APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1990, p. 1342–1346
0099-2240/90/051342-05$02.00/0

Vol. 56, No. 5

# Oligonucleotide Probes That Hybridize with rRNA as a Tool To Study *Frankia* Strains in Root Nodules

DITTMAR HAHN,* MARJO J. C. STARRENBURG, AND ANTOON D. L. AKKERMANS

*Department of Microbiology, Agricultural University, Hesselink van Suchtelenweg 4, NL-6703 CT Wageningen, The Netherlands*

Received 27 September 1989/Accepted 13 February 1990

**Oligonucleotide probes that hybridize with specific sequences in variable regions of the 16S rRNA of the nitrogen-fixing actinomycete *Frankia* were used for the identification of *Frankia* strains in nodules. *Frankia* cells were released from plant tissue by grinding glutaraldehyde-fixed root nodules in guanidine hydrochloride solution. rRNA was obtained after sonication, precipitation with ethanol, and purification by phenol-chloroform extraction. Degradation of rRNA, evident in Northern blots, did not affect hybridization with the oligonucleotides. Nodules of about 1 mg (fresh weight) provided sufficient rRNA for reliable detection of the *Frankia* strain. The utility of this rRNA extraction method was tested in a competition experiment between two effective *Frankia* strains on cloned *Alnus glutinosa* plants.**

The actinomycete *Frankia* is a nitrogen-fixing organism that forms root nodules in symbiosis with woody plants such as black alder (*Alnus glutinosa*) and sea buckthorn (*Hippophaë rhamnoides*) (2). In root nodules, typical *Frankia* strains form specialized cell clusters that can fix molecular nitrogen. The formation of these so-called vesicles is also obtained in pure cultures of *Frankia* strains grown under nitrogen-limited conditions (15, 16). Besides the typical strains, atypical *Frankia* strains exist that do not fix nitrogen, do not form vesicles, and are sometimes not able to infect their original host plants (10, 25). These atypical strains lack all morphological and physiological characteristics of typical *Frankia* strains and usually resist isolation.

Investigations on the ecology of *Frankia* strains are very often hampered by problems encountered with isolation and identification. In the presence of other bacteria, it is often impossible to obtain this organism in pure culture. In general, a *Frankia* strain is isolated from nodules which naturally enrich for this particular strain. Successful attempts to isolate *Frankia* strains from soil have been reported only once (1). Up to now, no selective isolation techniques have been developed, with the result that only a low percentage of isolation attempts has succeeded (23). The difficulties with isolation and identification of *Frankia* strains could be ameliorated by specific markers for identifying these bacteria without isolating them.

In a first attempt to identify *Frankia* strains in nodules, plasmids were used as molecular probes (21). Alternatively, strains in nodules can be identified by using restriction enzyme patterns of total DNA and subsequent hybridization using *nif* genes as probes (17, 18, 20). The application of plasmid and DNA probes is restricted to endophyte enrichments, i.e., to large nodules or pure cultures, allowing for sufficient DNA for direct hybridization experiments.

Another possible use of molecular markers is the application of synthetic oligonucleotides that hybridize with rRNA (5, 6, 19, 22). By using the reverse transcriptase sequencing method (4, 12; J. Smida, Ph.D. thesis, University of Kiel, Kiel, Federal Republic of Germany, 1988), the 16S rRNA nucleotide sequence of the effective (i.e., $Nif^+$) *Frankia* strain Ag45/Mut15 isolated from spore-negative nodules of *A. glutinosa* was analyzed in order to investigate the phylogenetic position of the specific *Frankia* strain (9). Variable regions of this typical *Frankia* strain showed several differences with homologous regions of two atypical, ineffective (i.e., $Nif^-$) strains. These regions were used to design complementary synthetic oligonucleotides. The resulting probes were suitable for use in hybridization experiments to discriminate between $Nif^+$ and $Nif^-$ strains and to discriminate strains within both physiologically different groups (8). Nodules that are metabolically highly active *Frankia* enrichments and consequently contain large amounts of *Frankia* rRNA are a good starting material for the application of these probes for identification.

