Low-Frequency Restriction Fragment Analysis of *Frankia* Strains (*Actinomycetales*)

MARTHA BEYAZOVA AND MARY P. LECHEVALIER†*

Waksman Institute, Rutgers University, Piscataway, New Jersey 08855

Low-frequency restriction fragment analysis of more than 100 strains of the genus *Frankia* showed that restriction enzyme *Dra*I (recognition site, TTT'AAA) gave rise to large DNA fragments (200 to 1,500 kb), which, when they were subjected to cluster analysis, reflected the host plants from which the strains were isolated. Our results support the conclusions of Lalonde and his colleagues (M. Lalonde, L. Simon, J. Bousquet, and A. Seguin, p. 671–680, in H. Bothe, F. J. de Bruijn, and W. E. Newton, ed., *Nitrogen Fixation: Hundred Years After*, 1988; P. Normand, P. Simonet, and R. Bardin, *Mol. Gen. Genet.* 213:238–246, 1988) and Fernandez et al. (M. P. Fernandez, H. Meugnier, P. A. D. Grimont, and R. Bardin, *Int. J. Syst. Bacteriol.* 39:424–429, 1989) that various biochemical and genomic analyses can give rise to groupings of *Frankia* strains that are consistent with the host plants from which the strains are isolated and that the resulting groups may form a basis for defining *Frankia* species.

In a very real sense, *Frankia* taxonomy has come full circle. A strain of these nitrogen-fixing actinomycete symbionts of higher plants was first isolated and maintained in pure culture only 14 years ago (15). As more pure strains were isolated, it became apparent that the taxonomic scheme of Becking (9, 10), which was formulated prior to the availability of the endophytes in pure culture, was not entirely satisfactory (27). Becking followed the lead of *Rhizobium* taxonomists in basing *Frankia* species definitions on host plant compatibility which was determined by using crushed-nodule inocula. When the pure cultures were used to determine infectivity, it became clear that Becking's host plant infectivity groups were not entirely correct. In addition, although Becking accurately described the morphology of frankiae from nodule sections, his attempt at describing the cell chemistry of these organisms was flawed because of the high levels of plant impurities present in his preparations. In addition, other workers found that *Frankia* isolates were not amenable to classification on the basis of the results of tests that are commonly used for assigning actinomycetes to species (14, 30). This was not only because of the exceedingly slow growth of these organisms (doubling times, 1 to 5 days) and their nutritional diversity, but also because many frankiae, like the slow-growing rhizobia, are relatively inactive, as assessed by dissimilation of carbohydrates and other substances. Consequently, in 1983 workers in the field agreed that it would be inappropriate to use Becking's species names until solid criteria could be developed to group the various isolates in a taxonomically meaningful way (27).

Recently, Lalonde and coworkers (24), summarizing a number of their own studies and studies of other workers, concluded that the evidence that has been accumulated to date points strongly to the plant host relationship as an important element in the definition of *Frankia* species. The data which were used in these studies included data on polypeptide patterns, serology, isozyme patterns, glycoside patterns, fatty acid composition, DNA homology, and *nif*-probed restriction fragment length polymorphisms. Although not always based on the same pool of strains, these

data supported the proposals that Becking's species *Frankia alni* and *Frankia elaeagnii* should be redescribed and that two subspecies should be created in *F. alni*, "*F. alni* subsp. *pommerii*" and "*F. alni* subsp. *vandijkii*." On the basis of DNA-DNA relatedness data, Fernandez et al. (21) subsequently proposed nine "genomic species" of the genus *Frankia* which also reflected host plant relationships. Genomic species 1 of Fernandez et al. was considered to be equivalent to *F. alni*. In this paper we report that on the basis of the distribution of high-molecular-weight restriction fragments of DNAs of members of this genus, we also identified clusters of strains which reflected the host plants from which the strains were isolated.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study and their origins are shown in Table 1. These strains were maintained in various liquid media, including L/2, S, S+Tw, HIML3, B/2, and L+S₂ (31), and were transferred every 4 to 6 months. For analysis the strains were grown in a defined medium (DPM, DPM/N, or DPM/N2Pyr [31]) under static conditions at 28°C and were harvested in the stationary phase. The length of incubation varied with the growth rate of the strains, but was usually 6 to 10 weeks.

Preparation of inserts. The cells were collected and washed with 10 mM Tris–1 mM disodium EDTA buffer (pH 7.4) (TE), ground, and then treated by shaking them at 65°C for 30 min with 2% sodium dodecyl sulfate–5 mM disodium EDTA. After the cells were washed with 5 mM disodium EDTA containing 1 mg of bovine serum albumin fraction V per ml, they were washed with decreasing concentrations of pH 8.4 disodium EDTA (0.5M, 0.25M, and 5 mM) to remove as much sodium dodecyl sulfate as possible; finally, the cells were extracted for 20 min at room temperature with chloroform-ethanol (2:1) and then with acetone by using a wrist action shaker. The solvents were discarded, and then the cells, after they were rehydrated in TE for 20 to 30 min, were ground with a Ten Broeck tissue grinder. Inserts for pulsed-field analysis were prepared in 0.5% (final concentration) low-melting-point agarose in TE and then incubated with gentle shaking at 37°C for 4 to 5 h or overnight in EC lysis buffer (38) (6 mM Tris hydrochloride [pH 7.6], 1 M sodium chloride, 100 mM EDTA [pH 7.5], 0.5% Brij 58, 0.2%

* Corresponding author.

† Present address: RR2, Box 2235, Morrisville, VT 05661.

