Micromonospora is a normal occupant of actinorhizal nodules

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Actinorhizal plants have been found in eight genera belonging to three orders (Fagales, Rosales and Cucurbitales). These all bear root nodules inhabited by bacteria identified as the nitrogen-fixing actinobacterium *Frankia*. These nodules all have a peripheral cortex with enlarged cells filled with *Frankia* hyphae and vesicles. Isolation in pure culture has been notoriously difficult, due in a large part to the growth of fast-growing contaminants where, it was later found, *Frankia* was slow-growing.

Many of these contaminants, which were later found to be *Micromonospora*, were obtained from *Casuarina* and *Coriaria*. Our study was aimed at determining if *Micromonospora* were also present in other actinorhizal plants. Nodules from *Alnus glutinosa*, *Alnus viridis*, *Coriaria myrtifolia*, *Elaeagnus x ebbingei*, *Hippophae rhamnoides*, *Myrica gale* and *Morella pensylvanica* were tested and were all found to contain *Micromonospora* isolates. These were found to belong to mainly three species: *Micromonospora lupini*, *Micromonospora coriariae* and *Micromonospora saelicesensis*.

Micromonospora isolates were found to inhibit some *Frankia* strains and to be innocuous to other strains.

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1. Introduction

Frankia is a nitrogen-fixing bacterium that establishes root nodules on 23 species of actinorhizal plants, thus allowing them to colonize nitrogen-poor biotopes and start ecological successions. *Frankia* is notoriously hard to isolate, which is why it took close to a century to obtain (Baker and Torrey 1979) the first isolate capable of fulfilling Koch's postulates (Callaham *et al*. 1978), if one ignores the *Alnus glutinosa* isolate obtained by Pommer but subsequently lost (Pommer 1959). The variety of contaminants obtained over the years is large, depending on the medium used, but contain unicellular bacteria, fungi, yeasts, as well as actinobacteria (unpublished). *Frankia* is now considered to contain four lineages (Normand *et al*. 1996), but has so far only one described species (Normand and Benson 2012) and one *Candidatus* Frankia coriariae (Persson *et al*. 2011).

The mechanisms underlying the symbiosis are still unknown. On the plant side, it is known that a Sym kinase responds to compounds synthesized by *Frankia* (Gherbi *et al*. 2008), and that homologs of the whole symbiotic cascade were present in an EST collection (Hocher *et al*. 2011). On the microbe side, however, not much is known beyond the fact that no canonical *nod* genes could be described in the complete genomes of three representative *Frankia* strains (Normand *et al*. 2007). Ability to synthesize the plant hormones phenylacetic acid (PAA) (Hammad *et al*. 2003) and cytokinin (Stevens and Berry 1988), a specific sugar 2-*O*-methyl-D-mannose (Mort *et al*. 1983), in some instances to synthesize lectins (Pujic *et al*. 2012), and an unidentified root hair deforming

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factor (Van Ghelue *et al*. 1997; Ceremonie *et al*. 1999) are postulated to help *Frankia* establish symbiosis with its hosts, but is without direct evidence so far.

Plant tissues are considered by most as microbe-free, except under conditions of disease, where pathogens invade plant tissues and eventually open barriers, permitting saprophytes to invade necrotized tissues. However there has been a recent series of papers describing a variety of bacterial isolates, in particular actinobacteria (Zhao *et al*. 2011; Kim *et al*. 2012a), and more specifically *Micromonospora* (Coombs and Franco 2003).

Micromonospora is a genus of mainly soil actinobacteria that contains 32 species in the latest version of the Bergey's (Genilloud 2012), but 50 species are validly described at the present moment. In the latest descriptions, a strong relationship of this genus with plant roots has been shown: four species from root nodules (Trujillo *et al*. 2006, 2007; Garcia *et al*. 2010), one species from leaves (Kirby and Meyers 2010), one species from roots (Li *et al*. 2012) and four from various rhizospheres (Wang *et al*. 2011; Carro *et al*. 2012b; Carro *et al*. 2013). There have been two reports on *Micromonospora* strains in actinorhizal nodules, one from *Casuarina* (Valdes *et al*. 2005), isolate L5 closely related to the species *Micromonospora aurantiaca* and one from a *Coriaria* plant described as *Micromonospora coriariae*. In addition, *Micromonospora* was found to be widespread in nodules of legume such as lupine (Trujillo *et al*. 2006) and peas (Carro *et al*. 2012a) and to synthesize a variety of bioactive compounds (Igarashi *et al*. 2007, 2011b). Moreover, Solans (2007) has shown how some of the compounds produced by a *Micromonospora* strain are able to improve the development of *Discaria trinervis* seedlings.

