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The C_4-dicarboxylate transport system of *Rhizobium meliloti* and its role in nitrogen fixation during symbiosis with alfalfa (*Medicago sativa*)

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Abstract. The *Rhizobium meliloti* C_4-dicarboxylate transport (Dct) system is essential for an effective symbiosis with alfalfa plants. C_4-dicarboxylates are the major carbon source taken up by bacteroids. Genetic analysis of Dct$^-$ mutant strains led to the isolation of the *dct* carrier gene *dctA* and the regulatory genes *dctB* and *dctD*. The carrier gene *dctA* is regulated in free-living cells by the alternative sigma factor RpoN and the two-component regulatory system DctB/D. In addition, DctA is involved in its own regulation, possibly by interacting with DctB. In bacteroids, besides the DctB/DctD system an additional symbiotic activator is thought to be involved in *dctA* expression. Further regulation of *dctA* in the free-living state is reflected by diauxic growth of rhizobia, with succinate being the preferred carbon source. The tight coupling of C_4-dicarboxylate transport and nitrogen fixation is revealed by a reduced level of C_4-dicarboxylate transport in nitrogenase negative bacteroids.
Key words. C_4-dicarboxylate transport; *dct* genes; energy source; regulation; *Rhizobium meliloti*; symbiosis.

Energy and carbon metabolism of rhizobia under free-living and symbiotic conditions

The symbiotic interaction between legumes and rhizobia is based on the development of root nodules. The root nodules are the sites of biological nitrogen fixation, which is known to be a highly energy-intensive process. Bacteroids thus profit from the plant compounds derived from photosynthetic activity and in response, reduced nitrogen is exported to the plant. The reduction of nitrogen to ammonia is catalyzed by the nitrogenase enzyme complex[40]. Under optimal conditions, nitrogen fixation requires 16 molecules of ATP per molecule of N_2 fixed, but the energy consumption can actually be as high as 42 molecules of ATP, according to growth yield measurements, as summarized recently[56]. The significance of symbiotic nitrogen fixation is reflected in the fact that legumes are important crop plants. The terrestrial flux of nitrogen from biological fixation has been calculated to range from 100 to 120 $\times$ 10^6 t N/year[58].

Fast growing *Rhizobium* species, including the alfalfa-nodulating *Rhizobium meliloti*, are capable of utilizing a wide range of carbon sources such as carbohydrates, organic alcohols, organic acids, and a variety of aromatic compounds (reviewed by Stowers[72]). Concerning the central catabolic pathways, it was reported that hexose degradation mainly proceeds through the Entner-Doudoroff (ED) pathway and that organic acids are directly metabolized via the tricarboxylic acid cycle[72]. It has been observed that growth with succinate as sole carbon source leads to the fastest growth rate of *R. meliloti*[73]. The generation time in minimal medium with succinate was determined to be 2.6 hours, compared to 3.2 hours in minimal medium with glucose[15].

In symbiosis, carbon metabolism and transport are considered to be of great importance in ensuring the tight physiological coupling between the two partners. At the end of the 1970s, the question of which carbon compound is used to run the nitrogen fixation process arose; that is, what compounds are taken up by the bacteroids? It was assumed that sugars may be the direct source of energy. However, *Rhizobium leguminosarum* bacteroids have been found to be unable to take up and metabolize hexoses or disaccharides[28]. Furthermore, *R. meliloti* and *R. leguminosarum* mutant strains with a defective sugar uptake or catabolism are still able to establish an effective symbiosis[13,28,29,51,62a]. It was concluded that only small amounts of sugars may be available to the bacteroids[51].

Since bacteroids do not show an active sugar catabolism it can be concluded that gluconeogenesis should occur at a high rate. Enhanced enzyme activities for gluconeogenesis have been detected in *R. leguminosarum* bacteroids[10,52]. Analysis of *R. meliloti* mutant strains defective in gluconeogenesis resulted in Fix$^-$ nodules, in which nitrogen fixation did not occur, thus confirming the importance of this pathway in the symbiotic state[11,23]. In summary, these observations reveal the necessity of gluconeogenesis for bacteroids. Analysis of *R. meliloti* and *R. leguminosarum* bv. *trifolii* mutants showed that furthermore an intact tricarboxylic acid cycle is essential for effective nitrogen fixation[20,24,25].

It has already been known for a long time that succinate can support bacteroid respiration and nitrogen fixation in *Bradyrhizobium japonicum*[4]. Similar results were also demonstrated for *R. meliloti*[54]. In 1979 it was suggested that tricarboxylic acid cycle intermediates like succinate

may be the major energy source for bacteroids[62a]. The first succinate uptake system active in free-living cells and bacteroids was demonstrated in *R. leguminosarum*[20,27]. At the beginning of our work with *R. meliloti*, C_4-dicarboxylate transport mutants had only been analysed for *R. leguminosarum* bv. *trifolii*, and *R. Leguminosarum* bv. *viciae*[1,20,21,63].

