Isolation of *Frankia* from nodules of *Casuarina equisetifolia*

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Accepted February 2, 1982

DIEM, H. G., D. GAUTHIER, and Y. R. DOMMERGUES. 1982. Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. Can. J. Microbiol. **28**: 526–530.

Using the simple serial dilution technique, five strains of actinomycetes were isolated from nodules of *Casuarina equisetifolia*. In spite of the fact that these strains did not nodulate the host plant, they were assumed to belong to the genus *Frankia* because they possessed the morphological and cultural characteristics now admitted as specific to this genus.

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En utilisant la technique de dilutions successives, on a isolé cinq souches d'actinomycète à partir des nodules de *Casuarina equisetifolia*. Bien que ces souches n'aient pas nodulé la plante-hôte, elles sont considérées comme appartenant au genre *Frankia* puisqu'elles possèdent les caractères morphologiques et culturaux propres à ce genre.

Introduction

Recently, strains of *Frankia* have been isolated from nodules of some plants using different methods, namely (1) enzymatic digestion, e.g., *Comptonia peregrina* (Callaham *et al.* 1978), (2) microdissection, e.g., *Alnus rubra* (Berry and Torrey 1979), (3) sucrose-density fractionation, e.g., *Elaeagnus umbellata* and *Alnus crispa* (Baker, Torrey, and Kidd 1979), (4) serial dilution, e.g., *Alnus crispa* (Quispel and Tak 1978; Quispel 1979), (5) Sephadex fractionation, e.g., *Myrica gale* and *Elaeagnus umbellata* (Baker, Kidd, and Torrey 1979), and (6) direct isolation from surface-sterilized pieces of nodules, e.g., *Alnus glutinosa* (Lalonde *et al.* 1981). With the exception of the last two methods isolation procedures were sophisticated and often associated with the use of complex media (e.g., Quispel and Tak 1978). Gauthier *et al.* (1981*a*) have reported that two *Frankia*-like actinomycetes isolated from nodules of *Casuarina equisetifolia* were able to reduce C_2H_2 *in vitro*. This paper describes in detail the procedures used for isolating *Frankia* strains from nodules of *Casuarina equisetifolia* from different sites throughout the world. Moreover, morphological and cultural characteristics of strains are presented.

Material and methods

Samples of nodules formed on *Casuarina equisetifolia* were taken from seven different locations: Park of Hann, Dakar, Senegal (D); Miami, FL (F); INRA Research Station, Petit-Bourg, Basse Terre, Guadeloupe (G1); INRA Research Station, Saint-François, Grande Terre, Guadeloupe (G2); IRRI Research Station, Los Baños, Philippines (P1); UPLB (Chemistry Building), Los Baños, Philippines (P4). Nodules were also taken from *Casuarina rumphiana* at Sampalac, Los Baños, Philippines (P2).

Seedlings of *Casuarina equisetifolia* were grown in test tubes containing a N-free nutrient medium (Vincent 1970) with the shoots free above an aluminium foil cap (Gibson 1963). Plants were grown in a growth chamber maintained at 28°C, light intensity 7000 lx, 14-h photoperiod. When seedlings were 1 month old their roots were inoculated with a suspension of crushed nodules, either freshly collected or stored for 3–6 months in a dry state. Nodulation occurred in both cases within 1–2 months. Nodules 0.5–2 mm in diameter from 3- to 4-month-old seedlings were cleaned thoroughly with tap water and surface sterilized with chloramine T (1% in distilled water) for 5 min and then rinsed several times with sterile distilled water before being aseptically crushed in sterile distilled water. The resulting suspension was diluted to 10^{-2}, 10^{-3}, and 10^{-4} with sterile distilled water. The dilutions were then used to inoculate either solid (1.5% agar) or liquid QMOD medium (Lalonde and Calvert 1979). For isolation on agar medium, 0.2 mL of each dilution was first spread onto the surface of the solidified medium in 9-cm-diameter petri dishes. In further experiments, the same amount of each dilution was mixed with approximately 2 mL of the agar medium which was maintained at 40°C and evenly spread by swirling over a bottom layer of the same agar medium. The agar medium has the advantage that bacterial contaminants remain spatially separated from the *Frankia* colonies; moreover, the method of double layers with the inoculum embedded in a thin top layer makes more uniform the growth conditions for *Frankia*. For isolation on liquid medium, fifty to one hundred 15-mL screw-capped vials, each containing 5 mL of medium, were inoculated with one drop of 10^{-3} or 10^{-4} dilution. After inoculation, plates and vials were incubated at 28–30°C.