TABLE 1. Strains analyzed

Strain	Frankia catalog no.	Original plant host or history ^a	Plant passage host	Country of origin	Source and/or reference(s)	LFRFA cluster
AgV4ch	ULF 010701008	<i>Alnus glutinosa</i>		France	F. Horrière, 28	5
AgIMal	ULF 010701101	<i>Alnus glutinosa</i>		France	28	9
AgIPal	ULF 0107XX	<i>Alnus glutinosa</i>	<i>Alnus incana</i>	France	F. Horrière	9
Aj1a	DDB 01161910	<i>Alnus jorullensis</i>		Mexico	D. Baker, 6, 28	4
AICF	ULF 0111XX	<i>Alnus incana</i> subsp. <i>incana</i>		France	F. Horrière	7
A18-2	UCS 0132XX	<i>Alnus incana</i> subsp. <i>incana</i>		United States	D. Benson	10
ARGP5Ag	ULQ 0132105009	<i>Alnus incana</i> subsp. <i>rugosa</i>	<i>Alnus glutinosa</i>	Canada	P. Normand, 24	NC ^b
CUSA		<i>Alnus incana</i> subsp. <i>rugosa</i>		United States	V. Ahmadian	8
32-30(Air12)	LLR 01322	<i>Alnus incana</i> subsp. <i>rugosa</i>		United States	28	8
32-43A	LLR 01327	<i>Alnus incana</i> subsp. <i>rugosa</i>		United States	M. P. Lechevalier	3
32-44(Air11)	LLR 01321	<i>Alnus incana</i> subsp. <i>rugosa</i>		United States	28	7
32-83	LLR 01325	<i>Alnus incana</i> subsp. <i>rugosa</i>		United States	M. P. Lechevalier	9
32-85	LLR 01324	<i>Alnus incana</i> subsp. <i>rugosa</i>		United States	M. P. Lechevalier	4
R52	LLR 013701	<i>Alnus incana</i> subsp. <i>tenuifolia</i>		United States	28	13
CWV Am-1	OSS 011802	<i>Alnus maritima</i>		United States	H. Vishniac	7
Ari12	DDB 01301610	<i>Alnus rhombifolia</i>		United States	D. Baker, 28	3
Ari13	HFP 013003	<i>Alnus rubra</i>		United States	A. Berry, 28	1
Ari13x(Ag)	HFP 0130XX	<i>Alnus rubra</i>	<i>Alnus glutinosa</i>	United States	J. G. Torrey	NC
Ari13x(Air)	HFP 0130XX	<i>Alnus rubra</i>	<i>Alnus incana</i> subsp. <i>rugosa</i>	United States	J. G. Torrey	5
Ari15	DDB 01310210	<i>Alnus rubra</i>		United States	D. Baker, 6, 28	7
UGL 013103	DDB 01310310	<i>Alnus rubra</i>		United States	D. Baker, 6, 28	7
131	UGL 013103	<i>Alnus rubra</i>		Scotland	C. Wheeler, 28	12
395	WEY 0131131	<i>Alnus rubra</i>		United States	28	8
442	WEY 0131395	<i>Alnus rubra</i>		United States	28	8
666	WEY 0131442	<i>Alnus rubra</i>		United States	28	8
888	WEY 0131366	<i>Alnus rubra</i>		Canada	28	8
AS-1	WEY 0131888	<i>Alnus rubra</i>		Canada	28	8
ACN1	LLR 0131301	<i>Alnus serrulata</i>		United States	M. P. Lechevalier	7
ACN1x(Ag)	ULQ 0102XX	<i>Alnus viridis</i> subsp. <i>crispa</i>		Canada	M. Lalonde	NC
ACX50h	ULQ 0102001007	<i>Alnus viridis</i> subsp. <i>crispa</i>	<i>Alnus glutinosa</i>	Canada	M. Lalonde, 28	NC
LLAvcl1	ULQ 010225008	<i>Alnus viridis</i> subsp. <i>crispa</i>		Canada	M. Lalonde and L. Simon	6
Wg1.1(WgAvcl1)	DDB 01020110	<i>Alnus viridis</i> subsp. <i>crispa</i>		Canada	6, 28	8
	DDB 01020130	<i>Alnus viridis</i> subsp. <i>crispa</i>		Netherlands	A. Akkermans	8
	(substrain of Avcl1)					
Avs12	DDB 01360530	<i>Alnus viridis</i> subsp. <i>sinuata</i>		United States	D. Baker, 28	6
Avs13	DDB 01360610	<i>Alnus viridis</i> subsp. <i>sinuata</i>		United States	D. Baker, 6, 28	8
Av soc	UFL 1014101601	<i>Alnus viridis</i> subsp. <i>viridis</i>		France	F. Horrière, 28	7
Av sts	UFL 0141XX	<i>Alnus viridis</i> subsp. <i>viridis</i>		France	F. Horrière	2
Alil1	HFP 022801	<i>Allocasuarina lehmanniana</i> (I) ^c		United States	J. G. Torrey, 28	13
Ccl2	HFP 020202	<i>Casuarina cunninghamiana</i> (NI) ^d		United States	28	NC
R3	LLR 02021	<i>Casuarina cunninghamiana</i> (NI)		United States	28	NC
R43	LLR 02022	<i>Casuarina cunninghamiana</i> (NI)		United States	28	15
R43A ₂	LLR 02023	Mutant of R43		United States	29	15
R436-ER-5	LLR 0202202021	<i>Casuarina cunninghamiana</i> (R) ^e		United States	M. P. Lechevalier	15
R436-ER-2	LLR 0202202022	<i>Casuarina cunninghamiana</i> (R)		United States	M. P. Lechevalier	15
R43Eu#2	LLR 0202213252	<i>Elaeagnus umbellata</i> (NI)		United States	M. P. Lechevalier	15
R43E2	LLR 02024	<i>Elaeagnus umbellata</i> (NI)		United States	M. P. Lechevalier	15

