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Role of Methylotrophy During Symbiosis Between *Methylobacterium nodulans* and *Crotalaria podocarpa*

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Some rare leguminous plants of the genus *Crotalaria* are specifically nodulated by the methylotrophic bacterium *Methylobacterium nodulans*. In this study, the expression and role of bacterial methylotrophy were investigated during symbiosis between *M. nodulans*, strain ORS 2060^T, and its host legume, *Crotalaria podocarpa*. Using *lacZ* fusion to the *mxaF* gene, we showed that the methylotroph genes are expressed in the root nodules, suggesting methylotrophic activity during symbiosis. In addition, loss of the bacterial methylotrophic function significantly affected plant development. Indeed, inoculation of *M. nodulans* nonmethylotroph mutants in *C. podocarpa* decreased the total root nodule number per plant up to 60%, decreased the whole-plant nitrogen fixation capacity up to 42%, and reduced the total dry plant biomass up to 46% compared with the wild-type strain. In contrast, inoculation of the legume *C. podocarpa* with nonmethylotrophic mutants complemented with functional *mxa* genes restored the symbiotic wild phenotype. These results demonstrate the key role of methylotrophy during symbiosis between *M. nodulans* and *C. podocarpa*.

Additional keyword: methanol.

The bacterial genus *Methylobacterium* belongs to the α subclass of the family *Proteobacteria*. It constitutes a group of strictly aerobic, gram-negative, rod-shaped, pink-pigmented facultative methylotrophic (PPFM) bacteria (Green 1992). Presently, the genus comprises 19 species isolated from a wide range of environments (Doronina et al. 2002; Gallego et al. 2005a, in press; Green 1992; Jourand et al. 2004; Van Aken et al. 2004).

The main feature of members of *Methylobacterium* is their ability to oxidize methanol, a methylotrophic property based on the presence of methanol dehydrogenase (MDH), a pyrroloquinoline quinone (PQQ)-linked protein with an $\alpha_2\beta_2$ tetramer structure (Anthony 1982; Anthony et al. 1994). The genes enabling methanol oxidation have been studied in the model strain *Methylobacterium extorquens* AM1 (Chistoserdova et al. 2003). The main gene cluster, containing 14 genes named *mxaFJGIRSACKLDEHB,* is transcribed in a single mRNA operon. Transcription is controlled by a methanol inducible promoter (Zhang and Lidstrom 2003). Within the gene cluster, *mxaF* and *mxaI* genes encode the largest α and the smallest β polypeptide subunits of MDH, respectively (Anderson et al. 1990; Nunn et al. 1989; Nunn and Lidstrom 1986a and b).

Although most *Methylobacterium* spp. have been regularly isolated from soils, water, or plants (Corpe and Rheem 1989; Gallego et al. 2005a; Green 1992; Holland 1997), little is known about the role of the methylotrophic function in the adaptation of bacteria to these various ecosystems. Only a few studies have suggested that *Methylobacterium* spp. were able to use methanol emitted by leaf stomata during their association with plants (Lidstrom and Chistoserdova 2003; Nemececk-Marshall et al. 1995).

Over the last 5 years, *Methylobacterium* spp. strains have been isolated from root nodules of legumes belonging to the genera *Crotalaria* and *Lotononis* (*Fabaceae* and *Crotalariae*) (Jaftha et al. 2002; Samba et al. 1999; Sy et al. 2001a and b). Nonpigmented strains isolated from three *Crotalaria* spp. (i.e. *Crotalaria glaucoides*, *C. perrottetii,* and *C. podocarpa*) were described as actually being a single novel *Methylobacterium* sp. (Sy et al. 2001a and b). This new species, which has the same methylotrophic function as other *Methylobacterium* spp., was named *M. nodulans* for its ability to nodulate and fix nitrogen specifically during symbiosis with *Crotalaria* spp. (Jourand et al. 2004; Sy et al. 2001a and b).

The aim of this work was to study the relationship between the methylotrophic function of *M. nodulans* during symbiotic processes with the host legume, *C. podocarpa*. The expression of methylotroph genes during symbiosis was investigated using *lacZ* fusion to *mxaF*, whereas the role of methylotrophy was determined by studying the effect of methylotroph mutants on the plant growth.

RESULTS

Methylotrophy of *M. nodulans* is expressed in root nodules formed during symbiosis with *Crotalaria podocarpa*.

To monitor the expression of methylotroph genes in planta, we constructed a *M. nodulans* ORS 2060^T recombinant strain bearing a *mxaF-lacZ* transcriptional fusion promoter (Fig. 1A).

The *mxaF-lacZ* fusion promoter was pretested in vitro by measuring the β-galactosidase (β-gal) activity of the wild-type

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strain or the recombinant strain after culture in yeast extract-manitol (YM) medium supplemented or not supplemented with methanol. β-Gal activity was detected only in cultures of the *M. nodulans* recombinant strain 2060 *mxaF-lacZ* (Table 1). Moreover, in the presence of methanol, its level was 20-fold higher than the basal activity detected in the control, thus confirming that methanol induces *mxaF* gene expression in *M. nodulans*. Such an *mxaF* induction by methanol previously was described in the two *Methylobacterium* model species, *M. extorquens* and *M. organophilum,* with induction levels ranging from 8- to 10-fold (Xu et al. 1993; Zhang and Lidstrom 2003).

