Dicarboxylate transport by rhizobia

Svetlana N. Yurgel a,*, Michael L. Kahn a,b

a Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA
b Department of Molecular Biosciences, Washington State University, Pullman, WA 99164-6340, USA

Received 11 September 2003; received in revised form 3 January 2004; accepted 4 April 2004
First published online 8 May 2004

Abstract

Soil bacteria collectively known as rhizobia are able to convert atmospheric dinitrogen to ammonia while participating in a symbiotic association with legume plants. This capability has made the bacteria an attractive research subject at many levels of investigation, especially since physiological and metabolic specialization are central to this ecological niche. Dicarboxylate transport plays an important role in the operation of an effective, nitrogen-fixing symbiosis and considerable evidence suggests that dicarboxylates are a major energy and carbon source for the nitrogen-fixing rhizobia. The dicarboxylate transport (Dct) system responsible for importing these compounds generally consists of a dicarboxylate carrier protein, DctA, and a two component kinase regulatory system, DctB/DctD. DctA and DctB/DctD differ in the substrates that they recognize and a model for substrate recognition by DctA and DctB is discussed. In some rhizobia, DctA expression can be induced during symbiosis in the absence of DctB/DctD by an alternative, uncharacterized, mechanism. The DctA protein belongs to a subgroup of the glutamate transporter family now thought to have an unusual structure that combines aspects of permeases and ion channels. While the structure of C4-dicarboxylate transporters has not been analyzed in detail, mutagenesis of S. meliloti DctA has produced results consistent with the alignment of the rhizobial protein with the more characterized bacterial and eukaryotic glutamate transporters in this family.

© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Rhizobium; Dicarboxylate transport; Glutamate transporter; DctA

Contents

1. Introduction ......................................................... 490
2. The use of dicarboxylic acids by free-living and symbiotic forms of rhizobium ................................. 491
3. Regulation of rhizobial dicarboxylate transport system ................................................................. 491
   3.1. The structure of dicarboxylate transport system ......................................................... 491
   3.2. The DctB and DctD two component regulatory system .............................................. 492
   3.3. Regulation of dctA expression in free-living conditions ............................................. 492
   3.4. Regulation of dctA expression during symbiosis ............................................................. 493
4. Substrate specificity of the Dct system ...................................................................................... 494
   4.1. DctA substrate specificity ................................................................................................. 494
   4.2. Mechanism of substrate recognition by DctA and DctB ............................................... 494
5. C4-dicarboxylate carrier ............................................................................................................. 495
   5.1. The family ....................................................................................................................... 495
   5.2. Membrane topology ....................................................................................................... 497
   5.3. Conserved motifs ............................................................................................................ 497

*Corresponding author. Tel.: +1-509-335-8327; fax: +1-509-335-8617.
E-mail address: syurgel@wsu.edu (S.N. Yurgel).
1. Introduction

Rhizobia are gram-negative soil bacteria that share the ability to form nitrogen-fixing symbiotic associations with legume plants. Most genera, like Sinorhizobium, Rhizobium, Bradyrhizobium, Mesorhizobium and Azorhizobium, belong to the β-proteobacteria order Rhizobiales but recently it was shown that some β-proteobacteria can also participate in this kind of relationship [1]. Establishing a fully functional symbiosis requires the successful completion of numerous steps that lead from recognition signals exchanged between the plant and bacteria to the differentiation and operation of root nodules, the plant organs in which nitrogen fixation takes place. The initial sensing of the two organisms by each other starts with the release of root exudates by the plant that include flavonoids and nutrients such as organic acids and amino acids. Rhizobia are chemotactic toward these exudates and respond to a characteristic plant flavonoid composition by initiating the synthesis of specific lipo-chitooligosaccharides, which can stimulate root cell division by the plant. In some tropical legumes, entry of the bacteria into the plant is enhanced by soybean bacteroids in vitro. Chemically derived mutants of Rhizobium leguminosarum biovar trifolii that were unable to transport C4-dicarboxylic acids were able to form nodules on clover but these nodules were unable to fix nitrogen and were described as ineffective [9]. This work triggered research in many systems showing that mutations that interfere with dicarboxylate transport universally block nitrogen fixation [10–20]. Although in some of these interactions, the defect in dicarboxylate transport leads to defects in nodule development, in many legumes the nodules appear to be filled with bacteroids and to develop normally until the time that nitrogen fixation would have begun. It has therefore been argued that the C4-dicarboxylates are the primary carbon source used by nitrogen fixing bacteroids in fixing nitrogen. We discuss the role of dicarboxylates in free-living and symbiotic forms of rhizobia in more detail below.

