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Symbiotic properties of *Methylobacterium nodulans* ORS 2060^T: A classic process for an atypical symbiont

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Abstract

Some legume species of the *Crotalaria* genus are specifically nodulated by methylotrophic bacteria belonging to the *Methylobacterium nodulans* species. The feature of this symbiotic bacterium is its ability to oxidize methanol, a property based on the presence of a methanol dehydrogenase enzyme. Despite a good knowledge of this property and its implication in symbiosis, the molecular dialogue between *M. nodulans* and *crotalaria podocarpa* leading to symbiosis is largely unknown, except the presence of a *nodA* nodulation gene in the genome of *M. nodulans* ORS 2060. To investigate if *M. nodulans* ORS 2060 produces Nod factors, molecules considered as the major bacteria-to-plant signals essential for the establishment of rhizobia–legume symbiosis, we identified and sequenced a *nodDABCUIJHQ* cluster from a genomic library of ORS 2060. Phylogenetic analyses of *nod* genes revealed that *M. nodulans* ORS 2060 form a branch together with *Burkholderia tuberum* STM678 and a strain of *Methylobacterium* sp. (4-46) isolated from *Lotononis*, and distinct from all the other rhizobia. To analyse the regulation of ORS 2060 *nod* genes, we constructed a *nodA–LacZ* promoter fusion to monitor the *nod* gene expression with various flavonoids. The flavone apigenin was found to be the strongest inducer of *nod* gene expression in *M. nodulans* ORS 2060. This latter flavonoid was used to induce ORS 2060, and Nod factors were purified by high-performance liquid chromatography (HPLC) and further characterized by mass spectrometry. One major Nod factor structure was identified as a pentamer of chitin substituted by C18:1 or C16:0 acyl chains on the non-reducing end and 6-O-sulphated on the other end, suggesting a classic symbiotic dialogue between *M. nodulans* and *C. podocarpa*.

Keywords: *Methylobacterium nodulans*; *Crotalaria* spp.; Lipo-chito-oligosaccharide (LCO); Nod factor; Flavonoids; Apigenin; Nodulation genes; Rhizobia

1. Introduction

The symbiosis between rhizobia and legumes results in the formation of N_2-fixing root nodules and has been described as a multi-step process mediated by signal molecules produced by both bacteria and plants (Spaink, 1992). The first apparent exchange of signals involves the secretion of phenolic compounds by legumes such as flavonoids and isoflavonoids (Peters and Verma, 1990). These polyphenolic compounds induce the transcription of bacterial nodulation genes leading to the biosynthesis of a bacterial signal, the nodulation Nod factor (NF) (Peters and Verma, 1990; Dénarié et al., 1996). NFs metabolites are lipo-chito-oligosaccharides (LCOs), mostly an oligomeric backbone of three to five β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues with the *N*-acetyl group replaced by an acyl chain on the terminal non-reducing end (Perret et al., 2000; D'Haeze and Holsters, 2002). The structure of the fatty acyl chain, the number of GlcNAc

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residues and the presence of extra substituents determine the host specificity of the bacterium (Dénarié and Cullimore, 1993; Dénarié et al., 1996). Nodulation genes can be classified into two categories: the common genes (*nodABCDIJ*) are functionally conserved across rhizobia and involved in the formation of the *N*-acylated chitin oligomer NF core while specific *nod* genes (such, as *nodZ*, *nodH*, etc.) encode enzymes involved in the synthesis and transfer of additional chemical compounds on the NF core, and are unequally distributed across rhizobia in relation to their host range (Downie, 1998).

NFs have been described as molecules participating in a various number of plant host physiological processes, including cell divisions forming nodule primordia, activation of gene expression, root hair deformation, and oscillations in cytoplasmic calcium levels (termed calcium spiking) (Minami et al., 1996; Spaink, 1996; Mitra et al., 2004; Kanamori et al., 2006).

The *Crotalaria* genus (Fabaceae family, Papilionoideae subfamily, Crotalarieae tribe) is composed of more than 600 species (Allen and Allen, 1981), located in the subtropical and intertropical regions (Polhill and Raven, 1981). Some species have agronomic significance as green manure or for their nematicid properties (Silva et al., 1989). In Senegal, *Crotalaria* species have been found to be nodulated by *Bradyrhizobium* strains (Samba et al., 1999). Surprisingly, *Methylobacterium* spp. strains have also been isolated from root nodules of three Senegalese species of *Crotalaria*, *Crotalaria glaucoides, Crotalaria perrottetii* and *Crotalaria podocarpa* (Samba et al., 1999; Sy et al., 2001b). These symbiotic strains belong to a single species, named *M. nodulans*, for its ability to nodulate and fix nitrogen specifically during symbiosis with *Crotalaria* spp. (Jourand et al., 2004). Interestingly, the three species of *Crotalaria* associated with *M. nodulans* are not nodulated by *Bradyrhizobium* sp. isolated from other species of *Crotalaria* (Samba et al., 1999, Sy et al., 2001b). If *Methylobacterium* strains have been found previously associated with plants at different levels, such as epiphytes (Omer et al., 2004) and endophytes (Elbeltagy et al., 2000), this was the first description of a *Methylobacterium* species as a legume symbiont. Later, another group of symbiotic *Methylobacterium* strains was isolated from various species of *Lotononis* in South Africa (*L. angolensis, L. bainesii, L. listii, L. solitudinis*) (Jaftha et al., 2002; Yates et al., 2007), which belong to a close species of *M. nodulans* (Yates et al., 2007). However, cross-inoculation studies showed that *M. nodulans* does not form nodules on *Lotononis* species (Yates et al., 2007).

