Isolation, characterisation (PCR-RFLP) and specificity of Frankia from eight Gymnostoma species endemic to New Caledonia.

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Abstract – Eight Frankia-Gymnostoma nitrogen-fixing symbioses endemic to New Caledonia were studied using a range of techniques including molecular typing of Frankia, infectivity/effectivity and host spectrum of the isolates. The study was conducted on 128 Frankia isolates from the eight Gymnostoma species. The RFLP analysis of the rRNA 16S-23S ITS allowed us to cluster the strains in four groups. Symbiotic characterization of the four groups failed to reveal any host specificity both in term of infectivity or effectivity. The characteristics of the strains were those of Eleagnaceae-infective Frankia strains. Co-inoculation of two Gymnostoma species with mixtures of strains evidenced 11% of mixed RFLP profiles. In such controlled conditions, this was interpreted as co-infections. This work evidenced original features of these Gymnostoma isolates: (i) they were closer to Eleagnaceae-infective Frankia than Casuarina-infective Frankia; (ii) closer to atypical Frankia from Casuarina; (iii) poorly host-specific; and (iv) they had a tendency of co-infection. They could represent a primitive group of Frankia able to survive in a wide range of habitats. © 2000 Éditions scientifiques et médicales Elsevier SAS

Gymnostoma / Casuarina / Eleagnaceae / infectivity / effectivity / nitrogen fixation / actinorhizal plants / ITS 16S-23S / co-infection

Résumé – Isolation, caractérisation (PCR-RFLP) et spécificité de Frankia provenant de huit espèces de Gymnostoma endémiques de Nouvelle-Calédonie. Une étude a été menée sur huit symbioses fixatrices d’azote Frankia-Gymnostoma endémiques à la Nouvelle-Calédonie. Cent vingt-huit souches de Frankia ont été isolées et étudiées par diverses techniques, dont le spectre d’hôtes, l’infectivité, l’effectivité et la PCR/RFLP de l’ITS 16S-23S. L’analyse par PCR/RFLP de l’ITS 16S-23S a permis de classer ces Frankia en quatre groupes qui n’ont pas montré de différences en ce qui concerne leurs capacités symbiotiques. La co-inoculation de deux espèces de Gymnostoma par des mélanges des quatre types de Frankia isolés a mis en évidence l’apparition de mélanges de profils RFLP dans les nodules ainsi obtenus. Dans de telles conditions contrôlées, ce résultat a été interprété comme une co-infection. Ce travail a mis en évidence chez les Frankia de Gymnostoma des caractéristiques originales : (i) ils sont plus proches des Frankia des Eleagnaceae que de ceux des Casuarina spp ; (ii) ils sont proches des Frankia atypiques des Casuarina ; (iii) ils sont peu spécifiques ; et (iv) ils sont capables de co-infection. Ils pourraient représenter un type de Frankia primitif capable de survivre dans des habitats très variés. © 2000 Éditions scientifiques et médicales Elsevier SAS

Gymnostoma / Casuarina / Eleagnaceae / infectivité / effectivité / fixation d’azote / plantes actinorhiziennes / ITS 16S-23S / co-infection
1. INTRODUCTION

Actinorhizal plants can fix atmospheric nitrogen through a symbiotic association with the actinomycete Frankia. Among the eight botanical families, Casuarinaceae comprise four genera: Ceuthostoma, Gymnostoma, Allocasuarina and Casuarina [10]. These genera are essentially tropical and subtropical, originating from the Australian, Melanesian and Malaysian regions.

The flora of New Caledonia is characterized by a very high level of endemicity (75 to 80 % at the species level) [8]. Casuarinaceae are the only actinorhizal plants native to New Caledonia, with one Casuarina and eight Gymnostoma species (among the eighteen existing), all endemic. Nitrogen fixation makes these species good candidates for the rehabilitation of degraded land as well as mine sites in New Caledonia.

The eight species of Gymnostoma grow on soils originating from ultramafic rocks, with the exception of G. nodiflorum which grows on alluvial soils of volcanic-sedimentary origin. Ultramafic soils are characterized by very low levels of P, K and Ca, high levels of Ni and Mn, a slow mineralization rate of organic matter and a poor level of available N. They can be either little transformed and rich in Mg (hypermagnesian inceptisol) or highly transformed in indurated oxisols. G. chamaecyparis is restricted to hypermagnesian soils and G. deplancheanum to indurated oxisols. The others species colonize a wider range of habitats such as forest (G. glaucescens, G. intermedium and G. poissonianum) or riparian ecosystems (G. leucodon, G. nodiflorum and G. webbianum) [9]. Their relative specificity towards adverse soils together with their nitrogen fixing capacity make these tree species almost obligate candidates in the revegetation processes of mine sites in New Caledonia.