Continued on following page

TABLE 1—Continued

Strain	Frankia catalog no.	Original plant host or history ^a	Plant passage host	Country of origin	Source and/or reference(s)	LFREA cluster
D1	ORS 020601	<i>Casuarina equisetifolia</i> (NI)		Senegal	H. Diem, 28	12
D11	ORS 020602	<i>Casuarina equisetifolia</i> (NI)		Senegal	H. Diem, 28	12
CeD1.82	ORS 020606	<i>Casuarina equisetifolia</i> (I)		Senegal	28	4
CeF1.82	ORS 020607	<i>Casuarina equisetifolia</i> (I)		Senegal	28	4
CeI2	DDB 02060210	<i>Casuarina equisetifolia</i> (NI)		United States	D. Baker, 6, 28	12
CeI5	UFG 02060605	<i>Casuarina equisetifolia</i> (I) ^y		United States	H. Berg	14
CeS15	RHB 0206606	Single-spore isolate from Cel5		United States	H. Berg	14
CeI5S51	HFP 0206XX	Single-spore isolate from Cel5		United States	J. G. Torrey	14
CeI5S52	HFP 0206XX	Single-spore isolate from Cel5		United States	J. G. Torrey	14
G2	ORS 020604	<i>Casuarina equisetifolia</i> (NI)		Guadeloupe	H. Diem	12
K105	CSF 0206XX	<i>Casuarina equisetifolia</i> (NI)		People's Republic of China	J.-S. Ruan	11
K114	CSF 0206XX	<i>Casuarina equisetifolia</i> (NI)		People's Republic of China	J.-S. Ruan	3
UFG 026105	UFG 0206105	<i>Casuarina equisetifolia</i> (NI)		United States	H. Berg, 28	13
UFG 026201	UFG 0206201	<i>Casuarina equisetifolia</i>		United States	H. Berg	4
32-76	LLR 020601	<i>Casuarina equisetifolia</i> (NI)		Bahamas	28	NC
CgI1	UFG 02088501	<i>Casuarina glauca</i> (I)		United States	H. Berg	14
CgI1 × Gp	HFP 0208XX	<i>Casuarina glauca</i> (I)		United States	J. G. Torrey	14
CgI4	HFP 020804	<i>Casuarina glauca</i> (I)		Egypt	32	6
CjI.82	ORS 021001	<i>Casuarina junghuniana</i> (I)		Thailand	28	4
GpI1	HFP 021801	<i>Gymnostoma papuanum</i> (I)		United States	J. G. Torrey	13
GpI1 × Gp	HFP 0218XX	<i>Gymnostoma papuanum</i>		United States	J. G. Torrey	13
CpI1	HFP 070101	<i>Comptonia peregrina</i>		United States	J. G. Torrey, 6, 28	1
CpI3	DDB 07010310	<i>Comptonia peregrina</i>		United States	D. Baker, 6, 28	8
R100	LLR 07011	<i>Comptonia peregrina</i>		United States	28	8
32-61	LLR 160401	<i>Myrica californica</i>		United States	28	9
McI2b	DDB 16060820	<i>Myrica cerifera</i>		United States	D. Baker, 6, 28	2
McI2c	DDB 16060830	<i>Myrica cerifera</i>		United States	D. Baker, 6, 28	8
McI3	DDB 16060910	<i>Myrica cerifera</i>		United States	D. Baker, 6, 28	2
K2129	CSF 1609XX	<i>Myrica esculenta</i>		People's Republic of China	J. S. Ruan	NC
M+gI5×(Gp)	HFP 1611XX	<i>Myrica gale</i>		United States	J. G. Torrey	9
M+gI5	HFP 161105	<i>Myrica gale</i>		United States	S. Racette	9
MgI7	DDB 16110410	<i>Myrica gale</i>		United States	D. Baker, 6, 28	4
MgI8	DDB 16110510	<i>Myrica gale</i>		Canada	D. Baker, 6, 28	6
R82	LLR 161101	<i>Myrica gale</i>		United States	28	15
RBR2	RBR 162002	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	11
RBR3	RBR 162003	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	6
RBR4	RBR 162004	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	3
RBR5	RBR 162005	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	11
RBR8	RBR 162008	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	11
RBR9	RBR 162009	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	4
RBR10	RBR 162010	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	NC
RBR13	RBR 162013	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	11
RBR14	RBR 162014	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	11
RBR18	RBR 162018	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	8
RBR20	RBR 162020	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	NC
RBR21	RBR 162021	<i>Myrica pennsylvanica</i>		United States	R. Bloom, 13	10

32-27(Mp11)	LLR 162001	<i>Myrica pensylvanica</i>	United States	28	M. P. Lechevalier	7
32-21	LLR 162007	<i>Myrica pensylvanica</i>	United States	7	M. P. Lechevalier	7
32-23	LLR 162009	<i>Myrica pensylvanica</i>	United States	7	M. P. Lechevalier	7
4-D(+)	LLR 162012	UV mutant of 32-27	United States	7	M. P. Lechevalier	7
8-120(-)	LLR 162013	NTG mutant of 32-27 ^g	United States	7	M. P. Lechevalier	7
32-63	LLR 162004	<i>Myrica pensylvanica</i>	United States	2	M. P. Lechevalier	2
32-74	LLR 162003	<i>Myrica pensylvanica</i>	United States	28	M. P. Lechevalier	2
Mu11	UFG 162201	<i>Myrica pubescens</i>	United States	H. Berg	NC	NC

^a The genera *Allocasuarina*, *Casuarina*, and *Gymnostoma* are members of the Casuarinaceae, the genus *Alnus* is a member of the Betulaceae, the genus *Elaeagnus* is a member of the Elaeagnaceae, and the genera *Comptonia* and *Myrica* are members of the Myricaceae.

^b NC, not clustered.

^c I, infective for the original host.

^d NI, not infective for the original host.

^e R, recovered from parenchymatous (nonnodular) tissue of *Casuarina cunninghamiana*.

^f I?, questionably infective for the original host.