In this review, we summarize the biochemical and genetic approach to studying the C_4-dicarboxylate transport system of *R. meliloti* and its role in symbiotic nitrogen fixation.

Genetic and physiological analysis of the *R. meliloti* C_4-dicarboxylate transport system in free-living cells and bacteroids

In order to investigate the C_4-dicarboxylate transport (Dct) of *R. meliloti*, and its impact on symbiotic nitrogen fixation, we isolated by random transposon mutagenesis *R. meliloti* mutants unable to grow on medium containing succinate as sole carbon source. All Dct$^-$ mutants obtained failed to grow with succinate, fumarate, and malate as sole carbon source, whereas growth with other caboxylic acids like pyruvate and glutamate, and different sugars, was similar to that of the wild type. All of these were defective in succinate and malate uptake under free-living conditions (Dctfl$^-$). Revertants of the mutants retained the ability to grow on all three C_4-dicarboxylic acids, thus indicating that the defects were caused by only one mutation. It was concluded that a common Dct system is responsible for uptake of succinate, fumarate, and malate in free-living *R. meliloti* cells[17]. Common Dct systems have also been shown for *R. leguminosarum*[20,63], *Bradyrhizobium japonicum*[50,68], *Escherichia coli*[38,45], *Bacillus subtilis*[26], *Azotobacter vinelandii*[62], *Pseudomonas putida*[12], and *Salmonella typhimurium*[39].

The *R. meliloti* Dct$^-$ mutants obtained were able to nodulate alfalfa plants. In the symbiotic state, the isolated Dct$^-$ mutants could be divided into two classes (fig. 1). Class I mutants induced small and white (leghemoglobin-free) nodules unable to reduce acetylene (Fix$^-$). After 3 weeks, inoculated plants showed clear symptoms of nitrogen starvation. Light microscopy of longitudinal sections of nodules revealed a small symbiotic and an extended senescent zone. This morphology is typical for Fix$^-$ nodules[19]. Similar results were also obtained by other groups[1,21,63,80]. The symbiotic zone in these nodules contained morphologically differentiated bacteroids indicating that the infection process is not affected. In addition, it was found that *R. leguminosarum* double mutants defective in C_4-dicarboxylate transport and sugar catabolism are able to infect nodules, which indicated that carbon sources other than C_4-dicarboxylic acids or common classes of sugars can be used to fuel the differentiation process[2].

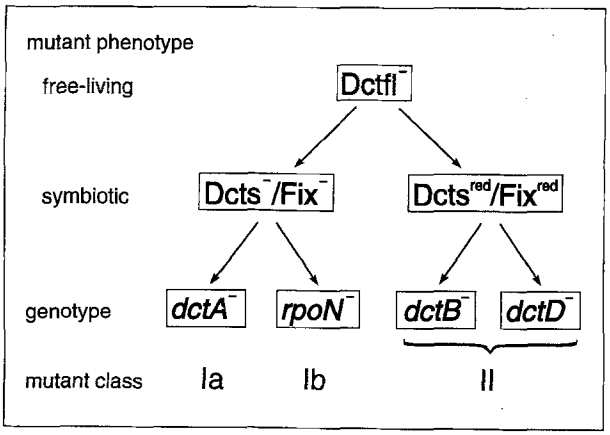

Figure 1. Classification of transposon-induced *R. meliloti* mutants defective in C_4-dicarboxylate transport. *R. meliloti* Dct$^-$ mutants split into two classes in the symbiotic state representing different C_4-dicarboxylate transport and fixation phenotypes. On the genetic level classes Ia, Ib, and II could be defined, each representing a different gene locus.
Abbreviations: Dctfl, C_4-dicarboxylate transport under free-living conditions; Dcts, C_4-dicarboxylate transport under symbiotic conditions; Fix, nitrogen fixation; red, reduced; *dctA*, *B*, *D*, C_4-dicarboxylate transport genes *A*, *B*, and *D*; *rpoN*, gene for the alternative sigma factor σ^{54}.

In contrast, it was reported that *R. leguminosarum* bv. *trifolii* mutants defective in succinate dehydrogenase are released from the infection thread during nodule development, but do not differentiate into bacteroids[25]. The bacteroids isolated from class I nodules were unable to take up succinate and malate (Dcts$^-$) indicating that in symbiosis the C_4-dicarboxylates are also transported by a common system[17].

