CHAPTER VI

Transporters involved in uptake of di- and tricarboxylates in \textit{Bacillus subtilis}.

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**Transport of TCA cycle intermediates in *Bacillus subtilis***

Di- and tricarboxylates found as intermediates in the Tri Carboxylic Acid (TCA) cycle (Citric Acid Cycle, or Krebs cycle) can be metabolized by many bacteria and serve as carbon and energy source. Under aerobic conditions the substrates are oxidized to CO₂ and H₂O. Under anaerobic conditions multiple pathways exist. The C₄-dicarboxylic acids fumarate and L-malate, that is dehydrated to fumarate by fumarase, serve as energy source through fumarate respiration. This respiration leads to the generation of a proton motive force (pmf) which drives the synthesis of ATP by FₒF₁-ATPases (for review see (14, 20)). Succinate produced during fumarate respiration is exchanged for external fumarate without cost of metabolic energy. The tricarboxylylate citrate can also be used under anaerobic conditions in different mechanisms as described extensively for enterobacteria (reviewed in (8)). In all cases, citrate is converted to oxaloacetate by citrate lyase. Bacteria that lack this enzyme, as is the case for *Bacillus subtilis*, do not grow anaerobically on citrate. After the conversion of citrate to oxaloacetate several pathways are possible. In *Escherichia coli*, oxaloacetate is converted to malate and subsequently to fumarate by malate dehydrogenase and fumarase, respectively. Fumarate is used as described above in fumarate respiration. A co-substrate like glucose is required to supply the reducing equivalents. In *Klebsiella pneumoniae*, no co-substrate is needed. Oxaloacetate is converted to pyruvate and formate by oxaloacetate decarboxylase. This membrane embedded enzyme functions as a Na⁺ pump, and conserves part of the free energy (estimated 0.3 mol ATP/mol citrate) derived from citrate fermentation in the form of a sodium ion gradient. This gradient can be used by secondary transporters, f.i. Na⁺ dependent transport of citrate in *K. pneumoniae*, or can be converted into a pmf by the action of Na⁺/H⁺ antiporters. Pyruvate is converted first to acetyl-CoA, which is subsequently converted to acetylphosphate and finally to acetate, a process which yields one mol ATP/mol citrate (8).

Lactic acid bacteria use fermentative pathways to metabolize the di- and tricarboxylates malate and citrate. In *Lactococcus lactis* malate is exchanged for its decarboxylation product lactate by a specialized transporter (MleP, see 2HCT family below) which leads to the formation of a membrane potential of physiological polarity. Furthermore, during the decarboxylation step a cytoplasmic proton is consumed which results in a pH gradient over the cell membrane. Taken together this malolactic fermentation results in a pmf that is large enough to drive ATP synthesis via the FₒF₁-ATPase (2). In a more complex manner, citrate is metabolized in *Leuconostoc mesenteroides* in a citrolactic fermentation (26). Similar as in malolactic fermentation, divalent citrate is exchanged for monovalent lactate (by CitP, see 2HCT family below). The pathway in the cytosol converting citrate into lactate requires three different enzymes and is coupled to glucose metabolism (25, 26). For all metabolic pathways described above it is essential that the di- and tricarboxylates are transported across the cytoplasmic membrane. All known and putative transporters of di- and tricarboxylates are secondary transporters; they derive energy from the chemiosmotic gradient of protons or sodium ions to accumulate the substrate against a concentration gradient.
Di- and tricarboxylate uptake in *B. subtilis* has been studied in the seventies and early eighties of the last century. For C$_4$-dicarboxylates two uptake systems were described. One is an L-malate uptake system that is induced by L-malate in the medium. It was reported to be responsible for growth on L-malate as sole carbon and energy source (45). The second system is less specific and claimed to transport L-malate, succinate and fumarate. It was found in cells grown on yeast extract (10), in the presence of citrate (9), or expressed constitutively (6). The latter study in membrane vesicles showed that the transporter was of the secondary type. Similarly, it was demonstrated that the uptake of the tricarboxylate citrate in *B. subtilis* is catalyzed by a secondary transporter (5). Remarkably, uptake of citrate was dependent on the availability of divalent metal ions (5, 28). Taken together these reports offer no information on the identity of the transporters that are responsible for the uptake of these solutes. The availability of molecular and biochemical techniques greatly facilitated the identification and characterization of the proteins involved in di- and tricarboxyate transport in *B. subtilis*. In addition, the complete genome sequence of *B. subtilis* (23) yielded valuable information about all the transport capabilities of this bacterium (35).