^g NTG, N'-nitro-N-nitrosoguanidine.

sodium deoxycholate, 0.1% *N*-laurylsarcosine) containing 2 mg of lysozyme per ml. The inserts were next treated with TE containing 1 mg of subtilisin Carlsberg (catalog no. P5380; Sigma Chemical Co., St. Louis, Mo.) per ml with shaking overnight; this was followed by two washes in TE at 56°C for 4 h or overnight and then two washes in TE at 56°C for 2 h. Restriction digestion with *Dra*I was carried out as described by Smith and Cantor (38), using the buffers recommended by the suppliers of the enzyme.

Pulsed-field electrophoresis. Pulsed-field electrophoresis was performed by using a 0.8% agarose gel in 1% Tris-borate buffer (0.1 M Tris base, 0.1 M boric acid, 0.2 mM disodium EDTA) in a Beckman Geneline transverse alternating field electrophoresis (TAFE) apparatus. The following program was used for 200- to 1,500-kb fragments: 12 h at 150 V with 70-s pulses, followed by 14 h at 150 V with 120-s pulses. For 50- to 500-kb fragments the following program was used: 20 h at 140 mA with 25-s pulses. The standards used for the DNA bands at the higher molecular weights were *Saccharomyces cerevisiae* 334 chromosomes (catalog no. 338404; Beckman Instruments, Inc.), and lambda concatemers (Beckman Instruments) were the standards used for the lower-molecular-weight range. Ethidium bromide-stained gels were visualized at 302 nm and were photographed with type 55 or 57 Polaroid film.

Data analysis. Similarity values were calculated by using the Taxan program (version 2.0) of David Swartz, Biotechnology Institute, University of Maryland (the simple matching [S_{SM}] algorithm and cluster analysis by the unweighted average pair group method [39]).

RESULTS

We analyzed the DNAs of more than 100 *Frankia* strains to determine their *Dra*I restriction patterns (recognition site TTT'AAA) by using pulsed-field electrophoresis (low-frequency restriction fragment analysis [LFRFA]). The strains were isolated from nodules from members of the plant genera *Alnus*, *Myrica*, *Comptonia*, *Casuarina*, *Allocasuarina*, and *Gymnostoma* growing in widely different areas of the world (United States, Canada, Scotland, Mexico, Egypt, France, Bahamas, Thailand, Senegal, Guadeloupe, and People's Republic of China) (Table 1). The sizes of the DNA fragments in the size range from 200 to 1,000 kb were determined by comparing them with a *S. cerevisiae* chromosomal standard, and the unweighted data were clustered by using the S_{SM} algorithm (39) (Fig. 1). Included in this comparison were wild-type strains, strains that were reisolated after plant passage, single-spore isolates, mutants, and co-isolates (different strains from a single nodule). We identified 15 clusters that were linked at levels of >75% and contained 90 strains; 11 strains did not fall into any cluster. In general, the clusters tended to be dominated either by strains that were isolated from members of the Casuarinaceae (Fig. 1) (e.g., clusters 12 through 15) or by strains that were isolated from *Alnus* spp. and members of the Myricaceae (e.g., clusters 1, 2, and 5 through 11). Clusters 3 and 4 were considered mixed, since they had no predominant group. The electrophoretic LFRFA patterns of some representative strains are shown in Fig. 2.

Table 2 shows the distribution of *Dra*I fragments in various size ranges (see above) for each cluster. Substrains (such as single-spore isolates) that were known to have been derived from one of the strains in a cluster and produced LFRFA patterns that were identical to the pattern of the parent were omitted from the calculations. Table 2 also

FIG. 1. Dendrogram constructed by performing an unweighted average linkage cluster analysis of S_{SM} values of DNA fragment sizes expressed as percentages. The DNA of each of the 101 *Frankia* strains obtained from the plant genera *Alnus*, *Myrica*, *Comptonia*, *Allocasuarina*, *Casuarina*, and *Gymnostoma* (see Table 1 for descriptions of the strains) was digested with *Dra*I, and the sizes of the resulting DNA fragments were calculated (see text). (A), strains obtained from *Alnus* spp.; (M), strains obtained from *Myrica* and *Comptonia* spp. (members of the family Myricaceae); (C), strains obtained from *Allocasuarina*, *Casuarina*, and *Gymnostoma* spp. (members of the family Casuarinaceae). N.C., not clustered.

TABLE 2. Distribution of *Dra*I fragments of various sizes within the LFRFA clusters of *Frankia* strains

Cluster	No. of strains	No. of strains with the following plant hosts:			% of <i>Dra</i> I fragments in the following size ranges:											
		<i>Alnus</i> spp.	Members of the Myricaceae	Members of the Casuarinaceae	<240 kb	240 to 269 kb	270 to 299 kb	300 to 329 kb	330 to 359 kb	360 to 389 kb	390 to 419 kb	420 to 449 kb	450 to 479 kb	480 to 509 kb	510 to 539 kb	540 to 569 kb
1	2	1	1	0	100	100	100	0	100	0	100	0	0	0	100	0
2	5	1	4	0	100	80	100	0	60	60	0	0	80	0	20	20
3	3	2	0	1	100	33	100	0	0	33	66	0	0	0	0	0
4	10	2	3	5	60	50	60	0	0	40	50	0	90	30	20	0
5	2	2	0	0	50	100	100	0	100	0	0	50	0	0	0	0
6	5	2	2	1	80	0	100	0	0	0	20	0	80	40	20	60
7	12	7	5	0	92	0	83	0	0	17	50	66	0	0	50	0
8	14	10	4	0	100	0	0	7	7	7	0	0	21	0	14	0
9	5	3	2	0	0	20	80	0	0	40	0	40	20	0	40	0
10	2	1	1	0	100	0	0	0	0	100	0	100	0	0	0	50
11	6	0	5	1	80	0	17	0	100	0	0	50	17	0	33	0
12	5	1	0	4	100	40	100	0	0	80	100	40	20	0	60	100
13	4	1	0	3	100	0	100	0	0	50	25	50	0	100	0	100
14	3	0	0	3	100	100	100	0	0	33	0	100	100	100	0	100
15	5	0	1	4	100	100	80	0	100	0	100	20	60	20	0	20

shows the average number of fragments for each cluster, as well as the range of number of fragments. The average number of *Dra*I fragments was higher for isolates obtained from members of the Casuarinaceae (average number of fragments, 9; range, 4 to 12) than for isolates obtained from members of the Myricaceae (average, 7; range, 3 to 14) or from *Alnus* spp. (average, 6; range, 3 to 10).