The specificity of the *Methylobacterium–Crotalaria* interaction has already been assessed by an evaluation of the role of the methylotrophic properties in the symbiotic process. Inoculation of *C. podocarpa* species with methylotrophic minus mutants resulted in a reduction of the nodule number and a drastic decrease of plant biomass (Jourand et al., 2005). However, no investigation has been carried out to determine which signal molecules are involved in the molecular dialogue between *M. nodulans* and its host plant. Previous genetic studies revealed the presence of the *nodA* nodulation gene in the type strain of *M. nodulans* (ORS 2060), suggesting that this bacteria may also produce the symbiotic LCOs (Sy et al., 2001a; Jourand et al., 2004).

The aim of this study was to determine which signal molecules are involved in the symbiosis between *M. nodulans* ORS 2060 and *C. podocarpa*, by the characterization of the bacterial nodulation gene cluster, identification of the flavonoids that induce their expression, and finally determination of the NF structures produced by the bacteria.

2. Materials and methods

2.1. Bacterial strains and cultures

M. nodulans strains, belonging to the bacterial collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes (Montpellier, France), were the wild-type strain ORS 2060 (Jourand et al., 2004) and the recombinant strain ORS 2060 *nodA–lacZ* (this study). All *M. nodulans* strains were grown in yeast-mannitol medium (Vincent, 1970) at 37 °C. *Escherichia coli* strains pCM132, S17-1 and XL1-MR were provided by M.E. Lidstrom (Marx and Lidstrom, 2001), R. Simon (Simon et al., 1983) and A. Sy (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 ($100\,\mu g\,ml^{-1}$) for all *M. nodulans* strains, kanamycin ($50\,\mu g\,ml^{-1}$) for *M. nodulans* ORS 2060 *nodA–lacZ* and *E. coli* strains.

2.2. Molecular techniques

Genomic DNA was prepared according to Chen and Kuo (1993). Plasmid and cosmid DNAs were isolated using Miniprep kits (Promega, Charbonnières, France). All reactions for DNA amplification by PCR or for sequencing, as well as methods for sequence analysis, were carried out as previously described by Sy et al. (2001a). DNA amplified 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 Southern blot hybridization, restricted DNA was blotted to positively charged nylon membranes by the alkali transfer procedure and hybridized with digoxigenin (DIG)-dUTP using the DIG labelling kit supplied by Roche (Meylan, France).

The study of *M. nodulans* nodulation genes was performed using the ORS 2060 genomic library obtained by Sy et al. (2001a). Screening of *nodA*-containing cosmids was performed by DNA amplification using the primer pair nodAf brady (5′-GTY-CAG-TGG-AGS-STK-CGC-TGG-G-3′) and nodAr brady (5′-TCA-CAR-CTC-KGG-CCC-GTT-CCG 3′). A selected clone, pSTM223, was

confirmed by hybridization with a *nodA* probe constructed by DIG labelling of the ORS 2060 580-bp *nodA* internal fragment. A 7.13-kb XmaI fragment of pSTM223 containing the putative *nod* genes was subcloned in pUC18. This clone, called pSTM520, was digested by *HindIII*, *SacI*, *SacII* and *BglII* and restriction fragments were cloned in pUC18 and sequenced using universal primer M13F (5′-GTT-TTC-CCA-GTC-ACG-AC-3′) and M13R-48 (5′-AGC-GGA-TAA-CAA-TTT-CAC-ACA-3′). Primer walk sequencing was performed to close the gaps of the pSTM520 insert. All sequences were performed using ABI chemistry on an ABI310 sequencer (Applied Biosystems).

2.3. Construction of an ORS 2060 nodA–LacZ transcriptional fusion

A 366-bp DNA fragment of the *M. nodulans* ORS 2060 genome was amplified by PCR with primers pmNodA 2060f (5′-GAA-TTC-GTT-TGC-GGC-CCC-TCA-TAA-TAA-ATA-GC-3′) and pmNodA2060r (5′-CGA-AGA-TCT-GGA-CAG-CTC-TCC-TGT-TCC-GGA-GCT-3′), corresponding to the putative promoter section of *nodA* (AF220764). This 366-bp DNA fragment was cloned into the *EcoRI* and *BglII* sites of the pCM132 plasmid vector (Marx and Lidstrom, 2001). The combined pCM132-*nodA-lacZ* plasmid was transferred by electroporation into conjugative *E. coli* S17-1 and then into *M. nodulans* ORS 2060^T strain by conjugation. Conjugative colonies were selected on a YM medium containing antibiotics (kanamycin at 50 μg ml^{-1} and nalidix acid at 100 μg ml^{-1}).