The application of oligonucleotide probes in the detection of specific *Frankia* strains in nodules depends on the development of a reliable isolation method for rRNA. Pure rRNA preparations must be obtained to ensure specific hybridization signals of enriched target sequences. Nonspecific binding to DNA or polyphenolic compounds must be avoided. Polyphenols, rather common in actinorhizal plant tissue, are quite difficult to remove from nucleic acid preparations and interfere with the quantification of hybridization signals (21). The presence of large amounts of polyphenols in *A. glutinosa* hinders the application of RNA extraction procedures commonly used with other plants (7, 26). Since large numbers of nodules per plant must often be investigated, a rapid rRNA extraction would be preferable. Additionally, the detection method must be sensitive to allow the identification of *Frankia* strains in smaller nodules.

The aim of our investigations was to develop a rapid RNA isolation method from nodules, ensuring high yields of pure rRNA of the endophyte that could be used in hybridization experiments to detect *Frankia* strains without the need for reisolation and cultivation. The method described below is rapid and sensitive enough to detect specific target sequences in small nodules.

## MATERIALS AND METHODS

**RNA extraction.** Root nodules of actively growing *A. glutinosa* plants were harvested and fixed in 1% glutaraldehyde. Nodules up to 1 mg (fresh weight) were ground in a mortar in 1 ml of 7.5 M guanidine hydrochloride–1 M Tris, pH 7.0, and centrifuged at 3,000 × *g* to precipitate plant cells

* Corresponding author.

and released *Frankia* cells. The pellet was suspended in 0.4 ml of guanidine hydrochloride solution and sonicated three to six times for 30 sec at 60 W (Sonifier B-12; Branson Sonic Power Co., Danbury, Conn.) to disrupt *Frankia* cells. To determine the extraction efficiency, pure cultures (fresh weight, 1 g) of *Pseudomonas putida* and a typical nitrogen-fixing *Frankia* strain, Ag45/Mut15, were sonicated under similar conditions. After centrifugation at 1,000 × *g* for 10 min to remove cell debris, ethanol precipitation with 2.5 volumes of 96% ethanol was performed. The resulting pellet was suspended in sterile distilled water and extracted twice with phenol-chloroform. Phenol was buffered with 10 mM Tris, pH 7.4. After two additional chloroform extractions, RNA was precipitated with 2.5 volumes of 96% ethanol, washed with 70% ethanol, dried, and dissolved in 10 μl of distilled water.

RNA isolations were checked on Northern (RNA) blots by hybridization with a universal eubacterial probe commonly used as primer in 16S rRNA sequencing reactions (primer 1115 [4]). Additionally, two probes that hybridize with 16S rRNA sequences specific to effective *Frankia* strains belonging to the *Alnus* compatibility group (3′-CCCTAGGCATTC CCAGGACG; probe EFP [8]) and with *Frankia* strains in general (3′-GACCACCACACCTTTCTAAATA; D. Hahn, unpublished data) were used to detect 16S rRNA sequences. The specificity of both probes has been demonstrated in dot blot hybridization experiments with immobilized rRNA of 23 *Frankia* strains belonging to different compatibility groups.

**Detection of *Frankia* strains.** RNA from different amounts of nodules (1 to 10 mg [fresh weight]) of *Frankia* strain Ag45/Mut15 was used in dot blot experiments with probe EFP to estimate the detection limit. In order to show the purity of the RNA preparation, i.e., the absence of polyphenols, temperature-dependent hybridization of this probe with 16S rRNAs of the effective *Frankia* strains Ag45/Mut15 and AgKG'84/4 was also tested in dot blot experiments. Probe EFP was designed on the basis of 16S rRNA sequences of *Frankia* strain Ag45/Mut15. Even under stringent conditions, this probe hybridized with 16S rRNA sequences of nitrogen-fixing strains obtained from alder plants (8).

**Competition experiments.** The usefulness of the RNA extraction method was tested in competition experiments with the effective *Frankia* strains Ag45/Mut15 and AgKG'84/4. Plants of *A. glutinosa* clone B II (10) were grown in 1.5-liter pots in the greenhouse with perlite as the substrate and inoculated with defined amounts of both *Frankia* strains. After 3 months of growth under natural light conditions, plant height, plant dry weight, and the number of nodules per plant were determined. Nodules were harvested, fixed in 1% glutaraldehyde solution and stored in this solution at 4°C until used for RNA isolation. About 20 nodules per plant were selected at random and used to determine strain compositions.