Probably due to their high endemicity, these Gymnostoma species and their symbiotic characteristics have been little studied. In 1989, Racette and Torrey [15] first isolated a Frankia strain from G. papuanum, then Savouré and Lim [18] isolated four strains from G. sumatranum. These strains were mostly studied for their symbiotic characteristics and their current availability can be seriously questioned. More recently, Navarro et al. [11] sequenced the rif DK ITS of six unisolated strains from six Gymnostoma species from New Caledonia and compared them to reference Frankia strains. These studies showed that Frankia infecting Gymnostoma nodules were indistinguishable from Eleagnaceae infective strains. However, there was still a need for a study (i) made on isolated Frankia strains, available as inoculum in field experiments, and (ii) on a significative number of strains susceptible to represent a wide genetic pool. The aim of this work was thus to isolate a large number of Frankia strains from a wide range of Gymnostoma species, and to characterize the isolates with molecular tools and by their symbiotic properties, i.e. infectivity, effectivity and host spectrum.

2. MATERIALS AND METHODS

2.1. Nodules harvest

Nodules were directly harvested from the roots of the Gymnostoma plants. Each lobe was individually rinsed under tap water, disinfected for 10 to 20 min in 30 % (wt/v) H2O2 and rinsed with sterile distilled water before being cut into small fragments. All the fragments from one individual lobe were incubated at 30 °C, in one tube containing 5 mL Nitrogen Free Medium (NFM) or Complex Medium (CM) [11], for 1 to 3 months. This method allowed to ascertain that each strain originated from one single original lobe. The isolated strains, identified microscopically by the presence of the Frankia characteristic morphological structures including hyphae, vesicles, sporangia and spores were then subsequently cultured in the CM medium.

2.3. Plant material

Seeds were sterilised by incubation in concentrated H2SO4 for 2 min. The seeds were rinsed out with sterile distilled water and germinated in sterile sand, in a shade house. We tested only seven species of Gymnostoma, G. glaucescens seeds being not available. The other actinorhizal plants tested were: Allocasuarina littoralis, A. stricta, A. torulosa, Casuarina collina, C. cunninghamiana, C. equisetifolia and Elaeagnus angustifolia. After 1 month, the seedlings were transferred into pots containing one of the three sterile soil (see below) from New Caledonia and watered every 12 h. Two-month-old plants were fertilized with 1 g·L−1 standard Welgro nutrient solution (Ulvir limited, Barcelona, Spain).

2.4. Soils

The three soils used in this study were from New Caledonia. They were all derived from ultramafic rocks, with low P, K and Ca and high Ni levels. The C/N ratio was about 15 with a low organic decomposition and a poor N availability. The soils were sterilized by autoclaving at 120 °C for 2 h twice at 24-h interval.
Characteristics of *Frankia* from *Gymnostoma*

2.4.1. Tontouta soil

Tontouta soil is a river sand (pH 6.6) from southern New Caledonia. It was considered as a control soil where all species can grow.

2.4.2. Nepoui soil

Nepoui soil is a hypermagnesian inceptisol (pH 6.7) toxic for most of *Gymnostoma* species where *G. chamaecyparis* can grow.

2.4.3. Prony soil

Prony soil is an indurated oxisol (pH 4.7) with high Fe, Ni and Mn levels, toxic for most of *Gymnostoma* species but where *G. deplancheanum* can grow.

2.5. DNA extraction

2.5.1. From cultures

DNA was extracted from 1-month-old *Frankia* strains according to Brenner et al. [2] and Simonet et al. [19].

2.5.2. From nodules

Nodule lobes were disinfected with 30% w/v H₂O₂ for 5 min, rinsed with sterile distilled water and kept at 20 °C. One nodule lobe was crushed in 500 mLTE buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, pH 8, 2% w/v CTAB (Sigma, St Louis, MO, USA) and 3% w/v PVPP (Sigma)). The mixture was incubated at 65 °C for 1 h and centrifuged at 3,000 x g for 5 min (20 °C). The supernatant was chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 10 mL TE buffer (pH 7.5).

2.6. PCR amplification

The 16S-23S InterTranscribed Spacer (ITS) rRNA fragment were amplified using primers FGPL2054' (5'-CCG-GGT-TTC-CCC-ATT-CGG-3') (beginning of 23S, universal) [14] and FGPS989e (5'-GGGGTCTG-GGAGGCT-3') (end of 16S, Eleagnaceae-specific) [1] or FGPS989ae (5'-GGGGTCCGTAGGGCT-3') (end of 16S, Casuarina-specific) [1] as described by Simonet et al. [20] and Nazaret et al. [12]. PCR were run on Perkin-Elmer 2400 GeneAmp PCR Systems.