In contrast to class I, class II mutants induced wild type-like nodules. Bacteroids isolated from these nodules showed a 30–50% reduction in symbiotic C_4-dicarboxylate transport, and fixed nitrogen at reduced levels (Dct^{red}/Fix^{red}). This Fix$^+$ phenotype correlates with the red colour of class II nodules. We concluded that the Dct system is essential for nitrogen fixation in *R. meliloti*[17,19]. Our results concerning class I mutants correlate with the findings of other groups which have also shown that *R. meliloti* Dct$^-$ mutants are unable to form effective nodules[6,32]. The existence of C_4-dicarboxylate transport systems and their necessity for nitrogen fixation has also been shown for slow growing rhizobia. Dct$^-$ mutants of *B. japonicum* and *Rhizobium* NGR234 are not able to form an effective symbiosis[14,71].

The Dct system of the wild type *R. meliloti* strain was investigated with respect to its kinetic characteristics. In competition studies with free-living cells of the *R. meliloti* wild type strain, uptake of radioactively labelled succinate was competitively inhibited by unlabelled succinate, fumarate, and malate, whereas glucose had no effect. This result confirms that the Dct system is used for transport of succinate, fumarate, and malate. The kinetic data for succinate uptake of free-living *R.*

meliloti and *R. leguminosarum* were determined. K_m values ranged from 2 to 5 μM, and V_{max} from 20 to 80 nmole/min/mg protein[17,20,21,32]. It was observed that V_{max} is somewhat reduced in bacteroids[53]. For *R. meliloti* it was shown that the Dct system is also able to transport aspartate, although the affinity is much lower[53,80]. Dct⁻ mutants were no longer able to grow on aspartate as sole carbon source[79]. A second high affinity aspartate transport system was identified in *R. meliloti* which was induced independently from the Dct system and is essential for cells growing on aspartate as the sole nitrogen source[79,81].

To determine whether the C_4-dicarboxylate transport system is an active or passive process, inhibition studies with the metabolic uncouplers DNP and azide were carried out. They indicated that the C_4-dicarboxylate transport systems in *R. meliloti* and *R. leguminosarum* are active transport systems depending on the energy status of the membrane[17,20,32].

In contrast to fast growing rhizobia, in *B. japonicum* there is evidence for the existence of two Dct systems for both the free-living and the symbiotic state which differ in their affinity for C_4-dicarboxylates[34]. Since *B. japonicum dct* mutants were isolated only recently[14] one can speculate that the existence of 2 Dct systems in this organism may complicate the isolation of *dct* mutants.

Analysis of the *R. meliloti* C_4-dicarboxylate transport genes: identification of the carrier gene *dctA*, the regulatory genes *dctB* and *dctD*, and the alternative sigma factor gene *rpoN*

Genetic complementation of the transposon induced Dct⁻ mutants with an *R. meliloti* cosmid gene bank led to the isolation of three cosmid clones. Their complementation pattern split class I mutants into class Ia and class Ib (fig. 1). One cosmid complemented Dcts⁻/Fix⁻ mutants of class Ia (fig. 1) as well as Dct^{red}/Fix^{red} mutants (class II) by restoring the wild type phenotype. Subcloning of the cosmid region resulted in the isolation of a 6 kb DNA fragment able to complement all mutants of class Ia and II. By further subcloning a 2.1 kb subfragment was identified which complemented only class Ia mutants. Two further cosmids, overlapping by ~90%, both complemented the remaining Dcts⁻, Fix⁻ mutants of class Ib. Subcloning revealed a 3.5 kb fragment to be the smallest complementing fragment. For mapping the Tn*5* insertion sites of class Ib Dct⁻ mutants, the cosmids were used to pick up Tn*5* carrying fragments from the mutant strains. Restriction fragment analysis allowed the identification of the Tn*5* insertion sites which were all located within the complementing fragments identified[9,17–19].

The DNA region coding for C_4-dicarboxylate transport was first analyzed in detail in *R. leguminosarum*[64,66]. In this species, the *dct* cluster contains the structural gene *dctA*, and the regulatory genes *dctB* and *dctD* organized in the two divergently transcribed operons *dctA* and *dctBD*. The phenotype spectrum of our Dct mutants concurred with the data obtained for *R. leguminosarum*. Therefore, class Ia mutants were assumed to be defective in the structural *dctA* gene, whereas class II mutants were assumed to be defective in regulatory *dct* genes. In order to reveal the identity of the genes isolated, we determined the nucleotide sequence of a 2.3 kb DNA fragment complementing class Ia mutants. The deduced amino acid sequence of the open reading frame identified on the fragment represents a very hydrophobic protein. The high homology of 86% to the *R. leguminosarum dctA* gene product suggested that the identified ORF represents the *dctA* gene. Due to its mutant phenotype, the *dctA* gene was assumed to encode the C_4-dicarboxylate permease located in the cytoplasmic membrane (fig. 2). Also present on the fragment was the 5′ part of a second gene which was identified by sequence homology to represent the *dctB* gene. We had thus isolated and identified the *dct* gene cluster of *R. meliloti*[19]. Later, it was shown by other groups that the *dct* region is located on the second megaplasmid of *R. meliloti*[8,80].

