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Identification of *Frankia* Strains by Direct DNA Hybridization of Crushed Nodules

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A hybridization procedure was developed to identify *Frankia* strains inside actinorhizae by direct probing of crushed root nodules. The probe consisted of an indigenous cryptic plasmid. This well-conserved, 8-kilobase plasmid was detected in *Frankia* isolates that were very close taxonomically (they possessed a very high DNA sequence homology). The probe did not hybridize to the DNA of *Frankia* isolates which did not carry the plasmid. Endophyte DNA was extracted by a modification of a technique originally developed for the detection of plasmids in *Frankia* isolates. The hybridization procedure applied to nodules collected in a stand of alder permitted determination of a distribution map of the plasmid-bearing *Frankia* strains.

In developing countries, tropical and subtropical actinorhizal plants have a great potential for playing a major role in protecting and regenerating disturbed or impoverished soils (5). However, the large-scale production of actinorhizal plants implies that there is a need to inoculate the seedlings with an appropriate *Frankia* strain before the seedlings are planted. It is therefore desirable to develop ecological markers to follow the viability and competitiveness of these inoculated strains. Because of problems associated with the isolation of *Frankia* strains in pure cultures and the definition of their growth requirements (they have a doubling rate of 24 h), a rapid method for directly fingerprinting strains in actinorhizae would be useful for understanding the competitiveness of *Frankia* strains that are inoculated into fields. In the *Rhizobium*-legume symbiosis, serological techniques often have been used to enumerate indigenous *Rhizobium* strains in the legume nodules (1, 13). However, these techniques have insufficient resolution to discriminate between specific *Frankia* strains (2) and therefore cannot provide information on the diversity of *Frankia* strains in the actinorhizae. Identification of *Rhizobium* strains in nodules has also been performed by hybridization of crushed root nodules with ^{32}P-labeled DNA probes (4, 6). These techniques have been demonstrated to be better than classical immunological and antibiotic resistance-marking techniques because they eliminate the culturing step and reduce cross-reactions.

We have previously described (P. Simonet, N. Thi Le, A. Moiroud, and R. Bardin, submitted for publication) the use of DNA hybridization for estimating the diversity of *Frankia* strains that were isolated from a single alder stand. The three indigenous *Frankia* strains harboring a similar 8-kilobase (kb) cryptic plasmid also possessed a very high DNA sequence homology. This indicates that plasmid sequences can provide powerful ecological markers for a class of very closely related frankiae. In this study a hybridization procedure based on the direct probing of crushed nodules was developed and applied to actinorhizae collected from a single alder stand in the Station d'Amélioration des Arbres Forestiers, Institut National de la Recherche Agronomique (INRA), Orléans, France (from which the plasmid-carrying *Frankia* strains were isolated). The hybridization procedure, with the 8-kb plasmid used as a DNA probe, permitted us to identify nodules that formed naturally with plasmid-bearing endophytes and allowed the creation of a distribution map of this group of *Frankia* strains.

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MATERIALS AND METHODS

Strains and media. All strains and plasmids used in this study are listed in Table 1. *Frankia* strains were cultured in Tween liquid medium at 28°C (16), and *Escherichia coli* strains were grown on LB medium (8).

DNA extraction. Total DNA from the isolated *Frankia* strains was obtained as described by Simonet et al. (15). For crushed nodule hybridization, DNA was extracted from nodules directly after they were collected from the fields or was obtained from *Alnus glutinosa* seedlings that were grown on soil samples collected from the alder stand. Nodules were also obtained from *Alnus glutinosa* seedlings that were inoculated with *Frankia* strains and grown in growth pouches as described by Normand and Lalonde (9). The nodules were kept at −20°C before preparation and were then washed in sterile water; each lobe was individually crushed in 50 μl of 50 mM Tris–20 mM EDTA disodium salt–15% (wt/vol) sucrose. The method for preparing endophyte DNA was based on the in situ lysis method described by Simonet et al. (14) for *Frankia* isolates. Vesicle clusters were sedimented by centrifugation (5 min, 12,000 × *g*) and then were digested with 50 μl of solution A (14), to which 10 mg of achromopeptidase (Wako Pure Chemicals, Dallas, Tex.) per ml was added. The cell suspension was incubated at 37°C for 30 min and laid directly on top of a 0.7% (wt/vol) agarose gel on which solutions B and C were added, as described by Simonet et al. (14). Electrophoresis was carried out at 5 V/cm for 30 min. When DNA preparations had to be deposited directly onto the nitrocellulose filter, vesicle clusters were digested in 50 μl of 50 mM Tris–20 mM EDTA, disodium salt (pH 8), with 10 μg of lysozyme (23,000 U/mg; Appligene, Illkirsch, France) per ml and 10 μg of achromopeptidase per ml. Lysis was completed by the addition of 20 μl of lauryl sulfate 20% (wt/vol) to the solution described above.

