Isolation of *Frankia* Strains from Alder Actinorhizal Root Nodules

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A simple procedure, based on the rapid filtration and washing of *Frankia* vesicle clusters, was devised for the isolation of *Frankia* strains from alder actinorhizal root nodules. Of 46 *Alnus incana* subsp. *rugosa* nodules prepared, 42 yielded isolates. A simple medium containing mineral salts, Casamino Acids, and sodium pyruvate proved to be the most effective for isolation. In general, colonies appeared 6 to 20 days after inoculation. On the basis of hyphal morphology, two distinct types of *Frankia* strains were characterized. Randomly selected isolates were tested for infectivity, and all formed root nodules on *A. glutinosa*. Because of its simplicity and efficiency, the procedure is an improved method for the study of *Frankia* diversity in alder root nodules.

Nitrogen-fixing actinorhizal root nodules are formed on nonleguminous plants by *Frankia* actinomycetes. The plants involved are important pioneer species in nitrogen-poor soil or disturbed environments (13). As such, they may be useful in biomass production, land reclamation, or forest restoration (12, 14). Studies of the diversity and distribution of *Frankia* strains have been hampered by the uncertainty of isolation from field-collected root nodules. A number of techniques have been employed for obtaining *Frankia* strains from nodules. These include treatment of nodule tissue with cellulase and pectinase plus microdissection (6), microdissection alone (3), serial dilution of a crushed nodule (7, 11), and Sephadex fractionation combined with sucrose density centrifugation (1, 2). Most of the procedures that have been described require laborious manipulation or special equipment. An additional disadvantage is the relatively low success rate for all of the methods.

Successful isolation procedures generally must accomplish the following: (i) toxic phenolic compounds must be removed from the nodule homogenate, (ii) the endophyte should be separated from contaminating microorganisms, and (iii) the endophyte should be separated from contaminating plant tissue. To add to the difficulty, *Frankia* strains appear to vary in their nutritional requirements, although few studies of utilisable carbon sources have been reported (4).

In this report, a simple procedure is described that has been used routinely to obtain *Frankia* isolates from root nodules of *Alnus incana* subsp. *rugosa*.

**MATERIALS AND METHODS**

**Root nodules.** *A. incana* subsp. *rugosa* (speckled alder) actinorhizal root nodules were collected from the Storrs, Conn. area. Usually, nodules measuring 0.3 to 1.0 cm in diameter were chosen. They were excised from alder roots in the field and assayed for acetylene reduction activity.

**Media.** The *Frankia* basal medium (FM) consisted of the following in 1 liter of distilled water: 3 g of K$_2$HPO$_4$, 2 g of KH$_2$PO$_4$, 0.2 g of MgSO$_4$·7H$_2$O, 0.3 g of NaCl, 0.16 g of ferric sodium-EDTA, 1 ml of a trace element solution described by Baker and Torrey (1), and 1 ml of a vitamin mixture described by Tjepkema et al. (15). To each liter of FM were added, as needed, 3 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) (FMC) and 3 g of a carbon source as indicated. Sodium pyruvate (Sigma Chemical Co., St. Louis, Mo.) was filter sterilized before use. The pH was adjusted to 6.9 with NaOH or HCl. Calcium carbonate at 0.05 mg/ml was added to the liquid cultures. The composition of *Frankia* broth (YD medium) was that described by Baker and Torrey (1), and the composition of QMOD medium was that described by Lalonde and Calvet (9).

**Inoculum preparation.** Root nodules were first washed in a stream of distilled water to remove loose soil. Nodule lobes were removed approximately 1 mm from the tip with a single-edged razor blade. The lobes were washed in distilled water, and 4 to 15 lobes from each nodule were placed in polypropylene microtube tubes (1.5 ml). I sterilized the lobe surfaces by adding 1 ml of 20% laundry bleach (1.05% sodium hypochlorite [final concentration]) to the tubes and agitating them for 5 min. The bleach was removed, and the lobes were washed four times, each time with 1 ml of sterile distilled water. All further manipulations were done aseptically.