**Filter hybridization.** Northern blots and dot blot hybridization experiments were performed on GeneScreen filters (Du Pont Co.). rRNA was applied with a Hybri.Dot manifold (Bethesda Research Laboratories, Inc.), immobilized by UV light, and hybridized by the method of Church and Gilbert (3). Northern blots (14) were hybridized under the same conditions, except that filters were incubated at 80°C for 30 min to remove formaldehyde prior to UV light immobilization. Oligonucleotide probes were 5′ labeled by using phage T4 polynucleotide kinase (Bethesda Research Laboratories) and 20 to 50 μCi of [γ-$^{32}$P]ATP (3,000 Ci/mmol; Amersham Corp.) (14).

Northern blots and filters containing rRNA from nodule preparations were always prewashed after rRNA immobilization in 0.1× standard saline citrate [1× standard saline citrate is 0.15 M NaCl plus 0.015 M sodium citrate], pH 7.0)–1% sodium dodecyl sulfate for 15 min at 80°C in order to remove possible contaminations with polyphenolic compounds.

The same washing procedure was also used to remove probes bound to immobilized target rRNA on the filters.

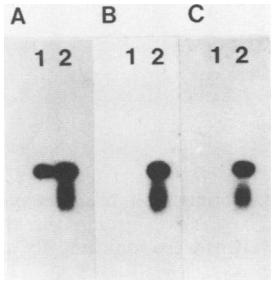


FIG. 1. Northern blot of RNA preparations (50 ng per slot) from pure cultures of *P. putida* (lanes 1) and *Frankia* strain Ag45/Mut15 (lanes 2) hybridized with a universal probe (A); probe EFP, specific for effective *Frankia* strains obtained from *Alnus* plants (B); and a *Frankia*-specific probe (C).

## RESULTS

**RNA extraction.** The RNA extraction method resulted in an rRNA preparation without detectable contamination by DNA. Northern blots of RNA from pure cultures of *P. putida* and *Frankia* strain Ag45/Mut15 showed distinct hybridization bands of 16S rRNA with the universal probe. Probe EFP normally showed cross-hybridization with DNA of *P. putida*. The specific hybridization of 16S rRNA of *Frankia* strain Ag45/Mut15 with probe EFP indicated the purity of the rRNA preparation (Fig. 1).

In the rRNA preparations isolated from pure cultures by this method, some degradation was observed. Degradation was even more pronounced when the rRNAs were isolated from nodules, most likely a result of mechanical disruption

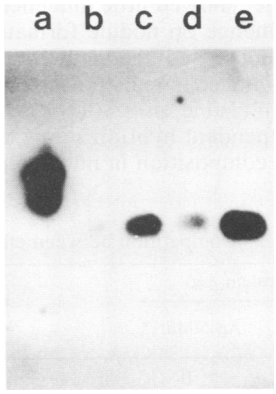


FIG. 2. Northern blot of RNA isolated from nodules induced by *Frankia* strains Ag45/Mut15 (b and d) and AgKG'84/4 (c and e). Preparations were done on the same sample after three (b and c) and six rounds (d and e) of sonication for 30 sec at 60 W and compared with rRNA isolation from a pure culture of strain Ag45/Mut15 after three rounds of sonication (a). Hybridization was performed with probe EFP.

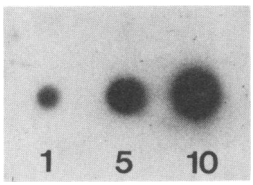


FIG. 3. Dot blot hybridization of immobilized RNA isolated from nodules formed by *Frankia* strain Ag45/Mut15. Nodules of 1, 5, and 10 mg (fresh weight) were sonicated six times for 30 sec at 60 W.

