DctBD-Dependent and -Independent Expression of the *Sinorhizobium (Rhizobium) meliloti* C₄-Dicarboxylate Transport Gene (*dctA*) During Symbiosis

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The *Sinorhizobium meliloti* C₄-dicarboxylate transport gene (*dctA*) is essential for symbiotic nitrogen fixation. Under free-living conditions, the expression of *dctA* is fully dependent on the cognate regulatory genes *dctBD*. However, during symbiosis with the *Medicago sativa* host plant, the *dctA* gene is efficiently expressed even in the absence of the *dctBD* genes. The spatial expression of the *dctA* gene has been monitored in situ in mature nitrogen-fixing nodules formed by wild-type and *dctD* mutant strains. In nodules induced by a wild-type strain, expression was observed in both the infection zone and the nitrogen-fixing zone of the nodule. DctD-independent expression of *dctA* was observed with a previously described *dctA::lacZ* fusion (pCU700) and was found to be confined to the fixation zone (zone III) of mature nodules. Therefore, the operation of the alternative system of symbiotic *dctA* activation (ASA) is concomitant with the onset of nitrogen fixation, which could be consistent with an increased need for transport of C₄-dicarboxylic acids by the nitrogen-fixing bacteroids. Sequences in the 5' part of the *dctA* coding region were found to be essential for the activation of the *dctA::lacZ* gene fusions by the ASA. Deletion of these sequences resulted in gene fusions that were found to be strictly dependent on *dctBD* for expression, under all conditions tested including symbiosis. Such gene fusions allowed us to establish that the DctBD-dependent *dctA* expression was occurring throughout the whole nodule.

Additional keywords: β -D-galactosidase, interzone II-III, metabolism, *nifH*.

During symbiosis between rhizobia and their host plant, energy needed for nitrogenase activity is provided by photosynthesis. Photosynthates are transported to the nodule in the form of sucrose but several lines of evidence indicate that nitrogen-fixing bacteria utilize C₄-dicarboxylates produced by sucrose metabolism in the nodule (Bergersen and Turner

1967; McRae et al. 1989; for a review, see Vance and Heichel 1991). In particular, strong genetic evidence was provided by the isolation of C₄-dicarboxylate transport *dct* mutants of *Rhizobium trifolii*. Nodules induced by such mutants contain differentiated bacteria or bacteroids similar to those found in wild-type nodules, but they are ineffective (Ronson et al. 1981). The *dct* locus has been characterized in several rhizobia, among them *Rhizobium leguminosarum* and *Sinorhizobium (Rhizobium) meliloti*. It consists of the divergently transcribed *dctA* and *dctBD* genes (Engelke et al. 1989; Jiang et al. 1989; Wang et al. 1989; Yarosh et al. 1989). *dctA* codes for a high-affinity permease (Finan et al. 1981) and the *dctBD* genes code for a two-component regulatory system belonging to the NtrBC family of regulatory molecules (Ronson 1988; Ronson et al. 1987a). The sensor DctB has an autophosphorylation activity and in the presence of inducer (C₄-dicarboxylic acids or the amino acid aspartate) in the environment, activates DctD by a mechanism of phospho-transfer (Giblin et al. 1995). DctD in turn activates the σ^{54} -dependent *dctA* promoter (Ronson et al. 1987b; Ledebur et al. 1990). Under free-living conditions, the regulatory *dctBD* genes and the *ntrA* gene coding for the sigma factor σ^{54} are indispensable for activation of the *dctA* promoter.

Strains bearing a mutation in the *dctA* gene induce nodules on their host plant, but fail to fix nitrogen (Engelke et al. 1989; Yarosh et al. 1989). In contrast, nodules induced by *S. meliloti* strains lacking the *dctBD* genes generally fix nitrogen at near wild-type rates (Watson et al. 1988; Engelke et al. 1989; Jiang et al. 1989; Wang et al. 1989; Yarosh et al. 1989). This implies that during symbiosis the *dctA* gene is efficiently expressed, even in the absence of the regulatory *dctBD* genes, and, therefore, that during symbiosis regulatory molecules other than DctBD are able to activate the expression of the *dctA* promoter. Despite intense studies, this alternative system of symbiotic activation (ASA) has not been identified to date. It is consequently also not known which mechanism, the ASA or the regular DctBD system, is primarily responsible for the expression of *dctA* during symbiotic N₂ fixation in wild-type nodules.