Restriction enzyme cleavage. Digestions of DNA with

TABLE 1. Bacterial strains and plasmids used in this study

Strain and plasmid	Relevant characteristic	Reference
Frankia		
ACoN24d	Wild-type isolate from *A. cordata*	14
ACoI8	Wild-type isolate from *A. cordata*	Simonet et al.[a]
Ar24H3	Wild-type isolate from *A. rubra*	Simonet et al.[a]
Plasmids		
pFQ56	8-kb indigenous plasmid from ACoN24d	14
pFQ121	Derived from pFQ56 (Ap^r)	Simonet et al.[a]

[a] Simonet et al., submitted.

restriction endonucleases (Boehringer GmbH, Mannheim, Federal Republic of Germany; Appligene; or Bethesda Research Laboratories, Gaithersburg, Md.) were accomplished as specified by the manufacturers.

Nick translation. DNA was labeled by nick translation (12) with [^{32}P]dCTP (3,000 Ci/mmol; Amersham, Buckinghamshire, England) (specific activities of 50 μCi/μg of DNA were obtained), or with Bio-11 dUTP (Bethesda Research Laboratories) in the case of the nonradioactive probes.

Southern transfer and hybridization. After agarose gel electrophoresis, Southern transfer to nitrocellulose filters was carried out (7). In other cases, the DNA solution was deposited directly onto the nitrocellulose filter, denaturated, and neutralized as described by Maniatis et al. (7) for colony hybridization. Hybridization (53°C, 65 h) was performed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution, 0.1% lauryl sulfate, and 50 μg of salmon sperm DNA per ml. Washing conditions were as follows: one change of 6× SSC, 0.1% lauryl sulfate, and 1× Denhardt solution at 65°C for 15 min and then six changes of 0.02× SSC and 0.1% lauryl sulfate at 65°C for 30 min. Hybridization and washing conditions were as described by the manufacturer in the case of the biotinylated probes.

RESULTS AND DISCUSSION

Specificity of the plasmid probe. The 8-kb plasmid pFQ56 was detected in *Frankia* sp. strain ACoN24d (14) and was cloned into the *E. coli* vector pBR322 (Simonet et al., submitted). The hybrid plasmid pFQ121 was nick translated. Genomic DNAs from three *Frankia* isolates were digested with the restriction enzyme *Bgl*II and hybridized to the ^{32}P-labeled pFQ121 plasmid probe. The probe hybridized strongly with pFQ56 sequences contained in ACoN24d DNA (Fig. 1, lane 3) and faintly to *Frankia* DNAs, which did not possess the 8-kb plasmid (Fig. 1, lanes 1 and 2). This indicates that plasmid sequences were not integrated into the chromosome for isolates which did not bear the 8-kb plasmid and that the hybridizing signal identified only plasmid-carrying strains. Endophyte DNA was also extracted from nodules that were obtained from *Alnus glutinosa* seedling roots after inoculation with a pure culture of *Frankia* and then were electrophoresed on an agarose gel. The lysis treatment that was applied to a single lobe of actinorhizae permitted the recovery of constant amounts of DNA, regardless of the *Frankia* inoculum used (Fig. 2A; lane 1, strain ACoN24d; lane 2, strain ACN1AG). Under the electrophoretic conditions used, the visualized band corresponded to the linear and the covalently closed circular forms of the 8-kb plasmid (to produce a stronger hybridizing band) and to

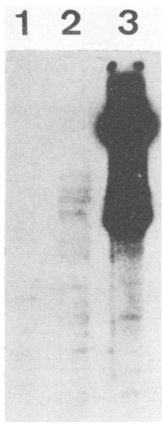

FIG. 1. Specificity of the plasmid probe. The autoradiograph was obtained from a filter membrane carrying *Bgl*II-digested total DNA after hybridization with the plasmid pFQ121 probe. DNAs of the following strains were loaded: *Frankia* sp. strain ACoI8 (lane 1), *Frankia* sp. strain Ar24H3 (lane 2), and *Frankia* sp. strain ACoN24d (lane 3).

chromosomal DNA from both the endophyte and the nodule. Endophyte DNA was hybridized to the ^{32}P-labeled plasmid pFQ121 probe. Results presented in Fig. 2B indicate that the probe again hybridized only to the ACoN24d endophyte DNA.