In this chapter an overview is given of the di- and tricarboxylate transporters found in *B. subtilis*. The protein families to which these transporters belong, and, if available, the data on functional properties, regulation of transcription and physiological function of the individual proteins are discussed.

**MeCit family**

The MeCit family currently consists of 12 homologous proteins that are found exclusively in bacteria. Some are found in typical pathogenic bacteria, like *Staphylococcus aureus* and *Neisseria meningitidis*, others in typical soil bacteria like *Streptomyces coelicolor* or *B. subtilis*. Until now, only proton-coupled symporters have been found, but only two members of the family have been functionally characterized (see below). The hydropathy profiles of members of the family indicate 11 to 12 hydrophobic regions, indicating 11 to 12 putative transmembrane segments (TMSs). The membrane topology of the family has not been determined experimentally. Based on the only two characterized members of this family, CitM and CitH of *B. subtilis*, the transporters in the family transport tricarboxylates in complex with divalent cations. *B. subtilis* contains in total three proteins that belong to the MeCit family, CitM, CitH and YraO, the latter of which has not been characterized.

**Characterization**

CitM and CitH, designated YfIN and YxiQ in the Subtilist database, respectively, and YraO of *B. subtilis* share 52 to 61% identical residues and, additionally, 18 to 25% similar residues. CitM and CitH have been successfully cloned and functionally expressed in *E. coli* (7, 22). However, functional expression of *yraO* in *E. coli* has failed sofar (Krom *et al*. unpublished results). Using Right-Side-Out (RSO) membrane vesicles, CitM and CitH were shown to be secondary transporters that are driven by the transmembrane pH gradient, ΔpH,
by the membrane potential, $\Delta \Psi$, inside negative (7). This observation indicates that at least one net positive charge is transported into the cell.

At first, CitH was identified as a transporter for free citrate (7). However, a more recent study has shown that CitH is a Ca$^{2+}$-citrate transporter that can also accept Ba$^{2+}$ and Sr$^{2+}$ complexed to citrate but not free citrate (22). The apparent affinities ($K_{\text{app}}$) for the different complexes are very similar, ranging from 33 to 50 µM. In contrast, the maximal rates of transport were at least 3 fold higher for Ca$^{2+}$ then for Sr$^{2+}$ or Ba$^{2+}$. It was shown that Ca$^{2+}$ is co-transported into the cell.

CitM has been shown to transport Mg$^{2+}$-citrate, but also Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$-citrate. The $K_{\text{app}}$'s of the cloned transporter for each complex were again very similar ranging from 35 to 63 µM and are in line with the $K_{\text{app}}$ of 40 µM measured in $B.\ subtilis$ vesicles for Mg-citrate (5). The maximal rate of transport ranged from 214 to 661 pmol/mg protein-min for Mn$^{2+}$ and Co$^{2+}$, respectively (22). It has been shown that the divalent cation is transported into the cell, i.e. the complex of metal and citrate is the transported species. Besides complexes of citrate with divalent metal ions, CitM transports also isocitrate in complex with divalent metal ions (Warner et al., unpublished results).

In conclusion, both CitM and CitH transport a metal-citrate complex, but with complementary metal specificity. It is possible that the two transporters distinguish metal-citrate complexes on basis of the ionic radii. CitH transports bulky ions with ionic radii greater than 0.98 Å (22). Since transport by both proteins was shown to be electrogenic and the Me-citrate complex is monovalent anionic, at least two protons are co-transported with the Me-citrate complex.