Further analysis of the *dctA* gene product revealed that its hydropathic profile corresponds to that of typical integral membrane proteins[19,46]. In order to test a model of the DctA topology deduced from the hydropathic profile, we made use of a genetic approach to elucidate the topology of membrane proteins by monitoring translational gene fusions[48,70]. Gene fusions of membrane proteins to the *E. coli* alkaline phosphatase (PhoA) and to the β-galactosidase (LacZ) allow subcel-

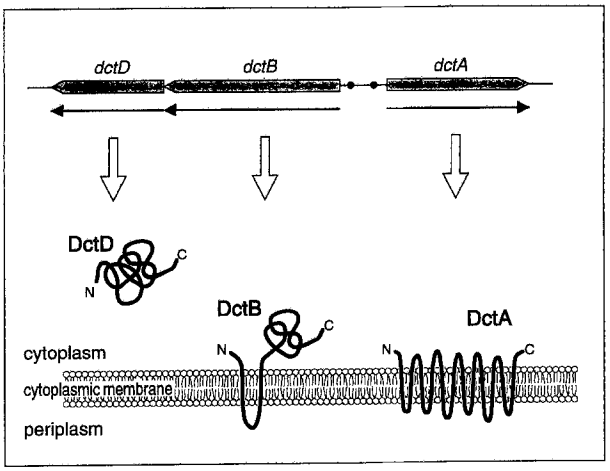

Figure 2. Organization of the C_4-dicarboxylate transport gene cluster and localization of the derived gene products. The organization of the *dct* gene region is presented. The carrier gene *dctA* and the regulatory genes *dctB* and *dctD* as well as their promoters (marked by filled circles), and their transcription scheme are shown. The locations of the derived gene products are indicated. DctA with 12 membrane spanning domains and DctB with 2 membrane spanning domains are considered to be membrane proteins, whereas DctD is located in the cytoplasm.

lular location of the fusion site, by means of the activity of the monitor proteins. Isolation and characterization of PhoA and LacZ fusions together with the analysis of the hydrophobic profile led to a two-dimensional model of DctA which comprised twelve transmembrane α-helices with the amino-termius and the carboxy-terminus located in the cytoplasm (fig. 2)[37].

Investigation of the amino acid sequence of the *dctB* gene product revealed two possible transmembrane passages allowing the protein to be anchored in the cytoplasmic membrane. The *dctD* gene product exhibits the hydropathy of a typical cytoplasmic protein (fig. 2). According to sequence homologies to *R. leguminosarum*, the *dctB* and *dctD* genes were suggested to code for a two-component regulatory system[55]. This regulatory system can be described as follows. DctB functions as a membrane-anchored sensor for C_4-dicarboxylates and is thought to activate the DctD protein by phosphorylation. The DctD protein contains a DNA binding site and in its phosphorylated form is able, together with the RpoN protein, to activate the expression of *dctA*[35, 64, 66, 67, 77, 79].

Partial nucleotide sequencing of the region adjacent to transposon insertion sites of class Ib mutants and comparison to the *rpoN* gene[67] revealed that class Ib mutants were defective in *rpoN*[9, 18]. Finan and coworkers showed that the *rpoN* gene is located on the chromosome[22]. The *rpoN* gene encodes the alternative sigma factor (σ^{54}) known to be responsible for the expression of many nitrogen regulated genes and a number of symbiotically expressed *nif* and *fix* genes. Phenotype analysis of class Ib mutants showed that the sigma factor RpoN is absolutely necessary for *R. meliloti* and *R. leguminosarum dctA* transcription both in free-living cells and bacteroids[18]. The *dctA* promoter is highly homologous with the RpoN-dependent consensus promoter sequence[19, 35]. The direct involvement of RpoN in *dctA* expression was shown by isolating Dct^+ revertants of transposon-induced *rpoN* mutants. Some of the revertants carried suppressor mutations linked to the *dct* locus and probably resulted in an RpoN-independent *dctA* promoter[83].

The transcriptional regulation of the C_4-dicarboxylate carrier gene *dctA* in free-lving cells

It was shown for *R. meliloti* that the Dct system constitutes an inducible process in free-living cells[17]. As described above, the two-component regulatory system consisting of DctB and DctD is responsible for responding to the presence of dicarboxylates and activating expression of the *dctA* gene (fig. 3A). Upstream of the *dctA* coding region we identified potential promoter elements, for example a potential RpoN dependent promoter was identified 60 bp upstream of the coding region. The RpoN protein, the alternative sigma factor σ^{54}, is known to function together with sensor/regulator pairs such as NtrB/NtrC and DctB/DctD. The regulator binds to an upstream activator sequence, usually located about 100 nucleotides upstream of the RpoN binding site, which resembles eukaryotic enhancers. The DNA region between these two regulators forms a loop