Cytological studies can provide valuable information about regulatory pathways operating in such intricate biological processes as nitrogen-fixing symbiosis. A mature indeterminate nodule is organized into different zones (Vasse et al.

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1990), which correspond to a temporal and spatial pattern of bacterial and plant gene expression. The most distal and youngest part of a mature nodule contains the apical meristematic zone, which is bacteria free. Zone II designates a region immediately behind the meristem containing the proliferating infection threads filled with dividing bacteria, followed by a region where the bacteria are released into the plant cells. Symbiotic N₂ fixation takes place in the distal part of zone III also called the fixation zone. After about 5 weeks, a zone of senescence develops in the most proximal region of the nodule (zone IV). The interzone II-III is characterized by a high level of starch deposition in the plastids of the invaded cells and is of special interest because expression of plant and bacterial genes needed for symbiotic nitrogen fixation, such as *nifA*, which encodes the transcriptional activator of nitrogenase genes, is induced sharply at this zone (de Billy et al. 1991; Soupène et al. 1995). Other genes, such as the *ropA* gene coding for an outer membrane protein of *Rhizobium leguminosarum*, are abruptly down regulated at this zone (de Maagd et al. 1994).

As a first step toward elucidating the physiological significance of the ASA as opposed to the DctBD system in regulating *dctA* expression during symbiosis, we set out to monitor the in situ activity of *dctA::lacZ* gene fusions, in mature nodules induced by wild-type and regulatory *dctBD* mutant strains of *S. meliloti*. The DctBD-dependent and -independent expressions of the gene fusions were found to display distinct patterns of temporal and spatial activity.

RESULTS

Construction of a “short” *dctA::lacZ* gene fusion.

The pCU700 *dctA::lacZ* gene fusion plasmid (Wang et al. 1989) was chosen as a starting point in these studies (Fig. 1B). The *dctA* fragment on this plasmid codes for the N-terminal part of DctA. The *S. meliloti* *dctA* gene contains two possible ATG start codons, only 33 bp distant from each other. Each ATG is preceded by a good consensus ribosome binding site (Engelke et al. 1989; Wang et al. 1989). The translational start of the *dctA* gene has not been determined, but only the second ATG is conserved between the *S. meliloti* and *R. leguminosarum* *dctA* genes (Jiang et al. 1989). Assuming that this second ATG is the start codon, the DctA-LacZ chimeric protein encoded by pCU700 contains the first 162 amino acids of the DctA protein and therefore consists of four of the 12 proposed transmembrane helices (Jording and Pühler 1993). This led to some concern about the fitness of the bacteria expressing this gene fusion. In addition, DctA is thought to be involved in the regulation of its own expression, possibly through direct interaction with DctB (Yarosh et al. 1989; Jording et al. 1992). Therefore, a truncated DctA protein could interfere with the normal regulatory pathway. To avoid these possible complications, a “short” *dctA::lacZ* gene fusion was constructed (pBBA2, Fig. 1C). Our intention was to construct a gene fusion that contained as little as possible of the *dctA* coding region, but conserved all the expression signals of *dctA*. Upstream of the *dctA* promoter, a *Pst*I restriction site, located just inside the coding region of the *dctB* gene, was used. This site is the same as was used in the construction of pCU700. A *Bcl*II restriction site conveniently overlapping the presumed initiator codon of *dctA* was used to construct the

new “short” gene fusion. The *dctA* expression signals were fused to the *lacZ* gene of pMC1403 with a suitable linker. This resulted in a translational gene fusion of the codon following the second ATG of the *dctA* gene, to the eighth codon of the *lacZ* reporter gene. Finally, this plasmid was linearized and combined with the broad-host-range plasmid pRK290, by the same procedure as that which led to the *nifH::lacZ* fusion plasmid pRKP9 (Fig. 1D).

Expression of the pBBA2 gene fusion under free-living conditions.