**Regulation of expression**

The expression of $citH$ was studied with the LacZ-fusion approach, with the promoter of $citH$ fused upstream of the gene for β-galactosidase ($\beta$-galactosidase). Growth of this strain on plates in the presence of many different di- and tricarboxylates did not result in any clear induction. However, after a prolonged incubation up to three days, some expression was observed. Either CitH is constitutively expressed at a very low level, or induction of expression is growth phase dependent. The first option is most likely since low citrate transport activity in the presence of Ca$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$ has been observed in $B.\ subtilis$ in the exponential growth phase (22). On the other hand, a DNA array study using 24 two-component regulatory systems detected on the genome of $B.\ subtilis$ indicated that the YufLM sensor kinase-response regulator pair is involved in CitH induction (19). Interestingly, many genes induced via YufLM are members of the ComK regulon involved in competence (19). The observed late expression of CitH could indicate that CitH expression is induced just before or during the development of competence.

The regulation of expression of $citM$ has been studied extensively. $CitM$ expression requires the presence of Mg$^{2+}$ and citrate or isocitrate in the medium (42, 46). Using the $citM$ promoter fused to lacZ it was shown that Mg$^{2+}$-citrate is by far the best inducer of expression. Ni$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ citrate were also capable of induction, albeit to lower levels (Krom et al., unpublished results).
The induction is mediated by a classical two-component signal transduction system, consisting of the sensor, CitS encoded by yflR, and the response regulator, CitT encoded by yflQ, both of which are located upstream of citM (Figure 1). CitT is the DNA binding transcriptional regulator and has been shown to bind to two regions in the promoter region of citM (46). The induction by citrate is overruled by the presence of carbon sources that are easier to metabolize, like glucose, glycerol and inositol (42). Also the combination of succinate and glutamate and some component(s) in Luria Bertani broth were shown to repress CitM expression (Warner et al. unpublished results). Repression is mediated by the classical Carbon Catabolite Repression system as has been shown for numerous other proteins in B. subtilis (12). A functional Catabolite Responsive Element (CRE) is located within the promoter region of CitM (27, 42, 46).

The third member of the McCit family in B. subtilis, encoded by yraO gene, could not be shown to be expressed under any of many conditions tested using the LacZ promoter fusion approach (Krom et al., unpublished results). Either this gene is expressed under very special conditions, or the gene is cryptic.

Physiological Function

B. subtilis grown in continuous cultures with glucose as sole carbon source results in acid production caused by overflow of the TCA-cycle, and consequently, rapid acidification of the medium which inhibits growth (11). Co-metabolism of glucose and citrate resulted in lower acid production and a higher growth yield due to a better coordination between glycolysis and the TCA cycle. It has been proposed that elevated levels of Ca\(^{2+}\) in the cell result in inhibition of pyruvate kinase activity, thus slowing down glycolysis. A specific Ca\(^{2+}\)-citrate transporter is reported to be responsible for this elevated level of Ca\(^{2+}\) in the cell. If this were indeed the case, CitH may very well be this Ca\(^{2+}\)-citrate uptake system.

CitM is the transporter involved in the growth of B. subtilis on citrate and isocitrate as sole carbon source (Warner et al. unpublished results, (46)). This was shown by using a CitM deletion strain. It also follows from the fact that CitM expression is induced by both carbon sources in the medium. Additionally, the catabolite repression exerted on CitM expression further confirms this role in the uptake of a carbon and energy source. The effect of CitM expression in the presence of citrate complexed to toxic heavy metals like Ni\(^{2+}\), Zn\(^{2+}\) and Co\(^{2+}\), on the growth of B. subtilis was studied (Krom, Huttinga, Warner and Lolkema, submitted for publication). It was shown that CitM activity in the presence of citrate complexed to toxic heavy metals dramatically increased the toxic effects of these metals. The fact that CitM expression is induced best by Mg\(^{2+}\)-citrate if compared to Ni\(^{2+}\), Zn\(^{2+}\) and Co\(^{2+}\)-citrate complexes, as well as the toxic effects in the presence
of these heavy metal-citrate complexes, indicate that Mg\(^{2+}\)-citrate is most likely the natural substrate for CitM.