For light microscopy observations, colonies of *Frankia* from cultures in liquid or solid QMOD medium were stained with 1% (w/v) trypan blue in lactophenol.

Casuarina equisetifolia seedlings used for the infection tests were grown either in plastic pouches (Lalonde 1979), tubes, or Leonard jars (Vincent 1970) containing a sterile mixture of

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vermiculite and sand (1:1, v/v) supplied with N-free Hoagland's solution (Hoagland and Arnon 1938). When the seedlings were 5 weeks old, they were inoculated with a suspension of washed, homogenized hyphae and spores from 4- to 6-week-old broth cultures. One to 3 months later, the root systems were periodically examined for the presence of nodules.

Results

Isolation on solid QMOD medium

After 10–15 days, the plates were carefully scanned under a dissecting microscope to detect the presence of *Frankia* colonies. At that time, the colonies were 100–200 μm in diameter, exhibiting a typical starfish shape (Fig. 1). If the nodules used for the dilution contained units able to form *Frankia* colonies on the medium (UFF), these colonies could be observed among a number of contaminant colonies, predominantly bacteria. The number of colonies of *Frankia* appearing in plates inoculated with 10^{-3} dilution was in the range of 1–20 per plate. Comparing the suspensions obtained from six different nodule lobes, only four lobes produced colonies of *Frankia* on the plates, suggesting a heterogeneous distribution of UFF in the nodules.

Isolation in QMOD liquid medium

After 4–6 weeks the 15-mL vials were examined for the presence of *Frankia* colonies. In most vials (80–90%) inoculated with 10^{-3} or 10^{-4} dilution, contaminants developed, making the medium turbid. These vials were discarded. In the other vials (10–20%) the medium remained clear, either devoid of any microbial growth or containing one or a few fluffy, chalk-white colonies. These colonies were usually located at the bottom of the vials and often adhering to the glass wall. Microscopic observations indicated that all the colonies exhibiting these features were *Frankia*.

Out of nodule samples corresponding to the seven sites mentioned above, *Frankia* colonies were obtained from four sites only: one site in Senegal (strains D1 and D11), two sites in Guadeloupe (strains G1 and G2), and one site in the Philippines (strain P1). Strains D1, D11, and G2 were obtained using liquid and solid QMOD media; strains G1 and P1 were obtained using the double-layer technique with the solid medium.

Morphology of the colonies

Most of the characteristics hereafter reported are related to strain D1; characteristics of other strains appeared to be similar except for the production of pigment, which varied greatly. Diameter of colonies of *Frankia* growing on the surface of QMOD solid medium increased linearly with time, reaching approximately 500 μm in 33 days. These colonies exhibited a central conical protuberance composed primarily of hyphae, sporangia, and vesicles embedded in mucilage. The outer region of young colonies was occupied by septate hyphae growing radially with a few sporangia and vesicles (Fig. 1). Since sporangia were more abundant at the center of the colonies, it was assumed that their initiation started from the center. Pigment was produced which precipitated as blood-red crystals.

Colonies growing in QMOD liquid medium were ellipsoidal, 0.5–1.0 mm in diameter, and 0.2 mm thick when 3 weeks old. Their inner parts were mucilaginous like the colonies growing in solid medium. Similarly, growing hyphae were located at the periphery of the colony (Fig. 2). When grown in liquid medium, *Frankia* did not cause turbidity, since the colonies remained undisrupted even when shaken. Furthermore, single colonies often flocked together forming clusters of one-layer colonies. After 6–7 weeks of incubation, the growth of peripheral hyphae stopped so that the whole colony was occupied by a mass of sporangia, vesicles, and lysing hyphae (Fig. 3). Trypan blue in lactophenol stained only the peripheral hyphae, whereas the central part of the colony remained colored by its own red pigment (not shown).