2.7. RFLP

Restriction analyses of the PCR products were performed with three enzymes: *HaeIII*, *MspI* and *CfoI*.

Digestion was performed on 10 μL PCR products at 37 °C for 2 h. Electrophoreses were made on 3% agarose gels (Sigma) at 140 mV for 1 h.

2.8. Cross inoculation studies

The seven *Gymnostoma*, three *Casuarina*, three *Allocasuarina* species and *Eleagnus angustifolia* were grown as already described, on the control sandy soil (Tontouta). They were inoculated 4 months after germination with 10 mg packed cell volume [13] of an 1-month-old *Frankia* culture. For each ITS group, one representative strain was arbitrarily selected and used for the experiment. The four selected strains were: Gd03 (ITS Group 1), Gg40 (ITS Group 2), GI03 (ITS Group 3) and Ge18 (ITS Group 4). Each treatment was repeated four times and two non-inoculated controls were used for each plant species. Because of very poor growth, *Gymnostoma* species were measured 11 months after inoculation. The other species were measured 6 months after inoculation. Infectivity was qualitatively estimated by the presence/absence of nodules for each plant, and effectivity was determined through the acetylene reduction method on the entire root system under standard procedures. Ethylene production was determined on a Carlo Erba GC6000 gas chromatograph. Results were expressed as μM C₂H₄ h⁻¹ g⁻¹ nodule dry weight.

2.9. Incidence of soil and host plant combination on symbiotic properties of the four ITS groups of *Frankia*

This study was conducted on two *Gymnostoma* species, *G. chamaecyparis* and *G. deplancheanum*, selected for their extremely opposite soil properties requirements. Moreover, these two species were the only ones to correspond to exclusive ITS group of *Frankia*. The three soils (Tontouta, Nepoui and Prony) were used as growth substrate. The growth and shade house conditions were as described above.

2.10. Infectivity and effectivity tests

Inoculations were performed with each ITS group using the same four selected strain as above, by delivering 10 mg (packed cell volume) of an 1-month-old culture. Four inoculated plants and two non-inoculated controls were used per treatment. Infectivity and effectivity were estimated as described above, 11 months after inoculation.

2.11. Co-inoculation study

Inoculation was performed by delivering to each soil/plant combination 10 mg inoculum. The inoculum was an equal volume (packed cell volume) mixture of
3. RESULTS AND DISCUSSION

3.1. Frankia strain isolation

The isolation method we used (one nodule lobe per tube, liquid medium) has two advantages: (i) it allows to quickly and easily eliminate contaminated lobes; and (ii) it ensures that isolates originate from one lobe.

About 2,100 nodule lobes had to be dissected to allow the obtention of 128 isolates from eight Gymnostoma and ten localities (Table I). This corresponds to about 6% of success of isolation among Gymnostoma species. With different method and different media, Rosbrook and Reddell [17] obtained isolation rates of 50% for Casuarina equisetifolia, 17% for C. glauca and 3% for C. cunninghamiana. The first strain of Frankia was isolated 20 years ago [3], however the isolation step is still a considerable bottleneck to obtain pure cultures, and a strong variability is observed among host plants and methods used. Isolation seemed to be more successful on nitrogen-free medium (90% of the successful isolations). This could be due to the early selection of nitrogen fixers (and the non-proliferation of contaminants) as well as a better compatibility between the culture medium and Frankia strains.

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Geographical origin</th>
<th>Frankia isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnostoma chamaecyparis</td>
<td>Nepoui</td>
<td>Ge01, 06, 10 to Ge16, 18</td>
</tr>
<tr>
<td>G. deplancheanum</td>
<td>Chute de la Madeleine</td>
<td>Gd33, 41, 51, 103</td>
</tr>
<tr>
<td></td>
<td>Creek Pernod</td>
<td>Gd02, 03, 17, 18</td>
</tr>
<tr>
<td></td>
<td>Prouy</td>
<td>Gd94, 95</td>
</tr>
<tr>
<td></td>
<td>Rivière bleue</td>
<td>Gd73</td>
</tr>
<tr>
<td></td>
<td>Yat (col de)</td>
<td>Gd19, 20, 21, 22, 30, 32, 35, 36, 88, 91, 93, 121, 130, 132</td>
</tr>
<tr>
<td>G. glaucescens</td>
<td>Dzumac</td>
<td>Gg01 to Gg30</td>
</tr>
<tr>
<td>G. intermediun</td>
<td>Étoile filante</td>
<td>Gg40 to Gg51</td>
</tr>
<tr>
<td>G. leucodon</td>
<td>Dzumac</td>
<td>Gg01 to Gg30</td>
</tr>
<tr>
<td>G. nodiflorum</td>
<td>Gt</td>
<td>Gn01 to Gn12</td>
</tr>
<tr>
<td>G. poissonianum</td>
<td>Dzumac</td>
<td>Gp01 to Gp04</td>
</tr>
<tr>
<td>G. webbianum</td>
<td>Rivière bleue</td>
<td>Gw01, 02, 07, 09, 12, 13, 20, 33, 34, 36, 40, 41, 51, 52</td>
</tr>
</tbody>
</table>