Bergsma and Konings studied Me\(^{2+}\) dependent citrate uptake in membrane vesicles prepared from citrate induced \textit{B. subtilis} cells (5). At that time it was not recognized that citrate transport in \textit{B. subtilis} could be mediated by at least three different transporter proteins. Most of the data reported by Bergsma and Konings is in line with the data found with the cloned system, i.e. the affinities of CitM for Mg-citrate are in the same range. The observed metal dependence is best explained by expression of both CitM and CitH at the same time. In the mentioned study the authors used a different \textit{B. subtilis} strain, \textit{B. subtilis} W23, which might explain the prominent presence of CitH that was not observed in our studies using \textit{B. subtilis} 168 (22).

\textbf{DctA family}

Several families of secondary transporters that transport C\(_4\)-dicarboxylates can be distinguished. In bacteria, four families are specific for C\(_4\)-dicarboxylates, the DctA family, members of which are expressed under aerobic conditions, and three Deu families, DeuA, DeuB and DeuC, members of which are expressed under anoxic or anaerobic conditions. No homologues of either one of the Deu families were identified in the genome of \textit{B. subtilis}, while one homologue of the DctA family was found, and, erroneously, designated DctP (1). The DctA family is actually a subfamily of the large dicarboxylate/aminoacid:cation symporter (DAACS (14, 34)) family of transporters found in bacteria, archaea and eukaryotes. The bacterial DctA subfamily homologues share at least 40% identical residues (36) and are found in Gram-positive and Gram-negative aerobic or facultative anaerobic bacteria. All members use either H\(^+\) or Na\(^+\) as coupling ion and are around 450 amino acids in length (for a recent review see (14)). For \textit{Rhizobium meliloti} DctA a 12 TMS model has been reported using the LacZ fusion method (15). Members of the glutamate transporter subfamily of the DAACS family share between 27% and 40% amino acid sequence identity with members of the DctA family. These proteins contain 8 TMSs and two reentrant loop-like structures that resemble the pore-loop structures found in ion channels (37, 38). Because of the high identity between GltT and the DctA subfamily and a very similar hydropathy profile, a similar membrane topology most likely applies to members of the DctA subfamily. For symbiotic \textit{Rhizobium} strains, for which the host provides malate and other C\(_4\)-dicarboxylates, DctA transporters are essential. DctA transporters of Gram-positive bacteria have not been characterized in great detail.

\textit{Characterization}

To date no cloning and functional characterization of DctP has been reported. Also no uptake experiments in whole cells using one of the deletion strains have been described. However, some characteristics of DctP can be deduced from a study with membrane vesicles of \textit{B. subtilis} (6). Transport of L-malate, fumarate and succinate in these membrane vesicles is sensitive to uncouplers. The
substrate specificity show an apparent affinity for L-malate of 13.5 µM, for fumarate of 7.5 µM and for succinate of 4.3 µM. It should be noted that the results are based on studies in a very undefined system in which it is unclear whether additional C₄-dicarboxylate transporters are present.

Regulation of expression

Expression of dctP can be found in minimal medium in the presence of a small amount of yeast extract. A functional CRE sequence located in the promoter region of dctP has been identified (27) indicating that expression is under control of carbon catabolite repression by f.i. glucose. Interestingly induction is also prevented by the addition of L-malate in the growth medium while this is one of the substrates of the transporter (1). It is remarkable to see that the substrates succinate and fumarate do not induce transcription of the transporter above levels found when B. subtilis is grown on minimal medium with a small amount of yeast extract. Possibly the amount of inducer in yeast extract is high enough to yield maximal expression levels. Induction of expression is regulated via a two-component sensor system, DctS and DctR, encoded by the ydbFG genes (Figure 2) and requires the periplasmic binding protein DctB. DctB, a homologue of the binding protein of the binding protein dependent secondary transporter family (or TRAP family), is essential for induction of the operon. It was postulated that this binding protein binds the substrate with high affinity and activates the two-component system (1, 32).