In 1984, Ronson and co-workers cloned and analyzed the genes that code for the Rhizobium leguminosarum biovar trifolii C4-dicarboxylate transport (Dct) system [21]. Three dct loci were identified, dctA, dctB and dctD. Ronson et al. suggested that dctA encoded a structural protein necessary for C4-dicarboxylate transport, whereas dctB and dctD encoded positive regulatory elements. Later work has provided complete support for these assignments and established that homologs of dctA, dctB and dctD are present in many (if not all) of the rhizobia [13,20,22–38]. DNA sequencing has also found proteins related to all three proteins in many other bacteria but, since DctA belongs to a family that includes transporters with a different substrate specificity and the two-component regulatory system family of proteins can have diverse regulatory functions, the functional significance of these proteins requires direct investigation.

The dctB and dctD genes code for a two-component regulatory system that responds to the presence of C4-dicarboxylates and regulates expression of the transport protein encoded by dctA, with DctB as the sensor pro-
tein and DctD as the response regulator. The structure and mechanism of action of both DctB and DctD proteins have been well studied [25,26,39–42]. On the other hand, only one paper has been published on the structure and membrane topology of the C4-dicarboxylate permease (DctA) [43], and there has been little direct information concerning the mechanism of substrate recognition and transport by DctA. In this review we will examine DctA in the light of work with more thoroughly studied members of the glutamate transport family, in which DctA is now classified. We also will discuss issues related to the substrate specificity of DctA and mechanism of substrate recognition by both DctA and DctB.

2. The use of dicarboxylic acids by free-living and symbiotic forms of rhizobium

C4-dicarboxylic acids, including malate, succinate, fumarate, aspartate and oxaloacetate, are common metabolites produced and consumed by various microorganisms. For aerobes like the rhizobia, these compounds can be used as an energy source, usually by oxidizing them to CO2 in the citric acid cycle. They can also be used as a carbon source through various reactions leading to pyruvate and gluconeogenesis. C4-dicarboxylates seem to play a special role in rhizobial metabolism as indicated by their ability to repress the utilization of other carbon sources [44,45]. Succinate, malate, and fumarate are also moderately potent chemotactants for rhizobia and appear to share a common chemoreceptor [46]. In some bacteria, fumarate, malate and aspartate can also be used as terminal electron acceptors during anaerobic growth but the recently published genomic DNA sequences of S. meliloti and M. loti do not contain homologs encoding fumarate reductase, a key enzyme in this use [47,48].

Rhizobium strains deficient in C4-dicarboxylate DctA permease fail to grow on the substrates listed above and are unable to accumulate [14C]-succinate above background levels [9,11–13,15,20,21,49]. In S. meliloti, this suggests that DctA is the sole uptake pathway for dicarboxylates and that the dctMQP-like [50] gene cluster in the S. meliloti genome and a dctO-like open reading frame found on pSymB are not involved in dicarboxylate transport. Our attempts to find pseudorevertants of a S. meliloti dctA deletion mutant able to grow on succinate or malate have been unsuccessful, which indicates that modifying these genes or others to substitute for DctA is a difficult task. dctA mutants are not impaired in their use of other carbon sources [14] and the only other phenotypic change that has been observed in the mutants is a slightly reduced chemotactic response to succinate [46]. This suggests that the C4-Dct system is peripheral to free-living carbon metabolism.

In contrast, C4-dicarboxylates appear to play a central role in symbiosis – they are a major carbon source provided to bacteroids by the host plant and, as noted above, are able to support the highest levels of nitrogen fixation in vitro by isolated bacteroids. In becoming bacteroids, rhizobia switch their metabolism to a completely different mode in which numerous genes are expressed that are silent during free-living growth, even in low oxygen conditions, and proteins are produced that appear to carry out unique bacteroid functions, including nitrogen fixation. At the same time, many genes used in free-living growth are repressed.