Expression of the *mxaF-lacZ* fusion promoter therefore was tested in planta by the histochemical localization of β-gal in nodule tissues. The β-Gal activity (Fig. 2A and A′, in blue) was detected in *C. podocarpa* root nodules inoculated with the *M. nodulans* recombinant strain 2060 *mxaF-lacZ*. Enzymatic activity clearly was localized in the apex of the nodule, as shown in thin longitudinal sections from fresh nodules (Fig. 2A). The blue-colored apex zone corresponding to β-gal activity also was viewed in half-nodule sections (Fig. 2A′). Interestingly, it appears that the apex zone was distinct from the medullar nitrogen-fixing zone, which is red-colored due to the presence of leghemoglobin (Balestrasse et al. 2004). In contrast, no β-gal activity was detected in nodules formed after inoculation with the *M. nodulans* wild-type strain (Fig. 2B and 2B′).

These results clearly showed that the *mxaF* gene of *M. nodulans* was expressed in root nodules during symbiosis and preferentially localized at the nodule apex. In vitro assays indicated that the *mxaF* gene of *M. nodulans* is inducible by methanol; therefore, its expression, observed in planta, could result from the presence of methanol produced during nodule development. To test this hypothesis, we measured residual

Table 1. β-Galactosidase (β-gal) total activity expressed in μmol of *o*-nitrophenyl-β D-galactoside (ONPG)/min/mg of total protein[z]

Strain	Control	Methanol
ORS 2060^T wild type	ND	ND
ORS 2060 *mxaF-LacZ*	0.15 ± 0.02 a	3.5 ± 0.08 b

[z] Both β-gal activity and total proteins were investigated on cell extracts prepared from *Methylobacterium nodulans* ORS 2060^T wild type or recombined *M. nodulans* ORS 2060^T *mxaF-LacZ*. ND = not detected. All data followed with the same letter are not significantly different ($P \leq 0.05$).