DctP is required for growth of B. subtilis on fumarate and succinate, but not on L-malate. The auxotrophic phenotypes for succinate and fumarate, suc⁻ and fum⁻, respectively, were also obtained using a binding protein deletion (dctB⁻) strain. However, this binding protein is not involved in a transport process. Expression from an inducible promoter of the transporter, DctP, alone could complement the fum⁻/suc⁻ phenotypes of B. subtilis strain dctB⁻ (1).

Physiological Function

DctP is likely the C₄-dicarboxylate transporter that is expressed in cells grown on yeast extract (6, 9, 10, 45). Interestingly in one of these studies a dicarboxylate binding protein has been identified, purified and characterized (18). This binding protein has an apparent molecular mass of 41 kDa and can be extracted from membrane vesicles with detergents indicating a membrane anchor. The purified binding protein was shown to bind L-malate. However, the role of this
protein in the transport of L-malate in vesicles of *B. subtilis* is unclear. The predicted molecular mass of DctB is 40 kDa and the protein has a very hydrophobic N-terminus indicative of a putative TMS. It is possible that DctB is the \( C_4 \)-dicarboxylate binding protein described previously (18).

**2HCT family**

The 2-hydroxycarboxylate transporter (2HCT) family currently consists of 17 members. They are found solely in bacteria and some members of the 2HCT family have been studied extensively. The family contains Na\(^+\) and H\(^+\)-symporters (CitS of *K. pneumoniae*, MaeN and CimH of *B. subtilis* and MaeP of *Streptococcus bovis* (17, 39, 44), but also citrate/lactate and malate/lactate exchangers found in lactic acid bacteria (CitP of *Lc. mesenteroides* and MleP of *L. lactis* (2, 25)). The latter transporters are involved in secondary metabolic energy generation under physiological conditions, (citrolactic and malolactic fermentation respectively (24, 26, 30)). Substrate specificity studies revealed that the symporters in the family are very specific for only citrate or L-malate, while the exchangers catalyze transport of a wide range of 2-hydroxycarboxylates (2).

Detailed studies have been performed on several members of the 2-HCT family. CitS has 12 hydrophobic regions in the amino acid sequence, but only 11 span the membrane (40, 41). Studies on the exchangers CitP of *L. mesenteroides* and MleP of *L. lactis* revealed that the last cytoplasmic loop and the C-terminal TMS are involved in substrate specificity (4). A conserved arginine within the last TMS was shown to be involved in substrate binding (Krom and Lolkema, submitted for publication, (3)). Two conserved residues that are close to this arginine are located in a cytoplasmic loop, which is accessible from the outside. It is proposed that this cytoplasmic loop forms a pore-loop structure (Krom and Lolkema, submitted for publication). Two members of the 2HCT family, MaeN and CimH, are found in *B. subtilis* and have been functionally characterized to different extents.

**Characterization**

MaeN, designated YufR in the Subtilist database, shows the highest identity to MaeP of *S. bovis*, a H\(^+\) dependent L-malate transporter (17). MaeN was cloned and functionally expressed in *E. coli* (44) and was shown to code for a Na\(^+\) coupled L-malate transporter. *E. coli* cells deficient in malate transport could be complemented for transport of L-malate by MaeN expression. The cells were unable to grow on minimal medium containing fumarate, succinate or citrate, indicating that these di- and tricarboxylates are not substrates of MaeN (44).