Analysis of the symbiotic phenotype of rhizobial dicarboxylate permease mutants showed the importance of C4-dicarboxylates in symbiotic nitrogen fixation. Mutants with a defective dicarboxylate transporter generally retain the ability to nodulate a host plant but the nodules formed are unable to fix nitrogen [10–12,15,19,20,49]. Legume plants inoculated with rhizobia that lack a dicarboxylate permease usually form nodules containing what appear to be normally differentiated bacteroids. (For an example where differentiation is not normal see van Slooten et al. [33]). However, nodules formed by dctA mutants senesce rapidly and are unable to fix nitrogen. When isolated from the nodules the bacteroids fail to accumulate labeled succinate [12,15]. The ineffective nodules contain more starch, located in both infected and uninfected plant cells, than do nodules induced by wild type rhizobia [9,10,15,20]. Thus, it appears that, while the plant is willing to make more carbon compounds available to mutant bacteroids, the carbohydrate precursors accumulate when bacteroids cannot act as a dicarboxylate sink.

Nodules induced on alfalfa by S. meliloti with partial ability to transport succinate accumulated even larger amounts of starch than nodules induced by strains completely unable to transport succinate [51]. This difference in starch accumulation suggested to us that the uptake and metabolism of dicarboxylates even at a low level allows S. meliloti to partially transform to functional bacteroids. If these partially defective mutants can carry out a more natural cross-talk with the host-plant, the plant may increase the amount of sucrose provided to the nodules even though the result is still a defective symbiosis.

3. Regulation of rhizobial dicarboxylate transport system

3.1. The structure of dicarboxylate transport system

The dicarboxylate transport system is similar among most members of the Rhizobiaceae family and consists of three genes, dctA, dctB and dctD. dctA is closely linked to but transcribed divergently from dctB and dctD [11,21,52]. In Rhizobium leguminosarum, dctB and
dctD compose a single transcriptional unit [53] while, in S. meliloti, dctD might have its own promoter [37,38,54]. Comparison of the promoter regions of S. meliloti and R. leguminosarum dctA revealed a high degree homology between the regions but, in contrast to R. leguminosarum, two possible ATG start codons are found in S. meliloti and each is preceded by a good consensus ribosome binding site. The second of these is the conserved ATG and it has been argued that this is the more likely start site [36].

Many members of the Rhizobiaceae have this single dicarboxylate transport system, but there are exceptions. Rhizobium sp. NGR234 has two systems that can transport C4-dicarboxylates. The homolog to dctA is located on the symbiotic plasmid but a second, uncharacterized locus that can confer growth on dicarboxylates is located on chromosome. No homology to dctB was found upstream of the NGR234 dctA promoter. Both systems support the growth of NGR234 on dicarboxylates but only the dctA-like system is able to support nitrogen fixation in symbiosis [33]. Rhizobium tropici, which has a dctABD system located on a megaplasmid, also has a second succinate transport system but again, there was no evidence presented that this system can enable symbiotic nitrogen fixation [52]. The Mesorhizobium loti genomic sequence has two genes with over 70% sequence identity to S. meliloti dctA and the B. japonicum DNA sequence has six genes with 45–55% sequence identity to S. meliloti dctA. Which of these contribute to dicarboxylate transport is unknown.

3.2. The DctB and DctD two component regulatory system

Since the rhizobial DctB/DctD system has recently been reviewed by Janausch et al. [55] and the mechanism of action of the $\sigma^{54}$ bacterial enhancer-binding protein family that includes DctD has been discussed by Morett et al. [56], our discussion of it will be brief. To adapt to their environment, bacteria must have systems that allow them to monitor relevant signals and respond appropriately to the changes they sense. Two-component regulatory systems consisting of a sensor kinase and cytoplasmic response regulator are common in rhizobia. The sensor kinase, which is often a transmembrane protein, interacts with an environmental cue and transmits a phosphorylation signal to the regulator, which then alters the expression of appropriate genes. A classic example of this kind of regulator/sensor couple are the DctB/DctD proteins which, in fact, were one of the first of these systems identified [57]. Related regulatory proteins include the ntrB/ntrC nitrogen regulatory system [58,59]; regulatory systems responding to osmolarity (envZ/ompR) [60], phosphate limitation (phoR/phoB) [61] in E. coli; genes controlling the virulence of Agrobacterium tumefaciens in response to plant exudates (virC) [62]; and genes regulating bacterial chemotaxis [63]. A particularly well studied member of the family is the rhizobial FixL/FixJ system, which regulates the expression of N2-fixation genes in rhizobia as function of the interaction of oxygen with a heme ligand in FixL [64–67].