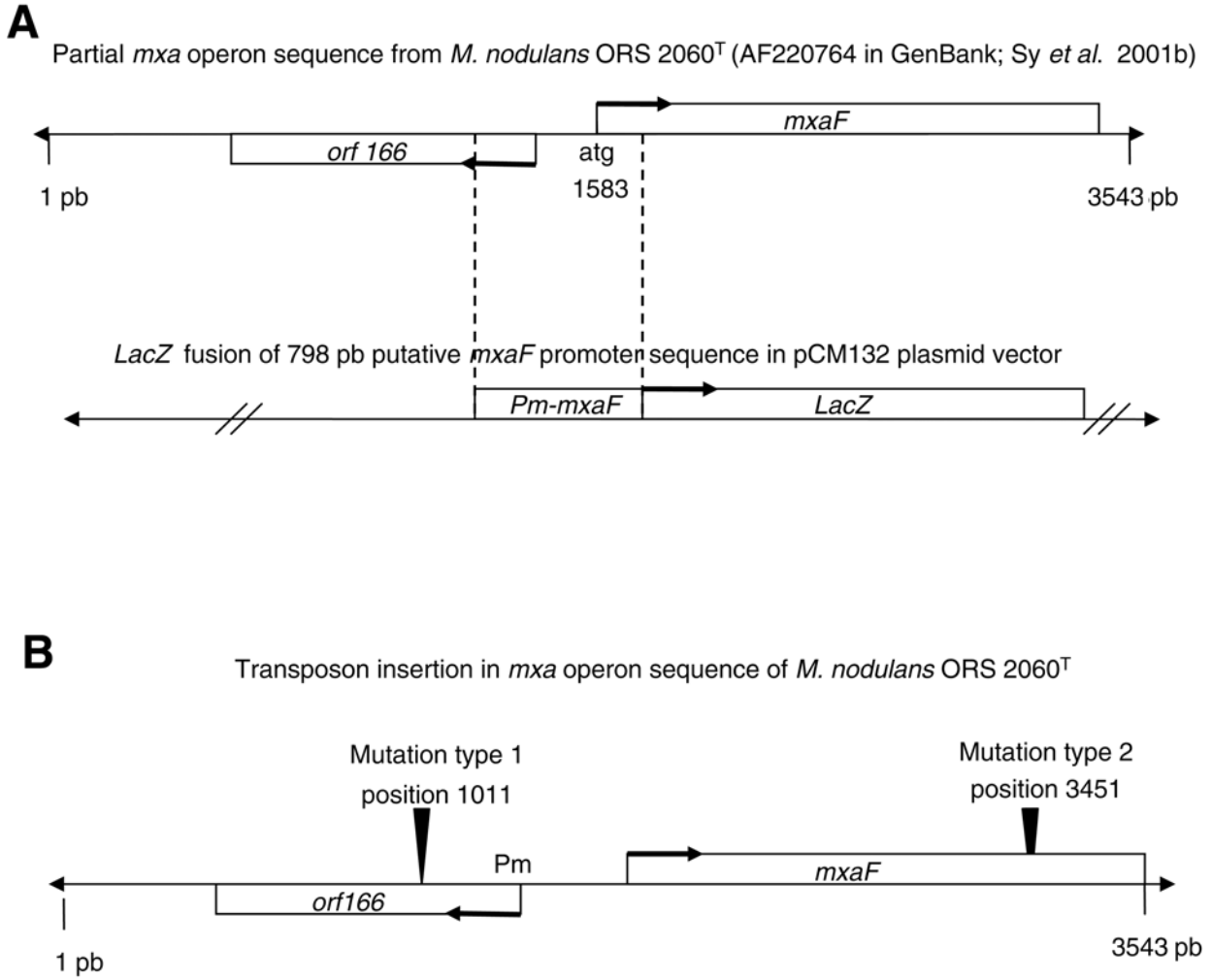

Fig. 1. Construction of *Methylobacterium nodulans* ORS 2060^T recombinant strain containing *mxaF-LacZ* transcriptional fusion promoter and ORS 2060^T negative methylotroph mutants by insertional mutagenesis of transposon *mTn5-GGm*. **A,** Physical map of the partial sequence of the operon *mxa* in *M. nodulans* ORS 2060^T wild type (AF 220764 in GenBank) according to Sy and associates (2001a), including the deduced *mxaF* putative promoter sequence position compared with the *M. extorquens* AM1 *mxaF* promoter sequence position as described by Zhang and Lidstrom (2003) and cloned into pCM132 plasmid vector (AF 327720 in GenBank) with *LacZ* gene reporter (Marx and Lidtsrom 2001). Abbreviations: Pm: promoter; Bp: DNA pair of base spacing. **B,** Mutations of *mxa* locus sequence of *M. nodulans* ORS 2060^T by insertion of transposon *mTn5-GGm* (AF080392) (Reeve et al. 1999): mutation type 1 upstream of the putative *mxaF* promoter sequence and mutation type 2 inside the *mxaF* gene.

methanol levels in nodules as well as in roots, leaves, and stems. We found low concentrations of residual methanol in *C. podocarpa* tissues which were two- to fivefold higher in nodules than in roots, leaves, or stems (data not shown), indicating that methanol is more specifically accumulated in root nodules.

Loss of methylotrophy strongly affects symbiosis.

Negative methylotrophic mutants of *M. nodulans* were constructed to further investigate the role of bacterial methylotrophy in the symbiotic processes. These mutants were obtained by insertional mutagenesis of the *mTn5-GGm* transposon (Reeve et al. 1999). They were selected on allyl alcohol, as described by Nunn and Lidstrom (1986a and b). Two of them were selected according to the position of the transposon insertion sites (Fig. 1B). The first one (mutation type 1) was close to the *mxa* operon, approximately 500 bp upstream of the putative start codon of the *mxaF* gene. The second one (mutation type 2) was inside the *mxaF* gene. Defective methylotrophic metabolism of the two mutants was checked by growth on M72 minimum mineral medium containing either sodium succinate or methanol as sole carbon source. Compared with the *M. nodulans* ORS 2060^T wild-type strain, both methylotrophic mutants presented normal growth on succinate but were unable to grow on methanol as sole carbon source (Fig. 3). In addition, MDH activity was tested according to Day and Anthony (1990): no MDH activity was detected in proteic extracts obtained from methylotrophic mutants, whereas this enzymatic activity is strongly detected in the wild-type strain protein extracts (data not shown). Two mutants were complemented with a conjugative cosmid containing a 35-kb DNA insert including the *mxa* operon of *M. nodulans* (Sy et al. 2001a). This complementation restored the methylotrophic capacity because the complemented mutants were able to grow on a M72 minimum mineral salt medium (MMS) with methanol as sole carbon source, and MDH activity also was detected (data not shown).

The growth of *C. podocarpa* plants inoculated with *M. nodulans* ORS 2060^T methylotrophic mutants type 1 or 2 was