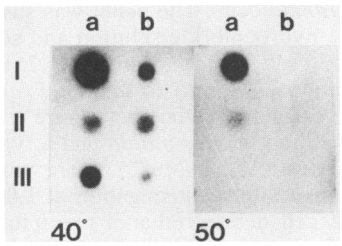


FIG. 4. Dot blot hybridization of RNA isolated from nodules (about 10 mg [fresh weight]) formed by *Frankia* strains Ag45/Mut15 (lanes IIa) and AgKG'84/4 (lanes IIb) compared with RNA preparations from pure cultures of Ag45/Mut15 with 10 (lanes Ia) and 1 (lanes Ib) ng and AgKG'84/4 with 10 (lanes IIIa) and 1 (lanes IIIb) ng. Hybridization with probe EFP shows temperature-dependent signal patterns, indicating the purity of the rRNA preparation.

during the sonication step (Fig. 2). A more intensive sonication yielded more rRNA without further degradation of the RNA. Small nodules (about 1 mg [fresh weight], about 1 mm in diameter) contained sufficient target sequences for clear hybridization signals (Fig. 3). The hybridization signals of isolated rRNA from nodules of 1, 5, and 10 mg (fresh weight) with probe EFP correlated more or less with that of 1, 5, and 10 ng of rRNA of *Frankia* strain Ag45/Mut15.

The isolated rRNA was pure enough to give specific hybridization signals (Fig. 4). Cross-reaction with contaminating polyphenols would result in nonspecific binding, disturbing signals even under high-stringency conditions. With rRNA of *Frankia* strain AgKG'84/4, no nonspecific binding was found. This rRNA contained one additional base in the target sequence (D. Hahn, unpublished observations). At high stringency, pure RNA preparations of this strain did not show any hybridization signal, whether specific or nonspecific. The same results, indicating the isolation of pure RNA, were obtained when probe IFP, specific for an ineffective *Frankia* strain, and probe EFP were used one by one (data not shown).

**Competition experiments.** Competition experiments with both effective *Frankia* strains that could be distinguished by temperature-dependent hybridization (8) were set up to investigate the applicability of the isolation and hybridization procedure in natural systems. Because of the restricted usefulness of this temperature-dependent hybridization which could not distinguish between strain Ag45/Mut15 and double infections with strains Ag45/Mut14 and AgKG'84/4, these experiments were used only to demonstrate how this technique works. Inoculations with each strain alone or with both strains together showed little influence on plant growth but significant influence on nodule formation. In all cases, dual inoculation decreased the amount of nodules when compared with identical inoculation amounts of the dominant *Frankia* strain (Table 1).

Temperature-dependent hybridizations used to determine the percent strain composition in nodules per plant showed that 75% of the nodules, obtained after inoculation with a mixture of AgKG'84/4 cells equivalent to 20 μg of total protein and Ag45/Mut15 cells (total protein, 2 μg) were formed by strain AgKG'84/4 (Table 1). This result was calculated from the disappearance of hybridization signals with probe EFP under high-stringency conditions. Nodule formation of this strain in any other inoculation combination was suppressed even when large amounts of cells were inoculated. Combined with identical amounts of strain Ag45/Mut15 (total protein, 20 μg), only about 5% of the nodules were formed by strain AgKG'84/4. In the presence of higher amounts of strain Ag45/Mut15, no nodules containing strain AgKG'84/4 could be detected. The strain composition of nodules showing hybridization signals even under high-stringency conditions was not investigated because of the lack of a second probe specific for strain AgKG'84/4.

## DISCUSSION

The simple and rapid isolation method for rRNA from nodules described here and the subsequent use of oligonucleotides for the detection of specific target sequences on rRNA is attractive for ecological investigations. The specificity of probes, low detection limit of target sequences, and simple application in hybridization experiments can avoid problems of reisolation and identification in pure culture. Nodules, being natural enrichments of *Frankia*, are a good starting material for these investigations. The relatively high sensitivity of the probe-target system allows in this case a reliable identification of the *Frankia* strain in very small nodules. The procedure permits the investigation of the strain composition in larger nodules consisting of several lobes. Even the strain composition of each lobe can be