Under the experimental conditions that are used now, not all frankiae can be shown to reinfect the hosts from which they were isolated (7, 18), and some frankiae are not

infective for any plant yet tested (8). For example, most isolates obtained from *Alnus*, *Myrica*, and *Comptonia* spp. are infective for their original hosts, but many strains obtained from other hosts, including *Casuarina*, *Elaeagnus*, *Hippophae*, *Ceanothus*, and *Purshia* spp., are not. As determined by LFRFA, noninfective strains obtained from members of the Casuarinaceae (found in clusters 3, 11, 12, and 15 or unclustered) (Fig. 1 and Table 1) did not cluster with infective strains (clusters 4, 6, 13, and 14). However, this was not true for either the noninfective strains obtained from *Alnus* spp. (strains Arh12 and R52) or the noninfective strains obtained from members of the Myricaceae (strains Mgl2b, Mgl2c, Mgl3, Mgl7, Mgl8, and R100). We also examined 22 strains (obtained from *Comptonia*, *Alnus*, *Myrica*, and *Casuarina* spp.) whose actual experimental infectivity characteristics (host compatibility groups [7, 8]) have been determined. The conclusion remained the same as the conclusion in the global analysis; the host plant from which a strain was isolated, not the infective capacity of the strain as determined under greenhouse conditions, largely dictated the cluster into which the strain fell (Table 3).

Relationship of LFRFA data to DNA-DNA relatedness data. With one exception (strain RBR5), a number of isolates obtained from a single plant host species (*Myrica pensylvanica*) whose DNA relatedness values have been determined previously (13) exhibited relationships that were very similar to relationships determined by DNA complementarity ranking when they were clustered by using LFRFA (Table 4). LFRFA cluster data for 11 strains common to this study and the DNA relatedness study of Fernandez et al. (21) showed that the clusters determined in the two studies were in substantial agreement (Fig. 3); 8 of 11 (73%) of the strains clustered similarly (the strains which exhibited differences were strains ARGP5^{Ag}, All11, and ACN1^{Ag}). The strains in LFRFA cluster A corresponded to genomic species 1 of Fernandez et al., but strain ACN1^{Ag}, which was also assigned to genomic species 1 by Fernandez et al., did not cluster in the LFRFA. Strains D11 and Cc12 did not cluster in either study. Strains ARGP5^{Ag} and All11 were members of LFRFA cluster B, but were assigned to two different genomic species (genomic species 3 and 9, respectively) by Fernandez et al. Strains CeF1.82, Cc13, CeD1.82, and Cj1.82 formed a single cluster in both studies.

FIG. 2. Gel obtained by pulsed-field electrophoresis of *Dra*I-digested DNAs from some representative *Frankia* strains. Lane A, standard (*S. cerevisiae* chromosomes; sizes [from bottom to top], 260, 290, 370, 460, 580, 650, 763, 835, 864, 991, 1,047, 1,285, and 1,500 kb); lane B, *Alnus* isolate Ar14; lane C, *Alnus* isolate 32-83; lane D, *Alnus* isolate Agv4ch; lane E, *Alnus* isolate ARGP5^{Ag}; lane F, *Casuarina* isolate Ce12; lane G, *Myrica* isolate RBR3; lane H, *Myrica* isolate RBR18; lane I, *Myrica* isolate 32-27.

TABLE 2—Continued

% of <i>Dra</i> I fragments in the following size ranges:															No. of bands	
570 to 599 kb	600 to 629 kb	630 to 659 kb	660 to 689 kb	690 to 719 kb	720 to 749 kb	750 to 769 kb	770 to 799 kb	800 to 829 kb	830 to 859 kb	860 to 889 kb	890 to 919 kb	920 to 949 kb	950 to 979 kb	980 to 1,000 kb	Avg	Range
0	0	100	0	0	0	0	100	100	100	0	0	100	0	0	11	
0	0	80	0	0	0	60	0	20	100	40	0	60	0	0	9	6–10
66	0	66	0	0	66	0	0	33	33	0	0	100	0	0	7	6–8
30	0	10	0	70	0	0	0	10	80	10	0	0	0	10	6	3–9
100	0	0	0	0	0	0	0	100	50	100	0	0	0	50	7	5–9
0	0	40	0	0	0	40	20	40	0	100	0	0	0	40	7	4–9
17	8	0	8	8	0	66	0	75	25	42	0	8	0	17	6	5–7
36	21	7	0	0	0	36	0	14	78	43	0	43	0	71	5	3–7
20	0	0	0	0	0	40	0	0	20	0	0	0	0	100	4	3–7
0	50	0	0	0	50	0	50	0	100	0	0	0	0	0	6	4–7
17	0	33	0	66	17	0	0	17	0	0	17	100	0	66	6	6–7
0	0	80	0	20	20	20	0	60	80	0	0	0	0	100	10	8–12
25	0	0	0	25	25	25	0	25	75	0	0	75	25	0	8	5–10
0	0	100	0	0	0	0	0	66	33	0	100	0	0	0	10	9–11
40	0	20	0	0	0	0	0	0	40	0	80	0	80	100	10	8–10

Such a relationship was less apparent for a groups of nine strains obtained from *Alnus* spp. and members of the Myricaceae and the Casuarinaceae whose DNA hybridization values have also been determined previously (2) (Table 5). A recent study (34) on the levels of DNA relatedness of 11 *Frankia* strains included only two strains that were also included in our study (strains K114 and ArI4). Shi et al. (34) found that the level of DNA relatedness between these two strains was very low (15%); as determined by our LFRFA, the level of relatedness was also low (66%).

