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Rhizobium meliloti Genes Encoding Catabolism of Trigonelline Are Induced under Symbiotic Conditions

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***Rhizobium meliloti trc* genes controlling the catabolism of trigonelline, a plant secondary metabolite often abundant in legumes, are closely linked to *nif-nod* genes on the symbiotic megaplasmid pSym [Boivin, C., Malpica, C., Rosenberg, C., Dénarié, J., Goldman, A., Fleury, V., Maille, M., Message, B., and Tepfer, D. (1989). In Molecular Signals in the Microbe-Plant Symbiotic and Pathogenic Systems. (Berlin: Springer-Verlag), pp. 401–407]. To investigate the role of trigonelline catabolism in the *Rhizobium*-legume interaction, we studied the regulation of *trc* gene expression in free-living and in endosymbiotic bacteria using *Escherichia coli lacZ* as a reporter gene. Experiments performed with free-living bacteria indicated that *trc* genes were organized in at least four transcription units and that the substrate trigonelline was a specific inducer for three of them. Noninducing trigonelline-related compounds such as betaines appeared to antagonize the inducing effect of trigonelline. None of the general or symbiotic regulatory genes *ntrA*, *dctB/D*, or *nodD* seemed to be involved in trigonelline catabolism. *trc* fusions exhibiting a low basal and a high induced β-galactosidase activity when present on pSym were used to monitor *trc* gene expression in alfalfa tissue under symbiotic conditions. Results showed that *trc* genes are induced during all the symbiotic steps, i.e., in the rhizosphere, infection threads, and bacteroids of alfalfa, suggesting that trigonelline is a nutrient source throughout the *Rhizobium*-legume association.**

INTRODUCTION

The symbiotic bacteria of the genus *Rhizobium* induce, on legumes, the formation of nodules in which they fix nitrogen (for a review, see Long, 1989). Metabolic exchanges between the two symbiotic partners involve exportation of the fixed nitrogen from the bacterium to the plant, while plant photosynthates supply the energy necessary for bacterial multiplication and nitrogen fixation. Although little is known about the nature of the plant metabolites available for the bacteria under symbiotic conditions, it has been shown that *Rhizobium* mutants affected in the transport of dicarboxylic acids such as succinate, malate, or fumarate induce the formation of nodules that are unable to fix nitrogen (Ronson et al., 1981; Engelke et al., 1987; Yarosh et al., 1989). From these results, it has been hypothesized that dicarboxylic acids are a major carbon source for bacteroids. Glutamate also seems to be an important nutrient source for bacteroids because mutants unable to grow on glutamate as sole carbon and nitrogen source in pure culture exhibit a reduced nitrogen fixing activity in nodules (Fitzmaurice and O'Gara, 1988). Plants also appear to supply amino acids, purines, and pyrimidines because most of the *Rhizobium* mutants auxotrophic for such compounds have a normal symbiotic behavior (Dénarié et al., 1976).

Rhizobium nutrition also involves plant secondary metabolites; for example, some *Rhizobium* strains are able to grow on rhizopines, which are compounds found exclusively in the nodules elicited by these strains (Murphy et al., 1987; Scott et al., 1987). More generally, rhizobia have evolved enzymatic functions to grow on secondary metabolites which are naturally present in plants. Thus, calystegins (Tepfer et al., 1988), L-homoserine (Johnston et al., 1988), trigonelline (A. Goldmann, C. Boivin, V. Fleury, B. Message, L. Lecoeur, M. Maille, and D. Tepfer, manuscript submitted; Boivin et al., 1989), and other betaines (Bernard et al., 1986) have been reported to be catabolized by *Rhizobium* strains. Calystegins are not present in legumes and their catabolism is thought to be involved in the survival of *Rhizobium* populations in the rhizosphere of nonhost plants (Tepfer et al., 1988). Unlike calystegins, L-homoserine and trigonelline are present in *Rhizobium* leguminous hosts, and the corresponding catabolic genes have been mapped on *Rhizobium* pSym symbiotic plasmids (Johnston et al., 1988; A. Goldmann, C. Boivin, V. Fleury, B. Message, L. Lecoeur, M. Maille, and D. Tepfer, manuscript submitted; C. Boivin, L. Barran, C. Malpica, and C. Rosen-

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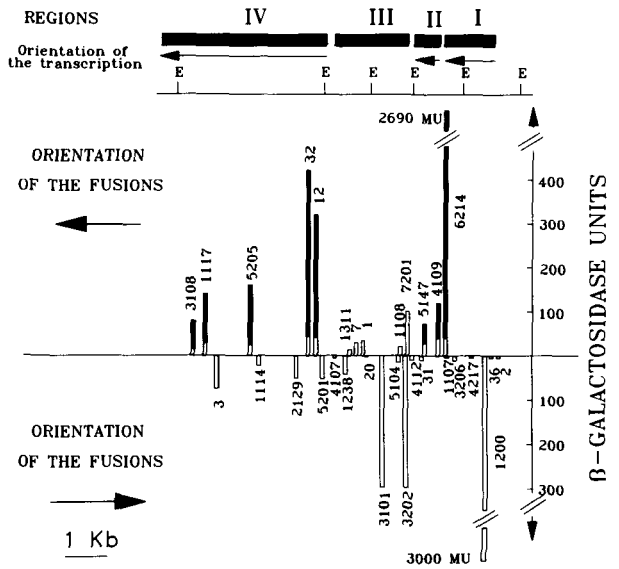


Figure 1. Location and Orientation of Tn5-B20 Insertions in the *trc* Region of the *R. meliloti* pSym.

The transcriptional activities at the Tn5-B20 insertion sites of strains GMI766 (pGMI471::Tn5-B20) in the free-living state are shown. The open bars indicate the β-galactosidase activity in Miller units (MU) of strains in VSG medium. The filled bars indicate the β-galactosidase activity in VSG medium supplemented with trigonelline at 0.5 g/L. Tn5-B20 insertions at which transcription activities are comparable are clustered in regions marked by Roman numerals (see text). E, EcoRI.

berg, manuscript in preparation). This location suggests a role for such catabolic functions in the symbiotic relationship with the plant. However, in vitro plant inoculation experiments with mutants altered in the ability to catabolize either of these compounds did not reveal symbiotic defects (Johnston et al., 1988; C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation). Nevertheless, the possibility that these catabolic functions might play a role in symbiosis under natural conditions cannot be ruled out.

In this paper, we address the question of a possible involvement in symbiosis of *R. meliloti* RCR2011 *trc* genes, which encode trigonelline catabolism functions. Using *Escherichia coli lacZ* as a reporter gene, we first investigated the expression of *trc* genes in *Rhizobium* pure culture and showed that some of them were specifically induced by trigonelline. Using such inducible *trc-lacZ* fusions, we monitored *trc* gene expression during the different steps of the symbiotic association with alfalfa. We demonstrated that *trc* genes were induced during all the symbiotic steps, i.e., in the plant rhizosphere, in infection threads, and in intracellular bacteroids.

