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Plasmids in *Frankia* sp.

PHILIPPE NORMAND,[1] PASCAL SIMONET,[1]† JEAN L. BUTOUR,[2] CHARLES ROSENBERG,[1] ANDRE MOIROUD,[3] AND MAURICE LALONDE[4]*

Laboratoire de Biologie Moléculaire, Institut National de la Recherche Agronomique-Centre National de la Recherche Scientifique, Castanet-Tolosan, 31320, France[1]; Laboratoire de Pharmacologie et de Toxicologie Fondamentales, Centre National de la Recherche Scientifique Toulouse 31400, France[2]; Laboratoire d'Écologie Microbienne, Université Lyon 1, Villeurbanne 69622, France[3]; and Département d'Écologie et Pédologie, Université Laval, Québec, Canada G1K 7P4[4]

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A method to achieve cell lysis and isolate *Frankia* sp. plasmid DNA was developed. A screening of *Frankia* sp. strains belonging to different host compatibility groups (*Alnus* sp., *Elaeagnus* sp., *Ceanothus* sp.) showed that, of 39 strains tested, 4 (strains CpI1, ARgN22d, ArI3, and EUN1f) possessed plasmids ranging in size from 7.1 to 32.2 kilobase pairs as estimated from agarose gel electrophoresis and electron microscopy. A total of 11 plasmids were detected.

Frankia sp., a pleiomorphic actinomycete, participates in a nitrogen-fixing symbiosis with a number of woody dicotyledonous plants, some of which have economically important applications. Alders (*Alnus* spp.), for example, have been used in the reclamation of mine spoils and other disturbed sites and in forest crop rotations (16), and autumn-olive (*Elaeagnus umbellata* Thunb.) has been used as nurse plant for black walnut (7).

The first isolation in pure culture of a *Frankia* sp. strain occurred only 5 years ago (5). Since then, research on the biochemistry of *Frankia* sp. has progressed (13, 17), leading to the evaluation and selection of strains for large-scale inoculation of plantations of host plants (8, 14).

A study of *Frankia* sp. genetics would offer powerful tools for the selection and improvement of strains and for a fundamental study of the actinorhizal symbiosis. A conventional genetic study is difficult, however, because of problems related to the physiology of *Frankia* sp. Its growth rate is slow, with a doubling time of at least 3 days (2) and even more in the case of type P (in vivo sporulating) isolates (14). Spore germination is poor and asynchronous (M. J. McBride, personal communication).

Plasmids can be used as cloning vehicles to promote sexual transfers and as strain markers, and in the case of *Streptomyces* spp., a close relative of *Frankia* sp. they have been developed as cloning vectors (9). Thus, *Frankia* sp. strains were screened for the presence of plasmids, following a report by D. Marvel (personal communication) that plasmids had been found in *Frankia* sp. strain CpI1.

MATERIALS AND METHODS

Growth conditions. The *Frankia* sp. strains studied were grown in a modified Qmod B medium (11) containing (per liter): K_2HPO_4, 0.3 g; $NaH_2PO_4 \cdot 2H_2O$, 0.26 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KCl, 0.2 g; yeast extract (Difco Laboratories), 0.5 g; Bacto-Peptone (Difco), 5 g; glucose, 10 g; H_3BO_3, 1.5 mg; $MnSO_4 \cdot H_2O$, 0.7 mg; $ZnSO_4 \cdot 7H_2O$, 0.6 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.02 mg; $CoSO_4 \cdot 7H_2O$, 0.001 mg; citric acid, 10 mg; ferric citrate, 10 mg; and L-α-lecithin (500 mg dissolved in 50 ml of ethanol and diluted to 100 ml with distilled water), 5 mg; pH 6.8 to 7.0. The cultures were grown in tubes (17 ml of medium in 35-ml tubes) or in 2-liter flasks (containing 1 liter of medium), incubated at 28°C, and agitated vigorously once a day to ensure homogeneous mycelial growth.