The newly constructed pBBA2 plasmid was conjugated from the *Escherichia coli* donor simultaneously into the *S. meliloti* 2011 wild-type strain and the *dct* mutant strains F642 (*dctA*), F332 (*dctB*), and F121 (*dctD*). In the wild-type background, the fusion was highly expressed when the strain was grown on complex medium. This is probably due to the presence in this medium of aspartate, which is known to be an effective inducer of the *dctA* gene. The *dctA* mutant strain, F642(pBBA2), also highly expressed the fusion. The *dctB* and *dctD* mutant strains, F332(pBBA2) and F121(pBBA2), respectively, did not show any expression of the gene fusion under these conditions.

To determine the effect of the inducer on the expression of the *dctA* gene fusion, the strains were pregrown for 23 h in complex medium. The β -galactosidase activity of these overnight cultures was measured (Fig. 2; time = 0) and an aliquot was used to inoculate at a 20-fold dilution mannitol minimal medium with and without malate. All cultures grew equally well on either medium and reached the stationary phase in less than 40 h. The final OD₆₀₀ obtained on minimal medium was only about half of that usually obtained on complex medium. The final OD₆₀₀ obtained on minimal mannitol medium, supplemented with malate, was similar for wild-type and *dct* mutant strains (data not shown). This indicates that the availability of carbon was not a growth-limiting factor in the minimal medium used. The levels of β -galactosidase obtained from the *dctD* (Fig. 2) and *dctB* (data not shown) mutant strains remained very low either with or without malate in the growth medium. The β -galactosidase activity from the wild-type strain that had been induced on the complex medium dropped back in the absence of malate within the first 24 h of incubation. The activity remained at a level about 10-fold higher than that of the *dctD* mutant for the rest of the experiment. The wild-type strain in the presence of malate showed a high level of β -galactosidase expression during the whole experiment. Apart from malate, the gene fusion in 2011(pBBA2) was found to be equally inducible by fumarate, succinate, and aspartate (data not shown). The *dctA* mutant strain showed high levels of gene fusion activity no matter whether malate was present in the medium or not.

All these observations were as would be expected for a properly regulated *dctA::lacZ* gene fusion. The patterns of expression obtained with the pCU700 gene fusion were similar (data not shown). However, the levels of expression obtained from the pBBA2 gene fusion were consistently three- to four-fold higher than those obtained with pCU700. On complex medium, maximum levels of β -galactosidase activity from 2011(pBBA2) reached close to 2,000 U at the late logarithmic growth phase. Maximum expression from 2011(pCU700) only reached to about 600 U. Similarly, in minimal medium, the

fusion activity from 2011(pBBA2), as judged by the rate of accumulation of β -galactosidase activity after addition of malate, was found to be about three times higher than that from 2011(pCU700). No difference was observed in the growth rate of the two strains. A plasmid, pBBBg1, in which the “short” fusion was cloned in the same vector as that used for constructing pCU700, displayed exactly the same expression characteristics as pBBA2 under free-living conditions.

Symbiotic expression patterns of the “short” and “long” *dctA* fusions in wild-type and *dct* regulatory mutant strains.

The *S. meliloti* 2011 wild-type strain, containing either pCU700 or the “short” fusion plasmid pBBA2 (or pBBBg1)

was inoculated onto alfalfa plants. As a comparison, the 2011 strain containing the *nifH::lacZ* fusion plasmid pRKP9 was inoculated in the same experiment because of the well-documented pattern of expression of *nifH* in alfalfa nodules (Labes et al.1993; Soupène et al.1995). At 15 days after infection, the nodules were well established and pink in color. Both the acetylene reduction assay (data not shown) and the vigorous aspect of the plants indicated a Fix⁺ phenotype. The spatial distribution of the gene fusion activities was observed after sectioning and histochemical staining of the nodules. As previously reported, the *nifH* fusion was induced sharply at the interzone II-III and remained highly expressed throughout zone III (data not shown). No expression of the *nifH* fusion could ever be observed at the early

Fig. 1. Genetic organization of the *dct* gene cluster and structure of the various gene fusions to the *lacZ* reporter gene, used in this work. **A**, *Sinorhizobium meliloti* *dct* cluster on the Rm *exo* megaplasmid (Ledebur et al.1990; Ledebur and Nixon 1992; Watson 1990). **B**, Plasmid pCU700 contains a 700-bp *Pst*I *dct* fragment cloned into pGD926. **C** and **D**, For the construction of pBBA2, the 260-bp *Pst*I-*Bcl*I fragment from pCU700 was used to replace, with a suitable linker, the *nifH* promoter fragment in pRKP9. Abbreviations: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; V, *Eco*RV; H, *Hind*III; Sp, *Sph*I; P, *Pst*I; NtrA, -24/-12 binding site (promoter); UAS, upstream activator site = binding site for the transcriptional activator (DctD; NifA).

stages of the symbiosis, such as during infection and in zone II of the mature nodules.