RESULTS

trc Genes Are Specifically Induced by Trigonelline

In a complementary study (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation), the plasmid pGMI471, which carries *R. meliloti* RCR2011 genes encoding trigonelline catabolism (*trc* genes), was mutagenized using transposon Tn5-B20, and 50 insertions affecting trigonelline catabolism were characterized. Because of the presence of a promotorless *lacZ* gene within the transposon Tn5-B20 (Simon et al., 1989), the transcriptional activity at the transposon insertion site can be measured, and we used the fusions created by this transposon to investigate the effect of trigonelline on *trc* gene expression. Figure 1 presents the levels of the β-galactosidase activity of 50 *R. meliloti* strains carrying the different pGMI471::Tn5-B20 plasmids in the presence or absence of trigonelline. Eight strains carrying a Tn5-B20 insertion exhibited an increased β-galactosidase activity when grown in the presence of trigonelline. The corresponding insertions were mapped to three regions, as shown in Figure 1.

Region I is characterized by fusion 6214, which produces a 50-fold increase of the β-galactosidase activity in the presence of trigonelline. All the *trc*::Tn5-B20 insertions located to the right of fusion 6214 are in the opposite orientation of fusion 6214, and their expression is not modified by the presence of trigonelline. Because complementation experiments indicated that all these insertions were located in the same transcription unit as fusion 6214 (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation), it can be hypothesized that a transcription unit extending from the right end of the *trc* region to the insertion 6214 is transcribed from the left to the right, as indicated on Figure 1.

Region II is characterized by the insertions 4109 and 5147. A twofold increase in the transcriptional activity of these insertions was observed in the presence of trigonelline. According to complementation experiments (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation), a potential transcription unit extends from insertion 4109 to insertion 5104. However, fusions 7201 and 1108, which are located in this transcription unit and in the same orientation as 4109 and 5147, are not induced by trigonelline. It is possible that the induction level, which is only twofold for fusions proximal to the promotor, is too low to be detected for fusions distal to the promoter.

In region III, none of the inserted transposons expressed trigonelline-inducible β-galactosidase activity, regardless of the orientation of the *lacZ* gene (Figure 1). We found that three fusions, 3101, 3202, and 1200, showed a high constitutive level of β-galactosidase activity. These results

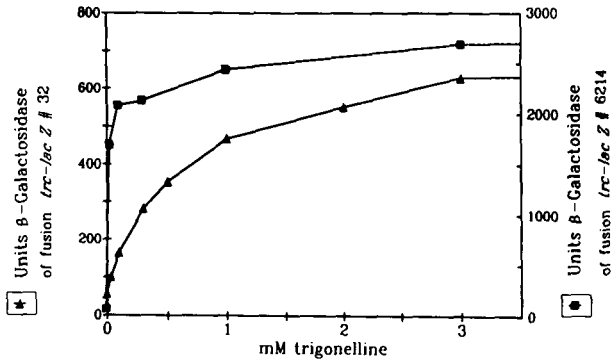


Figure 2. Effect of Trigonelline Concentration on *trc* Gene Induction.

Values are the average of three independent experiments. For both fusions, the β-galactosidase activity reached a plateau at a trigonelline concentration of 3 mM (optimal concentration for growth).

were not consistent with the transcriptional activities of very closely located insertions and, therefore, we thought they were artifactual.

Finally, insertions 3108, 1117, 5205, 32, and 12 were in the same orientation (leftward) and define region IV. Their β-galactosidase activities were fivefold to 10-fold higher in the presence of trigonelline than in noninduced conditions. Insertions in the opposite orientation (3, 1114, and 2129) did not show any induction by trigonelline.

We further investigated the regulation of the insertions 6214 and 32, which are representative of regions I and IV, respectively, and whose transcription is induced by trigonelline. The response of fusions 6214 and 32 to increasing concentrations of trigonelline is shown in Figure 2. Concentrations above 6 mM were not tested because they are toxic for *R. meliloti* RCR2011 (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation). A β-galactosidase activity twofold higher than that in noninduced cultures was observed at a trigonelline concentration of approximately 0.03 mM for the fusion 32, whereas an increase of the same order was observed at a concentration as low as 10 nM for the fusion 6214 (data not shown).

To determine whether trigonelline is a specific inducer of the *trc* genes, we assayed nicotinic acid and homarine, two compounds structurally related to trigonelline, and some other betaines, the structures of which are shown in Figure 3, as possible inducers. None of these compounds appeared to enhance the expression of fusions 32, 6214, and 4109 except for homarine, which showed a very slight inducing effect with fusion 6214 (data not shown).

The same compounds were also assayed for their ability to antagonize the inducing effect of trigonelline on *trc* fusions. The compounds were added at concentrations equimolar or at fivefold, 10-fold, or 100-fold molar excess to trigonelline. Results are presented in Table 1. The level of β-galactosidase activity of fusion 32 induced by 1 mM trigonelline is reduced by an equal concentration of any of the four betaines tested. An addition of a fivefold molar excess of these compounds resulted in an increasing inhibitory effect. The molecule with the strongest inhibitory effect was choline, which reduced the factor of induction from 5.8 to 2.3 when added at a fivefold molar excess to trigonelline. Nicotinic acid had no effect at 1 mM but was inhibitory at 5 mM. On the contrary, none of these compounds at concentrations up to a 10-fold molar excess to trigonelline inhibited expression of fusion 6214. Because glycine betaine, stachydrine, carnitine, and nicotinic acid are toxic at concentrations above 10 mM, only choline could be assayed at a 100-fold molar excess to trigonelline. At this molarity, choline showed some inhibitory effect on the expression of fusion 6214 (Table 1).

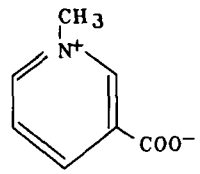
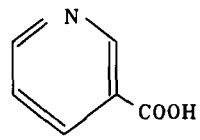
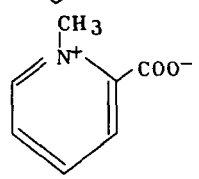
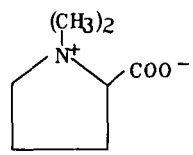
Compound	MW	Structure
Trigonelline	137	
Nicotinic acid	123	
Homarine	137	
Stachydrine (Proline betaine)	143	
Choline	121	$(CH_3)_3N^+CH_2CH_2OH$
Glycine betaine	134	$(CH_3)_3N^+CH_2COO^-$
Carnitine	161	$(CH_3)_3N^+CH_2CH-(OH)CH_2COO^-$

Figure 3. Structures of Trigonelline and Chemically Related Compounds.

Only trigonelline was able to induce *trc* gene expression.