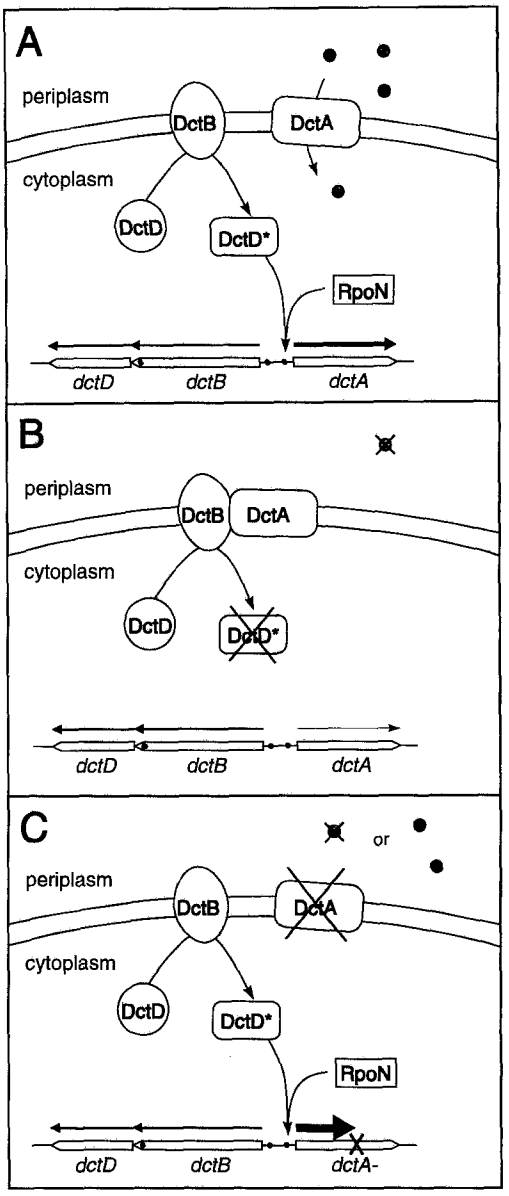

Figure 3. Model of the transcriptional regulation of the *R. meliloti* C_4-dicarboxylate carrier gene *dctA* in the free-living state. *A* In the presence of substrate (shaded circles) DctB is in an active state and able to activate, possibly by phosphorylation, the DctD protein. The activated DctD protein is able to stimulate, together with the alternative sigma factor RpoN, the transcription of the *dctA* gene.
B In the absence of substrate (crossed out circles) basic levels of DctA maintain DctB in an inactive conformation, possibly through protein-protein interaction, resulting in repression of the *dctA* gene.
C In a $DctA^-$ mutant background, DctB is in the same mode as in the presence of substrate (*A*) resulting in activity of the *dctA* promoter.
The approximate *dctA* transcription level is indicated by black arrows of different thicknesses.

structure. In *R. leguminosarum*, but not in *R. meliloti*, integration host factors (IHF) were found to be involved in forming this loop structure between the DctD and the RpoN binding sites[44]. Further investigation on the function of the DctD protein revealed that the presence of high amounts of active or inactive DctD proteins inhibits the expression of the *dctA* gene[41,44]. The inactive DctD may be able to bind to the DNA, but the activated form may be necessary to start the transcription[5].

We further studied *dctA* regulation using a phosphatase-active *dctA-phoA* monitor fusion. By measuring alkaline phosphatase activity of *dctA-phoA* carrying cells, the expression of the *dctA* promoter could be determined. We studied *dctA* expression in a $DctA^-$ background and in an otherwise isogenic, but $DctA^+$ background. In the $DctA^+$ background, the expression rate of the *dctA* gene was in response to succinate increased by about a factor of ten[36]. A ten-fold increase in succinate expression was also reported for the *dctA* gene of *R. leguminosarum*[65] and that of *R. meliloti*, grown in a micro-aerobic culture[77]. Surprisingly, in the $DctA^-$ background the *dctA-phoA* expression was no longer regulated by succinate, but was expressed constitutively and was about five times higher than under inducing condition[36]. Similar results have been obtained by other groups using a *dctA-lacZ* fusion in *R. leguminosarum*[65] and a *dctA-phoA* fusion in *R. meliloti*[83]. These results indicate that the *dctA* gene product probably plays a role in suppressing its own synthesis in the absence of C_4-dicarboxylates. It has been shown that this regulation is mediated by *dctB* and *dctD*[83]. It was suggested that *dctA* and *dctB* gene products might interact with each other in the cytoplasmic membrane in the absence of substrate in order to avoid activation of the C_4-dicarboxylate transport system (fig. 3B)[66]. A model suggests that DctB senses the active state of the DctA carrier and modulates the DctD regulator[83]. According to this model the consequence of the presence of an inactive DctA protein is a high, unregulated expression of the *dctA* promoter (fig. 3C). The same high *dctA* promoter expression rate was found in $dctA^-$ bacteroids, indicating that the effect which was observed in free-living cells, and is assumed to be caused by the interaction between DctA and DctB, is also important for the symbiotic state. Since the proposed interaction is a response to environmental signals, we assume that bacteroids are still able to respond to external stimulators, although the environment in the nodules is determined by the host plants[36].