2.4. Methylobacterium nodulans nodA gene induction by flavonoids

The *M. nodulans nodA–LacZ* fusion was tested with flavones (apigenin, luteolin), flavonols (kaempferol, quercetin), and isoflavones (daidzein, genistein). All these chemicals were provided by Extrasynthese (Genay, France). The flavonoid standard solutions were prepared in ethanol (10 mM) and not as usual in methanol because of the methylotrophic property of the *Methylobacterium* strains. Cultures of *M. nodulans* ORS 2060 *nodA–lacZ* reaching an A_{600} of 0.5 were supplemented with each signal compound at 5 μM. For each treatment, β-galactosidase activity was recorded until 102 h after induction using a specific protocol adapted from Miller (1972) for *Methylobacterium*. β-Galactosidase activity was measured on 20 ml aliquots of bacteria: cells were pelleted by centrifugation (10 min, 4000 rev min^{-1}), washed and resuspended in 1 ml of sterile water, then crushed by ultrasonication 2 times, 1 min at 20 W (Sonics Material, Danbury, USA). After treatment, cell suspension was centrifuged for 25 min at 14,000 rev min^{-1} and the crude supernatant was used to test both β-galactosidase activity (as described by Miller, 1972) and total soluble-protein content (using the Biorad Protein Kit, Biorad, Marnes la Coquette, France). The β-galactosidase activity was expressed in μmol min^{-1} mg^{-1} of soluble proteins. All recorded activities were processed using Statistica 6.0 software. The means were separated using one-way analysis of variance, and HSD Tukey post-hoc tests were then performed to constitute the induction groups shown in Table 1.

2.5. Nod factor extraction and purification

M. nodulans ORS 2060 was grown until the culture reached an A_{600} of 0.1. Then cells were induced to produce NFs by adding the best flavonoid inducer at 5 μM, and grown during 48 h at 37 °C. NFs were extracted from filtered culture supernatants by partition with *n*-butanol (Roche et al., 1991). Purification was performed on a high-performance liquid chromatography (HPLC) system

Table 1
Induction of the *Methylobacterium nodulans nodA* promoter by flavonoids[d]

Putative inducer	Hydroxylation pattern					Maximal response (mU β-gal min^{-1} mg^{-1})
	3	5	7	3′	4′	
Flavones						
Apigenin	–	OH	OH	–	OH	654 ± 23[a]
Luteolin	–	OH	OH	OH	OH	196 ± 6[b]
Flavonols						
Kaempferol	OH	OH	OH	–	OH	616 ± 21[a]
Quercetin	OH	OH	OH	OH	OH	1[c]
Isoflavones						
Genistein	–	OH	OH	–	OH	0[c]
Daidzein	–	–	OH	–	OH	0[c]
Control[e]						0[c]

Data are means of two independent determinations.
[a,b,c]Grouping is based on mean β-galactosidase activity and statistical results.
[d]Experiments were carried out as described in the text, with an induction time of 15 h (maximum activity was recorded at that time).
[e]Control was induction medium without added inducers.

Waters 2690 separation module (Waters, Milford, Massachusetts, USA) with a semi-preparative C_{18} reverse phase column (10 mm × 250 mm; 5 µm, Phenomenex Gemini) using an isocratic solvent A (water-acetonitrile, 70:30 [vol/vol]) containing 50 mM ammonium acetate for 20 min, followed by a 40 min linear gradient from solvent A to solvent B (100% acetonitrile) containing 50 mM ammonium acetate at a flow rate of 1 ml min^{-1}. The UV absorption was monitored at 206 and 260 nm. Ammonium acetate and solvents from the collected fractions were removed by two successive freeze–dryings.

2.6. Mass spectrometry

Each pre-purified HPLC fraction was analysed using a ESI-QqToF Ultima apparatus (Waters, Milford, Massachusetts, USA) using direct infusion. Spectra were recorded in both positive and negative modes. Peaks detected in the awaited range (m/z 1000–1500 for the simple charged species or 600–700 for the double charged ones) were submitted to MS/MS experiment to confirm their LCO nature.

Energies were the following: probe: 3 kV, cone: 100 V, Rf: 70 V, collision cell: 15 V for MS, 30 V for MS/MS. Collision gas: argon. Direct inlet: solvent AcCN/H_2O 1:1, 1% acetic acid, rate: 10 µl min^{-1}. Concentrations were about 10^{-4} M.