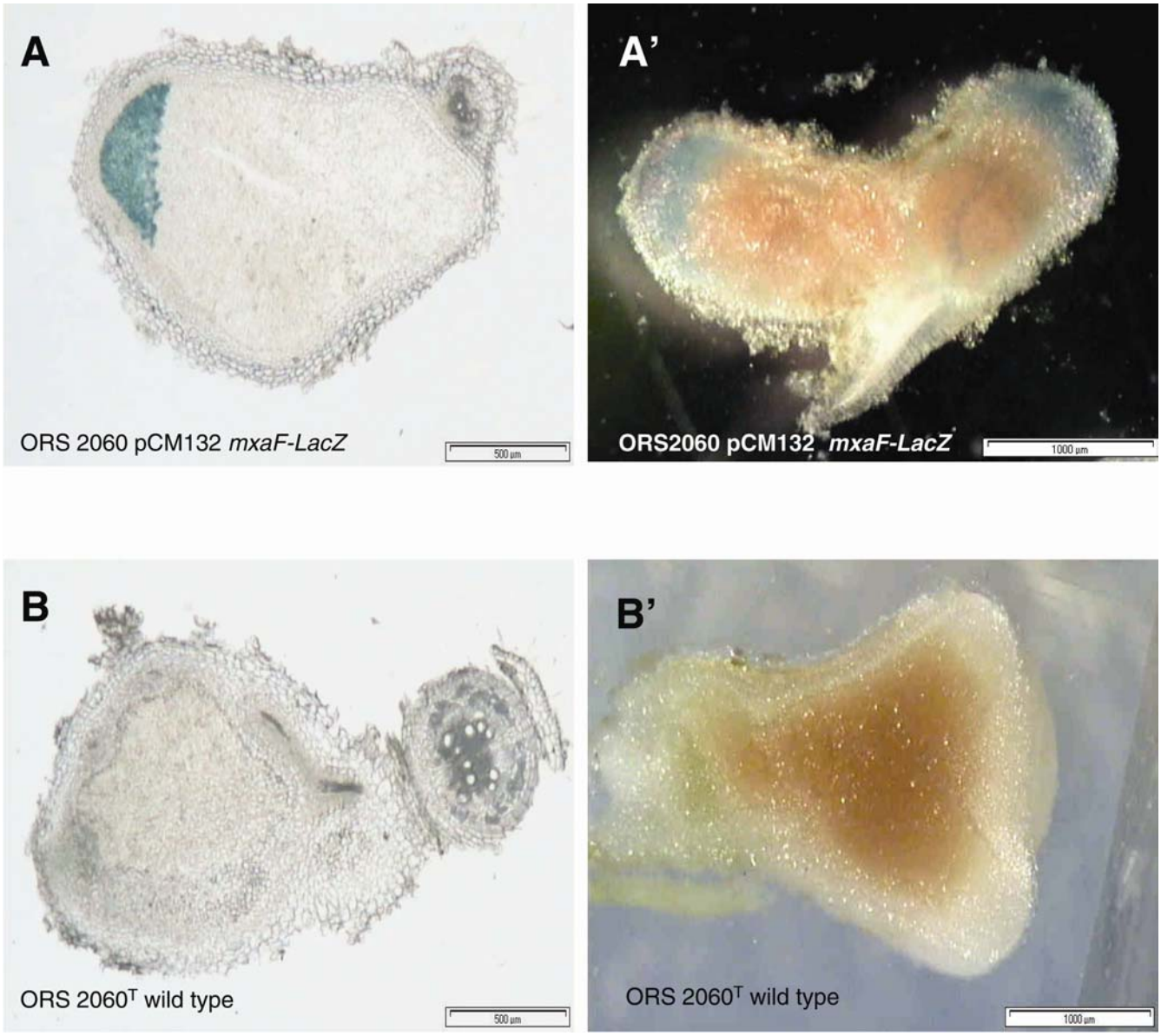

Fig. 2. A and **A′,** Histochemical localization of the β-galactosidase activity in 6-week-old *Crotalaria podocarpa* root nodules induced by the *Methylobacterium nodulans* ORS 2060^T recombinant strain containing the *mxaF-LacZ* transcriptional fusion promoter compared with **B** and **B′,** root nodules induced by *M. nodulans* ORS 2060^T wild type. **A** and **B,** Longitudinal nodule sections, 40 µm thick; **A′** and **B′,** longitudinal half of a *C. podocarpa* nodule with detection of leghemoglobine activity in red.

reduced substantially compared with that of plants inoculated with the wild-type strain (Fig. 4). This decrease in plant growth, which represented up to 46% biomass loss, was correlated with a drastic drop in the total root nodule number (up to 60%) and a significant reduction in the nitrogen fixation ability (up to 42%) (Table 2). Interestingly, if nodule number strongly decreased, the size of mutant nodules was bigger, leading to a nodule mass two to seven times higher than the wild type. In any case, for both mutants, nitrogen fixation per milligram of nodule appeared 1.2- to 5-fold weaker than the wild type (Table 2). We calculated that the average dry weight of nodules necessary to produce 100 g of plant biomass was two- to fivefold higher with the methylotrophic mutants than with the wild-type strain (Table 2). Altogether, these data indicate that the methylotrophic function of *M. nodulans* plays a major role during the symbiotic process between the bacteria and the legume. In contrast, inoculation of *C. podocarpa* with the complemented mutants restored the wild-type symbiotic phenotype (data not shown).

DISCUSSION

Although most *Crotalaria* spp. are nodulated by nonmethylotrophic *Bradyrhizobium* spp., only a few African species have the unusual ability to symbiotically and specifically associate with the newly described *M. nodulans* (Gao et al. 1994; Samba et al. 1999; You et al. 2002). Interestingly, another specific symbiosis with *Methylobacterium* spp. has been described in the genus *Lotononis*, which belongs to the same *Crotalariae* tribe (Jaftha et al. 2002). The symbiotic specificity observed between *Methylobacterium* spp. and some *Crotalariae* spp. raised the question as to the putative role of methylotrophy during this host–bacteria interaction. In this article, we clearly demonstrated that methylotrophy of *M. nodulans* is advantageous during symbiosis with the host legume *C. podocarpa*.