The present study was undertaken to study actinorhizal plants and determine if the presence of *Micromonospora* was an exception or the rule. Its purpose was also to position phylogenetically the recovered actinobacteria.

2. Materials and methods

2.1 *Plants*

The plants chosen for this study are listed in table 1. Nodules were harvested and kept at 4°C until treatment.

2.2 *Isolation procedure*

Nodules lobes (hereafter referred to as nodules) were washed under running water and surface sterilized using Vincent's method (Vincent 1970). Briefly, nodules were surface sterilized in $HgCl_2$ (2.5% w/v) for 2 min, rinsed with distilled water five times and crushed with a sterile glass pestle. Some of the nodules were also peeled after surface sterilization. The resulting slurry was plated onto solid yeast extract mannitol (YMA) medium (Vincent 1970) and incubated at 30°C in the dark. Pigmented colonies were subcultured onto yeast extract/malt extract agar (ISP2) medium. Isolates were kept in 20% ($vol.vol^{-1}$) glycerol solution at −80°C for long-term maintenance (Atlas 1993).

2.3 *Phylogenetic positioning*

DNA was extracted using the REDExtract-N-Amp Plant PCR kit (Sigma) according to Garcia *et al*. (2010). PCR amplification of 16S rRNA gene was carried out using primers 5′-AGAGTTTGATCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCANCCRCA-3′ (Rivas *et al*. 2003) yielding a 1500 nt amplicon. A highly variable fragment of 450 pb was sequenced directly (Biofidal, Villeurbanne) using the primer SR2 (Carro *et al*. 2012a). The resulting 16S rRNA gene sequences were compared to the databank using the EzTaxon-e server (*http://eztaxon-e.ezbiocloud.net/*; Kim *et al*. 2012b). All sequences were aligned using CLUSTAL_X (Thompson *et al*. 1997), and truncated to consider the same positions with the BioEdit program (Hall 1999). Pair-wise distances were corrected for multiple base substitutions with Kimura's two-parameter method (Kimura 1980), and phylogenetic trees were constructed with the neighbour-joining method (Saitou and Nei 1987). A bootstrap confidence analysis was performed with 1000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein 1985). The resulting trees were graphically represented using MEGA version 4 software (Tamura *et al.* 2007).

2.4 *Interactions*

Frankia strains were co-cultured with the different *Micromonospora* isolates on BAP agar plates containing 5 mM ammonium chloride (Murry *et al*. 1984). One *Frankia* strain for each infection groups was selected: for the *Alnus*-infective strains, it was *Frankia alni* strain ACN14a (Normand and Lalonde 1982); for the *Casuarina*-infective, *Frankia* sp. CcI3 (Zhang *et al*. 1984); and for the *Elaeagnus*-infective strains, *Frankia* Ea1-12 (Fernandez *et al*. 1989). The *Frankia* cells were repeatedly syringed with a 26G needle and the resulting homogenates were spread onto the agar surface, left to dry, before the *Micromonospora* cells were inoculated as a 1-cm-wide, 10-cm-long strip in the centre of the plates and grown at 30°C in the dark. Due to the slower development of *Frankia* strains on agar plates, their revelation was improved by adding ethidium bromide and photos were taken under UV light.

Table 1. List of actinorhizal plants described in the present study

	Biotope, soil	Locale	Coordinates[a]	No. of isolates	Code
Alnus glutinosa	River bank, clay	Le Montellier, France	45.930139885994556 N, 5.06770133972168E	15	AG
Alnus glutinosa	Lake shore, clay sand	Mimizan, France	44.22505899964523 N, 1.2224435806274414 W	17	AGM
Alnus viridis	River bank, sand	Crots, France	44.50097439840558 N, 6.455755233764648E	32	AV
Coriaria myrtifolia	River bank, clay sand	Avignon, France	43.9646503190861 N, 4.8126983642578125E	2	CMA
Elaeagnus x ebbingei	Road side, loam	Villeurbanne, France	45.7825490682828 N, 4.881277084350586E	17	EV
Elaeagnus x ebbingei	Forest border, sand	Mimizan, France	44.21893107033916 N, 1.2937688827514648 W	25	EEM
Hippophae rhamnoides	Road side, loam	Villeurbanne, France	45.78542212542394 N, 4.86445426940918E	28	HRF
Myrica gale	River bank, sand	Maskinonge, Canada	46.195250051965886 N, 73.00363540649414 W	2	MG40
Myrica gale	River bank, sand	Trois-Rivières, Canada	46.35409615684499 N, 72.52504348754883 W	2	MG3
Morella pensylvanica	Botanical garden, loam	Lyon, France	45.77895753863966 N, 4.855785369873047E	9	MPT