Hyphae, sporangia, and vesicles

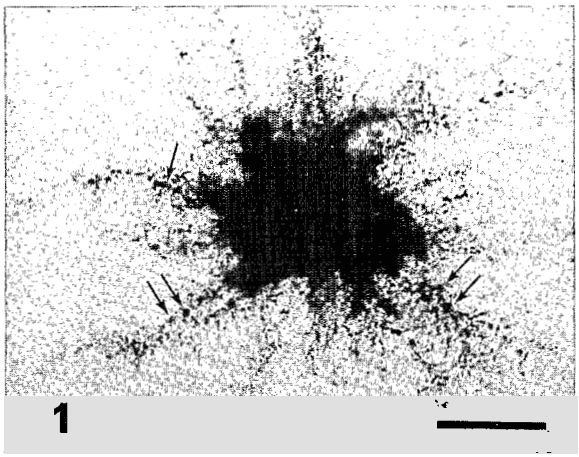
Young hyphae were typically branching, septate with a diameter approximately 0.4–0.9 μm. When older, hyphae could be up to 1.5–2.0 μm wide. Sporangia generally developed at the end of the hyphae but also in intercalary positions within hyphal filaments (Fig. 4). They were of many different sizes and shapes. Usually, terminally positioned sporangia were pear and club shaped but they could also be globose or subglobose, elongated, or exceedingly irregular (Figs. 5, 6, and 7). Club-shaped sporangia were 6–19 μm and globose ones were 13–19 μm, but sometimes much larger (up to 35 μm long) sporangia were found (Fig. 9). Spores (1.3–1.9 μm) were usually polyhedral, but were also irregular in form. They were easily released from mature sporangia (Fig. 9). Spore germination has not yet been observed in solid QMOD medium. Spherical vesicles (2.6 μm in diameter) were always formed terminally on short parental hyphae branching from hyphal strands (Fig. 8). Vesicles often remained attached to the parental hyphae even when lysis occurred (Fig. 3).

Infection tests with Casuarina equisetifolia

Despite repeated inoculation experiments, the formation of typical root nodules was never obtained with any of the tested strains.