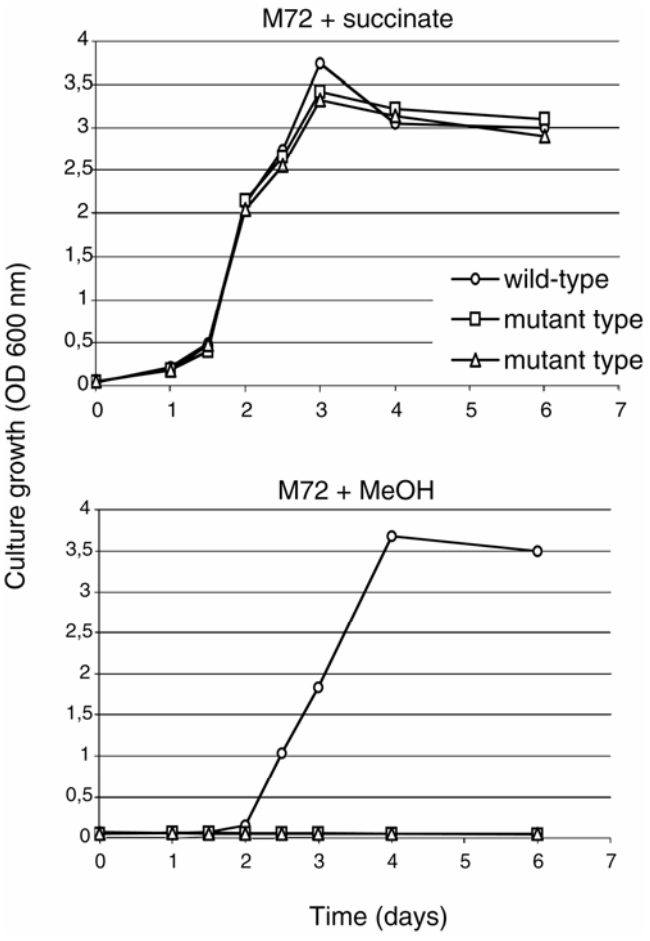

Fig. 3. Growth of *Methylobacterium nodulans* ORS 2060^T strains pregrown in succinate, harvested, and resuspended in minimum mineral medium containing either sodium succinate or methanol. Strains examined were methylotroph mutants *mTn5-GGm-pmmxaF* (mutation type 1) and *mTn5-GGm-mxaF* (mutation type 2) compared with the wild-type.

We first studied the expression of the *mxaF* gene, one of the key methylotrophic genes in *Methylobacterium* spp., both in vitro and in planta. We demonstrated that, in *M. nodulans*, this gene is not constitutively expressed but instead controlled by a methanol inducible promoter, as previously shown for *M. extorquens* and *M. organophilum* (Xu et al. 1993; Zhang and Lidstrom 2003). Close examination of the DNA sequence upstream of the putative start codon did not reveal any obvious DNA motif, which might be the target of a putative methanol-dependent regulator. This is in agreement with the previous study of Zhang and Lidstrom (2003), in which the promoter regions of four methanol-inducible operons were compared but no consensus sequence was observed. Therefore, the molecular basis of the control of *mxaF* operons in *Methylobacterium* spp. remains to be determined. Interestingly, the insertion of the Tn5 transposon 572 bp upstream of the putative *mxaF* start codon led to a negative methylotrophic metabolism. The *mxaW* gene was not detected in this region, as also was observed in *M. extorquens* (Springer et al. 1998; Zhang and Lidstrom 2003). Instead, this region was found to contain a putative open reading frame (ORF) encoding a protein of 163 amino acids, which did not display homology with any known proteins. The phenotype of this mutant may result from i) inactivation of this putative ORF, which could play a role in methylotrophy or ii) insertion of the Tn5 in the *mxaF* promoter region, which would extend more than 600 bp upstream of the *mxaF* putative start codon.

In planta, we observed that the *mxaF* gene was expressed in root nodules. In accordance with the in vitro study, this observation suggests that methanol is present inside the nodule and subsequently used by the bacteria. In agreement, a substantial amount of methanol was detected in the nodule tissue. In plants, methanol can be produced from different sources, such as protein repair pathways, lignin degradation, and cell-wall pectin demethylation (Downie et al. 2004). During symbiosis between *M. nodulans* and *Crotalaria* spp., we hypothesize that methanol could be produced from pectin cell-wall de-methylation. In agreement, we observed that the nodule apical tissue (i.e., the zone where the *mxaF* gene was expressed) was highly disorganized (data not shown). This could have resulted from either plant or bacteria pectinase production. Note that plants are known to produce several classes of pectinases (Prade et al. 1999), whereas there has not been any previous evidence that *Methylobacterium* spp. can degrade pectin. Further cytological observations and studies about pectin metabolism by *M. nodulans* might highlight the role of pectinolytic enzymes and interactions with bacterial methylotrophy during symbiosis between *M. nodulans* and *Crotalaria* spp.

The study of the plant response to inoculation with negative methylotrophic mutants highlighted the role of methylotrophy during symbiosis. Indeed, the absence of methylotrophy significantly affected *C. podocarpa* plant growth by up to 42%. This decrease of plant biomass was associated with a drastic reduction in total number of nodules and a drop in nitrogen fixation efficiency. The differences in nodule dry weight observed between the two mutants phenotypes could result from the different Tn5 insertions. Indeed, in mutant 1, the Tn5 insertion, located in the putative promoter region of *mxaF*, therefore could only partially affect the in planta methylotrophy

compared with mutant 2. This suggests that methylotrophy might be implicated at different levels of symbiosis.

During the first steps of infection, methylotrophy could be involved in the bacterial ability to colonize or invade plant roots. Indeed, some species of the *Crotalariae* tribe, including *C. podocarpa,* are reported to produce toxic methylated compounds such as pyrrolizidin alkaloids (Kinghorn and Smolenski 1981; Wink and Mohamed 2003). Based on their methylotrophy capacity, *Methylobacterium* spp. might be able to detoxify these toxic molecules. Thus, the symbiotic specificity observed between *Methylobacterium* and *Crotalariae* spp. could result from bacterial plant selection based on the rhizospheric excretion of these toxic molecules. Such plant selection already has been reported for *Rhizobium* strains resistant to mimosine in rhizospheres of *Leucaena leucocephala* (Soedarjo et al. 1994) and maybe also for *R. etli* in maize (Rosenblueth and Martinez-Romero 2004).