CimH, designated YxkJ in the Subtilist database, has been cloned and functionally expressed in *E. coli* (21). *E. coli* cells deficient either in malate or citrate transport could be complemented by expression of CimH, indicating that both citrate and L-malate are substrates of this transporter. CimH is capable of citrate and L-malate symport in membrane vesicles, while L-citramalate binds to the protein but is not transported. Kinetic analysis revealed a high \( K_{\text{app}} \) for citrate, but a low \( V_{\text{max}} \), while the \( K_{\text{app}} \) for L-malate is low, but the \( V_{\text{max}} \) high. CimH is therefore a high affinity low capacity citrate transporter and a low affinity high
capacity L-malate transporter. Citrate is transported in the free anionic state, in contrast to two other citrate transporters of *B. subtilis* (see MeCit family) that require divalent cations for transport. Like most 2-HCT members, CimH is highly stereoselective, recognizing the S-, but not the R-enantiomers of malate and citramalate. The driving force for transport has been shown to be the ΔpH component of the pmf and the ΔΨ appears to be not involved. This indicates that transport is electroneutral. At pH 6, the conditions of the experiments, divalent citrate and malate are the most prevalent anionic species, which suggests that two protons are co-transported.

**Regulation of expression**

![Figure 3. Schematic overview of the DNA region that contains maeN. Arrows indicate the direction of transcription, putative terminators of transcription are indicated by loop structures.](image)

A preliminary study on the expression pattern of *maeN* has indicated that expression is induced by L-malate in the medium (1). A two component sensor system has not been linked to the induction of MaeN expression, although the *yufLM* genes are located close to *maeN* on the chromosome (Figure 3). Based on amino acid sequence identity the sensor, YufL, is clustered in the CitA family of sensors for C₄-dicarboxylates (14) together with the C₄-carboxylate sensors DcuS of *E. coli* (35% identical residues), and citrate sensors CitS (31% identical residues) and CitA (25% identical residues) of *B. subtilis* and *K. pneumoniae*, respectively (16, 46). Remarkably, a DNA array study did not identify *maeN* as a potential target for YufL regulation (19). Although MaeN is most likely the transporter induced by, and responsible for growth of *B. subtilis* on L-malate (9, 10, 45) some observations remain unexplained. Firstly, none of 24 two component regulatory systems analyzed could be linked to induction of MaeN expression. Secondly, the reported induction by L-malate in the medium was subject to carbon catabolite repression (10), but no typical CRE sequence can be found in the *maeN* promoter region (27).

To study the expression of *cimH*, the promoter of *cimH* was fused upstream of the gene for β-galactosidase in the previously mentioned LacZ-fusion approach. Cultures on plates supplemented with di- or tricarboxylates did not show induction of expression. However, as observed for *citH* (see MeCit family), prolonged incubation of the plates resulted in LacZ activity irrespective of the carbon source used. Either *cimH* expression is growth phase dependent, or is expressed at a very low constitutive level. This last option seems likely since transport of anionic citrate in the presence of EDTA was observed in *B. subtilis* in the exponential growth phase (22). The promoter of *cimH* contains a functional CRE sequence suggesting that expression is subject to Carbon Catabolite
Repression (27). Interestingly, a DNA sequence can be identified in the promoter region of both citH, of the MeCit family, and cimH of the 2HCT family, which is located at exactly the same position before the translation starting point (Figure 4). Another two genes in B. subtilis share the same sequence at exactly the same position; dctB, the C₂-dicarboxylate periplasmic binding protein involved in induction of dctP (see DctA subfamily), and mleA, encoding the malolactic enzyme found in the mle operon (see NhaC family below). This identical region in the promoters of these four genes involved in carboxylate transport and metabolism could indicate that these genes are regulated in a similar fashion.

**Figure 4.** Alignment of 50 bp of the promoter region upstream of the ATG start codon (indicated with an arrow) of four ORFs involved in carboxylate transport and metabolism in B. subtilis. Alignment was prepared using the ClustalX program. Conserved nucleotides are marked with a star.