TABLE 1. Competition between effective *Frankia* strains AgKG'84/4 and Ag45/Mut15 on *A. glutinosa* clone B II[a]

| Inoculum (total protein, μg) | | Plant ht (cm) (mean ± SD) | No. of nodules (mean ± SD) | % Nodules formed by: | |
|---|---|---|---|---|---|
| AgKG'84/4 | Ag45/Mut15 | | | AgKG'84/4 | Ag45/Mut15 and/or AgKG'84/4 |
| 20 | 0 | 38 ± 9 | 55 ± 21 | 100 | 0 |
| 2 | 0 | 48 ± 7 | 27 ± 9 | 100 | 0 |
| 20 | 2 | 40 ± 8 | 14 ± 6 | 75 | 25 |
| 20 | 20 | 43 ± 10 | 95 ± 41 | 5 | 95 |
| 2 | 20 | 49 ± 7 | 110 ± 32 | 0 | 100 |
| 0 | 2 | 52 ± 3 | 134 ± 14 | 0 | 100 |
| 0 | 20 | 55 ± 7 | 282 ± 83 | 0 | 100 |

[a] $n$ = 15 plants, $t$ = 3 months.

studied separately. By using different specific probes, detection of double infections with two strains or competition experiments with defined strains may be feasible.

Combinations of strains differing in only one or two nucleotides in 16S rRNA, e.g., *Frankia* strains AgKG'84/4 and Ag45/Mut15, theoretically simplify the identification process because single hybridization and washing at two stringencies can distinguish between both strains. However, temperature-dependent hybridization cannot be used to study possible double infections in one nodule. Here, two specific probes are necessary to identify the infecting strains. In our experiment, the identification of *Frankia* strains in nodules obtained after dual inoculation of both *Frankia* strains at different concentrations indicates that strain Ag45/Mut15 is a superior competitor to strain AgKG'84/4. However, this assumption is based on a substantial amount of speculation since nothing is known about possible double infections. Furthermore, the application of two specific probes may be of limited value when the concentration of one of the infecting strains is below the detection limit.

The phenomenon of reduced nodule formation after dual inoculation of *Frankia* strains in *A. glutinosa* plants is similar to results found in competition experiments with *Rhizobium* strains (13, 28). More information on possible interference between both *Frankia* strains in nodule formation on *A. glutinosa* plants might therefore be obtained after independent inoculation of both *Frankia* strains at different times in combination with strain identification.

Group- or species-specific sequences of 16S rRNA have been found in various microorganisms (5, 6, 11). Analyses of variable regions of 16S rRNA of closely related organisms indicate variation sufficient to design probes of interest, despite the fact that DNA-DNA homology studies suggest these species might actually be one and the same. This makes rRNA sequences an attractive target for diagnostic research (11, 24, 27). The application of the RNA extraction method in combination with specific probes can supply rapid information on the occurrence and the establishment of recalcitrant organisms in natural populations, e.g., populations in natural environments like soil. Problems encountered with the isolation of RNA from soil samples are quite similar to that encountered with the isolation from nodules, i.e., the presence of large amounts of humic acids or polyphenols. During our isolation procedure, all polyphenols are removed from the rRNA sample. The usefulness of 16S rRNA sequences directly isolated from soil samples as targets for oligonucleotide probes should therefore only depend on the quality of the probe, thus ensuring the detection of small amounts of target sequences within large amounts of background rRNA.

## ACKNOWLEDGMENTS

These investigations were supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (NWO) and the Commission of the European Communities (EEC) [EN3B-0043-NL (GDF)].