Methylotrophy is a bacterial metabolic pathway used to assimilate carbon or retrieve energy (Chistoserdova et al. 2003). Therefore, during symbiosis, we could hypothesize that this function contributes to carbon or energy sources for the bacteria. Indeed, the bacteria could be able to recycle methanol produced through tissue degradation during nodule development and senescence, thus saving energy that the plant has to furnish to the bacteria for nitrogen fixation. In agreement, the marked drop in acetylene reduction activity observed with the methylotrophic mutants could reflect a decrease of energy supply to nitrogenase.

Methanol is known to be a key one-carbon signal metabolite regulating the expression of numerous genes involved in cell division, communication, metabolism, and defence of plants (Downie et al. 2004). Therefore, we cannot completely dismiss the possibility that bacterial methylotrophy interferes in nodule cell-wall rearrangements or defence mechanisms by suppressing the methanol signal.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

M. nodulans strains were i) ORS 2060^T (Jourand et al. 2004), called the wild-type strain throughout this study, ii) the constructed recombined strain ORS 2060 *mxaF-lacZ*, and iii) the methylotrophic mutants ORS 2060 *mTn5-GGm-mxaF* (mutation type 1) and ORS 2060 *mTn5-GGm-mxaF* (mutation type 2). All *M. nodulans* strains were grown in YM medium (Vincent 1970) for preparation of plant inoculum and in M72

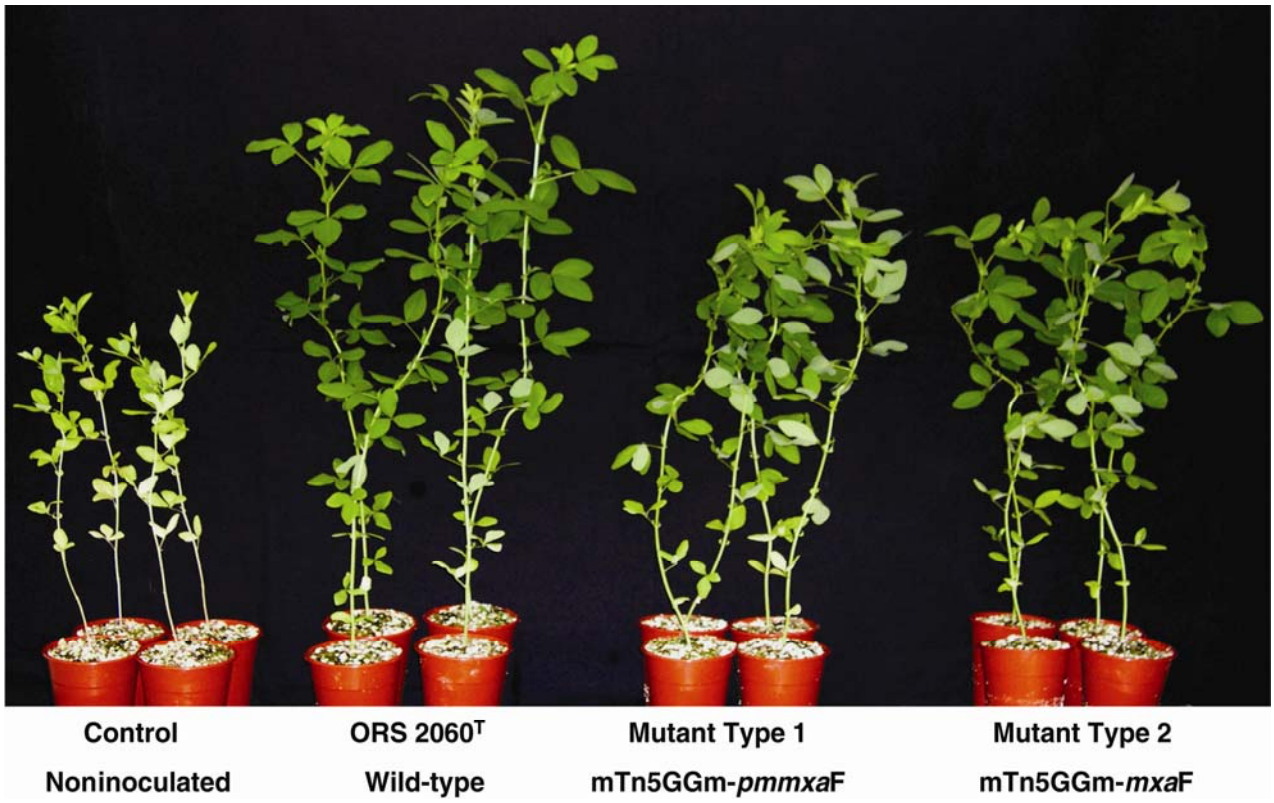

Fig. 4. Comparison of growth of *Crotalaria podocarpa* inoculated with either *Methylobacterium nodulans* ORS 2060^T wild type or ORS 2060^T negative methylotroph mutants compared with noninoculated control. Photographs were taken 5 weeks after inoculation.