[a] *http://garibou.perso.sfr.fr/GPS/googlemaps.htm*; *http://maps.google.fr/*

3. Results

All tested actinorhizal plants yielded microbial colonies after 14 days (figure 1). The total number of colonies recovered with typical *Micromonosora* morphology on plates was 1250, but the number of isolates obtained varied from 0 to 217 for each nodule (supplementary table 1; figure 2). The number of *Micromonospora* strains recovered seems to be dependent on the actinorhizal plant analysed; while a high number of colonies were recovered from nodules of *Alnus*, *Elaeagnus* and *Hippophae* (with a maximum per nodule of 217, 42 and 80, respectively) and only a few isolates were obtained for *Myrica*, *Morella* and *Coriaria* (with a maximum of 4 per nodule in all cases). From these colonies, 148 were selected for isolation and purification based on morphological diversity. These isolates developed in all instances to form orange-pigmented colonies which resembled the typical morphology of *Micromonosporaceae* (figure 1).

The pigmented isolates obtained were all positioned phylogenetically. They were all found to belong to

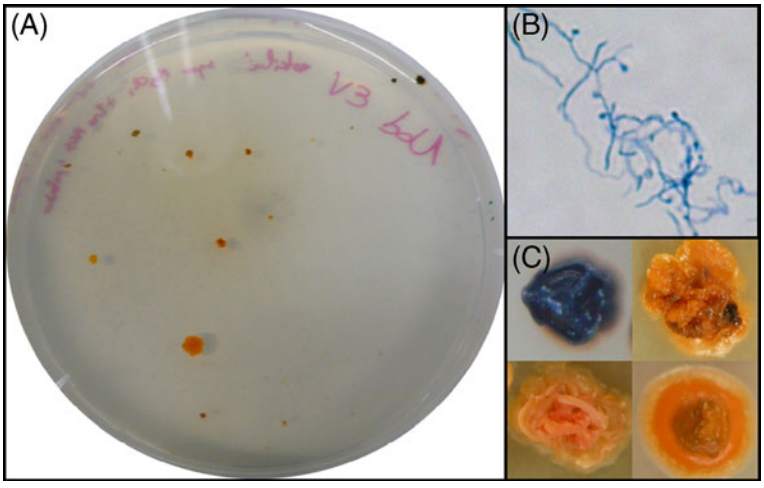

Figure 1. (**A**) Isolation plate of *Alnus glutinosa* nodule with *Micromonospora* colonies, (**B**) Gram stain of *Micromonospora* sp. colonies under light microscopy (objective 100×), (**C**) colonies of isolates EV18, AG11, AG12 and AG14 (from left to right and from top to bottom).

genus *Micromonospora*, except five which showed the highest similarity with *Plantactinospora*, a new genus of *Micromonosporaceae* close to *Micromonospora* genus. The closest neighbours and closest type species of each isolate are given in supplementary table 2, together with the percentage identity. When this identity percentage was above 99.5, the isolate was assigned to that species. In the other cases, further studies and a complete sequence determination would be necessary to determine the species to which it belongs. However, for the purpose of this article, the isolates were treated as belonging to the closest validly described species. In this way, most isolates were found to belong to species *M. saelicesensis*, *M. lupini* and *M. coriariae* with 60, 20 and 11 strains, respectively (figure 2). Interestingly, the type strains for these three most abundant species had also been isolated from nodules. In general, these proportions are maintained for the different actinorhizal plants analysed; however, as the number of recovered strains for *Myrica*, *Morella* and *Coriaria* is very small, it is difficult to determine a real relationship.