Identification of *Frankia* strains in field nodules. By using the DNA extraction, agarose gel electrophoresis, and transfer on nitrocellulose filter procedures described above, we tested for the presence of hybridizing signals on nodules collected from the alder stand of the Station d'Amélioration des Arbres Forestiers, INRA, Orléans, France (Fig. 3). The stand was divided into various plots, and inside each plot nodules were grouped into one or several lots. Another set of nodules was harvested from *Alnus glutinosa* seedlings which were grown in soil samples collected from the alder stand. Finally, we tested for the presence of plasmid in nodules from a location (Cantee stand) 30 km away from the original test stand. Seven lobes belonging to seven different nodules were tested in each lot (Table 2). Hybridization data indicated that in some lots, the seven nodules exhibited positive hybridizing signals (Table 2, plot L north, lot A14), indicating a widespread dispersion of plasmid-bearing endophytes in this area. It should be noted that the results obtained with nodules collected from the fields correlated well with those obtained from plants grown in soil samples from the alder stand; this technique remains the best way to evaluate the

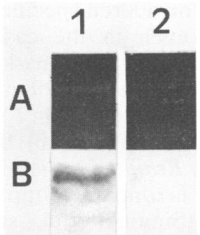

FIG. 2. Agarose gel electrophoresis (A) and hybridization (B) of crushed root nodules from *Alnus glutinosa* inoculated with an 8-kb plasmid-bearing strain (ACoN24d) (lanes 1) and ACN1AG (lanes 2). ACN1AG did not carry the 8-kb plasmid. The probe was the 8-kb plasmid pFQ56 cloned into the *E. coli* vector and nick translated with [^{32}P]dCTP (see text).

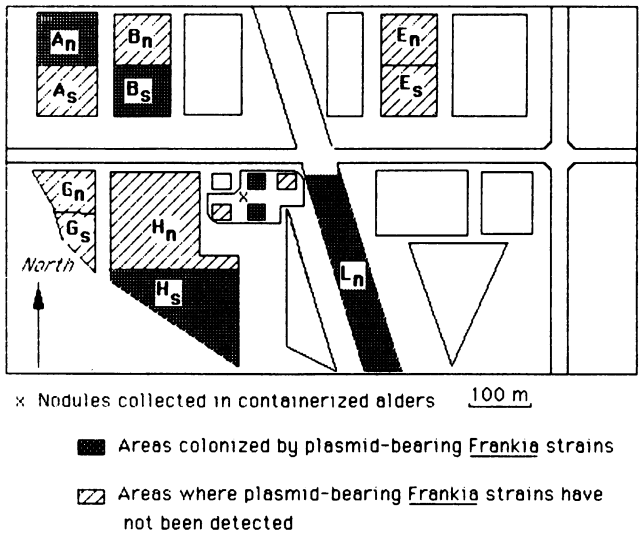

FIG. 3. Map of the INRA stand in the Station d'Amélioration des Arbres Forestiers, INRA, Orléans, France, showing plots in which plasmid-bearing endophytes were identified by hybridization of crushed nodules. The stand was divided into various plots (A, B, E, G, H, and L) with a north (n) and a south (s) area. Alders were also grown in containers (×). Subdivisions of each plot in various lots (Table 2) are not shown on the map. Open spaces indicate the areas that were not tested.

Frankia population in soils. In other lots only some of the nodules exhibited a positive band, indicating the presence of different *Frankia* strains (with and without plasmids) in the same area (for instance, plot A north). Finally, all the nodules of some plots failed to react with the plasmid probe. This is the case, for instance, with plots H north, E north, or E south in the INRA stand and in all the plots in the Cantee stand. These results allowed us to determine the distribution map of the 8-kb plasmid-bearing strains in the INRA stand (Fig. 3). The techniques for the direct identification of strains in nodules are particularly well suited for actinorhizal symbiosis, because *Frankia* strains grow very slowly and months are required to obtain a suitable biomass for experimentation. Moreover, in the case of several *Frankia* strains in the actinorhizae (11), isolation procedures can select for one strain. This preferential selection indicates the need for caution in interpreting results of competition studies with *Frankia* isolates.