**Physiological Function**

The 2HCT family contains proteins that function as symporters or that can function as precursor/product exchangers. The latter are involved in the generation of metabolic energy in lactic acid bacteria, (24, 26, 30). MaeN and CimH probably function as L-malate and citrate/L-malate symporters, respectively, under physiological conditions (21, 44). The observation that CimH is a high affinity low capacity citrate transporter and a low affinity high capacity L-malate transporter is remarkable. This could mean that the physiological function of CimH is two-fold. It could be a citrate transporter under conditions where the other citrate transporters, CitM and CitH (see MeCit family) are not sufficient because citrate concentrations are too low. It is known that microorganisms contain often both high and low affinity transporters for the same substrate and that these are differentially expressed dependent on the external concentration of the substrate (f.i. the iron transporters of Candida albicans, (33)). In a similar way, CimH could function as a L-malate transporter when higher concentrations of L-malate are present, while MaeN is present under conditions of low L-malate concentrations. These hypotheses remain to be tested.

**NhaC family**

In general, the function of Na⁺/H⁺ antiporters is believed to lie in regulation of internal pH in circumstances of alkaline external pH as well as in Na⁺ homeostasis. In addition Na⁺/H⁺ antiporters have a role in Na⁺ resistance (29). In B. subtilis only members of the NhaC family are present (35). The NhaC family of Na⁺/H⁺ antiporters is relatively small and found in Gram positive and Gram negative bacteria and in archaea. Two members have
been characterized (13, 44). The proteins are around 460 amino acids long and contain 12 putative TMSs. *B. subtilis* contains two proteins that are members of this family (35), YheL that functions as a genuine Na\(^+\)/H\(^+\) antiporter (and is not further discussed here), and MleN, that couples Na\(^+\)/H\(^+\) exchange to the exchange of malate and lactate (44).

**Characterization**

MleN, designated YqkI in the Subtilist database, was cloned and functionally expressed in *E. coli*. Everted membrane vesicles expressing MleN showed that Na\(^+\)-lactate uptake was strictly dependent on the presence of intravesicular L-malate. This exchange in everted membrane vesicles was ΔpH dependent, indicating that exchange involves movement of one or more protons. Furthermore, MleN catalyzed electroneutral exchange, between malate (outside) and lactate (inside). It was concluded that MleN catalyzes most likely 2H\(^+\)-malate\(^-\)/Na\(^+\)-lactate\(^-\) exchange.

**Regulation of Expression**

No information is available on the regulation of expression of *mleN*. Mostly based on the proposed physiological function (see below), it was suggested that *mleN* expression would be induced under low pmf conditions. Interestingly, the gene *mleA*, in the same operon as *mleN* (Figure 5) contains the same DNA sequence in the promoter region as found for *citH* of the MeCit family and *cimH* of the 2HCT family (Figure 4). This suggests a similar regulation of expression for these genes.
Physiological function

**Figure 5.** Schematic representation of the DNA region that contains the *mle* operon. The arrow indicates the direction of transcription. A putative terminator of transcription is indicated with a loop structure.

*MleN* is found in an operon with ORF *mleA* (Figure 5). *MleA* is predicted to be a malolactic enzyme that converts malate directly into lactate and CO₂. The following novel malolactic fermentation pathway was proposed. H₂-malate enters the cell via malate-lactate antiport catalyzed by *MleN* and is converted into lactate and CO₂ by *MleA*. The conversion of cytoplasmic malate to lactate requires the consumption of a cytoplasmic proton. Lactate⁻ is exchanged together with one Na⁺ for external H₂-malate in an electroneutral transport step. The net entry of one proton (two enter with malate and one is consumed by the decarboxylation step) result in acidification of the cytoplasm. The process results furthermore in extrusion of one Na⁺ that might be important for maintaining the Na⁺ homeostasis of the cell. It is proposed that *B. subtilis* uses this pathway as an alternative Na⁺/H⁺ antiport mechanism in Na⁺ resistance under conditions of low pmf (44). It has been shown that *B. subtilis* grown on malate under conditions of low pmf requires MleN activity for growth. Until now, this is the first and only report of coupling of Na⁺ efflux to product-precursor exchange.