DNA isolation. The method for preparation of *Frankia* sp. DNA was based on that described by Drocourt et al. (D. Drocourt, R. Ques, and G. Tiraby, *in 4th International Symposium on the Genetics of Industrial Microorganisms*, Kyoto, Japan, 1982, in press). For routine work, the mycelium from one tube (four in the case of slow-growing type P strains [18]) of late exponential-phase cells (2 to 3 weeks old) was washed in 50 mM Tris–20 mM EDTA–15% (wt/vol) sucrose (pH 8) and incubated at 34°C for 60 min with lysozyme (10 mg ml^{-1}) in 150 μl of the same buffer. After 60 min, 100 μl of a 10% (wt/vol) sodium dodecyl sulfate aqueous solution at 90°C was added, and the contents were vortexed. The tubes were kept at 60°C for 15 min. After the sodium dodecyl sulfate addition, the DNA content could be rapidly checked by adding 500 μl of phenol-chloroform mix (made up by adding 100 ml of chloroform, 40 ml of 0.5 M NaCl, 100 mg of 8-hydroxyquinoline, and 100 g of phenol [10]), vortexing, centrifuging at 12,000 × *g* for 5 min, and loading the clear supernatant fluid onto gels. For clearer gels

† Present address: Laboratoire d'Ecologie Microbienne, Université Lyon 1, Villeurbanne 69622, France.

with less linear DNA and with reduced streaking, the phenol-chloroform extraction step was omitted. Instead, 50 to 70 μl of 1 N NaOH was added to bring the pH to 12.2 to 12.4, and the contents were mixed and allowed to stand for 15 min at room temperature. The pH was lowered to 8 by adding 100 μl of 2 M Tris-hydrochloride (pH 7), and the contents were mixed and allowed to stand for 15 min at room temperature. Sodium dodecyl sulfate, cell debris, and denatured linear DNA were precipitated by adding one-fifth volume (usually 100 μl) of 5 M NaCl, vortexing, cooling to 4°C for at least 2 h, and centrifuging at 12,000 × *g* for 5 min. The supernatant fluid was transferred to another tube, and the DNA was precipitated by adding 1 ml of ethanol and leaving at −20°C overnight. The DNA was sedimented by centrifugation (5 min, 12,000 × *g*), redissolved in 0.05 M Tris–0.02 M EDTA (TE buffer; pH 8), and analyzed by gel electrophoresis, using 89 mM Tris–2.5 mM EDTA–89 mM H_3BO_3 buffer in 0.8% agarose at 4.5 V cm^{-1} for 150 min. The gels were stained in 0.4 mg of ethidium bromide $liter^{-1}$, destained in distilled water, and photographed with a 366-nm UV source (UV Products, San Gabriel, Calif.) and Polaroid type 55 N/P film.

Electron microscopy. Because of the low yields of *Frankia* sp. DNA, an electroelution method was tested with strain ArI3 plasmids whose intensity was highest (Fig. 1), but it was later found that large-scale isolation of DNA, followed by a CsCl-ethidium bromide isopycnic ultracentrifugation, yielded DNA that gave much better results. DNA was isolated from 2 to 5 liters of culture as described above. The ethanol-precipitated DNA was dissolved in 1 ml of TE buffer and transferred to 5-ml centrifuge tubes containing 4.04 g of CsCl in 3 ml of TE buffer. The contents were homogenized, and 0.25 ml of a 10-mg ml^{-1} ethidium bromide solution was added. The tubes were centrifuged in a Beckman VTi-80 rotor for 5 h at 65,000 rpm. The lower covalently closed circular DNA-containing band was removed, dialyzed against 0.10 M ammonium acetate, and adsorbed on carbon-coated copper grids, following a basic protein-spreading method (3). The grids were contrasted by rotary platinum shadowing and observed with a Philips 300 electron microscope. Pictures of molecules were taken at about ×10,000 magnification and the negatives were enlarged 10-fold. Measurements were done on tracings with a Hewlett-Packard digitizer-computer apparatus with an accuracy of ±1.5%. The lengths of the plasmid DNA molecules were determined by comparison with open circular phage PM2 DNA prepared as previously described (4) and spread as described above. Molecular weights were calculated by comparison with PM2 DNA molecules which have a contour length of 3.20 μm and a size of 9.63 kilobases (kb).