The transcriptional regulation of the C_4-dicarboxylate carrier gene *dctA* in bacteroids

The factors DctB, DctD, and RpoN, responsible for free-living *dctA* expression, are also involved in or necessary for symbiotic *dctA* expression. Appropriate mutant strains showed reduced or no *dctA* expression in the symbiotic state[16,74]. An additional symbiotic activator protein was therefore assumed to be involved in symbiotic *dctA* regulation (fig. 4), since mutant strains defective in the regulatory genes *dctB* and *dctD* still exhibited *dctA* gene expression in bacteroids[66]. When the nucleotide sequence of the *dctA* upstream region was analysed, a region with high homology to the NifA binding site was identified in addition to the consensus sequence for RpoN binding[19]. Since NifA is a regulator for many symbiotic genes, and functions together with the sigma factor RpoN, it was suggested that NifA might function as an additional symbiotic regulator[66,77]. This assumption could not be confirmed, since the symbiotic *dctA* expression was not influenced in the wild type or *dctB* background by an additional *nifA*-mutation. Further regulators of nitrogen fixation genes, FixL and FixJ, had also no effect on *dctA* expression[36,60]. Identification of the tandem DctD binding sites upstream of the σ^{54}-dependent *dctA* promoter revealed that the putative NifA binding site is part of the DctD binding site[35,43].

Investigations on the stimulation of the symbiotic activator (ASA, *a*dditional, *s*ymbiotic *a*ctivator) in the free-living state by Tn*5*-'promoter-out' mutagenesis of a *dctBD* mutant strain, and screening for free-living growth on succinate, have failed thus far[69]. It remains unknown whether the putative ASA factor consists of a *dct*-specific regulatory protein or represents cross-talk of other regulators. Labes and coworkers isolated a revertant of an *R. meliloti dctD*::Tn*5* mutant which

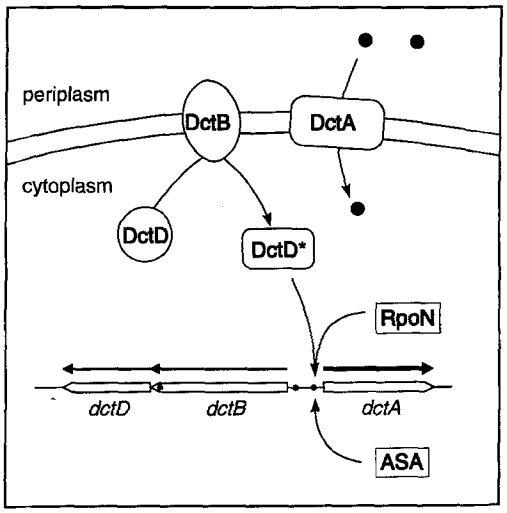

Figure 4. The transcriptional regulation of the *R. meliloti* C_4-dicarboxylate carrier gene *dctA* in the symbiotic state. Since substrates for the Dct system are present in the nodule, the situation is comparable to that of figure 3A. An additional symbiotic activator (ASA) is assumed to be involved in *dctA* expression together with DctD and the alternative sigma factor RpoN. For further details, see figure 3.

restored the ability to grow on succinate. Characterization of the revertant revealed a point mutation in the *ntrC* gene resulting in an NtrC mutant protein able to bind to the *dctA* promoter and also constitutively activate other RpoN dependent promoters[41].

Additional symbiotic activation may also be reflected by the different metabolic conditions of the endosymbiotic bacteroids. Batista and coworkers determined *dctA* expression under conditions of high (and unphysiological) NaCl concentration (0.25 M) (among other ionic and nonionic solutes) and found enhanced *dctA* expression. The *dctA* expression was also increased under low calcium conditions. The expression of *dctA* may also be influenced by the DNA topology, since *dctA* expression decreased in the presence of DNA gyrase inhibitors[3]. These or other environmental conditions may be present in the nodule. In addition to the activators known so far, further factors regulating symbiotic *dctA* expression are expected to exist.