The sensor kinase proteins in the family have a conserved region of about 200 amino acids in their C-termini and the regulators have a conserved region in their N-termini [31] (Fig. 1). Homology among the activator proteins is not restricted to the N-termini and, for example, the C-terminal region of DctD shares homology with the nitrogen regulatory protein NtrC and the nitrogen-fixation specific regulator NifA, which regulates the induction of several genes in response to microaerobiosis in S. meliloti and in several other Rhizobium and Bradyrhizobium species [31]. One of mechanism responsible for stimulus–response coupling involves altering the rate of autophosphorylation or dephosphorylation of the sensor protein at a histidine residue in response to the appropriate signal and transfer of the phosphate to an aspartate residue in the response regulator protein [68]. The phosphorylation status of the response regulator protein determines its ability of initiate transcription of regulated gene(s) [69]. This mechanism was shown to be involved in signal transduction between DctB and DctD [40].

3.3. Regulation of dctA expression in free-living conditions

A model for dctA regulation was first proposed by Ronson et al. [32] and has been developed in other work
[22,25,27,53,70,38]. In this model, DctB, DctD and RpoN (NtrA) are required to initiate expression of dctA in free-living rhizobia. The presence of RpoN is also required for transcriptional activation by NtrC and NifA. R. meliloti rpoN mutants exhibit a pleiotropic phenotype: they fail to grow on nitrate as sole nitrogen source or succinate as sole carbon source and form Fix− nodules [71]. The requirement of RpoN can be explained by the fact that RpoN encodes σ^54, a sigma factor that acts together with the core RNA polymerase to recognize specific promoter sequences [72,73].

Transcription from the dctA promoter is dependent on σ^54 RNA polymerase, which binds to the promoter site (−12, −24) [22], but in the absence of activated DctD is unable to denature the DNA and form an open transcription complex. In free living conditions, DctB and DctD are constitutively expressed at a low level [32]. When C4-dicarboxylates are present in the periplasm, DctB phosphorylates DctD [39] which then binds specifically and cooperatively to tandem upstream activator sequences (UAS) located in the −94 to −154 region upstream of the dctA start [25,26,74]. Phosphorylated DctD then activates transcription of dctA by catalyzing the ATP-dependent transition of a closed complex between σ^54 RNA polymerase to an open complex [28,34,35,71,75].

However, there are several nuances which must be taken into consideration. The first one is that in a dctA mutant background, the dctA promoter is constitutive. One possible explanation is that, in the absence of substrates, DctA and DctB interact with each other in the cytoplasmic membrane to prevent activation of DctB. Therefore, DctB would always be activated in a dctA strain [37,38,76]. It was later shown that DctA modifies signal detection by DctB such that the DctB ligand specificity is broader in the absence of DctA [53]. Blocking constitutive, DctB/DctD independent, transcription of dctA results in improper signaling by DctB/DctD. A S. meliloti strain carrying a transposon insertion in the dctA–dctB intergenic region was defective in nitrogen fixation. This mutant grew poorly on ammonia as nitrogen source when DctB/DctD were activated by the presence of C4-dicarboxylates. Increased expression of DctA restores control of DctB/DctD. This indicates that DctA contributes to the signaling specificity of DctB-DctD and attenuates cross-talk with other operons [53].

A second subtlety concerns cross-talk between two component regulatory systems. NtrC is the response regulator that corresponds to DctD in the NtrB/NtrC nitrogen regulatory system, [58,59]. In an E. coli background, NtrB/NtrC could affect transcription from a rhizobia dctA promoter under various physiological conditions [77]. Even though wild-type NtrC does not normally appear to activate transcription of dctA in Rhizobium, a spontaneous ntrC mutant was able to activate transcription from the dctA promoter and other σ^54 dependent promoters in S. meliloti [24]. Further analysis of the effect of the ntrC mutation on dctA expression in the presence and the absence of DctB/DctB suggested that in uninduced cells, inactive DctD bound to the dctA promoter and prevented its activation by the ntrC mutant. Moreover, overexpression of DctD prevented dctA expression and resulted in a Dct− phenotype [23].