In this study we used a cryptic indigenous plasmid as the strain marker. Plasmids are known to be agents of gene transfer and as catalysts for genetic recombination (3). The 8-kb plasmid can be considered specific for the strains which carry it. However, even in the case of nonconjugative systems, plasmids are not stable markers, as evidenced by bacterial strains that can be easily cured of their plasmids. Plasmid curing has been achieved in *Frankia* sp. strain ArI3 by regeneration from protoplasts (10), but it can also occur in nature. For instance, *Frankia* sp. strain ACoI8, which was classified in the same taxonomic group as the strains harboring the 8-kb plasmid (Simonet et al., submitted), could be a natural deletion mutant that was cured of its plasmids. This means that such deleted genotypes cannot be identified with the plasmid probe. An improvement of the technique would require the construction of probes from chromosomal DNA fragments which would be specific for one strain or a group of strains. However, detection with a chromosomal DNA probe could be lower than that found with the plasmid probe because of the high copy number of the plasmid. For instance, plasmid pFQ56 had a copy number of 50 (data not shown).

TABLE 2. Distribution of plasmid-bearing endophytes among nodules from two alder stands

Stand and plot	Lot	No. of nodules with a hybridization signal					
		Field-collected nodules			Soil-grown nodules		
		Total tested	Positive	Negative	Total tested	Positive	Negative
INRA							
A north	L1	7	0	7	7	0	7
A north	L3	7	5	2			
A north	L7	7	4	3			
A north	L8	7	5	2	7	4	3
A north	L10	7	4	3			
A north	L2				7	5	2
A north	L5				7	6	1
A north	L11				7	3	4
A south	L1				7	0	7
B south	L1				7	1	6
B north	L1				7	0	7
E north	L1				7	0	7
E south	L1				7	0	7
Conten.	2				7	0	7
Conten.	3	7	0	7			
Conten.	83	7	3	4	7	2	5
Conten.	120	7	6	1	7	5	2
L north	A14	7	7	0	7	7	0
L north	A32	7	0	7	7	0	7
H south	L1	7	1	6	7	0	7
H south	L3	7	0	7			
H north	L1				7	0	7
G south	L1				7	0	7
G north	L1				7	0	7
Cantee	L1	7	0	7	7	0	7
	L3	7	0	7	7	0	7
	L7	7	0	7	7	0	7
	L17	7	0	7	7	0	7
	L27	7	0	7	7	0	7

Direct transfer of the endophyte DNA solution. We also developed a hybridization procedure based on the direct probing of the endophyte DNA solution, thereby eliminating the electrophoresis step. A lack of strain specificity, which was not improved by increasing stringencies, was observed in this set of experiments. This could be due to the presence of phenol compounds in the actinorhizae, which would prevent normal conditions of hybridization, as has been noted by other workers (4). That means that electrophoresis by separating DNA from phenol compounds on agarose gels provides an additional purification step that is required for probe specificity.

Hybridization with a biotinylated DNA probe. *Frankia* sp. strain ACoN24d genomic DNA was digested with the restriction enzyme *Bam*HI and hybridized to the biotinylated pFQ121 DNA probe. The results presented in Fig. 4 (lanes A and A′) indicate that hybridization occurred with the pFQ56 homologous sequences contained in the ACoN24d genome. The same probe hybridized to endophyte DNA from crushed nodules that were formed with *Frankia* sp. strain ACoN24d (Fig. 4, lanes B and B′) and failed to hybridize to DNA from nodules that were formed with *Frankia* sp. strain ACNIAG (negative control; data not shown). The use of nonradioactive probes would be suitable for studying competition in

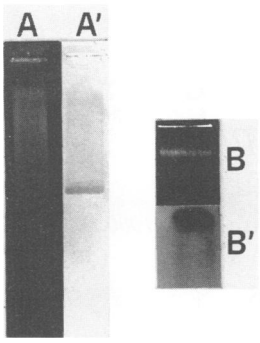

FIG. 4. Hybridization of isolate and endophyte DNA with the biotinylated 8-kb plasmid probe. DNA from the 8-kb plasmid-bearing *Frankia* sp. strain ACoN24d was digested with *Bam*HI, electrophoresed (lane A), and hybridized (lane A'). The hybridizing fragment corresponds to the covalently closed circular form of the plasmid pFQ56 (no site for the enzyme *Bam*HI) that is contained in strain ACoN24d DNA. Lanes B and B', Agarose gel electrophoresis and hybridization of crushed root nodules from *Alnus glutinosa* inoculated with *Frankia* sp. strain ACoN24d.

microbial ecology laboratories in which radioactive techniques are too difficult to be applied for routine investigations.

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