By contrast, in mature nitrogen-fixing nodules induced by the 2011 wild-type strain, expression of the *dctA::lacZ* gene fusions could be observed at all stages of bacteroid development and notably in infection zone II (Fig. 3B and D). However, no induction of the *dctA* gene could be detected in the infection threads immediately behind the apical meristem. Apparently, the fusions were induced as soon as the bacteria were released into the host cell cytoplasm. They remained strongly induced throughout the infection zone II, the interzone II-III, and into zone III. No senescence zone could yet be distinguished. The spatial distribution of fusion activity in the wild-type background was similar for the pCU700 (Fig. 3B) and the pBBA2 (Fig. 3D) gene fusions.

In order to examine the symbiotic expression of the two *dctA* gene fusions in an isogenic background, a *dctD* negative derivative of 2011 was constructed. Such a strain was obtained by transduction of the Tn5 insertion from F121 into 2011. One such strain (20D1) was taken for further studies. The *dctA::lacZ* fusion plasmids and the *nifH::lacZ* gene fusion plasmid (pRKP9) were introduced into 20D1 by conjugation. As expected for a *dctD* mutant strain, no expression of any of the *dctA* gene fusions could be obtained in 20D1 under free-living conditions.

Fig. 2. Free-living expression of the "short" *dctA* gene fusion carried by pBBA2 in wild-type and *dct* mutant strains. 2011 = wild-type; F642 = *dctA* mutant; F121 = *dctD* mutant. Cultures were pregrown overnight in rich medium, and were used to inoculate at time 0 minimal medium containing mannitol as sole carbon source (open symbols), or the same medium with malate added (closed symbols). One unit of β -D-galactosidase corresponds to 1 nmol of nitrophenol released at 37°C per min $\times$ OD₆₀₀.

Nodules induced by the 20D1 *dctD* mutant strain were efficient and only distinguishable from their parent strain after sectioning, by a higher level of starch depositions in zone III (see Figure 3C). The *nifH* promoter was highly expressed in both the wild-type and the *dctD* mutant strains. The mutation in the *dctD* gene had no effect on the temporal and spatial pattern of expression of the *nifH* gene fusion (data not shown).

The temporal and spatial pattern of expression from pCU700 in the 20D1 background closely resembled that observed for the *nifH* gene fusion. Strong expression of the "long" *dctA::lacZ* gene fusion could be observed in mature nodules (Fig. 3A). This DctD-independent expression was strictly confined to the nitrogen-fixing zone III of the nodules. No expression was ever observed at the early stages of colonization and infection, or in zone II of the mature nodules. It was induced sharply at the interzone II-III and remained strong all the way to the most proximal part of the nodules.

Expression of the pCU700 *dctA::lacZ* gene fusion was also monitored in nodules induced by the *ntrA* mutant strain 1681 (Ronson et al. 1987b). No activity of the gene fusion was observed (data not shown).

In contrast to the "long" *dctA::lacZ* gene fusion, no DctD-independent expression could be observed at any stage with the "short" fusions on pBBBg1 (Fig. 3C) or on pBBA2 (data not shown). This result was rather unexpected, as symbiotic activity of these fusions was readily observed in the wild-type background. Measuring the β -galactosidase activity from crushed nodules confirmed the complete absence of fusion activity (data not shown). This absence of ASA-promoted expression of the "short" fusion was also observed with nodules induced by the F121 *dctD::Tn5* donor strain and the F332 *dctB* mutant (data not shown). Furthermore, sequence analysis of the "short" *dctA* fragment cloned in pBBA2 and pBBBg1 did not reveal any difference with the corresponding genomic sequence. These data, together with the absence of β -galactosidase expression from either of the "short" fusion plasmids in the *dctD* background, were strong evidence that the absence of ASA-promoted expression was due to the absence of a *cis*-acting sequence from the "short" fusion rather than to a secondary genetic event.