Activation of a σ^54 dependent promoter requires interaction between σ^54 RNA polymerase and a response regulator that binds to a UAS, typically about 80–100 bases upstream of the promoter site. This brings the response regulator into the proper context with the closed promoter complex via a DNA loop [78,79]. This interaction can be influenced by the binding of integration host factor (IHF) to site(s) between the UAS and σ^54 dependent promoter [80]. Analysis of the dctA promoter region of Rhizobium leguminosarum identified several potential IHF binding sites adjacent to the promoter and it was shown that E. coli IHF stimulates DctD-mediated transcription from the R. leguminosarum dctA promoter. Proteins similar to the IHF subunits have been identified in the S. meliloti genome. These data suggest that R. leguminosarum has an IHF homolog that stimulates DctD-mediated transcription activation from the dctA promoter [81].

3.4. Regulation of dctA expression during symbiosis

In many rhizobia, mutations in dctBD lead to ineffective nodules but nodules induced by S. meliloti strains with mutations in the dctBD genes can fix nitrogen at nearly wild-type rate [13,37,38]. In this symbiosis DctA is expressed even in the absence of DctB and DctD, implying that operation of DctB/DctD not the only way to activate the dctA promoter. This alternative symbiotic activation (ASA) has been documented extensively but its mechanism has not been identified. A dctA::lacZ gene fusion is expressed in the nodules induced by dctD deficient S. meliloti [36,82] but the pattern of DctB/DctD-independent dctA expression during symbiosis differs from DctB/DctD-dependent expression. While DctB/DctD-dependent dctA expression occurs in the infection zone (II) and in the fixation zone (III) of the nodules, DctB/DctD-independent expression is not observed in zone II and starts in the interzone between II and III, where there is a transition from early to late symbiotic bacteroid development. Efficient activation of the dctA promoter by the ASA requires cis-acting regulatory elements located in the S′ one-third of the dctA coding region while, for DctB/DctD dependent activation, the presence of these sequences is not necessary [83].

One obvious candidate for a symbiotic activator of dctA is NifA, the transcriptional activator of the nif genes. NifA activated promoters have a high level of
expression during symbiosis even when their UAS sequences are removed [84,85]. Expression of the nifH structural gene of nitrogenase, which is under NifA control, also starts at the II–III interzone [83]. An observation that may be related to the ASA is that the presence of additional copies of nifA and dctABD increases symbiotic effectiveness in S. meliloti [86], an effect that is not observed when dctA is constitutively expressed [30]. The presence of a plasmid carried Klebsiella pneumoniae nifA and S. meliloti dctABD in S. inorhizobium fredii also improved symbiotic effectiveness in the strain [87]. On the other hand the presence of S. meliloti dctABD alone could improve nitrogen fixation efficiency of B. japonicum [82,88]. Based on its genome sequence, B. japonicum has several proteins that are similar to DctA and these observations raise the question of what unique features are similar to DctA and these observations raise the question of what unique features S. meliloti DctA or the regulatory proteins bring to the transgenic host.

4. Substrate specificity of the Dct system

4.1. DctA substrate specificity

The widely known and potentially physiologically important substrates for rhizobial Dct systems are succinate, malate, fumarate, aspartate, and oxaloacetate. Other compounds, including D-lactate, 2-methylsuccinate, malate, fumarate, aspartate, and oxaloacetate, are important substrates for rhizobial Dct systems, which are under NifA control, also starts at the II–III interzone [83]. An observation that may be related to the ASA is that the presence of additional copies of nifA and dctABD increases symbiotic effectiveness in S. meliloti [86], an effect that is not observed when dctA is constitutively expressed [30]. The presence of a plasmid carried Klebsiella pneumoniae nifA and S. meliloti dctABD in S. inorhizobium fredii also improved symbiotic effectiveness in B. japonicum [82,88]. Based on its genome sequence, B. japonicum has several proteins that are similar to DctA and these observations raise the question of what unique features S. meliloti DctA or the regulatory proteins bring to the transgenic host.