TABLE 3. Clusters of *Frankia* strains compared with host compatibility groups and original hosts

Cluster ^a	Strain	Host compatibility group ^b	Taxon of original host
I	CpI1	1	Myricaceae
	ArI3	1	<i>Alnus</i>
II	McI2b	3	Myricaceae
	CeD1.82	2	Casuarinaceae
	McI3	3	Myricaceae
	CcI3	2	Casuarinaceae
	CeF1.82	2	Casuarinaceae
	MgI8	4	Myricaceae
III	D11	3	Casuarinaceae
	G2	3	Casuarinaceae
	CeI2	3	Casuarinaceae
	CpI3	1	Myricaceae
IV	AirI1	1	<i>Alnus</i>
	MgI7	4	Myricaceae
	ArI4	1	<i>Alnus</i>
	ArI5	1	<i>Alnus</i>
	Avcl3	3	<i>Alnus</i>
	ArhI2	3	<i>Alnus</i>
V	32-30	1	<i>Alnus</i>
	R52	NI	<i>Alnus</i>
Not clustered	R100	NI?	Myricaceae
	R43	3	Casuarinaceae
	MgI1	4	Myricaceae

^a Clusters formed at levels of similarity of >75% (S_{SM}).

^b See reference 7. NI, noninfective; host compatibility group 1, infective for *Alnus* and *Myrica* spp.; host compatibility group 2, infective for members of the Casuarinaceae and *Myrica* spp.; host compatibility group 3, infective for members of the Elaeagnaceae and *Myrica* spp.; host compatibility group 4, infective only for members of the Elaeagnaceae.

Stability of LFRFA patterns. We also observed that the LFRFA patterns of a strain can vary spontaneously. An analysis of single-spore isolates obtained from *Frankia* sp. strain CeI5 (isolates CeSI5, CeI5SS1, CeI5SS2, and CeI5SS3) showed that two of them (isolates CeI5SS1 and CeI5SS2) differed from the parent by one or two fragments (Fig. 4). In contrast, nine single-spore isolates of *Frankia* sp. strain CcI3 were identical to the parent strain (data not shown). Plant passage could also affect LFRFA patterns. Substrains of *Frankia* sp. strain R43 passed through *Elaeagnus umbellata* (substrains R43EU2 and R43E2) and *Casuarina cunninghamiana* (substrain R43Er-2, which was reisolated from parenchymatous root tissue) differed from the original strain (Fig. 4). Despite their differences, the changed substrains clustered at levels of >75% with the parent. Other reisolates obtained from these same plant experiments were unchanged. No changes were found in substrains of strain Wg1.1 (substrains Wg1.1r4, Wg1.1r5, and Wg1.1r9) passed through *Alnus glutinosa*, a substrain of strain ACN1 passed through the same host (substrain ACN1^{Ag}), a substrain of strain CcI2 passed through *Gymnostoma papuanum* (substrain CcI2xGp), and a substrain of strain CcI3 passed through *Casuarina cunninghamiana* (substrain CcI3xCc). A substrain of strain ArI3 passed through *Alnus glutinosa* (substrain ArI3xAg) was the only plant-

TABLE 4. Levels of DNA relatedness and levels of LFRFA homology for *Frankia* strains obtained from *Myrica pensylvanica*

Strain	% DNA relatedness to strain RBR13 ^a	% LFRFA homology with strain RBR13 ^b
RBR13	100	100
RBR14	98	100
RBR10	98	90
RBR8	99	85
RBR18	59	65
RBR5	12	68
RBR20	54	68
RBR21	57	63
RBR3	48	60
RBR9	35	47

^a Data from reference 13.

^b As determined by using the S_{SM} .

FIG. 3. Dendrogram showing 11 *Frankia* strains obtained from *Alnus* spp. (strains Arl3 and ACN1^{Ag}) and members of the Myricaceae (strain Cpl1) and the Casuarinaceae (strains Ccl2, Ccl3, CED1.82, CEF1.82, Cj1.82, and D11) clustered for comparison with the genomic species assignments of Fernandez et al. (21). See text. NC, not clustered.

passed substrain that did not cluster reasonably closely with its parent (level of similarity, 67%) and may have been a contaminant.

It is perhaps worth noting that isolates Avcl1 and Wg1.1 (a substrain of strain Avcl1) clustered at a level of only 96% in our experiments. Strain Avcl1 was originally described by Baker and Torrey in 1980 (5) and was sent to the laboratory of A. Akkermans in Wageningen, The Netherlands, at about that time. After an interval of several years, the strain in the Netherlands (strain Wg 1.1 [= WgAvcl1]) was sent to the University of Tennessee, where An et al. (3) compared its DNA restriction patterns with those of the original strain. Using five different restriction enzymes, these authors identified several major new bands in strain Wg 1.1, showing that genetic variation had occurred. Using LFRFA, we observed that the original strain Avcl1 produced six major bands, whereas strain Wg1.1 produced only five bands, thus confirming the genetic variation observed by An et al.

Coisolates (strains isolated from the same plant nodule) sometimes varied slightly in their LFRFA patterns (for example, strains 32-63 and 32-74 obtained from *Myrica pensylvanica* in New Jersey [Fig. 5] and strains 32-37 and 32-21 obtained from *Myrica pensylvanica* in Massachusetts [Fig. 6]). Both of these pairs of strains clustered at a level of 92%. Even isolates obtained from two plants belonging to different species growing in the same region can be very similar; for example, strains Cg11 and Ce15, which were

isolated from *Casuarina glauca* and *Casuarina equisetifolia*, respectively, growing on the banks of the same river, were 93% related (Fig. 4). In contrast, strains 32-83 and 32-85, which were isolated from the same *Alnus rugosa* plant growing in Massachusetts, clustered at a level of only 78% (data not shown).