DISCUSSION

In order to investigate the physiology of C₄-dicarboxylic acid transport during symbiosis, we used a cytological approach that allowed us to visualize the in situ expression of the *dctA* gene during symbiosis. For this study, we used two different fusions with the *lacZ* reporter gene. The first, "long" gene fusion carried by the plasmid pCU700, contains the first 162 amino acids of the *dctA* coding region, assuming that the second ATG of the open reading frame is used as the translational start codon. The "short" gene fusion on pBBA2 or pBBBg1 only conserved this presumed ATG translational start codon and the second amino acid of the DctA protein. Our intention was to construct a gene fusion that contained as little as possible of the *dctA* coding region, but conserved all the expression signals of *dctA*.

Under free-living conditions, the "short" gene fusion behaved exactly as would be expected from correctly regulated *dctA::lacZ* gene fusion. It was expressed at a low level in the absence of C₄-dicarboxylic acids or aspartate in the growth

medium and induced strongly in the presence of any one of these inducers. Furthermore, the fusion was not expressed in the *dctB* or *dctD* regulatory mutant strains. Finally, it showed constitutive high levels of expression in the F642 *dctA* mutant background.

Both *dctA::lacZ* gene fusions were used to monitor *dctA* expression during symbiosis. Both fusions were efficiently expressed in mature nodules induced by the wild-type *S. meliloti* strain. Fusion activity could be detected as soon as the bacteria were released from the infection threads into the host cell cytoplasm. The fact that the fusions were not detectably expressed inside the proliferating infection threads immediately behind the meristem is in agreement with the observation that *dctA* mutants are infectious, suggesting that carbon sources other than C₄-dicarboxylic acids or aspartate are used by the dividing bacteria at the early stages of the symbiosis. Although *dctA* is expressed in the infection zone II, *dctA* mutants differentiate into bacteroids, which means that carbon compounds other than C₄-dicarboxylic acids can be used by differentiating bacteria in this prefixation zone. This is no longer the case in fixation zone III, as indicated by the fact that *dctA* mutants are ineffective. Therefore, during the symbiotic process, there is a gradual shift to exclusive utilization of C₄-dicarboxylic acids by endosymbiotic bacteria.

In contrast to strains mutated in the structural *dctA* gene, mutants in the *dctD* or *dctB* genes generally are effective for symbiotic N₂ fixation and have been shown to transport C₄-dicarboxylic acids. This implies that the *dctA* gene is expressed in such strains during symbiosis. We were able to confirm this DctBD-independent *dctA* expression with the "long" *dctA::lacZ* gene fusion on pCU700. However, in contrast to *dctA* expression in wild-type nodules, we observed that this DctBD-independent expression is confined to interzone II-III and zone III of mature nodules. Therefore, the alternative mechanism of symbiotic expression of the *dctA* gene (ASA) displays a typical late symbiotic phenotype. As a consequence of our observations, we can infer that *dctBD* mutant strains are effectively DctA⁻ at the early stages of the symbiosis. The difference between the regulatory *dctBD* mutants and the structural *dctA* mutant strains is that, at the later stages of bacteroid differentiation, the activity of the ASA suppresses the *dctA* negative phenotype of the *dctBD* mutants. Therefore, although C₄-dicarboxylic acids are present at the early stages of bacteroid development, their efficient uptake is not essential for bacteroid differentiation resulting in the expression of the ASA.

No DctBD-independent symbiotic induction of the *dctA* gene at the late symbiotic stage was observed with the "short" gene fusions on pBBA2 (or its equivalent pBBBg1). It thus appears that the "short" fusion does not respond to the ASA, even though under free-living conditions it behaved as would be expected from correctly regulated *dctA::lacZ* gene fusion. Probably, efficient activation of the *dctA* gene by the ASA re-