**Miscellaneous transporters**

The genome of *B. subtilis* (23) contains many proteins with unknown functions. Based on homology to proteins with known function, putative transporters of di- and tricarboxylates may be assigned. Only one additional transporter was identified that could potentially be involved in growth of *B. subtilis* on di- and tricarboxylates. Two other ORF’s are annotated in the Subtilist database as putative α-ketoglutarate transporters, but are probably not.

*YflS* is located close to the *citM* gene (see Figure 1) on the genome of *B. subtilis*. The product of this gene belongs to the ubiquitous family of divalent anion:Na⁺ symporters (DASS (35)) and all members are around 450 amino acids in size. They contain 12 hydrophobic regions indicative of 12 putative TMSs (31). Members of this family transport inorganic anions, like sulphate and phosphate, but also organic anions like di- and tricarboxylates and acidic amino acids. The product of *yflS* is homologous to CitT, the citrate-succinate antiporter of *E. coli* that is expressed under anaerobic condition (31). CitT is located in an operon involved in the metabolism of citrate under anaerobic condition similar to the operon for anaerobic citrate metabolism in *K. pneumoniae*. In the latter case, CitS, belonging to the 2HCT family is the citrate transport protein. Genes for anaerobic citrate
metabolism, like citrate lyase that converts citrate into oxaloacetate (8), and oxaloacetate decarboxylase involved in Na$^+$ pumping in K. pneumoniae, are not present in the genome of B. subtilis. This means that B. subtilis is probably not capable of anaerobic growth on citrate which makes it unlikely that YflS is involved in citrate-succinate antiport as is CitT of E. coli. YflS has the highest identity with SOT1 found in chloroplasts of Spinacia oleraceae (35% identity to CitT, 53% to SOT1). SOT1 has been extensively characterized (43) and was shown to import α-ketoglutarate in exchange for stromal malate. Besides malate also succinate, fumarate and α-ketoglutarate could be used as counter ions. Possibly YflS transports one of the substrates.

Two gene products, YoaB and CsbX, are homologous proteins that have been annotated in the Subtilist database (http://genolist.pasteur.fr/SubtiList/) to transport α-ketoglutarate. However, no sequence homology can be found with known α-ketoglutarate transporters e.g. KgtP of E. coli. Homology searches using BLAST show that the proteins are more homologous to the ribitol transporter, RbtT of E. coli and K. pneumoniae and arabinitol transporter DalT of K. pneumoniae. We therefor conclude that both CsbX and YoaB are most likely not involved in transport of di- or tricarboxylates in B. subtilis.

**Conclusion**

Transport of TCA intermediates in B. subtilis is catalyzed by both proton and sodium dependent secondary transporters. A variety of protein families are involved and overlapping substrate specificities between several transporters has been found (Table 1). Multiple transport systems thus could be involved in growth on a certain substrate as sole carbon and energy source. No known or putative transport system for oxaloacetate, cis-aconitate and α-ketoglutarate could be identified in the genome sequence of B. subtilis (23).

<table>
<thead>
<tr>
<th>TCA intermediate</th>
<th>Confirmed transporter</th>
<th>Possible transporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>citrate</td>
<td>CitM, CitH and CimH</td>
<td>YraO and YflS</td>
</tr>
<tr>
<td>cis-aconitate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>isocitrate</td>
<td>CitM</td>
<td>CitH and YraO</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>succinate</td>
<td>DctP</td>
<td>YflS</td>
</tr>
<tr>
<td>fumarate</td>
<td>DctP</td>
<td>YflS</td>
</tr>
<tr>
<td>malate</td>
<td>MaeN, MleN, DctP and CimH</td>
<td>YflS</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In conclusion, DctP probably mediates the observed transport of all three C₄-dicarboxylates, while the L-malate induced system probably represents MeaN activity. The C₄-dicarboxylate binding protein (18), most likely DctB, is involved in regulation of expression of DctP but not in the transport process. For citrate the Me$^{2+}$ mediated uptake is mediated by two transporters CitM and CitH of the MeCit family, while the uptake of free citrate is mediated by CimH.
References