Two mutants of strain 32-27, strain 4-D(+), which was produced by near-UV irradiation (366 nm) in the presence of 8-methoxypsoralen, and strain 8-120, which was produced by treatment with *N*-nitro-*N'*-nitrosoguanidine, clustered with the parent at a similarity level of 82% (Fig. 6). A

FIG. 4. Pulsed-field gel of the *Dra*I-restricted DNAs of *Frankia* isolates obtained from members of the Casuarinaceae. Lane A, *S. cerevisiae* standard (see the legend to Fig. 2 for the sizes of the chromosomes); lane B, strain Cg11; lane C, strain Ce15; lane D, strain Ce15SS1; lane E, strain Ce15SS2; lane F, strain R43E2; lane G, strain R43Er-2; lane H, strain R43 (parent). See text.

TABLE 5. Levels of DNA relatedness and levels of LFRFA homology for various *Frankia* strains

Strain	% DNA relatedness to strain Arl4 ^a	% LFRFA homology with strain Arl4 ^b
Arl4	100	100
ACN1 ^{Ag}	94	67
Cpl1	89	67
32-27(Mp11)	72	78
32-44(Air11)	72	81
Avcl1	67	78
32-30(Air12)	27	70
G2	36	59
D11	28	59

^a Data from reference 2.

^b As determined by using the *S*_{SM}.

FIG. 5. Pulsed-field gel of *Frankia* coisolates obtained from *Myrica pensylvanica*. Lane A, *S. cerevisiae* standard (see the legend to Fig. 2 for the sizes of the chromosomes); lane B, strain 32-63; lane C, strain 32-74.

spontaneous mutant of strain R43, strain R43A₂, whose phenotypic behavior has been studied extensively (29), was identical to its parent in its LFRFA patterns.

DISCUSSION

In this study our aim was to cast some light on the problem of species definition in the genus *Frankia*. Because of the evolutionary constraints that the symbiotic state undoubtedly imposes, we felt that groups that could be equated with species might be delineated. We also felt that in organisms that have DNAs with such high guanine-plus-cytosine contents (4), the AT-rich sequences must have substantial significance for the organisms. Using *Dra*I, a restriction enzyme that has a TTT'AAA recognition site, we generated DNA digests which contained large fragments (200 to 1,000 kb) that were separable by using pulsed-field electrophoresis. Using this technique, we found that each strain produces a DNA fragment "fingerprint" that is characteristic of the strain. This was also the result that was obtained by other workers who used restriction enzymes that yielded many smaller fragments (3, 13, 16, 19). However, like Bloom et al. (13), we found that our organisms tended to fall into groups, and like Dobritsa and Stupar (20), we found that some coisolates differed very slightly in their patterns. In studies of other bacteria, species-specific restriction patterns have been found for high-molecular-weight fragments of *Brucella* DNA (1), *Mycobacterium tuberculosis* DNA (37), and DNAs of certain *Streptomyces* species (11).

Because the number of our *Frankia* restriction fragments was relatively small, we calculated the sizes of the fragments and subjected them to a numerical analysis. Of the 15 clusters that were identified at the >75% similarity level (Fig. 1), 9 were dominated (80 to 100%) by isolates obtained from *Alnus* spp. or members of the Myricaceae, 4 were dominated (75 to 100%) by isolates obtained from members of the Casuarinaceae, and 2 were mixed. Many, but not all, of the strains that were actually infective for *Casuarina* spp.

FIG. 6. Pulsed-field gel of mutants and coisolates of *Frankia* sp. strain 32-27 obtained from *Myrica pensylvanica*. Lane A, *S. cerevisiae* standard (see the legend to Fig. 2 for the sizes of the chromosomes); lane B, strain 32-27; lane C, coisolate 32-21 (note the loss of a small band at <260 kb); lane D, coisolate 32-22; lane E, mutant 8-120 (note the loss of a doublet at ca. 760 kb); lane F, mutant 8-30(+); lane G, mutant 8-30(-); lane H, mutant 4D₂; lane I, mutant 4D₂(+) (note the loss of a doublet at ca. 760 kb and the gain of a fragment at ca. 860 kb).

(strains CeD1.82, CeF1.82, CcI3, and Cj1.82) were found in the major mixed cluster (cluster 4). (As discussed above, many strains isolated from members of the Casuarinaceae and most of the strains isolated from this plant family which we analyzed are not infective for their original hosts.) Thus, our results provide additional evidence which supports the conclusion of Lalonde et al. (24) and Fernandez et al. (21) that many types of analyses (DNA-DNA relatedness, cell polypeptides, isozymes, and fatty acid composition) produce groups of *Frankia* strains that are consistent with the host plants from which the strains are isolated.