2.7. Phylogenetic analyses

The multiple nucleotide sequence alignments were generated using ClustalX (Thompson et al., 1997) and optimized manually. All phylogenetic analyses were performed using PAUP version 4.0b10 (Swofford, 1998). A concatenation of the *nodABCIJ* genes from *Methylobacterium* ORS 2060 and 4-46 (genome available at JGI, http://genome.jgi-psf.org/) strains plus nine strains (*Mesorhizobium loti* R7A (accession number AL672111) and MAFF303099 (NC_002678), *R. etli* CFN42 (NC_007761), *Sinorhizobium* sp. NGR234 (NC_000914), *S. meliloti* 1021 (NC_003037), *R. leguminosarum* 3841 (AM236084), *Burkholderia phymatum* STM815 (NZ_AAUG00000000), *B. japonicum* USDA110 (NC_004463) and *Azorhizobium caulinodans* ORS 571 (L18897)) from which all these genes were available was constructed. Partition homogeneity tests were calculated between each marker partition, and homogeneity was found between all markers (P value = 1−(704/1000) = 0.296). A *nodA* ML phylogeny was also built, using 50 representative strains of the rhizobial diversity. The best-fit model for *nodA* selected by MODELTEST3.6 (Posada and Crandall, 1998) was the HKY + I + G model, with parameters base = (0.1966 0.3043 0.3003), Nst = 2, Tratio = 1.6522, rates = gamma, shape = 1.2320 and Pinvar = 0.2330.

For *nodABCIJ* and *nodA* phylogenies, bootstrapping analyses were performed using heuristic searches with 100 and 1000 replicates, respectively.

3. Results

3.1. Organization and analysis of nod genes in M. nodulans ORS 2060

A 7.13-kb region of the pSTM223 cosmid (from a *M. nodulans* ORS 2060 genomic library constructed by Sy et al., 2001a), showing a positive hybridization signal to the *nodA* probe, was fully sequenced and analysed (see Materials and methods for details). The full DNA sequence is available in GenBank under accession number AM712915, and annotation of the fragment is presented in Fig. 1. The analysis of the nucleotide sequence of the cosmid fragment revealed the presence of six open reading frames (ORFs). These ORFs showed high nucleotide sequence identity with known rhizobial nodulation genes. ORF_1 (215 amino acids) encodes a protein with 81% identity with NodA from *B. tuberum* STM678. ORF_2 (220 aa) presented 74% of identity with NodB from *Burkholderia* sp. STM 678 (AC42488). ORF_3 (485 aa) showed 76% of identity with NodC from *Rhizobium* sp. N33 (AAB16897). ORF_4 (305 aa) revealed 81% of identity with NodI from *S. meliloti* 1021 (AAK65130). ORF_5 (264 aa) revealed 77% of identity with NodJ from *R. tropici* (CAC39129). The sixth ORF (250 aa) showed 71% of identity with NodH of *Rhizobium* sp. strain N33 (AAB16900). Upstream of these six ORFs, a 262 bp fragment similar to the 5′-end part of *nodD* (encoded on the reverse strand), is present but is interrupted by the end of the cosmid. In addition, an ORF with homology to the 3′ end part of *nodQ* (56% identity with the 180 amino-acid long C-terminal part of *R. tropici* NodQ, P52978), as well as relics of *nodU* and transposases were detected.

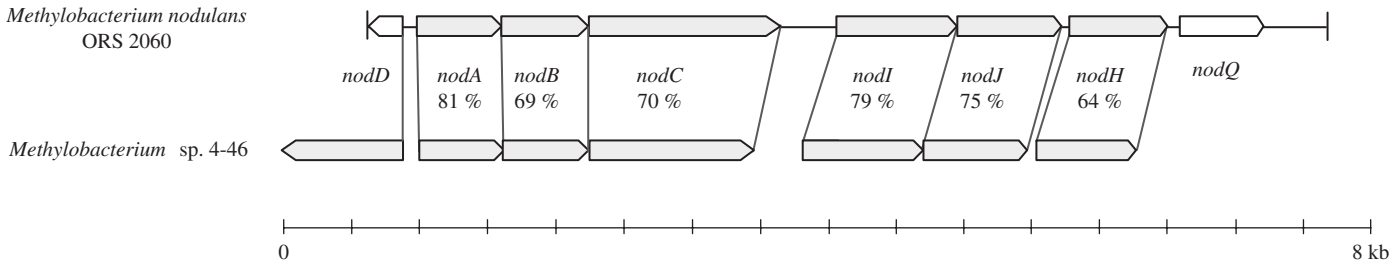

Fig. 1. Comparison of nodulation genes operons in *Methylobacterium* sp. 4-46 (A) and *Methylobacterium nodulans* ORS 2060 (B). % indicated are % of identities at the amino-acid level.

During the preparation of our article, the complete genome of *Methylobacterium* sp. 4-46, a *Lotononis* symbiont, became available (http://genome.jgi-psf.org/draft_microbes/met_4/). We thus included a comparison of both *nod* genes operons. Both strains exhibit a very similar organization of the *nod* cluster (see Fig. 1). The NodDABCIJ proteins share 69–81% identities between ORS 2060 and 4-46 strains (Fig. 1). In *Methylobacterium* sp. 4-46 genome, no additional *nod* gene was found (using blast searches of *B. japonicum* and *S. meliloti nod* gene sets).