quires some *cis*-acting regulatory elements located in the 5' one-third of the coding region of the *dctA* gene. Such sequences may have been deleted partially or in their entirety when the "short" gene fusion was constructed. A gene fusion containing this truncated *dctA* regulatory region appears to monitor correctly the activity of the DctBD system but displays a mutant phenotype when tested for its response to ASA. It is worth noting that the exclusive use of the "short" fusion would have led to conclusions about symbiotic expression of *dctA* in the *dctBD* mutants in contradiction to the data that led to the ASA hypothesis. On the other hand, since the "long" fusion can be activated by either the cognate DctBD system or the ASA, we could not distinguish which mechanism was primarily responsible for the late symbiotic expression of this gene fusion. By contrast, the "short" gene fusion allowed the monitoring of the DctBD-driven expression of the *dctA* gene during symbiosis, without interference of the ASA. Therefore, the use of these "short" fusions made it possible to show that DctBD activation of *dctA* expression was operating in the whole nodule and notably in the nitrogen fixation zone. Although ASA-driven expression of *dctA* could be demonstrated only in the absence of a functional DctBD system, we speculate that it could play a role in the wild-type background by augmenting the level of *dctA* expression in nitrogen-fixing bacteroids.

A final point of interest concerns the identity of the ASA. The fact that the ASA does not activate the expression of *dctA* in an *ntrA* mutant background indicates that an activator of the σ^{54} RNA polymerase holoenzyme is involved either directly or indirectly in the alternative symbiotic activation of *dctA*. Furthermore, our finding that DctBD-independent expression of *dctA* occurs in the interzone II-III and in fixation zone III is consistent with the hypothesis that the ASA could be the transcriptional activator NifA, since *nifH* expression, which is under the control of NifA, also starts very abruptly at the interzone II-III. NifA could mediate an increase in *dctA* expression coupled with the building up of the nitrogen-fixing apparatus. Jording et al. (1992) reported that *dctA* expression was not altered in *nifA* mutant bacteroids. This result can be explained by our finding that the *dctBD* system of regulation is operating in bacteroids.

MATERIALS AND METHODS

Microbiological techniques.

Bacterial strains and plasmids are listed in Table 1. Complex medium for *E. coli* and *S. meliloti* was Luria broth (Gibco BRL, Paisley, Scotland). Minimal medium for *S. meliloti* was as described by Vincent (1970), supplemented with nitrate at 6 mM as a source of nitrogen and biotin at 1 nM. Carbon sources were added at 0.2% wt/vol. Plasmids were transferred from the *E. coli* donors into *S. meliloti* by triparental matings with pRK2013 as helper plasmid. Exconju-

Fig. 3. In situ *dctA::lacZ* gene fusion expression in 16-day-old nodules: **A**, DctD-independent activation of the "long" gene fusion in a mature nodule induced by a *dctD* mutant strain. Strain: 20D1 (pCU700); 16 days post infection (dpi); coloration: X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) for 2.5 h. **B**, Activity of the "long" gene fusion in a wild-type nodule. Strain: 2011 (pCU700); 16 dpi; coloration: X-Gal for 2.5 h. **C**, Absence of DctBD-independent expression of the "short" fusion. Strain: 20D1 (pBBBg1); 16 dpi; coloration: X-Gal for 1 h. Staining periods for up to 20 h did not reveal any significant coloration. **D**, DctBD-dependent expression of the "short" gene fusion. Strain: 2011 (pBBA2); 16 dpi; coloration: X-Gal for 2.5 h. Small arrowheads indicate position of the interzone II-III.

gants were selected on minimal medium supplemented with suitable antibiotics and purified on complex medium. Transduction experiments with the N3 transducing phage were carried out as described by Martin and Long (1984).

Construction of the “short” gene fusion.