![Fig. 2. Structure of some compounds recognized by DCT system. (1) succinate; (2) fumarate; (3) maleate; (4) aspartate; (5) orotate; (6) succinamide.](https://example.com/fig2.png)
succinate can mimic both the “stretched/trans” and “folded/cis” conformations. A similar distinction between “stretched” and “folded” versions of glutamate analogs has been noted for substrates of the glutamate transporter members of the transporter family to which DctA belongs [100].

In support of this idea, aspartate is an excellent inducer of DctA and a low-affinity substrate for DctB. Although it is expected to be less rigid than maleate, aspartate also favors a folded conformation with both carboxyl groups on the same side of molecule. While this folded conformation is not locked in place by a double bond, the folded configuration may help make it a poor substrate for DctA with an approximately 600-fold lower affinity for DctA than succinate. In contrast, orotate, which has a rigid stretched conformation between the carboxyl group and the carbonyl group is recognized by DctA but not DctB.

5. C4-dicarboxylate carrier

5.1. The family

Rhizobia DctA proteins belong to a subgroup of the glutamate transporter family called the bacterial C4-dicarboxylate transporter family or the dicarboxylate/cation symporter (DAACS) family [100]. The proteins of the family are secondary transporters that use the free energy of solute or ion gradients to drive transport across a membrane. The typical transporter of this family is made of a single polypeptide that forms several transmembrane α-helices connected by loops. There is micrographic evidence that a pentameric complex of this monomer is the active form of the protein [101]. Members of the glutamate transporter family are found in neurons and glial cells in the mammalian central nervous system where they are essential for terminating synaptic excitation and for maintaining extracellular glutamate concentration below a neurotoxic level. Activation of the N-methyl-D-aspartate receptors by excessive extracellular glutamate is associated numerous neurological disorders. These animal proteins have attracted considerable research attention and, until recently, their bacterial relatives (including the bacterial C4-dicarboxylate and amino acid transporters) were less prominent. In the last part of the review we will look at rhizobial dicarboxylate transport proteins as members of the family and draw a parallel between structure and mechanism of action of DctA and other well studied proteins of the family.

The glutamate transporter family consists of three groups based on their substrate specificity: bacterial 

C4-dicarboxylate transporters, bacterial and eukaryotic glutamate/aspartate transporters and neutral-amino-acid transporters. As we discussed above, the substrates for bacterial C4-dicarboxylate transporters are malate, fumarate, succinate, aspartate, orotate and some of their analogs. Eukaryotic neutral amino acid transporters are divided on two groups. One group can transport alanine, serine, cysteine, and threonine while another has wider substrate specificity, with substrates also including asparagine and glutamine. Eukaryotic and bacterial glutamate transporters can transport glutamate, aspartate and, in addition, some glutamate analogs [102]. The eukaryotic glutamate transporters catalyzed the symport of the substrates with two or three sodium ions and one proton, while a potassium ion is antiported [103]. The bacterial glutamate transporters catalyze the electrogenic symport of glutamate with at least two cations [104]. It is not known which cations are coupled to the transport of substrates by the bacterial dicarboxylate proteins or how many accompany a transport event.

At the present time more than sixty proteins from Eucarya, Bacteria and Archaea belonging to a glutamate transporter family have been identified [100,105]. The length of the proteins varies between 400 and 600 residues, with the bacterial proteins generally shorter than the eukaryotic proteins. Multiple-sequence alignment shows that the difference in the length results mostly from a gap in the loop connecting transmembrane domains four and five but, in addition, there are several gaps in the loop connecting α-helices one and two (Fig. 3). The C-terminal part of the proteins are more highly conserved between members of the family and were important in determining the structure of the phylogenetic tree [100]. The tree includes a subset of 35 members of the family and presented eukaryotic and bacterial glutamate transporters, bacterial C4-dicarboxylate transporters, eukaryotic neutral amino acid transporters and bacterial serine transporters.