The phylogenetic tree shows the high diversity found in the isolates (figure 3; high resolution version of the figure in supplementary material). Isolates are scattered throughout

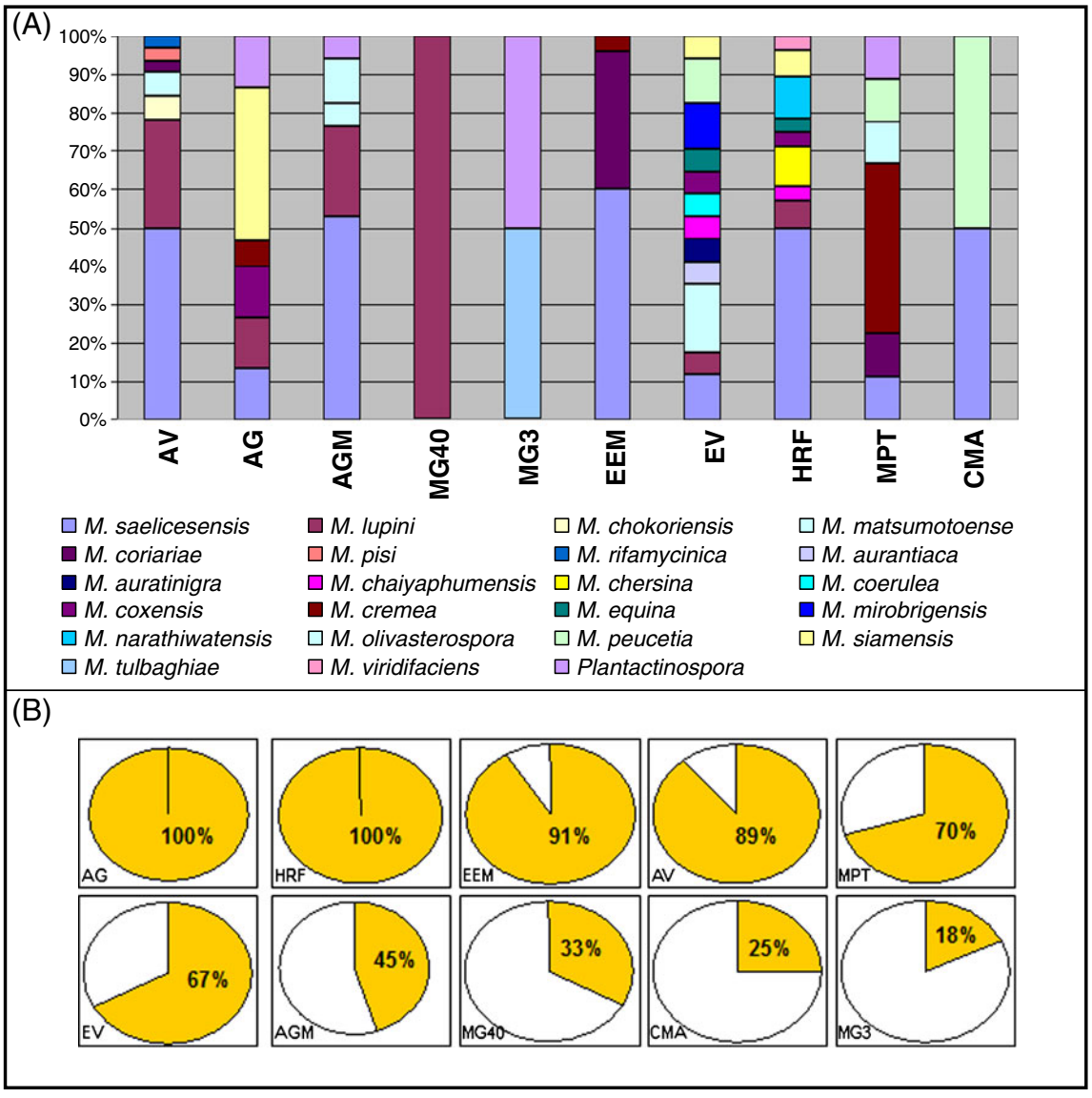

Figure 2. (**A**) Percentage of *Micromonospora* species per tree and (**B**) percentage of nodules with *Micromonospora* strains. AV: *Alnus viridis* (tree 1, 2 or 4); AG: *Alnus glutinosa* (Montellier); AGM: *Alnus glutinosa* (Mimizan); MG3: *Myrica gale* (Trois-Rivières); MG40: *Myrica gale* (Maskinonge); EEM: *Elaeagnus x ebbingei* (Mimizan); EV: *Elaeagnus x ebbingei* (Villeurbanne); HRF: *Hippophae rhamnoides*; MPT: *Morella pensylvanica*; CMA: *Coriaria myrtifolia*.