Table 2. Symbiotic phenotypes of *Methylobacterium nodulans* ORS 2060^T wild-type and negative methylotrophic mutants on *Crotalaria podocarpa*[z]

Strain	Plant dry wt (g/plant)	No. of nodules/plant	Average nodule dry wt (mg)	Acetylene reduction (C_2H_4 µmol/h/plant)	Acetylene reduction/mg of nodule/plant	Average nodule dry wt (g) to produce 100 g of plant
ORS 2060^T wild type	2.8 ± 0.2 a	30 ± 11 a	5.6 ± 0.7 a	11.4 ± 0.2 a	0.067 ± 0.002 a	6.0 ± 0.6 a
ORS 2060^T *mTn5GGm-mxa*						
Mutation type 1	1.5 ± 0.1 b	12 ± 4 b	13 ± 2 b	8.6 ± 0.3 b	0.055 ± 0.001 b	10.4 ± 0.8 b
Mutation type 2)	1.6 ± 0.2 b	13 ± 5 b	38 ± 4 c	6.6 ± 0.2 b	0.013 ± 0.001 c	30.7 ± 0.8 c
No inoculum (control)	0.2 ± 0.03 c	0	0	0	0	0

[z] Plant dry weight (dry wt) biomass, nodule number and dry wt, and nitrogen-fixing activity expressed by acetylene reduction assay were performed 5 weeks after inoculation. All data followed with the same letter are not significantly different ($P \leq 0.05$)

minimum mineral medium (Green 1992) supplemented with either sodium succinate (10 mM) or methanol (50 mM) for both the methylotrophic tests and the in vitro total β-gal activity. *Escherichia coli* strains (i.e., pCM132, BW 20767 containing plasmid pCRS548 with mini-transposon *mTn5-GGm*, conjugative strain S17-1, and strain STM 217) were provided by M. E. Lidstrom (Marx and Lidstrom 2001), W. G. Reeve (Reeve et al. 1999), and R. Simon (Simon et al. 1983) and the strain collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes (Montpellier, France) (Jourand et al. 2004; Sy et al. 2001a), respectively. Standard methods were used for growth of *E. coli* in Luria-Bertani (LB) medium (Sambrook et al. 1989). All media were supplemented with appropriate antibiotics: nalidixic acid (100 μg/ml) for the three types of *M. nodulans* strains; kanamycin (50 μg/ml) for *M. nodulans* ORS 2060^T *mxaF-lacZ* and *E. coli* pCM132; and gentamycin (30 μg/ml) for *M. nodulans* methylotrophic mutants (type 1 and type 2) and *E coli* BW 20767. Strains were grown aerobically, at 37ºC under gyratory shaking (170 rpm) in complete darkness, for the appropriate time to obtain one unit of optical density (OD) at 600 nm.

General DNA technology.

Genomic DNA was prepared according to Chen and Kuo (1993). Plasmid and cosmid DNA were isolated with a Miniprep kit (Promega, Charbonnières, France). Polymerase chain reaction (PCR) products were purified with a Qiaquick gel extraction kit (Qiagen, Courtaboeuf, France). Restriction endonuclease and ligase reactions were performed according to the manufacturer's specifications (Eurogentec, Angers, France). For DNA amplification, sequencing and analysis, the primers used are described in Table 3. All reactions for PCR or sequencing as well as the methods for DNA sequencing and analysis were carried out as previously described by Sy and associates (2001a).

Construction of the *M. nodulans* ORS 2060^T recombinant strain containing the *mxaF-LacZ* transcriptional fusion promoter.

From the total genomic DNA of *M. nodulans* ORS 2060^T wild type, a 798-bp DNA fragment was amplified with primers 1 and 2 (Table 3) corresponding to the DNA putative promoter section of the *M. nodulans* sequenced gene *mxaF* as described in Figure 1A (AF220764 sequence in GenBank) (Sy et al. 2001a). This 798-bp DNA fragment was cloned into the *Eco*RI and *Bgl*II sites of the pCM132 plasmid vector (Marx and Lidstrom 2001). The combined plasmid pCM132-*mxaF-lacZ* vector was transferred by electroporation (transformation) into conjugative *E. coli* S17-1 using an electroporator (Biorad, Ivry, France) and then transferred by conjugation into *M. nodulans* ORS 2060^T wild type. Conjugative colonies were selected on YM medium containing antibiotics (kanamycin at 50 μg/ml and nalidix acid at 100 μg/ml). Recombined *M. nodulans* ORS 2060 *mxaF-lacZ*-positive clones were controlled for *lacZ* in vitro expression after promoter *mxaF* methanol induction in M72 minimum mineral medium supplemented with MeOH (50 mM) and the same two antibiotics mentioned above and then compared with the *M. nodulans* ORS 2060^T wild-type strain.

Construction of *M. nodulans* ORS 2060^T methylotrophic mutants.