Diauxic growth of *R. meliloti* in a medium containing succinate and glucose

R. meliloti dctA expression in free-living cells was further characterized in the following experiment. We tested *R. meliloti* growth and carbon utilization in the

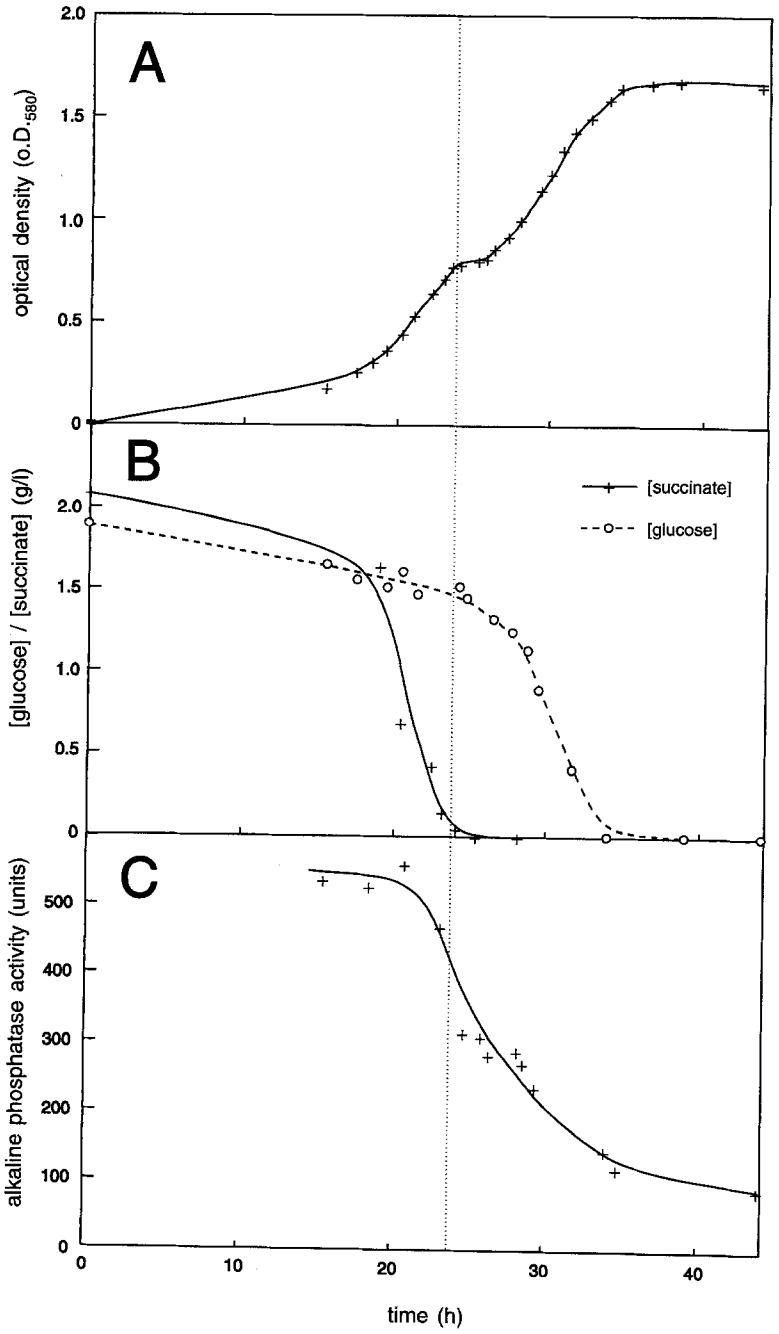

Figure 5. Diauxic growth of *R. meliloti* in a medium containing succinate and glucose. The *R. meliloti* wild type strain was grown in liquid minimal medium containing 2 g/l succinate and 2 g/l glucose. The cell growth in the culture was determined by measuring optical density (*A*). The succinate and glucose concentrations in the culture supernatant (*B*) and the expression of the *dctA* promoter determined through alkaline phosphatase activity of a *dctA-phoA* fusion (*C*) are shown. The dotted line indicates the start of the second growth phase.

presence of the two carbon sources succinate and glucose. *R. meliloti* showed diauxic growth, with the utilization of succinate during the first and glucose during the second growth phase (fig. 5). The *dctA* gene is highly expressed only during the first growth phase, indicating that the preference for succinate is regulated on the transcriptional level[74]. Further catabolite-repression-like phenomena were observed in *R. meliloti* cells in a medium containing succinate plus lactose[73] and succinate plus glucose or fructose[31,33]. Furthermore, malate was found to repress glucose transport in *R. leguminosarum*[76]. No diauxic growth was observed with lactose, plus either maltose, sucrose, glucose, or histidine[73].