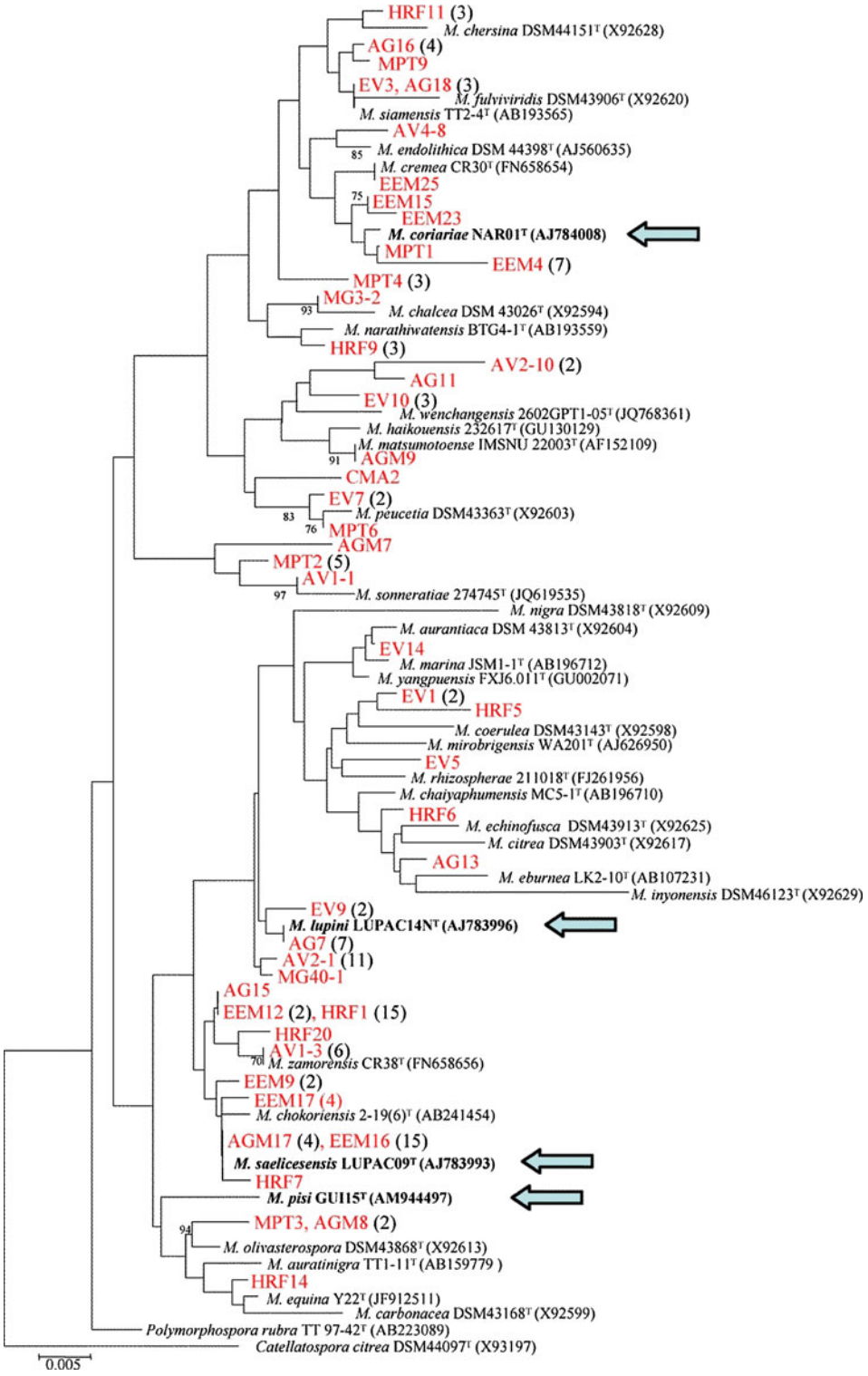

Figure 3. Phylogenetic tree of partial 16S rRNA gene reconstructed by the neighbour-joining method. The numbers in parentheses after the isolates represent the number of similar sequences.

the tree, however, most of them form small groups, of 1 to 4 leaves. On the other hand, isolates similar to *M. saelicesensis* LUPAC09^T and *M. lupini* LUPAC14N^T form a large group with a monophyletic origin.