Insertional mutagenesis of the *M. nodulans* DNA *mxa* operon was carried out by insertion of the *mTn5-GGm* transposon using conjugation between the *M. nodulans* ORS 2060^T wild type and *E. coli* BW20767 containing the *mTn5-GGm*. After conjugation, conjugants first were selected on YM medium supplemented with antibiotics (nalidixic acid at 100 μg/ml and gentamycin at 30 μg/ml). Then, methylotroph mutants were selected by cultivating conjugants on microplates containing M72 minimum mineral medium with the same two antibiotics mentioned above, and i) sodium succinate (10 mM), ii) MeOH (50 mM), or iii) sodium succinate (10 mM), MeOH (50 mM), and allyl alcohol added to a final concentration at 0.2% (wt/vol) was used to select *Methylobacterium* methylotroph mutants as described by Nunn and Lidstrom (1986a). All chemicals were provided by Sigma-Aldrich (St-Quentin, France). Colonies growing on M72 + succinate and also on M72 + succinate + MeOH + allyl-alcohol but not growing on M72 + MeOH were selected as methylotroph-negative clones, which were controlled by PCR for the presence of *mTn5-GGm* transposon using primers 4 and 5 (Table 3). Among the positive *mTn5-GGm* clones, a new screening was carried out with PCR using primer pairs (Table 3) to identify the position of the insertional transposon mutagenesis in the *mxaF* promoter sequence and in the *mxaF* gene. Positions of *mTn5-GGm* (Fig. 1B) were obtained by DNA sequencing. Selected clones of *M. nodulans* ORS 2060 *mTn5-GGm-mxaF* (mutation type 1) and ORS 2060 *mTn5-GGm-mxaF* (mutation type 2) were grown on M72 + succinate or M72 + MeOH to verify the loss of the methylotrophy capacity by measuring bacterial growth with a UV-visible spectrophotometer at OD 600 nm (Varian, Courtaboeuf, France).

Complementation of methylotrophic mutants.

For complementation experiments, we used the STM217 clone of *E. coli* XL1-MR from the genomic library of the *M. nodulans* ORS 2060^T wild type as described by Sy et al. (2001a) and containing the 35-kb *mxaF* DNA fragment cloned into the cosmid SuperCosI (Stratagene, La Jolla, CA, U.S.A.). After growth of the STM 217 clone on LB supplemented with kanamycin (50 μg/ml), the cosmid was extracted using the DNA Miniprep Kit as described above and then transferred to the conjugative strain of *E. coli* S17-1 by electroporation. Transformed clones were selected on LB + kanamycin (50 μg/ml). Then, the cosmid was transferred for complementation by conjugation between the transformed conjugative *E. coli* S17-1 and both *M. nodulans* methylotrophic mutants type 1 and type 2. Complemented *M. nodulans* methylotrophic mutants were selected on M72 minimum mineral medium containing nalidixic acid (100 μg/ml) + gentamycin (30 μg/ml) + kanamycin (50 μg/ml) and MeOH (50 mM) as sole carbon source. Their symbiotic phenotypes were tested on *C. podocarpa* plants.

Plant tests.

C. podocarpa seeds were obtained from IRD (Bel-Air, Dakar, Senegal) and identified as reported by Jourand and associates (2004). Germination and cultivation of *C. podocarpa* were conducted in the greenhouse as previously described by

Table 3. Primers used for DNA amplification and sequencing

Name	Sequence	Target gene	Reference
pm *mxa*F 2060f	5′-GAA-TTC-CAG-AGG-ACA-AGA-GGT-TGA-TGT-TG-3′	Promoter *mxa*F	This study
pm *mxa*F 2060r	5′-ACA-GAT-CTC-TTA-CCC-AGC-ATG-TGC-GTC-TCC-CA-3′	Promoter *mxa*F	This study
WIL3	5′-GAA-TGC-CCA-CAG-GCC-CTG-GAG-3′	Transposon mTn5-GGm	Reeve et al. 1999
TAC-105F	5′-GCA-TCT-AGC-CCG-CCT-ATT-G-3′	Transposon mTn5-GGm	Reeve et al. 1999

Giraud and associates (2000) and Sy and associates (2001a). At day 15 after germination, plants were root inoculated with 5 ml of culture of either the *M. nodulans*, ORS 2060^T wild type, ORS 2060 *mxaF-lacZ*, ORS 2060 methylotrophic mutants, or ORS 2060 complemented methylotrophic mutants. Sterile distilled water was used for control. All the inocula were prepared according to Giraud and associates (2000). Fifteen replicates were grown for each experimental treatment. Six weeks after inoculation, plants were harvested. Nitrogen-fixing activity was estimated by measurement of acetylene-reducing activity on the entire plant according to Hardy and associates (1968). The dry plant biomass and the number and dry weight of root nodules were evaluated. All data were subjected to a one-way analysis of variance (ANOVA) and means were compared with the Newman-Keuls multiple range test ($P \leq 0.05$) using the Super ANOVA computer software package (Gagnon et al. 1989).

Enzyme assays, methanol dosage, and light microscopy.

Crude extracts of total soluble proteins were prepared by sonication, as reported by Day and Anthony (1990), from i) *M. nodulans* cultures ORS 2060^T wild type and ORS 2060^T pCM132 *mxaF-lacZ* grown in M72 mineral minimum medium with methanol (50 mM) or succinate (10 mM) supplemented with the appropriate antibiotics or ii) *C. podocarpa* nodule tissues. β-Gal activity was assayed in vitro using the standard method described by Miller (1972). MDH microassays were performed as described by Day and Anthony (1990). Total proteins were quantified using the Lowry method Biorad kit (Biorad, Ivry, France). Methanol dosage was performed as reported by Sy and associates (2001a). All data were subjected to a one-way ANOVA, and means were compared with the Newman-Keuls multiple range test ($P \leq 0.05$) as described above. In planta tests were performed on 40-μm and whole half-thick sections of fresh nodules (VT1000S Vibratome, Leica, France), then stained for histo-β-gal assays as described by Boivin and associates (1990). After mounting in water, nodule sections were observed and photographed under light microscopy as reported by Sy and associates (2001a).

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