This is in contrast to observations in *E. coli*, which preferably utilizes glucose. In *E. coli* diauxic growth is regulated by catabolite repression and involvement of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). However, in rhizobia no phosphotransferase system has been identified[72]. Furthermore, cAMP was reported not to be taken up by rhizobia[7], and succinate was found to repress glucose catabolism enzymes whereas cAMP did not[47]. Mutants defective in adenylate cylcase have been isolated from *R. meliloti*, but they are still able to synthesize wild-type levels of cAMP[57]. Wang and coworkers suggested an influence of an as yet unidentified *crp*-like protein on *dctA* expression, since the *E. coli* cAMP receptor protein represses the *R. meliloti dctA* promoter in an *E. coli* background and in vitro[78]. The preferred utilization of succinate by *R. meliloti* reflects that succinate is also an important carbon source under free-living conditions. Little is known about the regulation of diauxic growth in species other than *E. coli*. For example, in *Pseudomonas putida* diauxic growth does not appear to involve a cyclic-AMP-mediated mechanism[59]. Recently, a *Pseudomonas aeruginosa* mutant was isolated which is no longer able to repress transport and catabolism of carbohydrates in the presence of succinate and an inducer[82]. Analysis of this mutant may help to understand catabolite-repression-like phenomena in rhizobia and other bacteria.

The role of C_4-dicarboxylates for symbiotic nitrogen fixation

As it was shown in the preceding sections, C_4-dicarboxylate transport is essential for the nitrogen fixation process, and the rate of C_4-dicarboxylate transport has an influence on the rate of this fixation. Hence, it was of interest to ascertain whether nitrogen fixation also reacts upon the C_4-dicarboxylate transport rate. Therefore, we studied *dctA* expression and C_4-dicarboxylate activities directly in a series of different *R. meliloti* Fix^- bacteroids. To this end, alfalfa plants were inoculated with the *R. meliloti* wild type strain and different Fix^- mutant strains, one of which was defective in the nitrogenase reductase enzyme. Bacteroids isolated from the Fix^- nodules were not influenced in their *dctA* expression rate compared to those from Fix^+ nodules. In contrast, C_4-dicarboxylate transport activities were reduced to approximately 50% of the wild-type transport activity in both regulatory and structural Fix^- bacteroids. The reduced transport rate in all Fix^- bacteroids indicates that the nitrogen fixation process reacts upon the actual C_4-dicarboxylate transport rate. This regulation may be mediated by a direct modification of the DctA protein or may be due to a metabolic effect of reduced energy consumption in Fix^- bacteroids. We assumed that the amount of C_4-dicarboxylates transported in wild-type bacteroids is partitioned to maintain the nitrogen fixation process on the one hand, and the basic metabolism of the bacteroids on the other[36].

The effect of nitrogen fixation on C_4-dicarboxylate transport indicates that nitrogen fixation may limit the C_4-dicarboxylate transport rate, and not vice versa. This assumption is supported by the finding that C_4-dicarboxylate transport and nitrogen fixation cannot be enhanced by amplified *dct* genes or constitutive expression of *dctA*[16,61]. Therefore it can be concluded that other, perhaps more general, factors are limiting nitrogen fixation.

In addition to their role as the energy source for symbiotic nitrogen fixation, other possible functions of C_4-dicarboxylates should be taken into consideration. Catabolism of dicarboxylates is highly oxygen-consuming, and may be involved in reducing the oxygen pressure in the nodule in order to protect the nitrogenase enzyme complex from oxygen damage. However, in nodules induced by *dctA* mutant strains a low oxygen pressure, comparable to that of the wild-type, was determined by oxygen electrode measurements[49]. It may be possible that remaining, undetectable amounts of oxgyen are consumed by respiration of dicarboxylates. Furthermore, the Dct system may also be involved in transporting other specific factors essential for nitrogen fixation, such as leghemoglobin components. Competition studies on δ-aminolevulinate (a precurser of heme synthesis) and malate uptake of *R. leguminosarum* and *B. japonicum* indicated that δ-aminolevulinate transport of bacteroids is mediated by the C_4-dicarboxylate carrier[30].

Finally, C_4-dicarboxylate as major carbon source for bacteroids may be an adaptation of the bacteroids to the nodule's metabolism. Nodule carbon metabolism may be similar to that of other oxygen-limited plant tissues. An alternative reductive branch may redirect glycolysis to the formation of C_4-dicarboxylic acids through incorporation of CO_2 into oxaloacetate[75]. This would result in high concentrations of succinate, fumarate, and malate, as was determined by McRae and coworkers[53].

Conclusion

Our studies on C_4-dicarboxylate transport, together with data obtained from other groups, comprise a step forward in understanding the Dct system in rhizobia, in both free-living cells and in bacteroids. C_4-dicarboxylate transport has been shown to be embedded in a complex regulatory network. However, better knowledge of carbon and nitrogen metabolism in bacteroids and nodules may help in identifying the specific role of the Dct system and in understanding the coupling between C_4-dicarboxylate transport and the nitrogen fixation process.

Acknowledgements. We are grateful to Tanja Dammann-Kalinowski and Mathias Keller for helpful discussions and Loraine Heydeloff and Uwe Dresing for critical reading of the manuscript. This work was supported by a grant from the BMFT (project No. 0318968A + B) through KFA Jülich.

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