Table 1. Effect of Trigonelline-Related Compounds on *trc* Gene Expression

	Expression of *trc*::Tn5-B20#32 in RCR2011 (pGMI584) at 1 mM trigonelline		Expression of *trc*::Tn5-B20#6214 in RCR2011 (pGM1579) at 0.1 mM trigonelline		
	% Inhibition in the presence of a *second compound* at the ratios (trigonelline/compound)		% Inhibition in the presence of a second compound at the ratios (trigonelline/compound)		
Compound	1/1	1/5	1/1	1/10	1/100
Glycine betaine	46 (519 ± 35)	55 (455 ± 9)	16 (1069 ± 21)	8 (1164 ± 60)	ND[a]
Choline	31 (620 ± 10)	72 (336 ± 7)	8 (1172 ± 91)	8 (1164 ± 92)	33 (868 ± 94)
Carnitine	13 (743 ± 30)	37 (578 ± 13)	7 (1183 ± 60)	5 (1201 ± 6)	ND
Stachydrine	21 (687 ± 18)	38 (570 ± 11)	4 (1213 ± 99)	4 (1215 ± 33)	ND
Nicotinic acid	0 (832 ± 16)	39 (567 ± 12)	2 (1244 ± 73)	2 (1240 ± 58)	ND

For inhibition assays, trigonelline was added at 1 mM for fusion *trc*::Tn5-B20#32 and 0.1 mM for fusion *trc*:Tn5-B20#6214 because these concentrations produced a good stimulatory effect on the respective target fusions (see Figure 2). Miller units and standard errors are given in parentheses. β-Galactosidase activities of fusions 32 and 6214 are 146 ± 4 and 49 ± 5, respectively, in the absence of any compound, and 834 ± 19 and 1267 ± 10, respectively, in the presence of trigonelline alone. At 10 mM, all compounds except choline affect the growth of the *R. meliloti* cells. Values are the average of two independent experiments done in duplicate.

[a] ND, not determined.

Are *trc* Genes under the Control of General or Symbiotic Regulation Circuits?

In *R. meliloti*, several general regulation mechanisms have been identified. The σ factor NtrA controls the expression of genes involved in nitrogen assimilation (Ronson et al., 1987; Kustu et al., 1989). Furthermore, in association with the two-component regulatory system DctB/DctD, NtrA is also involved in the regulation of the genes controlling the transport of dicarboxylic acids (Yarosh et al., 1989). To determine whether *trc* genes are controlled in this general fashion, pGMI471 derivatives carrying the fusions 32 and 6214 were introduced into RCR2011 mutant strains whose *ntrA*, *dctB*, or *dctD* gene was mutated. None of these mutations appeared to significantly affect the basal level of expression of the fusions or their expression in the presence of trigonelline (data not shown). Another global regulation system has been described for the *R. meliloti* *nod* genes, which control the early symbiotic steps. In *R. meliloti*, *nod* gene expression depends on the presence of both regulatory genes of the *nodD* family and plant inducers, such as the flavone luteolin (Mulligan and Long, 1985; Peters et al., 1986; Honma et al., 1990), or the 4,4′-dihydroxy-2′-methoxy chalcone (Maxwell et al., 1989). The possibility that the expression of the *trc* genes could be under the control of *nodD* was ruled out because the *trc* fusions were still induced by trigonelline in GMI766 (pGMI471*trc*::Tn5-B20) strains, in which none of the regulatory genes of the *nodD* family is present. This indicated that *nodD* is not involved in the induction of the *trc* genes by trigonelline.

Interestingly, it has been reported that trigonelline exhibits some inducing activity on *R. meliloti* AK631 *nodA* gene expression (Schmidt et al., 1986). Because *nod* genes can be induced by trigonelline, the question arose whether the *trc* genes, like the *nod* genes, could be induced by flavones in the presence of *nodD*. The expression of fusions 32 and 6214 was not modified by the addition of 10 μM, 25 μM, or 50 μM luteolin in the culture medium, regardless of the location of the Tn5-B20 on the pSym plasmid or on the plasmid pGMI471 (data not shown). Furthermore, because it has been reported that the activating effect of luteolin on the expression of the *nodABC* genes was much higher when a *nodD* gene was present on an IncP1 plasmid (Mulligan and Long, 1985), we introduced IncP1 plasmids carrying either *nodD1* or *nodD3* *syrM* genes into strains carrying fusions 32 or 6214 on their pSym. The level of β-galactosidase activity of these constructs was not modified by the addition of luteolin (data not shown).

Influence of the Replicon Carrying the *trc-lacZ* Fusions

Tn5-B20 insertions were recombined into their pSym location to examine the influence of the replicon carrying the fusions on the β-galactosidase activity. Results are presented in Table 2. In the absence of trigonelline, the β-galactosidase activity of fusion 6214 was approximately similar, regardless of the location of the fusion on pGMI471 or in the *trc* region of the pSym (Table 2, lines 3 and 4). However, the inducing effect of trigonelline was much higher when the fusion was located on pGMI471 than when carried by the pSym (Table 2, lines 3 and 4). Thus, in the case of fusion 6214, there might be a gene dosage effect, and/or a response of the promotor to the super-

Table 2. Expression of *trc*::Tn5-B20 Fusions in Various Genetic Backgrounds

Strain	*trc* Genotype		β-Galactosidase Units (-fold Induction)	
	pSym	pGMI471 Derivative	No Inducer	Trigonelline
1. RCR2011 (pGMI584)	+	*trc*::Tn5-B20#32	112	754 (6.7)
2. GMI6122 (pGMI471)	*trc*::Tn5-B20#32	+	17	567 (33)
3. RCR2011 (pGMI579)	+	*trc*::Tn5-B20#6214	35	1739 (48)
4. GMI6272 (pGMI471)	*trc*::Tn5-B20#6214	+	36	107 (3)
5. RCR2011 (pGMI567)	+	*trc*::Tn5-B20#4109	47	148 (3.1)
6. GMI6275 (pGMI471)	*trc*::Tn5-B20#4109	+	49	119 (2.4)

Response of *R. meliloti* bearing *trc*::Tn5-B20 fusions to 2.9 mM trigonelline. The host strain is written in the left column, plasmids are indicated in parentheses. The genotype for pSym is designated + when the megaplasmid is wild type. Vector-borne *trc* loci are designated + if *trc* genes are wild type. Values represent averages of at least three independent experiments.

coiling level of the replicon. Fusion 32 has a different behavior: in the absence of trigonelline, its β-galactosidase activity was higher when the fusion was present on pGMI471 than when it was carried by the pSym (Table 2, lines 1 and 2), which could also suggest a gene dosage effect. However, such a gene dosage effect is not observed in the presence of trigonelline (Table 2, lines 1 and 2), indicating that under these conditions, the presence of extra copies of the *trc* genes is not the limiting factor for the expression of fusion 32. Expression of fusion 4109 showed no difference in response to a pSym or a pGMI471 location (Table 2, lines 5 and 6).