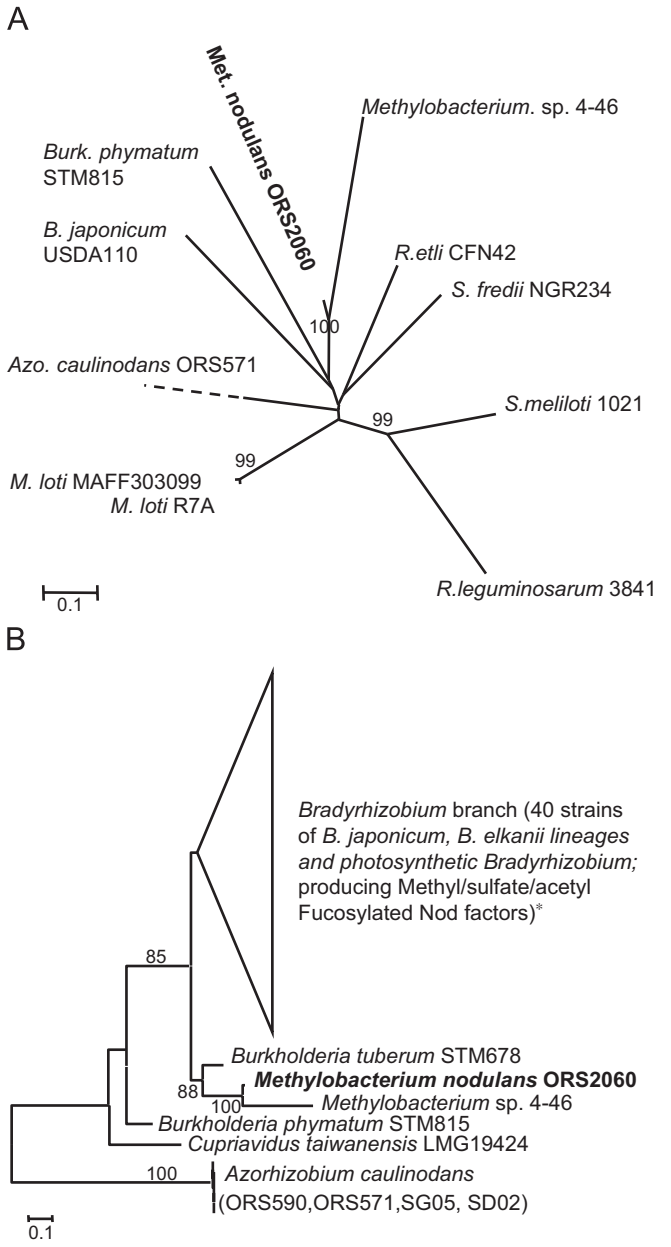

Fig. 2. Maximum likelihood (ML) phylogenies of *nodABCIJ* concatemered alignments (A) and *nodA* gene (B). Bootstraps indicated at tree nodes (when >50%) were calculated under ML criterion with 100 replicates. **Bradyrhizobium* branch shown in (B) includes 40 strains belonging to several *Bradyrhizobium* genomic species; for details see Fig. 2 of Moulin et al. (2004); Nod factor structure was deduced from structural studies and *nod* gene content. Abbreviations: *B.: Bradyrhizobium*, *Burk.: Burkholderia, S.: Sinorhizobium, R.: Rhizobium, M.: Mesorhizobium, Azo.: Azorhizobium.*

A phylogenetical analysis of the *nodABCIJ* operon was then investigated using a partition of these five *nod* genes, and using a maximum likelihood approach (see Materials and methods). We included in the analysis all rhizobia from which all *nodABCIJ* genes had been previously sequenced (nine strains, see Fig. 2A). The tree obtained (Fig. 2A) shows that *M. nodulans* ORS 2060 form a distinct branch together with *Methylobacterium* sp. 4-46 supported by a high bootstrap (100%), though their sequences accumulated mutations since the speciation event.

As the *nodA* gene has been previously used as a symbiotic marker of rhizobia (Moulin et al., 2004; Stepkowski et al., 2005), we also built a *nodA* phylogeny including 50 rhizobial strains representative of the rhizobial diversity (except *Sino-Meso-Rhizobium* strains, as they constitute separate *nodA* lineages from the *Methylobacterium–Bradyrhizobium* branch, Stepkowski et al., 2007) to detect the closest phylogenetical symbiotic neighbours of *M. nodulans,* presented in Fig. 2B. *M. nodulans nodA* gene grouped strongly (100% bootstrap) with *Methylobacterium* sp. 4-46 and *B. tuberum* STM 678 (88% boostrap), strains isolated from nodules of *Lotononis bainesii* and *Aspalathus carnosa,* respectively (Moulin et al., 2001; Jaftha et al., 2002).

3.2. The expression of nodA is inducible by the flavone apigenin

We tested a number of commercially available flavonoids for their ability to induce the *nodA* promoter of *M. nodulans* ORS 2060. As shown in Table 1, addition of apigenin or kaempferol results in a high β-galactosidase activity of the *nodA–lacZ* promoter fusion and can thus be considered as strong *nod* gene inducers in strain ORS 2060. Their activities were not significantly different despite an additional C-3 hydroxylation of the kaempferol compared to apigenin. Luteoline (C-3′OH-apigenin) gave a β-galactosidase activity that was three-fold lower as compared to apigenin. The flavonone quercetin (C-3′OH-kaempferol) did not give any detectable β-galactosidase activity (Table 1). Thus, the presence of a hydroxyl on position C-3′ of the flavonoid skeleton (as in luteolin or quercetin) reduces greatly the induction of *nod* genes expression.