RESULTS

In the course of our research for indigenous plasmids in *Frankia* sp. strains, many different DNA isolation methods were tested. The most critical step appears to be the lysis of the cells. Thus, lysozyme, proteases, neuraminidase, and crude preparations of cellulase, helicase, and mutanase were all tried, but none effectively lysed a significant proportion of cells as determined by phase-contrast microscopy. Consequently, very small amounts of DNA were recovered when the pretreated cells were analyzed. No bands besides that of chromosomal DNA were visualized in gels. A pretreatment of strain ARgN22d with lysing enzymes from basidiomycetes (Sigma Chemical Co.) or with lysozyme and EDTA, followed by in situ lysis according to Eckhardt (6), permitted the visualization of a fuzzy satellite band. The sharpness of that band was increased when an alkaline lysis procedure was used (10), but this treatment was not reproducible, often producing nicks that resulted in open circular forms which have a much reduced mobility.

The physiological condition of the cells also was found to be critical. Young (3 weeks old), actively growing cells were preferred and subcultured regularly with large (10%) inoculations of mycelium broken by repeated passage through a 23-gauge needle, producing mycelium growth that responded well to our treatments. The presence of $CaCO_3$ in the original Qmod B medium (11) was found to interfere with DNA isolation and so was omitted.

For detection of plasmids, the treatment which produced the best results was a combination of enzymatic attack by lysozyme in the presence of EDTA, followed by a chemical treatment with 10% (wt/vol) sodium dodecyl sulfate at 90°C (Drocourt et al. in press). With that method, we screened 39 *Frankia* sp. strains of varied geographical and host plant origin and found 4 which harbored one or more plasmids (Table 1). Strain ArI3, isolated from *Alnus rubra* (1), contains two plasmids with sizes of 7 and 19 kb (Fig. 1), as determined by measurement of contour length after electron microscopy (Table 1). Strain CpI1, isolated from *Comptonia peregrina* (5), yielded plasmids of similar sizes (Table 1) although their intensities were much lower than those of ArI3 (Fig. 1), probably as a result of lower plasmid copy numbers since the intensities of the chromosomal bands were similar (Fig. 1). D. Marvel (Biology of *Frankia* Conference, Madison, Wis., 1982) also detected plasmids in these two strains.

Strain ARgN22d isolated from *A. rugosa* (14) was found by electron microscopy to contain six plasmids of 7.9, 16.2, 20.2, 23.3, 27, and 32.2 kb (Table 1). Initially, only one band could be seen in gels, corresponding to the 27-kb plasmid (data not shown). Later on, other plasmids appeared sequentially. This could have been due to improvements in the technique or to a genetic event such as excision of nonessential sequences of plasmid pFQ25, as has been found to occur in plasmids of *Streptomyces lividans* (10). Electron microscopy of CsCl-ethidium bromide centrifugation-purified supercoiled DNA finally

TABLE 1. Plasmid-bearing *Frankia* sp. strains

Strain	Designation of plasmids[a]	Size (kb)	
		Agarose gel electrophoresis	Electron microscopy
CpI1	pFQ11	7	7.1 ± 0.3 (14)[b]
	pFQ12	19	14.5 ± 0.3 (34)
ARgN22d	pFQ21	7	7.9 ± 0.3 (22)
	pFQ22	—[c]	16.2 ± 0.4 (6)
	pFQ23	19	20.2 ± 0.2 (25)
	pFQ24	23	23.3 ± 0.3 (23)
	pFQ25	26	27.0 ± 0.8 (15)
	pFQ26	—[c]	32.2 ± 0.4 (9)
ArI3	pFQ31	7	8.3 ± 1.1 (31)
	pFQ32	19	18.3 ± 1.1 (18)
EUN1f	pFQ41	14	—[d]

[a] Plasmid nomenclature according to Novick et al. (15).

[b] Numbers in parentheses indicate numbers of molecules measured.