The lobes were homogenized in 5 ml of FM in a Ten Broeck tissue homogenizer (7.5 ml) to release the endophyte vesicle clusters. Vesicle clusters originate in single cells of host plants. They consist of a central mycelial mass surrounded by hyphal distortions known as vesicles. I gravity filtered the homogenate through nylon screens with nominal mesh openings of 52 and 20 μm (Tetko, Inc., Elmsford, N.Y.), using the apparatus diagrammed in Fig. 1. Large particles re-
tained on the 52-μm screen were washed once with 30 ml of FM. *Frankia* vesicle clusters retained on the 20-μm screen were washed twice with 30 ml of FM. The initial homogenate often clogged the 20-μm screen; I overcame this problem by repeatedly aspirating the homogenate into a Pasteur pipette and ejecting it against the screen. The vesicle clusters were collected with a Pasteur pipette from the 20-μm screen and placed in sterile test tubes. The suspension was diluted to the desired number (usually 8 × 10^6 vesicle clusters ml⁻¹), and the endophyte was poured plated at a density of 1 × 10^6 to 4 × 10^6 vesicle clusters per plate. At least two media were used for each isolation attempt. Three pour plates of each medium were used per nodule. Before the agar solidified, the plates were swirled to give a spiral gradient of vesicle clusters. To prevent drying, I wrapped the plate edges in Parafilm before incubating the plates at 30°C.

Colony development was followed microscopically to ensure that each isolate originated from a vesicle cluster. When visible colonies were formed, they were removed from the plate with a sterile spatula, homogenized in a Ten Broeck homogenizer, and inoculated into 1 ml of FM containing 0.3% sodium pyruvate.

**Nitrogenase assay.** I determined the nitrogenase activity by observing the reduction of acetylene to ethylene (5). Ethylene was quantitated with a Shimadzu Mini-II gas chromatograph fitted with a Porapak R column (1.4 m). All incubations were carried out after equilibration to room temperature.

**Nodulation experiments.** *Frankia* isolates were subcultured several times in FMC plus pyruvate. They were homogenized in a Ten Broeck tissue homogenizer and used to inoculate *A. glutinosa* seedlings grown on nitrogen-free nutrient solution (8) in Dispo growth pouches (Scientific Products, Inc., Bedford, Mass.). Uninoculated plants served as controls.

**RESULTS**

**Acetylene reduction activity.** Except for two, all nodules collected during the growing season showed substantial acetylene reduction activity (Table 1). The two that showed no nitrogenase activity were nodules from which strains Ail4-1, Ail4-2, and Ail5-1 were isolated. The range of activity varied widely: between 0 and 22 μmol of C₂H₂ reduced h⁻¹ g of nodule⁻¹ (Table 1).

In the fall and winter, after the growing season, acetylene reduction assays of randomly selected nodules were performed, and no activity was detected.

**Efficacy of isolation procedure.** The filtration device (Fig. 1) was easy assembled and stable to autoclaving and could be reused several times. Using the apparatus had a number of advantages: (i) washed vesicle cluster suspensions were obtained in 1 to 2 min, (ii) washing removed the bright-orange oxidized plant phe- nolic compounds as well as large and small particles of plant tissue, and (iii) washing removed contaminating bacteria and fungi small enough to pass through the 20-μm screen. In general, contaminants appeared at an approximate frequency of 1/4 × 10⁶ vesicle clusters. Since pour plates were used, contaminants rarely grew over the surface of the plates.

Of the 46 *A. incana* subsp. *rugosa* nodules used in this study, 42 yielded isolates (91% success rate). To improve the chances of obtaining a successful subculture from a plate, I transferred three separate colonies. Thus, the total number of strains obtained was considerably greater, but many were duplicates from the same nodule. Isolates from the same nodule were designated as in the following example: Ail1-1, Ail1-2, and Ail1-3.

**Frankia colony development.** To ensure that the organisms obtained from the plates were *Frankia* strains, I observed hyphal outgrowth from vesicle clusters on inverted petri plates by microscopy. Hyphae generally grew from several points on the vesicle cluster (Fig. 2). It was not possible to determine if the hyphae originat-

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**Table 1. Representative rates of acetylene reduction by root nodules from *A. incana* subsp. *rugosa***