It appeared from these results that fusion 32 recombined into its genomic location on the pSym had a low basal expression, but that this expression was increased 30-fold in the presence of trigonelline. This construct was chosen to study *trc* gene expression in association with plants.

trc Genes Are Induced during Nodule Development

Because we had shown that *trc* gene expression was inducible, one way to address the question of the possible role of the *trc* genes in symbiosis was to monitor the expression of *trc*::Tn5-B20 fusions under symbiotic conditions. Alfalfa was used for studying *trc* gene expression during symbiosis because it has been reported to contain trigonelline (Sethi and Carew, 1974), and for all the fusions whose transcription was induced by trigonelline, addition of alfalfa seed exudate resulted in an increase in the β-galactosidase activity approximately half of that observed under induction by 3 mM trigonelline (data not shown). Fusions 12 and 32, which are located in region IV, were chosen for monitoring *trc* gene expression because when carried by the pSym plasmid, their in vitro expression was increased 30-fold to 50-fold by the addition of 2.9 mM trigonelline (Table 2). Insertion 3 was used as negative control: in the absence of trigonelline, this insertion has a β-galactosidase activity similar to that of fusions 12 and 32. Furthermore, it is located in the same region, but in the opposite orientation to the orientation of fusions 12 and 32 and, hence, is not induced by trigonelline. To ascertain that the β-galactosidase activity was exclusively related to the expression of the *trc-lacZ* fusions, Tn5-B20 insertions 3 and 32 were introduced into the pSym of strain GMI211, an RCR2011 derivative whose endogenous *lac* gene is inactivated by a Tn5 insertion (David et al., 1988). β-Galactosidase activity was visualized by light microscopy observation of the pigment resulting from the degradation of the indigogenic substrate X-Gal. Experimental conditions were adapted to minimize the blue staining resulting from the plant β-galactosidase activity (see Methods). Entire plants inoculated with RCR2011, GMI211, or their derivatives carrying either insertions 3, 12, or 32 were collected from day 2 to day 10 after inoculation and observed for β-galactosidase activity.

No indigo accumulation was observed in plant controls inoculated either with RCR2011, GMI211, RCR2011*trc*::Tn5-B20#3, or GMI211*trc*::Tn5-B20#3. In the two latter strains, however, a very low staining could occasionally be seen in a few infection threads observed generally on the top of emerging nodules (results not shown). On plants inoculated with strains carrying insertions 12 or 32, we observed an accumulation of indigo in bacterial flocs in the vicinity of or in contact with roots (result not shown). Figures 4A and 4B show indigo accumulation in the middle of the crook of a curled root hair, in infection threads developing in root hairs, and in the plant cortex. Figure 4C shows developing nodules that emerged at the surface of plants collected 6 days to 10 days after inoculation. They appeared to be subdivided in a central core, which contained the infected cells and where indigo was concentrated, and in peripheral tissues, which remained unstained. These results demonstrated that the *trc* genes are induced during the different steps of the infection, i.e., when the bacteria multiply in the plant rhi-

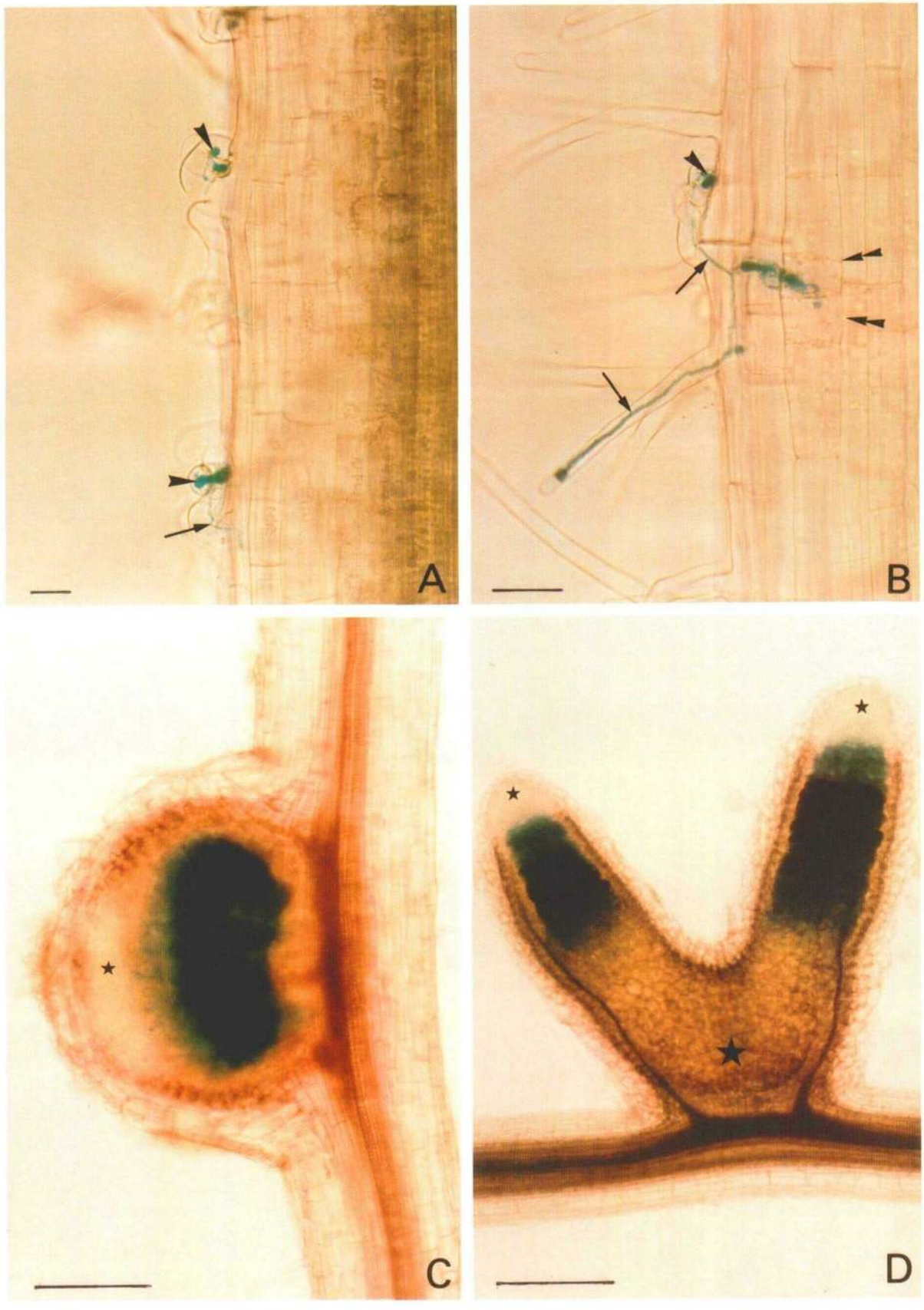


Figure 4. Histochemical Localization of β-Galactosidase Activity in Undissected and Cleared Alfalfa Roots Inoculated by Various *R. meliloti* RCR2011 Derivatives.