The strains isolated from Gymnostoma exhibited all the characteristic morphological structures of the genus Frankia including hyphae, sporangia, spores and vesicles. Vesicles were very numerous in the NFM medium and still present after ammonium complementation. All the strains presented an orange to blood-red pigmentation. These characteristics are close to those of (i) the five already isolated Gymnostoma strains [15, 18], (ii) atypical strains from Casuarina (i.e. strains unable to nodulate the host of origin but which nodulate species belonging to Eleagnaceae and Rhamnaceae families) [5, 6, 22] and (iii) strains isolated from Eleagnaceae or Rhamnaceae described by Clawson et al. [4], Gauthier et al. [7] or Nazaret et al. [12]. The 128 isolates represented a wide genetic pool suitable to evaluate diversity of the strains nodulating the eight species of Gymnostoma.

3.2. Molecular characterization of the bacterial rRNA operon

3.2.1. PCR amplification

The DNA extraction protocol yielded DNA that was pure enough to obtain amplification with primer FGPS989c, which is Eleagnaceae group-specific. No amplification was obtained with primer FGPS989ac, which is Casuarina group-specific. The amplified fragments were about 1,000 bp long. Similar grouping of non-isolated Frankia from Gymnostoma (in nodule) with those of Eleagnaceae was already described by Navarro et al. [11] by using specific primers which confirms the particular position of Gymnostoma nodulating Frankia among the Casuarinaceae family.

3.2.2. RFLP

Discriminant patterns were observed when PCR products were digested with HaeIII and MspI. Each enzyme produced three profiles which, in combination, allowed to discriminate four different ITS groups named 1, 2, 3 and 4. Groups 2 and 3 were predominant: 59 and 26%, respectively. These two groups were widely represented in the different species of Gymnostoma in their various ecological habitats. Groups 1 and 4 represented only 8.6 and 6.3% of the isolates and were found to exclusively occur in G. deplancheanum and G. chamaecyparis, respectively, in their natural stands (Table II). Working with non-isolated strains of Gymnostoma nodulating Frankia, the RFLP analysis of the ITS rRNA operon allowed the discrimination of four groups corresponding to those already described by Navarro et al. [11] by using specific primers which confirms the particular position of Gymnostoma nodulating Frankia among the Casuarinaceae family.

<table>
<thead>
<tr>
<th>Host plant</th>
<th>ITS groups (16S-23S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. chamaecyparis</td>
<td>Number 1234</td>
</tr>
<tr>
<td>G. deplancheanum</td>
<td>10 28 42 3</td>
</tr>
<tr>
<td>G. glaucescens</td>
<td>11 11 15 12</td>
</tr>
<tr>
<td>G. intermedium</td>
<td>11 8 1 13</td>
</tr>
<tr>
<td>G. leucodon</td>
<td>14 1 13</td>
</tr>
<tr>
<td>G. nodiflorum</td>
<td>Total 128</td>
</tr>
<tr>
<td>G. poissonianum</td>
<td>11 76 33 8</td>
</tr>
</tbody>
</table>
lated strains of six Gymnostoma species, Navarro et al. [11] compared mainly sequences of the nif DK intergenic spacer, which did not allow a direct comparison with our results.

3.3. Symbiotic characterization of each group

3.3.1. Host spectrum of each group

The four ITS groups were found to be infective and effective on the seven Gymnostoma species tested and on Eleagnus angustifolia. They were non-infective on the three Casurina and three Allocasuarina species tested. No significant difference was found in either infectivity or effectiveness between the different groups. Re-isolation and PCR/RFLP characterization of a representative strain from the four Frankia groups allowed to confirm Kochs postulate for each group. Twenty-one arbitrarily chosen strains from the different groups were additionally tested, mainly on G. chamaecyparis and G. deplancheanum, which allowed us to confirm the lack of specificity between the groups and the species (data not shown).