The approximately 700-bp *HindIII*-*Bam*HI *dctA* promoter fragment was excised from pCU700 and cloned into pBluescript SK⁻. This plasmid (pSKA9) was used as a source of DNA to construct gene fusions to the *lacZ* reporter genes. A *Bcl*I restriction site coinciding with the ATG start codon was chosen to fuse the *dctA* expression signals to the reporter gene. An *E. coli* *dam* strain (GM48) was used to prepare unmethylated DNA for restrictions with *Bcl*I. The 260-bp *Pst*I-*Bcl*I fragment from pSKA9 was cloned into a purposely made vector (pSKBBB) containing the compatible *Bcl*I, *Bgl*II, and *Bam*HI restriction sites in three different reading frames. This resulted in a plasmid designated pSK4. The *dctA* promoter fragment was excised from pSK4 as an *Eco*RI-*Bgl*II fragment and used to replace the *nifH* promoter in pVSP9. This resulted in an in-frame gene fusion of the second triplet codon of the *dctA* gene to the eighth codon of *lacZ* (ATG/ATC/ACA/GAT/*lacZ*). Finally, this plasmid, designated pSVPA, was linearized with the unique *Eco*RI restriction site and cloned into the *Eco*RI site of the broad-host-range plasmid pRK290. One such plasmid, designated pBBA2, was used for this work. Another plasmid, pBBBg1, with similar characteristics, was constructed by directly cloning the 267-bp *HindIII*-*Bgl*II *dctA* promoter fragment into plasmid pGD926.

dctA induction assays.

To verify fusion activity under free-living conditions, the *Sinorhizobium* strains were grown in minimal medium with

mannitol as a non-inducing carbon source. To induce the *dctA* promoter, malic acid (pH adjusted to 6.5) was included at 0.2%. On complex medium, the *dctA* promoter is efficiently induced, presumably because of the presence of aspartate. Expression of the reporter genes was usually followed regularly until well into the stationary phase. Peak levels of expression usually occur in the mid-log growth phase. However, depending on the gene fusion, β -galactosidase levels may continue to rise a long time after the stationary phase has been reached.

The β -D-galactosidase assays were a modification of the Miller (1972) assays. In a typical assay, 250 μ l of a cell suspension (in growth medium, or diluted in 0.1 M phosphate buffer pH 7.0) was mixed with 250 μ l of assay buffer (0.1 M NaHPO₄ pH 7.0; 50 mM β -mercaptoethanol; 0.2% sodium lauryl sarkosine; 0.2 % Triton X-100; 10 mM KCl and 1 mM MgSO₄) as used for the β -D-glucuronidase assays described by Gallagher (1992). The samples were pre-incubated at 37°C for 1 min, before the addition of 50 μ l of substrate (4 mg of *o*-nitrophenyl- β -D-galactopyranoside per ml). Incubation proceeded at 37°C until a yellow color developed (between 3 and 15 min). The reaction was stopped by the addition of 200 μ l of 1 M NaCO₃. The release of *o*-nitrophenol was measured at 420 nm and the enzyme activities expressed in terms of nmol of nitrophenol released per min $\times$ OD₆₀₀. The effect of the increased temperature (37°C instead of 28°C) on the β -D-galactosidase assay was evaluated. The levels measured at 37°C were about 50% higher than those measured at 28°C.

Plant assays and histochemical techniques.

In our experimental set-up the nodules are fully mature and functional within 14 days after infection. We have focused on symbiotic expression of the *dctA* gene in 14- to 21-day-old