zosphere, at the infection point in the crook of the curled hairs, inside the infection threads located in root hairs and in the plant cortex, and in the core of developing nodules.

trc Genes Are Expressed in Mature Nitrogen-Fixing Nodules

We looked for β-galactosidase activity in 3-week-old to 7-week-old nodules. Figure 4D shows nodules still attached to the root and Figures 5A to 5D show 80-μm-thick longitudinal sections of nodules. Whatever the strain inoculated, no staining was observed in the bacteria-free tissues such as the meristematic zone I, or in the nodule peripheral tissues including the outer cortex, the endodermis, the vascular bundles, or the nodule parenchyma, and in the proximal zone IV, where bacteroids have senesced (Figures 4D to 5D). However, it should be mentioned that in 5-week-old or older nodules, the vascular bundles could sometimes stain (Figures 5A and 5C). In these cases, indigo never deposited in the distal part of the vasculature, but rather in its proximal part, which is adjacent to the central zone III (Figure 5A) and/or the unreactive, senescent zone IV (Figure 5C). It is clear that this staining accumulation in noninfected tissues, which takes place whatever the bacterial strain inoculated, including the control strain GMI211, which has no functional *lac* gene, reflects a β-galactosidase activity of plant origin.

With nodules elicited by strains carrying insertions 12 or 32, indigo was concentrated in the zones of the nodules where plant cells were invaded by bacteroids. These zones included (1) the prefixing zone II (Vasse et al., 1990), the distal part of which is enriched with infection threads that reacted intensely (Figures 5A and 5B). In this zone, the plant cells showed a progressive staining as a function of the level of cytoplasm occupancy by bacteria, whereas noninvaded cells remained unstained (Figure 5B); (2) the interzone II–III (Vasse et al., 1990) where a slight increase in staining was noticed; and (3) all along zone III, where indigo deposited widely and evenly (Figure 5A). It is noteworthy that the nonfixing bacteroids of the inefficient part of zone III (Vasse et al., 1990) still exhibited a β-galactosidase activity. Controls were performed by observing nodules elicited by RCR2011 or GMI211 and their derivatives carrying Tn5-B20#3. In the nodules elicited with strains RCR2011*trc*::Tn5-B20#3 or GMI211*trc*::Tn5-B20#3, a faint staining occurred in the interzone II–III and in the central zone III, whereas the apical zone I, zone II, zone IV, and the peripheral tissues remained unstained (Figure 5C). No β-galactosidase activity was detected in the central tissue of nodules elicited by RCR2011 or GMI211, regardless of the age of the nodules (Figure 5D). These results show that *trc* genes are induced during late symbiotic steps, in bacteria still enclosed in the infection threads, as well as in all the zones of the nodule where the plant cells are invaded by alive bacteroids and, thus, independently of the nitrogen-fixing ability of the bacteroids.

It is known that within nodules, infecting bacteria are subjected to microaerobiosis (Appleby, 1984) and increased osmolarity. To determine whether *trc* gene induction observed in nodules could result from these environmental changes, we tested the effect of microaerobiosis and elevated osmolarity on the expression of fusion 32 in free-living bacteria. For this purpose, well-aerated early log-phase cultures of strain GMI6122, which carries fusion 32 on the pSym, were subjected to microaerobic conditions by bubbling with 2% O_2. No induction could be detected, although under the same conditions, the control strain GMI6043 carrying on the plasmid pWF10 a *fixK*::*lacZ* fusion, which is only expressed in microaerobic conditions, showed a high induction of its β-galactosidase activity (data not shown).

The effect of NaCl concentrations of 0.1 M, 0.2 M, 0.3 M, and 0.4 M was also tested on strain GMI6122. No induction of β-galactosidase activity could be detected in response to this increase of the osmotic strength of the growth medium (data not shown).

DISCUSSION

By using transcriptional *lacZ* fusions, we analyzed the regulation of expression of *trc* genes encoding trigonelline catabolism functions in *R. meliloti* RCR2011. In free-living rhizobia, *trc* genes appear to be regulated at the transcriptional level, with trigonelline acting as an inducer. Analysis of the expression of the *trc*::Tn5-B20 fusions in the absence and presence of trigonelline allowed us to define

Figure 4. (continued).

(A) Early symbiotic steps, 5 days after inoculation with RCR2011*trc*::Tn5-B20#12. Hac (arrowheads) and Inf (arrows) phenotypes. Bar = 100 μm.

(B) Early symbiotic steps, 5 days after inoculation with RCR2011*trc*::Tn5-B20#32. Hac (arrowheads) and Inf (arrows) phenotypes are shown. Note the division of cortical cells (double arrowheads) in the front of advancing infection threads. Bar = 100 μm.

(C) Ten-day-old developing nodule elicited by RCR2011*trc*::Tn5-B20#32. The nodule core is stained, but not the apical meristem (small star). Bar = 100 μm.

(D) Five-week-old mature nodules elicited by RCR2011*trc*::Tn5-B20#12. Staining is visible in the central tissue of nodules, but not in the apical meristem (small stars), in the senescent zone (large star), or in peripheral nodular and plant tissues. Bar = 500 μm.