The isoflavones differ from the flavones in that the B ring is attached to C-3 instead of C-2. As daidzein and genistein are not able to provoke a promoter response (Table 1), we conclude that the attachment of the B ring to C-2, as is found in flavones and flavonols, is of crucial importance for induction. As apigenin was the most active inducer, this flavone was used to conditions to obtain a maximum expression of *nod* genes. Addition of 5 μM apigenin to a *M. nodulans* culture reaching an A_{600} of 0.1 led to the highest β-galactosidase activity, and this condition was used to produce NFs.

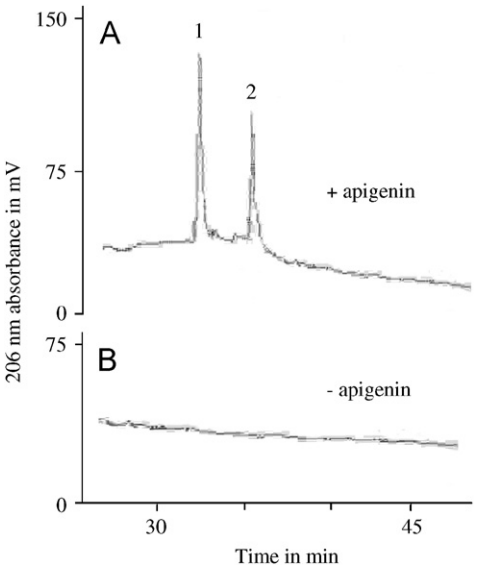

Fig. 3. HPLC profiles of *n*-butanol extract induced with apigenin (A) or not induced (B) from *Methylobacterium nodulans* ORS 2060 culture. Separation was performed with the presence of ammonium acetate as described in Materials and methods, with monitoring of the absorption at 206 nm.

3.3. *Methylobacterium nodulans ORS 2060 produces pentameric LCOs 6-O sulphated at the reducing end*

The NFs produced by *M. nodulans* were purified using the protocol described in Materials and methods. Fig. 3 shows the comparison of two HPLC profiles of NFs extraction from two different *M. nodulans* ORS 2060 cultures: the first one was obtained after induction with apigenin and showed the presence of putative LCO compounds (Fig. 3A), the second one was obtained without an inducer (Fig. 3B). In the absence of apigenin, no NF could be detected on the HPLC profile (Fig. 3B). HPLC analysis revealed the presence of two chromatographic peaks absorbing at 206 nm, in the induced cultures (Fig. 3A). Peaks were eluate at 57% and 65% acetonitrile, respectively.

Fractions corresponding to the two peaks detected in the HPLC profile were then collected and analysed by ESI–MS. The positive ion spectra of compounds in fraction 1 exhibited a complex series of protonated $[M+H]^+$ (*m*/*z* 1336 with a minor *m*/*z* 1310) and alkali cationized molecules assigned to $[M+Na]^+$ (*m*/*z* 1358), $[M+K]^+$ (*m*/*z* 1374 and 1338, respectively,) and $[M-H+2Na]^+$ (*m*/*z* 1380) or $[M-H+Na+K]^+$ (*m*/*z* 1396 and 1370, respectively). Presence of such dialkali ions is characteristic of acidic functions. Samples were therefore submitted to a negative ionization mode analysis. Only two compounds have been observed, the first very weak at *m*/*z* 1308 and the second intense at *m*/*z* 1334, allowing to attribute the molecular masses at M 1309 and 1335 Da. The mass difference between both is 26 amu, indicating that the predominant one might exhibit a $C_{18:1}$ and the minor one a $C_{16:0}$ chain. Molecular masses obtained from the ESI–MS