Table 1. Bacterial strains, plasmids, and bacteriophage used in this study

Designation	Relevant characteristics ^a	Reference or source
Bacteria		
<i>S. meliloti</i>		
2011	SU47, wild-type, Nod ⁺ , Fix ⁺ on <i>Medicago</i> spp	Rosenberg et al. 1981
F121	<i>dctD</i> ::Tn5	Yarosh et al. 1989
F332	<i>dctB</i> ::Tn5	Yarosh et al. 1989
F642	<i>dctA</i> ::Tn5	Yarosh et al. 1989
20D1	<i>dctD</i> , Tn5 insertion of F121 transduced into 2011	This study
<i>Escherichia coli</i>		
DH5	<i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> , <i>supE44</i> , λ -, <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>deoR</i>	Hanahan 1983
GM48	F ⁻ , <i>thr</i> , <i>leu</i> , <i>thi</i> , <i>lacY</i> , <i>galK</i> , <i>galT</i> , <i>ara</i> , <i>fnuA</i> , <i>tsx</i> , <i>dam</i> , <i>dcm</i> , <i>supE44</i>	Marinus and Morris 1973
Plasmids		
pCU700	<i>dctA</i> :: <i>lacZ</i> gene fusion in pGD926, broad-host-range replicon, Tc ^r , mobilizable (Mob ⁺)	Wang et al. 1989
pBluescript SK ⁻	ColE1 replicon, multiple cloning site, Ap ^r	Stratagene, La Jolla, CA
pSKBBB	pSK ⁻ derivative with <i>Bam</i> HI, <i>Bgl</i> II, and <i>Bcl</i> I restriction sites in three different reading frames, Ap ^r	This study
pSKA9	700-bp <i>Bam</i> HI- <i>Hind</i> III <i>dctA</i> promoter fragment of pCU700 cloned into pSK ⁻ , Ap ^r	This study
pSK4	260-bp <i>Pst</i> I- <i>Bcl</i> I <i>dctA</i> promoter fragment cloned into pSKBBB, Ap ^r	This study
pMC1403	Ap ^r	Casadaban et al. 1980
pVSP9	pMC1403 derivative bearing the <i>nifH</i> expression signals fused to the <i>lacZ</i> reporter gene, Ap ^r	Sundaresan et al. 1983
pRK290	Broad-host-range replicon, Tc ^r , mobilizable (Mob ⁺)	Ditta et al. 1980
pRKP9	pVSP9 and pRK290 joined at their <i>Eco</i> RI sites, Ap ^r , Tc ^r	Szeto et al. 1987
pGD926	pRK290-based translational <i>lacZ</i> probe plasmid, parent plasmid of pCU700, Tc ^r , mobilizable (Mob ⁺)	Ditta et al. 1985
pSVPA	<i>nifH</i> promoter of pVSP9 replaced by the 267-bp <i>Hind</i> III- <i>Bgl</i> II <i>dctA</i> promoter fragment of pSK4, Ap ^r	This study
pBBA2	pSVPA and pRK290 joined at their <i>Eco</i> RI sites, Ap ^r , Tc ^r	This study
pBBBg1	267-bp <i>Hind</i> III- <i>Bgl</i> II <i>dctA</i> promoter fragment of pSK4 cloned into pGD926	This study
pRK2013	Tra ⁺ helper plasmid for conjugations, narrow host range, Nm ^r	Ditta et al. 1980
Bacteriophage		
N3	Transducing phage of <i>S. meliloti</i>	Martin and Long 1984

^a Ap, ampicillin; Cm, chloramphenicol; Nm, neomycin; Tc, tetracycline; r, resistant; s, sensitive.

nodules. The patterns of in situ gene expression and the levels of fusion activity were found to be stable during this period. Levels of β -galactosidase measured from mature nodules around 21 days post infection did not differ significantly from those measured at 15 days (data not shown). Histochemical staining confirmed a similar distribution of gene fusion activity in these nodules.

Alfalfa (*Medicago sativa* cv. Europe) seeds were sterilized and plants were grown as described by Ardourel et al. (1994). Three-day-old seedlings were infected with a light suspension of inoculant strains freshly grown on L-plates in the presence of antibiotics to ensure plasmid maintenance. Nodules were harvested in 2 ml of 0.1 M sodium-phosphate buffer pH 7.0. Triton X-100 was added to 0.1% and the nodules were placed under vacuum for at least 30 min to expel the free air from the nodule cortex. The samples were then fixed in 1.25% glutaraldehyde in 0.1 M phosphate buffer pH 7.0, for 45 min under vacuum. This step also inactivates the β -galactosidase activity of plant origin (Teeri et al. 1989). After fixation the nodules were taken up in phosphate buffer and either crushed for immediate β -D-galactosidase assays, or stored at 4°C for histochemical staining. For sectioning the nodules were stuck onto a carrot slice with Loctite superglue-3. They were cut under phosphate buffer on a Microcut H 1250 vibratome (Elexience, Verrieres le Buisson, France) into 80- μ m longitudinal slices and stained in 0.1 M sodium-phosphate buffer pH 7.0, 5 mM $K_3[Fe(CN)_6]$ and 5 mM $K_4[Fe(CN)_6]$ and 0.02% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. The staining proceeded for various lengths of time, depending on the strength of the fusions and the intensity required. Typical staining times for nodule sections varied from 5 min for the highly expressed *nifH::lacZ* fusions to about 2.5 h for very weak fusions. The samples were treated with acetone to stop the coloring reaction and to remove the glue. They were taken up in water and mounted between glass object and cover slides for microscopy. The specimens were observed by brightfield microscopy and photographed with a Zeiss Axio-phot photomicroscope.

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