![Fig. 3. Model for the membrane topology of the glutamate transporters (Modified from Slotboom et al. [100]). Putative transmembrane domains enclosed in boxes are indicated by numbers 1–8. The regions indicated as 6a and 7a are thought to be intermittent pore domains that can cross the membrane under some circumstances. Motifs A–D are highly conserved regions within the family of related transporters. For more precise positions of the motifs see Fig. 4.](https://academic.oup.com/femsre/article-abstract/28/4/489/553060)
Fig. 4. Multiple sequence alignment of the members of different subfamilies of glutamate transporter family. DCTA_RHIME: C4-dicarboxylate transport protein [Sinorhizobium meliloti]; DCTA_ECOLI: aerobic C4-dicarboxylate transport protein [Escherichia coli]; EAA1_CAEEL: excitatory amino acid transporter (sodium-dependent glutamate/aspartate transporter) [Caenorhabditis elegans]; AAAT_BOVIN: neutral amino acid transporter (sodium-dependent neutral amino acid transporter type 2) [Bos taurus]; SATT_HUMAN: neutral amino acid transporter A (SATT) (alanine/serine/cysteine/threonine transporter) (ASCT1) [Homo sapiens]; GLTP_ECOLI: proton glutamate symport protein (glutamate-aspartate carrier protein) [Escherichia coli]; YGJU_ECOLI: bacterial serine transporter [Escherichia coli]. Consensus key: * – single, fully conserved residue, : – conservation of strong groups, . – conservation of weak groups, – no consensus.
5.2. Membrane topology

Since no three-dimensional structure of these secondary transporters has been solved yet, models presenting their structure are based on computational analysis of their amino acid sequences and on biochemical data. However, the membrane topology of the eukaryotic and bacterial glutamate transporters has been extensively studied by a combination of mutagenesis and labeling approaches [100]. Several studies predicted six hydrophobic segments in the N-terminal part of the protein. The C-terminal half of the protein does not contain clear alternating regions of high and low hydrophobicity and there is still some controversy in the models of this part of the protein. In the most generally accepted model the six N-terminal α-helices are followed by a reentrant loop entering the membrane from the cytoplasm, a seventh membrane-spanning helix, a re-entrant loop entering the membrane from the periplasm and, finally, an eighth amphipathic α-helix, leaving the C-terminus in the cytoplasm (Fig. 3). However, recent evidence was presented that transmembrane domain seven might be reassigned to the reentrant loop [106].

In contrast to the membrane topology of bacterial and eukaryotic glutamate transporters, the structure of C4-dicarboxylate transporters has not been analyzed in detail. Work on the S. meliloti DctA protein by Jording and Puhler [43] using computation analysis and DctA-alkaline phosphatase (PhoA) and DctA-β-galactosidase (LacZ) fusions led them to construct a two dimensional model of DctA that contained 12 transmembrane α-helices with both the amino-terminus and the carboxyl-terminus located in the cytoplasm. While this differs from the eight transmembrane structure presented by Slotboom et al. [100], the novelty of the re-entrant pore motif identified in the latter work, together with structure perturbation by the protein fusions used by Jording and Puhler [43], allow these two structures to be rationalized.

5.3. Conserved motifs

Due to the inclusion of distantly related sequences, the glutamate transporter family does not contain many residues that are completely conserved (Fig. 4). However several motifs conserved to some degree within the family have been identified [100]. The most conserved is Motif A, which is located in reentrant loop 6a and appears to be important for transporter function. Amino acids in the loop were shown to be protected from extracellular modifying agents under most circumstances, leading to the conclusion that the residues are intracellular but under other transport conditions residues in this motif become accessible from the outside and were hypothesized to be a part of the substrate-binding site [107].

Motif B, located at the cytoplasmic interface of membrane helix 7, is present in all functionally characterized transporters and is involved in cation binding. Mutagenesis studies of Motif B in the rat glutamate transporter (GLT-1) have shown that Tyr403 and Glu404 (corresponding to S. meliloti DctA positions 330 and 331 respectively) are involved in binding potassium ions [108, 109]. Moreover, Tyr400 behaves as if it is alternately accessible to both sides of the membrane [110]. Replacement of each of these five residues by cysteine in glutamate transporter GLT-1 abolished its transport activity.

A defective phenotype was also obtained with the S. meliloti DctA protein by substituting Asp325 with glycine [51]. The protein lost its ability to transport C4-dicarboxylates as indicated by radioactive succinate transport assays and an inability to grow on succinate, but it retained partial sensitivity to the toxic DctA substrate fluoroorotate. Replacement of the corresponding aspartate in GLT-1 with cysteine, asparagine, glycine or glutamate also blocked transport by the protein [110] and controls showed that the protein was present and properly targeted. Asp325 is highly conserved in the dicarboxylate transporter branch of the glutamate transporter family (Fig. 4). Aspartate in the position corresponding to Asp325 in S. meliloti is present in all members of the family with the only exception of the bacterial serine transporter.

