarity along with the experimental evidence suggests that Dmd1 or Dmd2 may also contribute to transcriptional regulation.

Finally, we herein suggest a nomenclature for naming each member of the CitMHS family. Each member is assigned the capital Cit followed by the subscripted initials of the bacterium from which the protein came (e.g. Cit_{Sc} from *Streptomyces coelicolor*). In the case of additional proteins from the same organism, each protein is assigned in chronological order of discovery (e.g. Cit_{Bs1} and Cit_{Bs2} from *Bacillus subtilis*). Organisms that share similar initials must add letters to the species name to distinguish from already named organisms (e.g. putative Cit_{Bc} from *Bacillus cereus* and putative Cit_{Bco} from *Bacillus coagulans*).

Conclusions and future directions

While extensive research has been conducted into citrate transport across biological membranes, there has been a relative dearth of investigation of membrane protein systems that can transport complexed, metal-bound citrate. Of particular importance is the use of these systems to access Fe in the form of Fe–citrate. Given the importance of citrate in metal sequestration this represents a gap in our knowledge of a fundamental biological process. This is of particular significance with regard to microorganisms that cause disease in humans. Growth in such environments has demanded that these organisms develop diverse ways of obtaining Fe, overcoming host measures to prevent this.

Isolation and purification of the unique CitMHS transporters may allow for development of new vaccines and/or detection methods. New vaccines would help to combat bacteria that are becoming resistant to antibiotics, such as *Staphyloccocus aureus*, *Bacillus anthracis* and *Streptococcus pyogenes*.

Alternatively, CitMHS members may be used for bioremediation. Radioactive waste is a prime target for bioremediation. Nuclear power plants use fission to generate energy, and produce spent fuel cells containing Pu-239, U-238, Sr-90 and Cs-127. *Kineococcus radiotolerans* is a bacterium belonging to the order *Actinomycetes*, and was discovered in a shield cell where it was exposed to highly radioactive waste at the Savannah River site in Aiken, SC. This organism is resistant to high gamma radiation, making it an ideal candidate for radioactive bioremediation. *K. radiotolerans* encodes for a putative CitMHS member that is very similar to Cit_{Sc} and may take up Sr^{2+}–citrate or Pb^{2+}–citrate, making it an intriguing organism for studying isolation of such metals from radioactive waste.

Whether CitMHS family members occur in/across eukaryotes (e.g. algae) also remains an open question. Another question is whether a symbiosis exists between CitMHS-containing bacteria and citrate-excreting plants.

Future work in the field of the CitMHS family promises to be rich with new discoveries, both fundamental and applied.

Declaration of interest

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