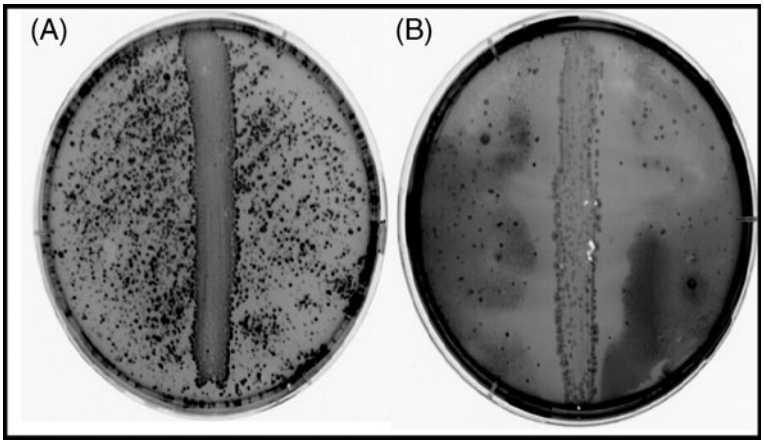

Figure 4. Inhibition plates for *Micromonospora* sp. AG4 isolated from *Alnus glutinosa* nodules grown in the presence of (**A**) *Frankia alni* ACN14a and (**B**) *Frankia* sp. Ea1.12.

Representative *Micromonospora* strains obtained in this work were selected to undertake inhibition test against *Frankia* strains. The selected strains were AG4, AV4-18, EEM4, EEM6, EEM11, EEM13, EV8, EV9, HRF2, MG40-1 and MG40-2 isolated from *Alnus glutinosa*, *Alnus viridis*, *Elaeagnus x ebbingei*, *Hippophae rhamnoides* and *Myrica gale*. The *Micromonospora* isolates were never inhibited by the *Frankia* isolates. However, growth of the *Frankia* strains was sometimes inhibited (figure 4). The most sensitive strain among those tested was *Frankia* sp. Ea1.12, which was inhibited by most of the strains not isolated from *Elaeagnus*, and also from some of the isolates isolated from this plant, while the most resistant strain was *Frankia alni* ACN14a (table 2).

4. Discussion

Actinobacteria are emblematic of soils. They have a number of morphological and physiological adaptations that make them good competitors in soils, such as hyphae to bridge soil cavities, spores to withstand episodes of desiccation, and secondary metabolites to ward off competition. Most actinobacteria have been isolated from soils except for some animal pathogens (*Mycobacterium*, *Nocardia*, *Tropheryma*), commensals/symbionts (*Bifidobacterium*) or plant pathogens (*Clavibacter*, *Streptomyces*) and symbionts (*Frankia*) and more recently from stone surfaces (*Modestobacter*) and marine sediments (*Salinispora*). However, each of these genera is not homogeneous as lineages have specialized to exploit the diverse niches.

Frankia, after its first isolation in pure culture (Callaham *et al*. 1978), was initially considered essentially a genus of plant symbionts (Benson and Silvester 1993) before isolates were obtained that were unable to reinfect the plants (Mirza *et al*. 1991). Genus *Frankia* is now divided into 4 clusters, 3 of which are symbiotic and 1 containing, in particular, non-symbiotic strains (Normand *et al*. 1996), all of which have been isolated from efficient nodules. In the same manner, *Frankia* strains belonging to Cluster 3 have sometimes been isolated from *Casuarina* (Gauthier *et al*. 1981), now known to be in symbiosis with Cluster 1 strains. Actinorhizal nodules thus contain, besides *Frankia*, not only other atypical strains unable to reinfect their host

Table 2. Inhibition test of *Frankia* strains versus *Micromonopora* isolates

	Frankia alni ACN14a	*Frankia* sp. CcI3	*Frankia* sp. Ea1.12
AG4	0	-	- -
AV4-18	0	-	- -
EEM4	0	0	-
EEM6	0	0	0
EEM11	0	0	0
EEM13	0	-	- -
EV8	-	- -	- -
EV9	- -	-	- -
HRF2	- -	-	- -
MG40-1	-	-	- -
MG40-2	0	0	0

0: no inhibition; -: less than 0.5 cm inhibition; - -: more than 0.5 cm inhibition.