<table>
<thead>
<tr>
<th>Nodule no.</th>
<th>Strain no.</th>
<th>Acetylene reduction rate (μmol of C₂H₂ reduced h⁻¹ g (fresh wt)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Ail4-1</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>Ail20-3</td>
<td>1.6</td>
</tr>
<tr>
<td>22</td>
<td>Ail22-2</td>
<td>5.8</td>
</tr>
<tr>
<td>16</td>
<td>Ail6-1</td>
<td>11.2</td>
</tr>
<tr>
<td>43</td>
<td>Ail43-2</td>
<td>22.0</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Apparatus used to isolate vesicle clusters from *A. incana* subsp. *rugosa* root nodule homogenates. A, Cut 30-ml plastic syringe barrel; B, 50-ml plastic syringe barrel; C, 52-μm mesh nylon screen heat annealed to the syringe barrel; D, 20-μm mesh nylon screen heat annealed to the syringe barrel.
ed from vesicles or from internal hyphae in the cluster. Sporangia typical of *Frankia* strains usually developed within 7 days after hyphal emergence on QMOD medium (Fig. 2). On plates containing pyruvate or succinate, sporangia were formed only after an extended incubation of 20 to 30 days.

The earliest that hyphal outgrowth was detected microscopically was 3 days after inoculation. Macroscopically visible colonies were observed as early as 6 days after inoculation on FMC with pyruvate as the carbon source. More commonly, *Frankia* strains from nodules obtained during the growing season showed microscopically detectable outgrowth from vesicle clusters within 10 days of inoculation, and colonies were macroscopically visible and ready to be transferred by 20 days after inoculation. *Frankia* strains from 10 nodules collected after leaf fall required at least 3 weeks of incubation before outgrowth was observed.

**Seasonal effects on isolation.** Isolates were obtained at the same frequency regardless of season. Nodules were harvested from soil that was extremely wet, dry, or frozen. The only difference noted was the longer incubation time required for outgrowth of *Frankia* strains from nodules collected in late November through winter.

**Effect of medium on isolation.** Media used in other studies to isolate *Frankia* strains were compared at various times with FMC containing pyruvate, succinate, or Tween 80. Table 2 gives the rates of successful isolation, as determined by examination of the plates 3 weeks after inoculation. These results represent isolations performed between June and August 1981. FMC with succinate and QMOD were virtually equivalent in effectiveness, although in some cases, *Frankia* strains from individual nodules did not grow on QMOD but grew well on FMC with succinate. No isolates were obtained from plates containing FMC with Tween 80 or YD. The success rate for the *Frankia* strains isolated on FMC plus pyruvate was 92%. In some cases, isolates were obtained on FMC alone, although only after an extended incubation of 4 to 5 weeks.

The most efficient method of obtaining isolates was to use at least two media in pour plates. The media of choice were FMC with succinate or pyruvate and QMOD. Overall, using the multiple-media approach, I achieved a 91% success rate.

**Morphology of *Frankia* isolates.** The *Frankia* strains obtained were all filamentous actinomycetes with a hyphal diameter that varied between 0.4 and 1.0 μm. They could be separated into two morphological types. Type 1 (Fig. 3a) was characterized by very sinuate hyphae with extensive branching. The following strains had type 1 morphology: Ai2-1, Ai2-2, Ai3-1, Ai4, Ai11, Ai11-1, Ai11-2, Ai12-2, Ai13-2, Ai13-3, Ai14-1, Ai14-2, Ai16-1, Ai16-3, Ai18-1, Ai18-2, Ai20-3, Ai21-1, Ai22-2, Ai24, Ai25-2, Ai26-2, Ai29-2, Ai31-1, Ai31-2, Ai37-1, Ai37-2, Ai38-1, Ai39-1, Ai40-1, Ai42-2, Ai43-2, Ai45-1, Ai45-3, Ai46-1, Ai48-1, Ai48-2, Ai50-2, Ai57-2, Ai58-2, and Ai62-1. Type 2 was characterized by smooth hyphae that rarely branched and were slightly sinuate (Fig. 3b). The following strains had type 2 morphology: Ai17, Ai17-1, Ai20-4, Ai21, Ai22, Ai23-2, and Ai28-1. Organisms of types 1 and 2 produced sporangia that consisted of packets of spores in a terminal or intercalary position in the hyphae (Fig. 3c). Type 1 organisms were the most abundant, accounting for ca. 84% of the total. Several of the isolates produced a bright-yellow pigment of unknown composition in liquid cultures.