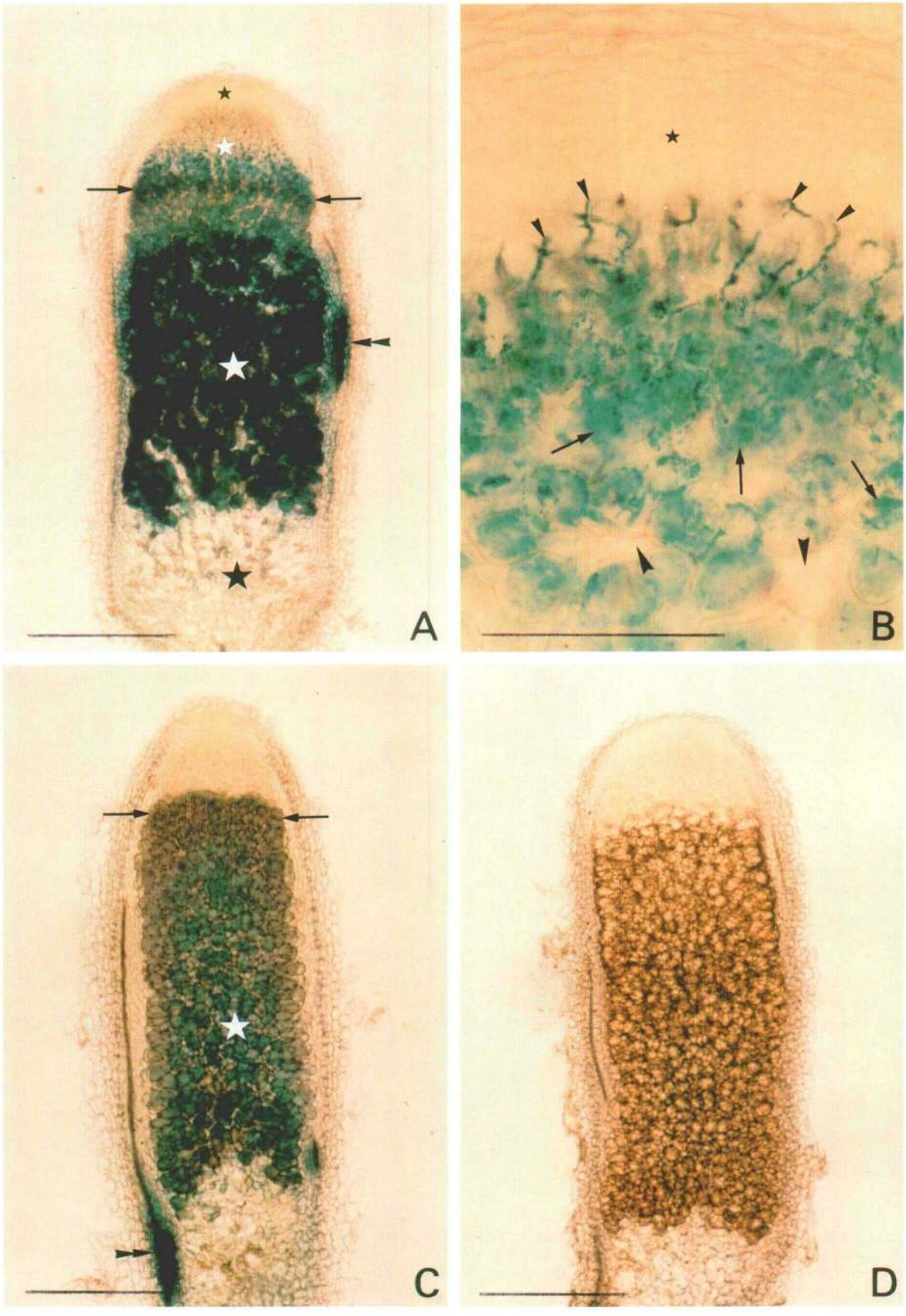


Figure 5. Histochemical Localization of β-Galactosidase Activity in 6-Week-Old Alfalfa Nodules.

regions I, II, and IV, for which addition of trigonelline results in a 50-fold, twofold, and 10-fold increase of the β-galactosidase activity, respectively, and region III, in which it is constitutively expressed at a low level. These four regions, defined on gene expression criteria, are compatible with the three potential transcription units predicted on the basis of functional complementation experiments, regions I and II corresponding to transcription units 1 and 2, respectively (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation).

Results of complementation experiments involving Trc^d mutants (which grow slowly on trigonelline as sole C and N source) were difficult to interpret, and did not enable regions III and IV to be distinguished. However, on the basis of expression studies, it appears that transcription unit 3 is, in fact, composed of two independent transcription units: region III, in which Tn5-B20 fusions are not induced by trigonelline and result in a Trc^- phenotype, and region IV, in which fusions are inducible by trigonelline and result in a Trc^d phenotype. When the fusions are recombined into their genomic location on the pSym, the level of β-galactosidase activity is modified, but the inducing effect of trigonelline is still observed. Compounds closely related to trigonelline are not able to induce *trc* gene expression. The fact that nicotinic acid and homarine, which differ from trigonelline by the methylation of the N atom or by the position in *meta* of the carboxyl group, respectively (Figure 3), do not induce *trc* genes shows the importance of these radicals as determinants of induction specificity.

All of the trigonelline-related compounds tested so far were able to antagonize the effect of trigonelline on the expression of fusions in region IV. It has been shown that trigonelline and various other betaines are competitors for proline betaine transport (Gloux and Le Rudulier, 1989). Furthermore, glycine betaine is a competitor for the transport of choline and choline-related compounds (Pocard et al., 1989). Thus, the antagonistic effect of trigonelline-related compounds on *trc* gene induction by trigonelline could be due to competition for transport. Such a hypothesis is supported by the different behavior of fusion 6214 in region I: contrary to fusions in region IV, induction by trigonelline of fusion 6214 was not affected by these compounds except when a 100-fold molar excess of choline was added. This difference could reflect the fact that the fusion in region I, which is fully induced at a trigonelline concentration much lower than that required for the full induction of region IV fusions, would be less affected by a lower internal trigonelline concentration resulting from competition for transport.

The question of a possible control of *trc* gene expression by general circuits of regulation was addressed by introducing *trc* fusions into strains carrying mutations affecting these circuits. Neither the *ntrA* gene, which controls expression of genes involved in nitrogen metabolism (Ronson et al., 1987; Kustu et al., 1989), nor the *dctB/dctD* genes controlling the transport of dicarboxylic acids (Yarosh et al., 1989) seem to be necessary for *trc* gene expression in free-living bacteria. Similarly, the NodD protein, which controls the expression of the *nod* genes (Mulligan and Long, 1985), is not required because *trc* gene expression was not modified in a *R. meliloti* background lacking all the regulatory genes *nodD1*, *nodD2*, *nodD3*, and *syrM*. Furthermore, although it has been reported that *nod* genes are induced by trigonelline (Schmidt et al., 1986), *trc* genes are not induced by luteolin, one of the most potent inducers of *R. meliloti nod* genes. However, the fact that both *nod* and *trc* genes are induced by the same molecule suggests some similarity in the two regulatory mechanisms. According to the hypothesis proposed by Peters and Verma (1990), such a similarity would imply some common evolutionary origin, the symbiotic regulatory genes being derived from catabolic or metabolic regulatory genes involved in the plant relationship.

We adapted the method described by Teeri et al. (1989) to our experimental conditions to study the expression of inducible *trc* fusions in plant tissues under symbiotic conditions using the *E. coli lacZ* gene as a reporter gene. This technique appeared to be very efficient for monitoring the expression of bacterial genes during the different symbiotic steps and should be of great value in the future for studying the expression of various *Rhizobium* symbiotic genes for which *lacZ* fusions are already available. Our results show that induction takes place at a very early stage, at the rhizosphere level, and continues throughout the successive symbiotic stages, i.e., the infection thread formation and development, and all along the five steps in bacteroid differentiation (Vasse et al., 1990), independently of their nitrogen-fixing capacity. The staining observed in bacter-

Figure 5. (continued).

Eighty-micrometer-thick longitudinal sections cleared with Na hypochlorite.

(A) Inoculation with *R. meliloti* RCR2011*trc*::Tn5-B20#32. Indigo is seen in central infected cells of zone II (small white star), interzone II-III (arrows), and zone III (large white star), but not in the apical meristem (small black star) or in the senescent zone IV (large black star). β-Galactosidase activity is observed in some parts of the vasculature (double arrowheads).

(B) Inoculation with *R. meliloti* RCR2011*trc*::Tn5-B20#32. Detail of the non-nitrogen-fixing zone II. Infection threads (small arrowheads) and infected plant cells (arrows) are stained. The noninvaded host cells (large arrowheads) do not react. Star = apical meristem.