[c] These plasmids were detected by electron microscopy of DNA extracts purified by CsCl-ethidium bromide centrifugation only. They were not visualized in gels due to their low copy numbers and because of the other plasmids with similar sizes present in this strain.

[d] This plasmid was shown to be a covalently closed circular molecule of 14 kb by two-dimensional agarose gel electrophoresis (not shown). The plasmid DNA of strain EUN1f was not measured by electron microscopy.

permitted the detection of six plasmids present in strain ARgN22d. Agarose gel electrophoresis only permitted the visualization of those plasmids with a relatively high apparent copy number (Fig. 1).

Strain EUN1f, isolated from *Elaeagnus umbellata* (12), yielded one band upon gel electrophoresis (Fig. 1) corresponding to a 14-kb plasmid. DNA extracts of *Frankia* sp. strains belonging to the *Elaeagnus* sp. host compatibility group always showed intense streaking, although this problem could be alleviated by additional ethanol precipitation once or twice. The streaking at first prevented the vizualization of the 14-kb plasmid (Fig. 1). Nucleases more active in strains of the *Elaeagnus* sp. group may degrade linear DNA or the presence of single-stranded DNA may cause the streaking (T. Kieser, personal communication). The 14-kb plasmid of strain EUN1f has been confirmed to be a covalently closed circular DNA by two-dimensional agarose gel electrophoresis (10) (data not shown). The plasmid DNA from *Frankia* sp. strains belonging to the *Elaeagnus* sp. group could not be evenly spread on the grids; therefore, contour length measurements could not be done.

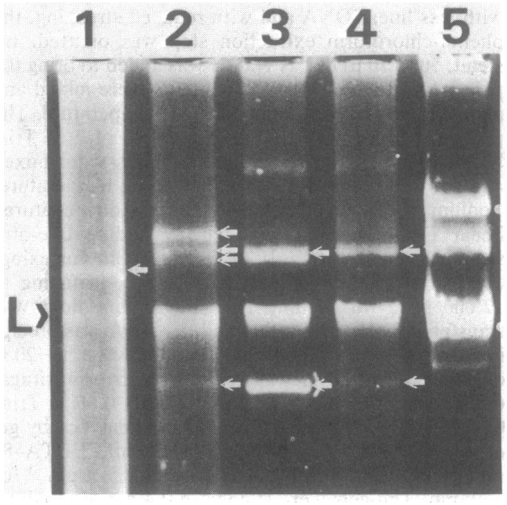

FIG. 1. Agarose gel electrophoresis of *Frankia* sp. strain DNA. L, Linear DNA. (Lane 1) Strain EUN1f DNA with a 14-kb plasmid (arrow); the intense streaking was found in DNA extracts from all strains belonging to the *Elaeagnus* sp. group. (Lane 2) Strain ARgN22d DNA with four plasmids of 7.9, 20.2, 23.3, and 27 kb (arrows); two other plasmids with sizes of 16.2 and 32.2 kb but with low copy numbers were found in DNA extracts purified by CsCl-ethidium bromide centrifugation. (Lane 3) Strain ArI3 DNA with 8.3- and 18.3-kb plasmids (arrows); the upper bands would correspond to open circular and linear forms with reduced mobilities. (Lane 4) Strain CpI1 DNA with 7.1- and 14.5-kb plasmids (arrows). (Lane 5) Reference plasmids of 11.9 and 47 kb (dots). Electrophoresis was carried out at 4.5 V cm^{-1} for 150 min in 0.8% agarose. The gel was stained in 0.4 mg of ethidium bromide $liter^{-1}$, destained in distilled water, and photographed with a 366-nm UV source.

DISCUSSION

No phenotype has been ascribed to the 11 plasmids found in four *Frankia* sp. strains (Table 1). They are unlikely to be involved in the symbiosis since 35 *Frankia* sp. strains in which no plasmid DNA was detected had similar symbiotic properties. Whatever their function, these small plasmids, being indigenous to *Frankia* sp. strains of widely different host plant and geographical origin, may prove valuable in the genetic study of such an economically important microorganism.

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