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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