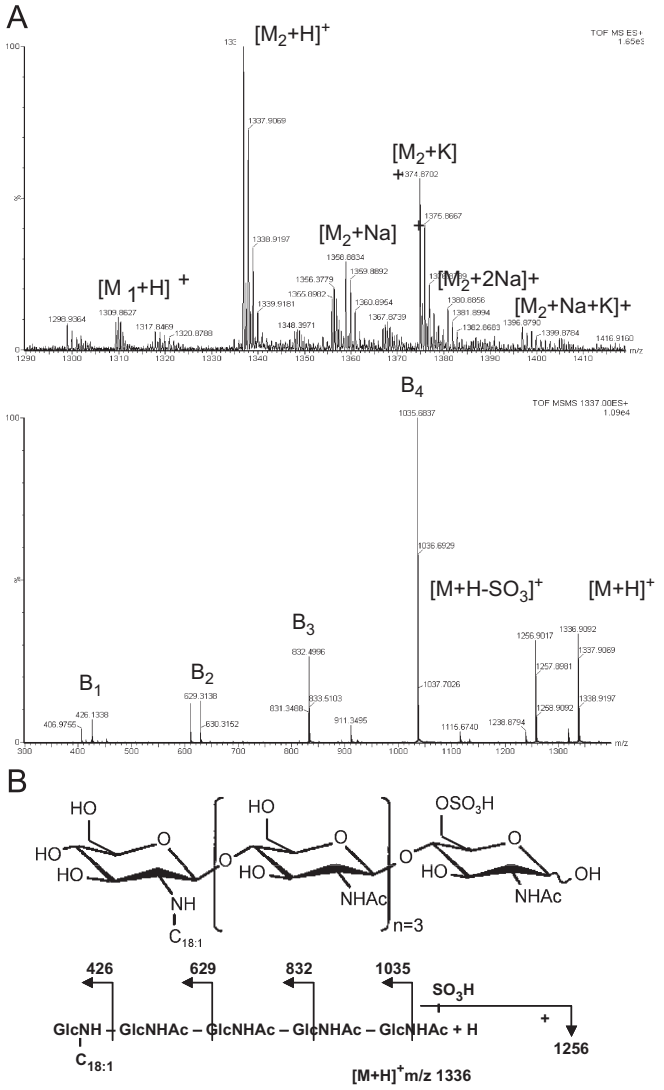

Fig. 4. Results of the mass spectrometric analysis of the C_{18} high-performance liquid chromatography fractions (A) and structure of *Methylobacterium nodulans* ORS 2060 Nod factor (B).

spectra are compatible with a chitopentamer substituted by a $C_{18:1}$ (or $C_{16:0}$) fatty acyl and a sulphate. Presence of this sulphate has been assessed by ionization in the negative mode.

These structures have been confirmed through MS/MS experiments. Collision energy has been set to 30 V. A classical B fragmentation pattern has been observed, corresponding to disruption of the osidic junction between the glucosamines (Fig. 4A). This fragmentation produces a regular profile with a mass difference of 203 amu between each ion corresponding to the GlcNAc subunits (221 amu for the reducing end). Fragmentation of the molecular ions $[M+H]^+$ produced a loss of sulphite (SO_3) (80 amu) directly from the molecular species, indicating that sulphation occurs on the reducing end. The profiles ended with B1 ions at *m*/*z* 410 and 426, respectively, for the two NFs, indicating that the non-reducing end is substituted only by a $C_{16:0}$ or mostly a $C_{18:1}$ acyl. From the MS/MS

experiments, the structure presented in Fig. 4B can be deduced. Localization of substitutions was deduced from already known NF structures. No NF compounds were found in fraction 2.

4. Discussion

Although most *Crotalaria* sp. are nodulated by non-methylotrophic *Bradyrhizobium* sp., few African species (*C. glaucoides, C. perrottetii* and *C. podocarpa*) are found associated with bacterial symbionts belonging to the *M. nodulans* species (Samba et al., 1999; Sy et al., 2001b). In comparison to many other rhizobia, relatively little is known about the *M. nodulans nod* genes content and the symbiotic molecules involved in the molecular dialogue between the two partners. In this study, we show that the type strain ORS 2060 harbours a set of *nod* genes organized in a single operon with six ORFs (encoding NodA, NodB, NodC, NodI, NodJ and NodH proteins), as well as gene fragments of *nodD*, *nodU*, *nodQ* and of a transposase.

M. nodulans harbours *nod* genes similar to the other rhizobia, although they constitute a separate cluster in the *nod* gene phylogenies together with *Methylobacterium* 4-46 (isolated from *Lotononis bainesii* (Norris, 1958) genome, which has been recently sequenced at the Joint Genome Institute) and *B. tuberum* STM678 (isolated from *A. carnosa,* Moulin et al., 2001). As most *Methylobacterium* species are not symbiotic, it is highly probable that symbiotic abilities in this genus are not an ancestral character but have been acquired by lateral transfer. The presence of transposase relics close to the *nod* gene operon supports this idea. The host origin of the transfer remains unknown, as no close homolog of the *M. nodulans nod* genes has been characterized so far, except for *B. tuberum* that groups with the *Methylobacterium* branch with a high bootstrap value (98%) in the *nodA* tree. *M. nodulans* ORS 2060 and *Methylobacterium* sp. 4-46 are close species (97% 16S rDNA identity), with almost-identical *nod* genes organization and rather close *nod* gene sequences (Fig. 1). Thus, the transfer of *nod* genes must have occurred in the ancestor of these two species, followed by vertical transmissions and coevolution of each species with their plant hosts, conducting to a high specificity between the symbiotic partners *M. nodulans*/*Crotalaria* sp. and *Methylobacterium* sp./*Lotononis* sp. (*M. nodulans* does not nodulate *Lotononis* species, Yates et al., 2007).