AV: *Alnus viridis*; AG: *Alnus glutinosa*; MG: *Myrica gale*; EEM: *Elaeagnus x ebbingei* (Mimizan); EV: *Elaeagnus x ebbingei* (Villeurbanne); HRF: *Hippophae rhamnoides*.

plant but also numerous 'contaminants' (Baker and Torrey 1979), many of which were actinobacteria labeled *Actinomyces alni*, *Streptomyces alni*, *Nocardia hippophae*, etc. Some of these systematic inhabitants of the actinorhizal nodules could have a biological function that remains unknown for the moment.

Micromonospora was defined as a genus of ray-fungi, as actinobacteria were called at the time (Orskov 1923). Most of its species are isolated from soils, mainly alkaline or neutral ones, and to a lesser extent from aquatic environments. Lately, several studies aimed at characterizing endophytic communities have yielded *Micromonospora* isolates, for instance, from wheat (Coombs and Franco 2003). Among the factors postulated to select for *Micromonospora* is their ability to synthesize secondary metabolites that inhibit pathogens such as *Pythium* or *Phytophthora* (Coombs and Franco 2003). The genus is described as able to synthesize a large number of antibiotics (Berdy 2005) and is second only to *Streptomyces* in this respect (Genilloud 2012), synthesizing up to 500 different molecules with varying properties (Furumai *et al*. 2000, 2002; Igarashi *et al*. 2000, 2002, 2005, 2007, 2011a, b). Lately *Micromonospora* isolates have also been recovered from *Casuarina* (Valdes *et al*. 2005) and from *Coriaria* (Trujillo *et al*. 2006). They were the first descriptions of the presence of *Micromonospora* strains inside these kinds of nodules, but they were considered sporadic occurrences. The results of this study, however, indicate that the presence of micromonosporas in nodules is common and widespread for actinorhizal plants.

The high proportion of isolates with a strong similarity to *M. saelicesensis* and *M. lupini* type strains is remarkable. These type strains were isolated from *Lupinus* surface-sterilized nodules, a leguminous plant (Trujillo *et al*. 2007). In previous analyses of many *Micromonospora* isolates from nodules of legumes, this high proportion of these two species was also noted (Trujillo *et al*. 2010; Carro *et al*. 2012a). The proportion of isolates recovered with a high similarity with these type strains is quite important. Also, *M. coriariae* strains appear in high proportion, but less so than the other two species. These results could indicate that these species have coevolved or co-speciated with plants as shown for some *Frankia* lineages (Simonet *et al*. 1999), and it is probable they are better adapted to live inside nodules. Although other species of *Micromonospora* appear to be like other endophytic bacteria, we could find, inside plants, strains of the group of *M. saelicesensis*, and *M. lupini* could have a specific role for plants and more specifically in symbiosis.

On the plant side, the results obtained in this work show that the *Micromonospora* relationship is intense with *Alnus*, *Elaeagnus* and *Hippophae* and not so intense with *Coriaria*, *Morella* and *Myrica*. It was relatively easy to recover a large number of colonies from the first ones, but not from the others. The low number of colonies recovered from *Coriaria*, *Morella* and *Myrica* are evocative of normal endophytic bacteria, whereas for the other host genera, it seems there is a strong association. In this way, the small number of *Micromonospora* isolates in preliminary results of roots isolation (data not shown) indicates that *Micromonospora* finds a more comfortable habitat inside nodules of some plants and is a normal occupant of actinorhizal nodules.

The function of these *Micromonospora* isolates is unknown at the moment. They are probably a minority because 16S rRNA amplicon sequencing of nodules have not yielded mixed readings, only *Frankia* ones, and so the amount of *Frankia* cells should be much higher than that of *Micromonospora* cells. Yet, these *Micromonospora* are ubiquitous in actinorhizal nodules, either because they have the necessary determinants to enter alone, or they can squeeze in together with *Frankia*. Regardless, this ability would appear unique as few other actinobacterial genera have been recovered from actinorhizal nodules. *Micromonospora* cells would not trigger the host defence reactions and could even help the plant fight off the numerous soil pathogens with their rich array of secondary metabolites, to which the symbiotic *Frankia* would have grown used to.

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