Discussion

All the strains isolated from nodules of *Casuarina equisetifolia* possessed the morphological characteristics now admitted as specific to the genus *Frankia*: prokaryotic septate hyphae, polymorphic sporangia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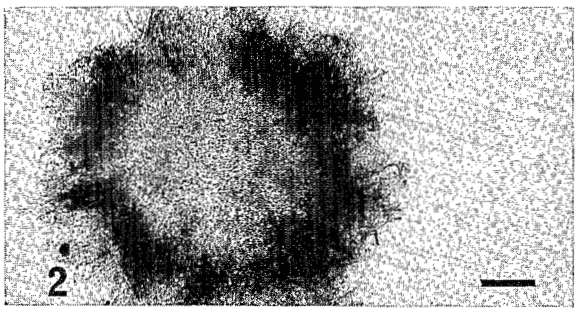
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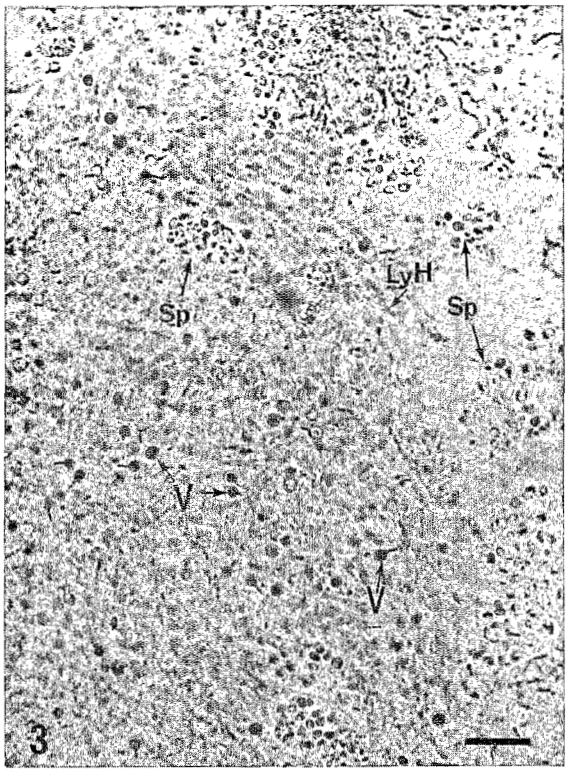
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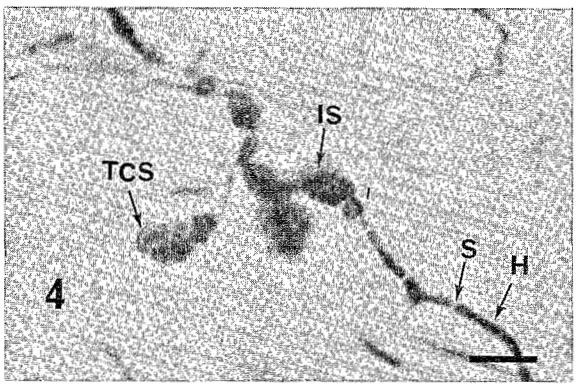
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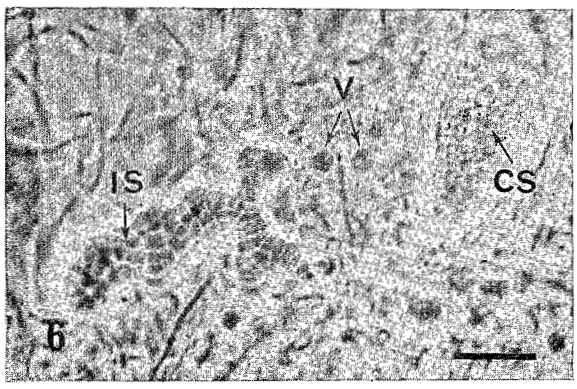
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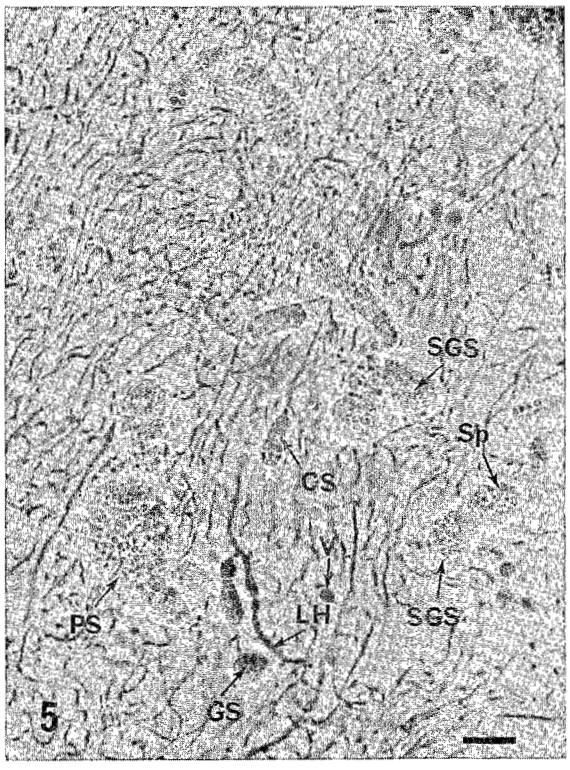
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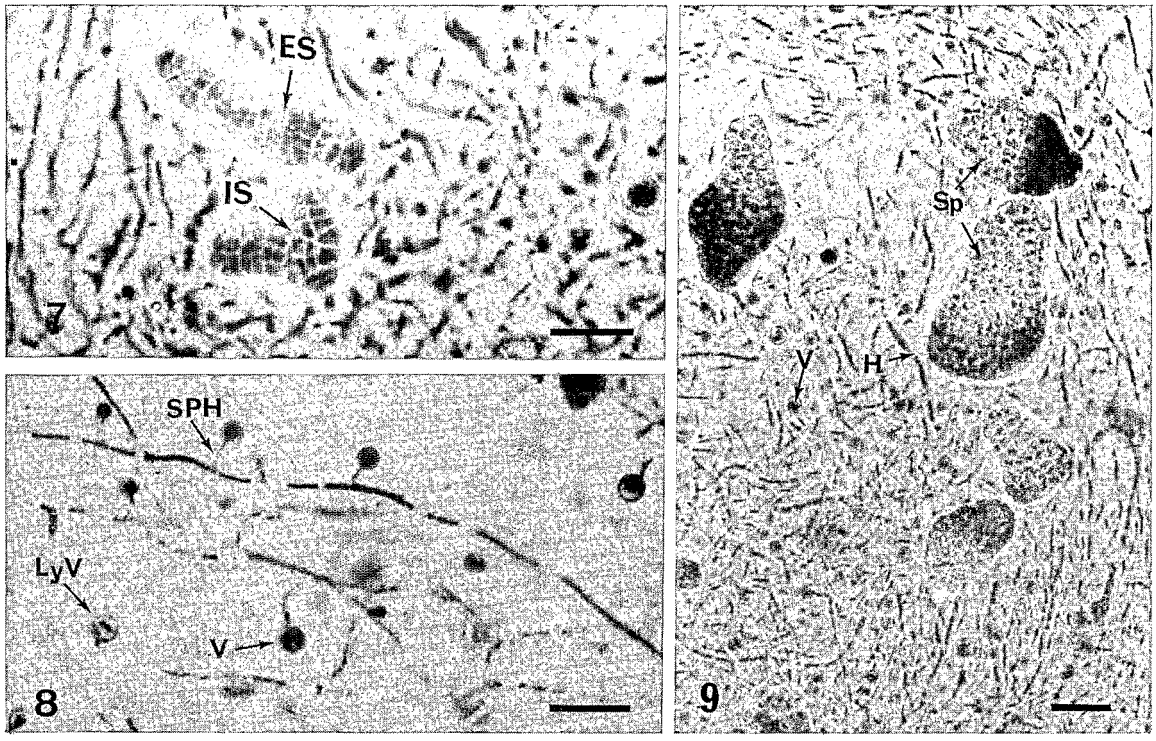