Previous studies have shown that the *nodA* gene phylogeny was correlated to some NF structural features (presence of additional fucose and/or arabinose on the NF backbone) produced by the bacteria, and to some extent to the host plant systematics (Debellé et al., 2001; Moulin et al., 2004). In the *nodA* tree, *M. nodulans* ORS 2060 groups together with *B. tuberum* (formerly *Bradyrhizobium aspalati,* Moulin et al., 2001) that produces unfucosylated NFs (Boone et al., 1999), and not with the *Bradyrhizobium* branch, whose strains produce NFs carrying a fucose (Fig. 2B, Moulin et al., 2004). It is also interesting to note that the host plant of the *Methylobacterium–Burkholderia* branch belongs to the same Crotalarieae tribe (i.e. *Aspalathus*, *Crotalaria*, *Lotononis* species). This legume tribe may have a predisposition to form symbiosis with taxonomically uncommon rhizobia, and future diversity studies of Crotalarieae tribe genera symbionts would confirm this assumption true or not.

A number of *nod* genes inducer compounds secreted from legume roots have been characterized so far. Most of these compounds belong to the flavonoid group, and share a common phenylpropanoid biosynthetic pathway. In order to purify and determine the chemical structure of LCOs produced by *M. nodulans* during the symbiotic interaction with *C. podocarpa*, we tried to identify strong inducers of *nod* genes of *M. nodulans* ORS 2060 using a *lacZ* fusion in the promoter region of the *nodA* gene. We monitored the action of several known nod-gene inducers by following β-galactosidase activity. Apigenin was found to be one of the most powerful. This result is consistent with the identification of apigenin 7-O-β-d-apiofuranosyl $(1\rightarrow6)$-β-glucopyranoside in the aerial parts of *C. podocarpa* (Wanjala and Majinda, 1999). Apigenin is thus likely the natural inducer of *M. nodulans nod* genes in the rhizosphere.

Isoflavonoids (as genistein) have been shown to be common inducers of *Bradyrhizobium nod* genes (Gillette and Elkan, 1996; Zhang et al., 1996; Souleimanov et al., 2002). In this study, we found that *M. nodulans* ORS 2060 *nod* genes are not induced by this flavonoid subfamily. This first level of specificity between rhizobia and legumes could explain the host range and cross-inoculation results reported by Yates et al. (2007).

NF metabolites trigger the formation of nodules on legume roots (Dénarié et al., 1996). These compounds harbour various substitutions differing between rhizobia and related to their host specificity. The NF produced by *M. nodulans* is a pentamer of chitin carrying a sulphate on the reducing glucosamine residue and substituted by fatty acids, either vaccenic acid ($C_{18:1}$) or palmitic acid ($C_{16:0}$), on the non-reducing glucosamine residue. This NF can be considered as having a simple structure compared with rhizobial NFs reported so far (around 60 strains have been characterized for their NFs structure). Such kind of structure has only been reported as a minor part of the NF mixture produced by *R. tropici* CIAT899, the major part being NFs with additional mannose on the reducing end and various fatty acyl chains (Folch-Mallol et al., 1996). Several rhizobial strains produce sulphated NFs as intermediates of NF biosynthesis, the final product carrying always additional substitutions on the NF backbone, such as fucose, arabinose, methyl or carbamoyl (D'Haeze and Holsters, 2002). The symbiosis between *Methylobacterium* and its plant host is specific, since *Bradyrhizobum* isolated from other species of *Crotalaria* (*C. comosa*, *C. goreensis*, *C. hyssopifolia*, *C. lathyroides, C. ochroleuca,* and *C. retusa*) do not nodulate the three African species nodulated by *Methylobacterium*

(Samba et al., 1999; Sy et al., 2001b). Even the broad host-range rhizobium NGR234, nodulating more than 350 species of legumes (Pueppke and Broughton, 1999) and producing a complex mixture of NFs (Jabbouri et al., 1995), do not nodulate *C. podocarpa* (data not shown). The *Bradyrhizobium* isolated from *Crotalaria* species should produce fucosylated NFs (like all *Bradyrhizobium* NFs described so far), as Moulin et al. (2004) reported the presence of *nodZ* (encoding NF fucosyl tranferase) in the genomes of several *Crotalaria*-isolated strains.

Crotalaria symbionts described so far thus seem to belong to two inoculation groups: most of *Crotalaria* species are nodulated by slow-growing *Bradyrhizobium* that may produce a mixture of fucosylated NFs, while the three species *C. podocarpa, C. glaucoides* and *C. perrottetti* are specifically nodulated by *M. nodulans* producing a simple NF structure, sulphated on the reducing end. Specificity of this model may be present at two steps of the interaction: first, the nature of the flavonoid *nod* gene inducer, being apigenin for *Methylobacterium* and not genistein, a common inducer of *Bradyrhizobium nod* genes; and second, at the NF structure level since *C. podocarpa* seems to discriminate between sulphated and fucosylated NFs. This symbiotic molecular dialogue appears thus classic, likewise the other rhizobia. *Methylobacterium* is an uncommon genus among symbiotic nitrogen-fixers, and occurrence of symbiosis in this genus may be the result of a more complex interaction, via specific adaptations of bacterial species to the *C. podocarpa* rhizosphere, these legumes being known to produce alkaloids (Arzt and Mount, 1999). The complete genome sequencing of *M. nodulans* ORS 2060 (underway at the Joint Genome Institute in 2007) will surely enhance our understanding of the intimate symbiosis between *Methylobacterium* and *Crotalaria*.

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