On the other hand S. meliloti DctA Thr327 was not as critical for function as Thr400 appears to be in rat GLT-1. A mutant DctA with a Thr432 Ala substitution could transport succinate at about 30–50% of the wild type level but was insensitive to fluoroorotate [51]. Thr400 was shown to play critical role in sodium binding by mammalian glutamate transporters and thereby in protein function. It may be significant that threonine is not conserved at this position in all members of the glutamate family. While it is present in the bacterial C4-dicarboxylate transporters and eukaryotic glutamate transporters, the neutral amino acid transporters have alanine at this position, and the bacterial glutamate transporters have serine (Fig. 4).

Motif C, which is also located in transmembrane domain 7, was shown be involved in substrate binding [111]. Motif C is a part of a domain that is involved in binding a glutamate analog dihydrokainate, which is an inhibitor of glutamate transport [112].

Motif D, which is located on the predicted transmembrane amphipathic helix eight, is conserved to some degree between all members of the family; however its exact amino acid composition varies along with the substrate specificity of the transporters [100]. Arg447 located within Motif D of rat GLT-1 (Arg405 on S. meliloti DctA) was shown to control the binding of the...
γ-carboxyl group of glutamate [113]. Substituting Arg447 in a neuronal glutamate transporter with a neutral or negatively-charged amino acid completely abolished transport of glutamate and aspartate without impairing cysteine transport. It was suggested that Arg447 plays a pivotal role in the sequential interaction of acidic amino acids and potassium with the transporter.

Mutagenesis of *S. meliloti* DctA identified a residue on transmembrane domain three that might also play an important role in the protein function [51]. The domain has never been designated as carrying amino acids directly involved in transport of dicarboxylates. A Gly114 Asp substitution led to a ten-fold decrease in a level of succinate uptake. Multiple sequence alignment of more than 60 members of the glutamate transporter family revealed Gly in the position corresponding to *S. meliloti* Gly114 in nearly all members of the family [51] (Fig. 4). The only exception was in the bacterial serine transporters and the eukaryotic alanine/serine/cysteine/threonine transporters, which have alanine in this position.

6. Concluding remarks

The rhizobial dicarboxylate transport system has been subject of intensive study because of the crucial role the protein plays in symbiotic nitrogen fixation, but relatively little of this effort has been directed at the DctA protein itself. Regulation of DctA expression in free-living cells is fairly well understood but there are some questions which still have to be answered, including the constitutive expression of dctA mutants. For example, it is still not obvious which ATG is a start codon for dctA in *Sinorhizobium meliloti* and whether the dctA translation starts at the same ATG in free-living cells and during symbiosis. Based on the differences in substrate recognition by DctA and DctB/DctD, it is reasonable to ask whether DctB/DctD might be involved in regulation of other genes. It is still not clear how the components of Dct system interact with each other or whether there may be accessory proteins that interact with other members of the glutamate transporter family to modify their activities or specificities. The mechanism of regulation of dctA expression in the *S. meliloti* symbiosis with alfalfa is still not understood and identifying the mechanism of ASA is needed. Mutagenesis of *S. meliloti* DctA was consistent with the alignment of the rhizobial dicarboxylate transport protein with better studied bacterial and eukaryotic glutamate transporters. Constructing DctA proteins with substituted residues should help to better understand the mechanisms of DctA action.

DctA is unusual in having a large number of physiologically important substrates that can affect free-living growth. It is not yet clear how important this broad substrate specificity is for the operation of DctA in symbiosis – while it seems likely that malate is an important substrate in root nodules, the ability to transport other compounds may also be important. Altering DctA through the generation of mutants with more limited substrate specificity would allow this issue to be investigated directly. Such an investigation would also allow additional tools of bacterial physiology and metabolism to be used to investigate DctA-mediated transport as a model for the unusual glutamate transporter family.

Acknowledgements

This work was supported by grants #98353056553 from the United States Department Agriculture-Competitive Research Grants Office and MCB-0131376 from the National Science Foundation.

References