FIGS. 7–9. Morphological characteristics of *Frankia* strain D1 isolated from nodules of *Casuarina equisetifolia* and stained with trypan blue. Fig. 7. Elongated (ES) and irregularly formed (IS) sporangia. Bar = 10 μm. Fig. 8. Septate vesicle (V) formed in a 25-day-old colony grown in solid QMOD medium, with short parental hyphae (SPH) and lysing vesicle (LYV). Bar = 10 μm. Fig. 9. Large sporangia with mature spores (Sp). Vesicle (V) and hyphae (H) formed in a 21-day-old colony grown in liquid QMOD medium. Bar = 10 μm.

producing polyhedral spores, and septate vesicles. According to Torrey *et al.* (1980), these typical structures were described by Pommer for the first time. Strains isolated from other host plants (Callaham *et al.* 1978; Baker, Torrey, and Kidd 1979; Berry and Torrey 1979; Quispel 1979; Lalonde *et al.* 1981) always exhibited hyphae and sporangia but vesicles appeared only on some of the media used, namely QMOD medium and the N-free medium used by Tjepkema *et al.* (1980).

Strains described in this paper produced pigment(s) and mucilage. Production of pigment by an *Elaeagnus* isolate was reported by Baker, Torrey, and Kidd (1979). In contrast with this pigment, the pigment produced by our strains did not diffuse in the medium, but sometimes precipitated as microcrystals. The production of mucilage was also observed by Berry and Torrey (1979) in strains from *Alnus rubra*.

Since *Frankia* cultures from nodules of *Casuarina equisetifolia* were unable to nodulate the host plant, they theoretically could not be considered as endophytes from this plant species according to the requirements of Koch's postulate. However, Lechevalier and Lechevalier (1979) considered as members of the genus *Frankia* free-living actinomycetes producing vesicles and spore-containing sporangia even if they had no known

FIGS. 1–6. Morphological characteristics of *Frankia* strain D1 isolated from nodules of *Casuarina equisetifolia* and stained with trypan blue. Fig. 1. Starfishlike colony (25 days old) grown on solid QMOD medium. Sporangia on branching and radial hyphae (arrow). Bar = 50 μm. Fig. 2. Ellipsoidal 21-day-old colony grown in liquid QMOD medium. Peripheral actively growing hyphae are well stained, whereas the centre of the colony is only slightly stained. Bar = 100 μm. Fig. 3. Centre of a 49-day-old colony grown in liquid QMOD medium, with aggregates of mature spores (Sp), vesicles with short parental hyphae (V), and lysing hyphae (LYH). Bar = 25 μm. Fig. 4. Intercalary sporangia (IS), terminal club-shaped sporangia (TCS), septa (S), and hypha (H). Bar = 10 μm. Fig. 5. Club-shaped (CS), pear-shaped (PS), globose (GS), and subglobose (SGS) sporangia and released mature spores (Sp), vesicle (V), and large hypha (LH). Bar = 10 μm. Fig. 6. Irregularly formed (IS) and usually club-shaped (CS) sporangia and vesicle (V). Bar = 10 μm.

nodule-forming capacity. The morphological and cultural characteristics described in this paper in addition to the ability to reduce C_2H_2 *in vitro* observed in two strains, D11 and G2 (Gauthier *et al.* 1981*a*), support the fact that actinomycetes isolated in this work are true members of the genus *Frankia*. The question remains whether these strains of *Frankia* can be considered as endophytes of *Casuarina equisetifolia*. Since during the isolation experiments we did not handle any N_2-fixing nonlegume other than *Casuarina equisetifolia* or any exogenous *Frankia* strains, we assume that the strains described in the present paper are derived from *Casuarina equisetifolia* nodules. Further experiments reported elsewhere (Gauthier *et al.* 1981*b*) showed that strains D11 and G2 were able to nodulate *Hippophaë rhamnoides*, suggesting that they had lost their ability to infect *Casuarina equisetifolia*, but could still infect *Hippophaë rhamnoides*. This modification of the infective characteristics of strains D11 and G2 was attributed to alterations occurring during isolation and cultural procedures, an hypothesis reminiscent of that proposed by Burggraaf *et al.* (1981) for some *Frankia* strains isolated from *Alnus*.

Acknowledgments

This investigation was supported in part by grant DFR/1216/FG-LAT-R9 from Délégation Générale à la Recherche Scientifique et Technique, Paris. We acknowledge Marc Boureau for the photographs.

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Volume 28 • Number 5 • 1982

Pages 526–530

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