## LITERATURE CITED

1. **Baker, D., and D. O'Keefe.** 1984. A modified sucrose fractionation procedure for the isolation of frankiae from actinorhizal root nodules and soil samples. Plant Soil **78**:23–28.
2. **Bond, G.** 1983. Taxonomy and distribution of non-legume nitrogen-fixing systems, p. 55–87. *In* J. C. Gordon and C. T. Wheeler (ed.), Biological nitrogen fixation in forest ecosystems: foundations and applications. Martinus Nijhoff/Dr W. Junk Publishers, The Hague, The Netherlands.
3. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
4. **Embley, T. M., J. Smida, and E. Stackebrandt.** 1988. Reverse transcriptase sequencing of 16S ribosomal RNA from *Faenia rectivirgula*, *Pseudonocardia thermophila* and *Saccharopolyspora hirsuta*, three wall type IV actinomycetes which lack mycolic acids. J. Gen. Microbiol. **134**:961–966.
5. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. J. Bacteriol. **170**:720–726.
6. **Goebel, U. B., A. Geiser, and E. J. Stanbridge.** 1987. oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. J. Gen. Microbiol. **133**:1969–1974.
7. **Govers, F., T. Gloudemans, M. Moerman, A. van Kammen, and T. Bisseling.** 1985. Expression of plant genes during the development of pea root nodules. EMBO J. **4**:861–867.
8. **Hahn, D., M. Dorsch, E. Stackebrandt, and A. D. L. Akkermans.** 1989. Use of synthetic oligonucleotide probes in identification of *Frankia* strains. Plant Soil **118**:211–219.
9. **Hahn, D., M. P. Lechevalier, A. Fischer, and E. Stackebrandt.** 1989. Evidence for a close phylogenetic relationship between members of the genera *Frankia*, *Geodermatophilus*, and "*Blastococcus*" and emendation of the family *Frankiaceae*. Syst. Appl. Microbiol. **11**:236–242.
10. **Hahn, D., M. J. C. Starrenburg, and A. D. L. Akkermans.** 1988. Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. Plant Soil **107**:233–243.
11. **Kohne, D., J. Hogan, V. Jonas, E. Dean, and T. H. Adams.** 1986. Novel approach for rapid and sensitive detection of microorganisms: DNA probes to rRNA p. 110–112. *In* L. Leive (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
12. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S rRNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82**: 6955–6959.
13. **Lie, T. A., G. J. Nijland, and S. H. Waluyo.** 1988. Competition between nodulating and non-nodulating *Rhizobium* strains: delay of nodulation, p. 127–136. *In* F. O'Gara, S. Manian, and J. J. Drevon (ed.), Physiological limitations and the genetic improvement of symbiotic nitrogen fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Meesters, T. M.** 1987. Localization of nitrogenase in vesicles of *Frankia* sp. Cc1.17 by immunogoldlabelling on ultrathin cryosections. Arch. Microbiol. **146**:327–331.
16. **Meesters, T. M., S. T. van Genesen, and A. D. L. Akkermans.** 1985. Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.7 and Cp1.2. Arch. Microbiol. **143**:137–142.
17. **Normand, P., and M. Lalonde.** 1986. The genetics of actinorhizal *Frankia*: a review. Plant Soil **90**:429–453.
18. **Normand, P., P. Simonet, and R. Bardin.** 1988. Conservation of Nif sequences in *Frankia*. Mol. Gen. Genet. **213**:238–246.
19. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40**:337–365.
20. **Simonet, P., P. Normand, and R. Bardin.** 1988. Heterologous hybridization of *Frankia* DNA to *Rhizobium meliloti* and *Klebsiella pneumoniae* Nif genes. FEMS Microbiol. Lett. **55**:141–146.
21. **Simonet, P., N. Thi Le, E. Teissier du Cros, and R. Bardin.** 1988. Identification of *Frankia* strains by direct DNA hybridization of crushed nodules. Appl. Environ. Microbiol. **54**:2500–2503.
22. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079–1084.
23. **St.-Laurent, L., and M. Lalonde.** 1987. Isolation and character-

ization of *Frankia* strains isolated from *Myrica gale*. Can. J. Bot. **65**:1356–1363.
24. **Tenover, F. C.** 1988. Diagnostic deoxyribonucleic acid probes for infectious diseases. Clin. Microbiol. Rev. **1**:82–101.
25. **van Dijk, C., and A. Sluimer-Stolk.** 1984. An ineffective strain of *Frankia*-Ag in the dune area of Voorne. Prog. Rep. Inst. Ecol. Res. Verh. Kon. Ned. Akad. Wetensch., 2e Reeks **82**:56–59.
26. **Verwoerd, T. C., B. M. M. Dekker, and A. Hoekema.** 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. **17**:2362.
27. **Viscidi, R. P., and R. G. Yolken.** 1987. Molecular diagnosis of infectious diseases by nucleic acid hybridization. Mol. Cell. Probes **1**:3–14.
28. **Winarno, R., and T. A. Lie.** 1979. Competition between *Rhizobium* strains in nodule formation: interaction between nodulating and non-nodulating strains. Plant Soil **51**:135–142.