**Infectivity of *Frankia* isolates.** I chose several *Frankia* strains of each morphological type for nodulation trials, using *A. glutinosa* seedlings as
test plants. All isolates tested, including Ai2-2, Ai4, Ai11, Ai12, Ai13-3, Ai17, Ai18-2, Ai20-4, Ai21, Ai22, Ai23-2, Ai43-2, and Ai48-1, produced nodules on *A. glutinosa*. Acetylene reduction assays were not done. However, at 2 months after infection, the nodulated plants grown in nitrogen-free nutrient solution were obviously green and well developed, whereas the uninoculated controls had died.

**DISCUSSION**

A rapid and convenient method for obtaining *Frankia* isolates from *A. incana* subsp. *rugosa* actinorhizal root nodules was developed. Previously described methods for other plant species involve lengthy preparation procedures (see above). The filtration method is rapid and requires only simple equipment. One worker can easily prepare and inoculate *Frankia* suspensions from 20 nodules in 1 day.

Other advantages to this method include the following: (i) the rapid washing of vesicle clusters to free them from plant phenolic compounds, (ii) the removal of contaminating microorganisms small enough to pass through a 20-μm filter, and (iii) the removal of the majority of plant material. The technique relies on the fact that the vesicle clusters are actually masses of actinomycete hyphae derived from individual cells of infected plants. The vesicle clusters are released from the cells upon homogenization and are usually between 20 and 50 μm in diameter. Similar techniques have been used to prepare vesicle cluster suspensions for biochemical studies (16).

My overall success rate with this method was 91%. This rate would have been higher had pyruvate been added to isolation plates more often. The best results were obtained when two or three media were used to pour plate the vesicle cluster suspensions. The success rate may also reflect the high densities used to inoculate the plates. In some cases, only 10 to 20 colonies of the endophyte were obtained, although each plate received between $1 \times 10^6$ and $4 \times 10^6$ vesicle clusters as an inoculum. Thus, some isolates would probably have been missed at higher dilutions.

The reason for the relatively low outgrowth rates for some nodule endophytes is unknown; perhaps the surface sterilization procedure killed the endophyte, or the *Frankia* strains had a variable inability to withstand the temperature of molten agar. Alternatively, the percentage of viable vesicle clusters may vary from nodule to nodule. I cannot rule out the possibility that individual nodules may be inhabited by more than one *Frankia* strain. Evidence that this may in fact be the case was provided by strains Ai20-3 and Ai20-4, which were taken from the same nodule. Strain Ai20-3 had type 1 morphology, and strain Ai20-4 had type 2 morphology. Individual legume root nodules often contain more

**FIG. 3.** Nomarsky interference contrast micrographs of *Frankia* strains of different morphological types. (a) Type 1. Sinuate hyphae of strain Ai4 grown in FMC plus pyruvate at 14 days are shown. (b) Type 2. Smooth hyphae of strain Ai17 grown in FMC plus pyruvate at 14 days are shown. (c) Strain Ai17 hyphae and sporangia after 60 days of incubation in FMC plus pyruvate. Bar represents 10 μm.
than one strain of *Rhizobium* (10), so it is not surprising that a similar situation may be true for actinorhizal nodules. The fact that 9% of the isolation attempts were unsuccessful may mean that some strains have nutritional requirements not met in the media provided.

Of particular interest is the observation that isolates could be obtained from root nodules collected from frozen ground. To my knowledge, this is the first report of isolation of *Frankia* strains from dormant root nodules. Endophytes therefore remain viable within nodules over the winter, and reinfection is probably not necessary.

The *Frankia* isolates were of at least two morphological types. Type 1 organisms accounted for 84% of the successful isolations, and type 2 organisms accounted for 19%. Organisms of both morphological types were obtained within an area of several centimeters, indicating that there may be considerable *Frankia* diversity in an environment. Whether *Frankia* morphological similarity indicates genetic similarity is not known at present. Further studies on the diversity and distribution of *Frankia* isolates should be possible if the isolation techniques described here are used.

The development of a rapid and reliable *Frankia* isolation procedure is an important development in the study of actinorhizal plants. The procedure can be extended to other plant species, and my co-workers and I have used it successfully to obtain hyphal outgrowth from vesicle clusters derived from the root nodules of *Comptonia peregrina*, *Myrica gale*, and *Myrica pensylvanica* (data not shown), although the conditions for these types of nodules have not been optimized. For other plant species, it is possible that alternate carbon sources or growth factors may be required.

ACKNOWLEDGMENTS

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LITERATURE CITED


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