(C) Inoculation with *R. meliloti* RCR2011*trc*::Tn5-B20#3. Slight staining is observed in interzone II–III (arrows) and in central zone III (white star). β-Galactosidase activity is also observed in some parts of the vasculature (double arrowheads).

(D) Inoculation with *R. meliloti* GMI211. No indigo deposit is observed.

Bars = 500 μm.

oids does not result from a remaining β-galactosidase activity induced previously in the infection threads. Rather, it reflects a genuine induction because the same technique has revealed that another type of mutant, carrying a *lac* fusion in an early symbiotic gene, presents a similar indigo accumulation in the infection threads but no coloration in the bacteroids (G. Truchet, unpublished results). Furthermore, we have shown that this induction observed in nodules is not likely to result from an increase in the osmotic strength of the environment or from microaerobic conditions.

Trigonelline, which has been reported to be present in legume seeds, roots, and root exudates (Kuo et al., 1982; Evans and Tramontano, 1984), is a specific inducer of the *trc* genes in free-living Rhizobium. This set of results highlights trigonelline as a probable inducer of *trc* genes in association with plants as well, and suggests that trigonelline could be used as a nutrient source by the bacteria during all the stages of the symbiotic relation, in the rhizosphere, and in the nodules of the host plant. According to this hypothesis, trigonelline must be able to get through the peribacteroid membrane, which is likely to limit the substrates available for the bacteroids (Herrada et al., 1989). Among these substrates, dicarboxylic acids are abundant in nodule cytosol and, more particularly, succinate, which is known to be responsible for catabolic repression in *R. meliloti* (Ucker and Signer, 1978; Arias et al., 1982). The fact that *trc* genes are expressed in the bacteroids is in agreement with our finding that in vitro, *trc* genes are not repressed by succinate (data not shown). Because it has been shown that mutants affected in dicarboxylic acid transport induce the formation of Fix^- nodules (Engelke et al., 1987), trigonelline, at least at the concentrations present in alfalfa, cannot be an alternative substrate able to support nitrogen fixation. Nevertheless, an involvement of trigonelline in nodule development cannot be ruled out. The *trc* gene expression during the different symbiotic stages and the location of the *trc* genes in a symbiotic region of the pSym strongly suggest a role for these genes in the relationship with the plant. This is apparently in contradiction with the absence of symbiotic defects of the *trc* mutants (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation). Similarly, inactivation of the genes encoding L-homoserine catabolism in *R. leguminosarum* bv *viciae*, which are pSym borne, do not affect symbiotic properties in laboratory conditions (Johnston et al., 1988). Because each *Rhizobium* strain is able to nodulate various potential hosts, which synthesize different secondary metabolites, it would seem logical that each strain would evolve different sets of catabolic enzymes to take advantage of these substrates. In this case, inactivation of one of these sets of catabolic enzymes might affect the symbiotic properties of the strain so slightly that it could not be detected in laboratory conditions. However, it can be hypothesized that in natural conditions the presence of such catabolic genes could confer on the strain a selective advantage for the colonization of the plant rhizosphere and/or the plant infection.

METHODS

Bacterial Strains, Plasmids, Phage, and Media

The strains, plasmids, and phage used in this study are listed in Table 3. Mutagenesis of the plasmid pGMI471 by the transposable element Tn5-B20 is described elsewhere (C. Boivin, L. Barran, C. Malpica, and C. Rosenberg, manuscript in preparation). Localization of some of the corresponding Tn5-B20 insertions is presented in Figure 1. Luria broth (LB) (Sambrook et al., 1989) was used as rich medium for growth of *Escherichia coli* and *Rhizobium meliloti* strains. For *R. meliloti* strains, the minimal medium, without C and N source (V medium), was as described by Vincent (1970). Antibiotics were used at the following concentrations: 30 μg/mL kanamycin (Km), 100 μg/mL neomycin (Nm), 10 μg/mL tetracycline (Tc), and 200 μg/mL spectinomycin (Sp).

Microbiological Techniques

The method for conjugal crosses was as described by Truchet et al. (1985). Initially, pGMI471 derivatives were introduced into *E. coli* strain S17-1 by mobilization with the helper plasmid pRK2013. Resulting transconjugants were then used as donors for transfer of these plasmids into *R. meliloti* strains. Tn5-B20 inserts were recombined into their genomic location by the method described by Debellé et al. (1986), and the presence of the Tn5-B20 inserts was verified by examining the ability of the resultant strain to grow on V minimal medium containing 0.5 g/L trigonelline as sole C and N source.

Chemicals

Glycine betaine, choline, trigonelline, and carnitine (γ-amino-β-hydroxybutyric acid betaine) were purchased from Sigma. Homarine (pipecolinic acid betaine), stachydrine (proline betaine), and luteolin were from Extrasynthese (Genay, France). Nicotinic acid was from Hoffmann-La Roche et Cie. (Paris, France).

Plant Assays

Seeds of *Medicago sativa* cv Gemini were obtained from Tourneur Frères (F77120 Coulommiers, France). Seeds were surface sterilized and germinated as previously described (Truchet et al., 1985), and the seedlings were aseptically grown in test tubes on Fåhraeus nitrogen-free (Fåhraeus, 1957) agar slants. *R. meliloti* strain RCR2011 and RCR2011 derivatives carrying a fusion of *trc* genes to the *E. coli lacZ* reporter gene were grown for 48 hr on LB or LB agar medium plus neomycin, resuspended in sterile water, and added to each tube containing 2-day-old to 3-day-old seedlings at a density of 10^6 bacteria per tube.