3.3.2. Incidence of soil and host plant combination on symbiotic properties of the four ITS groups

Considering the relative exclusivity of groups 1 and 4 on G. deplancheanum and G. chamaecyparis and the specificity of the soils they colonized (Prony and Nepoui), it seemed particularly interesting to test infectivity and effectiveness of the four ITS groups on these two species on their respective soil. A less extreme soil (Tontouta) was also used as control. Despite numerous attempts, it was impossible to maintain heterologous soil-plant combinations such as G. deplancheanum/Nepoui and G. chamaecyparis/Prony. Table III illustrates the results which did not allow to symbiotically differentiate the four groups whatever the tested combinatorial situation. Even in extreme edaphic conditions, it is thus not possible to establish a clear relationship between the origin of a given strain and the symbiotic performances of the association. In a control sandy soil where both plant species can grow, there is no evidence of a clear advantage for any strain/plant combination.

3.3.3. Co-inoculation of the four groups in the different soil/plant combinations

Co-inoculation of two Gymnostoma species (table IV) with mixtures of strains allowed to evidence 11% of mixed RFLP profiles. In such controlled conditions, this was interpreted as co-infections. Such co-infections have been reported by several authors [16, 21] and were already observed in the Frankia-Gymnostoma symbiosis through: (i) isolation of mixtures of strains originates from one nodule lobe (Gauthier, unpubl.); and (ii) observation of more than one ITS pattern in some nodule lobes harvested in the field (data not shown). Table IV presents the percentage of the different ITS groups of Frankia detected within the nodules in the different soil/plant combinations. A similar number of nodule lobes was analysed for each plant species. All the groups appeared to be equally represented within the nodule for any combination. No comparative advantage could thus be detected for any of the ITS groups in term of competitiveness in any of the situations.

Groups 1 and 4 are not more adapted to their original plants whatever the soil, and the predominance of groups 2 and 3 cannot be related to any symbiotic ability. One can thus speculate that the relative higher frequency of groups 2 and 3 among isolates is attributable to a better isolability. It can be questioned whether the isolation steps underestimate the biodiversity of the strains by selecting some groups above others. Using the same strategy (PCR/RFLP on 16S-23S ITS) with non-isolated Frankia from Gymnostoma (in nodule), it

Table III. Incidence of soil and Gymnostoma spp. combinations on symbiotic properties of the four ITS groups of Frankia. For each species, four repetitions were carried out. I, infectivity (% of nodulation); E, effectiveness (as ARA: µmoles C₂H₄ h⁻¹ g⁻¹ nodule dry weight, extreme values).

<table>
<thead>
<tr>
<th>Frankia</th>
<th>Soil and plants tested</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Tontouta soil</td>
</tr>
<tr>
<td>ITS group 1</td>
<td>G. chamaecyparis</td>
</tr>
<tr>
<td>Gd03</td>
<td>I: 100</td>
</tr>
<tr>
<td>ITS group 2</td>
<td>Gd40</td>
</tr>
<tr>
<td>ITS group 3</td>
<td>Gd50</td>
</tr>
<tr>
<td>Gc18</td>
<td>I: 12–52</td>
</tr>
<tr>
<td>ITS group 4</td>
<td>Gc18</td>
</tr>
</tbody>
</table>

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Eleagnaceae, and to atypical strains of Casuarina spp. poorly host-specific, widely represented, easily isolat-
rapidly in vitro even on nitrogen-free media; they are
norhizal symbiosis and to produce inoculum for field
Tontouta soil
[58x422]ing capacity and a putative ability of saprophytic life
infect a wide range of species; (ii) they have a strong
ability to survive in soils both through a high sporulat-
Frankia strains is essential for the studies of acti-
was possible to determine seventeen ITS groups
(Navarro, pers. comm.). However, the isolation of
Frankia strains that have in common certain characteristics: these
strains sporulate easily; they are pigmented, they grow
in vitro even on nitrogen-free media; they are
poorly host-specific, widely represented, easily isolat-
able and able to co-infect. They are close to strains of
Eleagnaceae, and to atypical strains of Casuarina spp.
Together with strains from Eleagnaceae and atypical
strains, they could represent a primitive group of
Frankia able to survive in a wide range of habitats
to a high adaptability: (i) they can infect or co-
infect a wide range of species; (ii) they have a strong
ability to survive in soils both through a high sporulat-
capacity and a putative ability of saprophytic life
(that could be reflected by their high isolability).

Acknowledgements
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