Table 3. Bacterial Strains, Plasmids, and Phages

Strain, Bacteriophage, or Plasmid	Relevant Characteristics	Source or Reference
Escherichia coli		
S17-1	F^-, lambda$^-$, *recA*, *hsdR*, carrying a modified RP4 ($Ap^sTc^sKm^s$) integrated in the chromosome	Simon et al. (1983)
TB1	*ara*, delta (*lac-pro*), *strA*, *thi*, phi80 *lacZ* delta (M15), *hsdR*, Sm^r	Boucher et al. (1987)
Rhizobium meliloti		
RCR2011 = SU47	Wild type; Nod^+ Fix^+ Trc^+	Rosenberg et al. (1981)
GMI766	Nod^- delta (*nod nif A*) *766*, Sp^r Trc^-	Truchet et al. (1985)
GMI6278	RCR2011 *trc*::Tn5-B20#12, Nm^r	This work
GMI6122	RCR2011 *trc*::Tn5-B20#32, Nm^r	This work
GMI6272	RCR2011 *trc*::Tn5-B20#6214, Nm^r	This work
GMI6275	RCR2011 *trc*::Tn5-B20#4109, Nm^r	This work
GMI6101	RCR2011 *trc*::Tn5-B20#3, Nm^r	This work
GMI211	RCR2011 Lac^-, Sm^r	David et al. (1988)
GMI6301	GMI211 *trc*::Tn5-B20#3	This work
GMI6302	GMI211 *trc*::Tn5-B20#32	This work
GMI5941	GMI766 carrying the pGMI471 plasmid, Sp^r Trc^+	Boivin et al. (1989)
GMI6043	RCR2011 *rif-2 recA*::Tn5 233	This laboratory
Rm1021	Sm^r derivative of RCR2011	Mulligan and Long (1985)
RmF121	Rm1021 *dctD16*::Tn5, Nm^r	Finan et al. (1988)
RmF332	Rm1021 *dctB18*::Tn5, Nm^r	Finan et al. (1988)
1681	Rm1021 *ntrA2*::Tn5, Nm^r	Ronson et al. (1987)
IncP1 plasmids		
pLAFR1	cosmid carrying *cos*, *rlx*, *ori*(RK2), Tc^r	Friedman et al. (1982)
pGMI471	pLAFR1-prime, 30-kb insert of pSym RCR2011 carrying *trc* genes, Tc^r	M. H. Rénalier (this laboratory)
pGMI584	pGMI471 *trc*::Tn5-B20#32, Tc^r Nm^r	C. Boivin, L. Barran, C. Malpica, and C. Rosenberg (manuscript in preparation)
pGMI579	pGMI471 *trc*:Tn5-BN20#6214, Tc^r Nm^r	C. Boivin, L. Barran, C. Malpica, and C. Rosenberg (manuscript in preparation)
pGMI567	pGMI471 *trc*::Tn5-B20#4109, Tc^r Nm^r	C. Boivin, L. Barran, C. Malpica, and C. Rosenberg (manuscript in preparation)
pMH901	pWB5A-prime, 2.7-kb insert from pSym Rm1021 carrying $nodD_1$, Tc^r	Honma et al. (1990)
pMH682	pWB5A-prime, 8-kb insert from pSym Rm1021 carrying $nodD_3$ and *syrM*, Tc^r	Honma et al. (1990)
pWF10	pGD926 derivative, *pfixK-lacZ* translational fusion, Tc^r	F. Waelkens (this laboratory)
Other plasmid		
pRK2013	Helper plasmid for mobilization of pLAFR1 derivatives, *tra* (RK2) *ori* (ColE1) Km^r	Figurski and Helinski (1979)
Phage		
N3	Transducing phage of *R. meliloti*	Martin and Long (1984)

β-Galactosidase Activity in Free-Living Bacteria

The screening of the different fusions for their ability to be induced by trigonelline was performed in V medium supplemented with saccharose (0.1%) and sodium glutamate (0.1%) as C and N sources (VSG medium). In further induction experiments, sodium succinate (0.1%) was added to VSG medium (VSGS medium) because it has been shown to repress endogenous β-galactosidase activity of *R. meliloti* (Ucker and Signer, 1978) but does not affect *trc* gene expression (this work). Bacteria were grown overnight to mid-log-phase in the induction medium and then the bacterial suspension was diluted in fresh medium to $A_{600} = 0.1$. A solution containing either the potential inducer, or, for inhibition experiments, both trigonelline and a potential inhibitory compound (see Table 1), was added. Unless stated otherwise, trigonelline was added to 2.9 mM (0.5 g/L) and induction was performed at

28°C for 3 hr, 30 min. Induction experiments using alfalfa seed exudates were performed under the same conditions. The alfalfa seed exudate, prepared as described by Mulligan and Long (1985), was added at 1:10 dilution.

The background level of endogenous β-galactosidase activity in wild-type *R. meliloti* is less than 5 units and is affected neither by the addition of trigonelline to the medium nor by the presence of the plasmid pGMI471.

To test the inducing activity of luteolin, bacteria were grown overnight to mid-log-phase. Bacterial suspensions were diluted to $A_{600} = 0.004$ and luteolin was added to 10 μM, 25 μM, or 50 μM. Induction was performed at 28°C for 16 hr and enzyme activity was assayed as described above.

The experiments to test the effect of microaerobic conditions on *trc* gene expression were performed as described by David et al. (1988).

To test a possible effect of high osmotic strength on *trc* gene expression, cells were incubated in VSGS medium supplemented with 0.1 M, 0.2 M, 0.3 M, or 0.4 M NaCl. After 3 hr and 5 hr of incubation, the cells were washed by centrifugation, resuspended in distilled water, and assayed for β-galactosidase activity.

β-Galactosidase assays were carried out as described by Miller (1972) with the following modifications: 0.15 mL to 0.25 mL of bacterial suspension was added to Z buffer to a final volume of 0.75 mL. Cells were permeabilized with 100 μL of chloroform and 50 μL of sodium dodecyl sulfate (0.1%). The reaction was started by adding 0.3 mL of ONPG (4 mg/mL) and stopped by addition of 0.375 mL of Na_2CO_3 (1 M).

β-Galactosidase Assay in Association with Plants

The histochemical staining is a modification of the method described by Teeri et al. (1989). The enzymatic activity was assayed throughout the different steps in nodule development either on whole undissected plants or on nodule slices 80 μm thick. Using plants inoculated with strain GMI211, which is devoid of β-galactosidase activity, we first adapted our experimental conditions to suppress the background plant endogenous β-galactosidase activity by fixing the plants with a glutaraldehyde solution, buffered either with Z (Miller, 1972), Z′ (Teeri et al., 1989), or sodium cacodylate. In such assays, the concentration of glutaraldehyde, the pH of the final solution, and the time of fixation have been considered. In further experiments, entire plants inoculated with the various *R. meliloti* strains were fixed with 1.25% glutaraldehyde buffered with 0.2 M sodium cacodylate (pH 7.2; 15 min under vacuum followed by 1 hr at atmospheric pressure) and then rinsed with the buffer (pH 7.2; 2 × 15 min). After rinsing, the following treatments were applied either to whole plants or to 80-μm longitudinal slices of fixed nodules (Microcut H 1200, Bio-Rad). The specimens were transferred to the staining solution: 800 μL of sodium cacodylate, 0.2 M, pH 7.2; 50 μL of $K3[Fe(CN)_6]$, 100 mM; 50 μL of $K4[Fe(CN)_6]$, 100 mM; 40 μL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 2% in *N,N*-dimethylformamide (X-Gal). After the incubation, usually overnight at room temperature, the specimens were successively rinsed with buffer (3 × 10 min) to remove the staining solution, with distilled water (2 × 5 min), and finally mounted in water between a slide and a coverslip. The specimens were finally observed by bright-field microscopy with an Olympus Vanox light microscope.

To improve the contrast between the reacting tissues and the nonreactive tissues, the specimen could be briefly cleared (30 sec to 60 sec) with an aqueous solution of sodium hypochlorite with 12% active chloride (Truchet et al., 1989), and rinsed with distilled water (4 × 5 min) before being mounted and observed by light microscopy.

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