However, there are a number of problems. The first is that the strains which we analyzed differed from the strains that were studied by Lalonde et al., with five exceptions (strains ACN1^{AG}, ARgP5^{AG}, ArI3, CpI1, and ACX50h). According to our data and the data of other workers, strains ArI3 and CpI1 are 100% homologous on the basis of LFRFA results (this paper), cell polypeptide data (23), isozyme data (22), *nif* sequence data (33), and plasmid data (36). As both of these strains were isolated in the same laboratory, we believe that it is possible that strain ArI3 is a reisolate of strain CpI1. However, strain CpI1, which Lalonde et al. (24) designated the type strain of "*F. alni* subsp. *pommerii*," did not cluster closely with the 36 *Alnus* isolates used in our analyses (Fig. 1), although it did cluster in fatty acid and isozyme analyses with the *Alnus* isolates used by Lalonde and his colleagues (22, 35). It is possible that if we submitted the strains of Lalonde et al. that were used in the studies discussed above to LFRFA, we would find that they are very similar to strain CpI1; however, on the basis of our results obtained for the few common *Alnus* isolates analyzed both by us and by

Lalonde et al., this seems unlikely. For example, both in the isozyme study and in the fatty acid study, the authors placed strain Cp11 in the same group as strain ACX50h (although these strains were actually in two different subgroups). In the same studies, strain Cp11 was clearly segregated from strain ARgP5^{AG}. In our study, strain Cp11 was very distant from both strain ACX50h (level of similarity, 63%) and strain ARgP5^{AG} (level of similarity, 52%). In the isozyme study (22), strain ACN1^{AG} was identical to strain Cp11; in our analyses, these strains exhibited only a very low level of similarity (48%). In a study of the conservation of *nif* sequences (33), strains ACN1^{AG} and Ar15 were shown to be 100% related to strain Cp11; in our study these strains were related at levels of 48 and 55%, respectively. In the DNA hybridization experiments of Fernandez et al. (21), strains Cp11 and Ar13 were found to be related to a central strain (strain ACON24d) at levels of 81 and 82%, respectively, but no relatedness data for the combination of strains Cp11 and Ar13 were reported. Strain ARgP5^{AG} was related at a level of only 2% to this same central strain, underlining its uniqueness among the *Alnus* isolates. It is clear that on the basis of the criteria of Lalonde et al., strain Cp11 is closely related to the *Alnus* isolates which these authors used, whereas in our study, in which we used LFRFA, this strain did not cluster closely with the strains which we examined. The apparent identity of strains Cp11 and Ar13 in all of the studies appears to rule out a difference in the strain Cp11 cultures used by the two groups of workers. It seems possible that LFRFA is more sensitive to differences among strains than the biochemical tests used by Lalonde et al.

In a study of levels of DNA relatedness among 43 *Frankia* strains, Fernandez et al. (21) proposed nine genomic species on the basis of their results. Although our LFRFA data for a small number of strains common to both studies yielded results that were reasonably congruent with the results of Fernandez et al. (Fig. 3), more LFRFA analyses of the strains of Fernandez et al. will be necessary to determine whether the genomic groups of Fernandez et al. are truly supported by the results of our method. In addition, it is difficult to make a final judgment about the validity of the groups of Fernandez et al., since of the 1,849 possible combinations (43 times 43) in their study, they reported test results for only 121 combinations. Thus, the distinctiveness of their various genomic species has not been fully validated by actual hybridization data.

A second problem concerns the fact that in setting up the taxon "*F. alni* subsp. *pommerii*" (24), according to the *International Code of Nomenclature of Bacteria*, Lalonde et al. automatically created the taxon *F. alni* subsp. *alni* (25), but they may not have been aware that they had done so. Of course, *F. alni* subsp. *alni* would be the type subspecies of the genus. Thus, a type strain should be designated for this taxon. Furthermore, it is clear from studies of other workers and our studies that not enough data have been generated for a common pool of strains to warrant designation of a type strain at this time. We confirmed the finding of Lalonde et al. that strain ARgP5^{AG} is distinct from strain Cp11; however, we did not confirm that strain Cp11 is a representative of the *Alnus* host group in general. We do not believe that our results stem from inadequate sampling; our strains came from more diverse regions than the strains of Lalonde et al. However, strain Cp11 is undoubtedly representative of certain types of isolates obtained from *Alnus* spp. and members of the Myricaceae and should thus be retained as the type strain of "*F. alni* subsp. *pommerii*."

The third problem is that some *Frankia* strains appear to

be somewhat mutable (e.g., strains R43, Ce15, and 32-27) when they are assessed by LFRFA, whereas other strains (e.g., strain Cc13) appear to be quite stable. This mutability may be inherent in certain strains. Our observation that the LFRFA patterns for single-spore isolates from a given parent strain (e.g., strains CeS15, Ce15SS1, and Ce15SS2) can vary from the pattern of the parent may well indicate the basic mechanism for the similar observed variability in some plant-passed reisolates. Mutations in frankiae may be the result of amplifications or deletions of certain sequences, a phenomenon common in other actinomycetes (12, 17). They could also be due to base methylation or transposable elements. Nonetheless, mutants and variants of *Frankia* strains in this study generally clustered closely with their parents (e.g., clusters 7 and 13). However, given that our overall similarity calculations might be termed a snapshot in time, it is possible that repeated passages through various hosts may cause profound changes as assessed by LFRFA, but we have not performed such experiments. However, as our strains included not only recent isolates (e.g., strain R43EU2, which was analyzed immediately following isolation) but also isolates maintained by transfer in vitro for as long as 14 years (strain Cp11), it is significant that our clusters reflect host origin as well as they do. The role of the sequence TTT'AAA in the plant host-endophyte relationship can only be speculated on. However, the compatibility of our findings with those of other workers leads us to propose that LFRFA data should be seriously considered as a valid criterion for grouping *Frankia* strains into species.

Although we do not feel that we can propose a specific strain as the type strain of *F. alni* subsp. *alni* at this time, we believe that such a strain should meet the criteria described below. It should be infective for *Alnus* sp. and be effective (*nif*⁺). It should be morphologically typical of the genus and have appropriate cell wall chemistry (i.e., type III sensu Lechevalier and Lechevalier [31]) and whole-cell chemistry. In addition, it should be reasonably homologous with a large representative group of strains isolated from *Alnus* spp. growing in very diverse regions of the world as determined by the following criteria: DNA relatedness data, LFRFA data, isozyme analysis data, fatty acid analysis data, cell sugar analysis data, data on restriction fragment length polymorphisms probed with *nifH*, and cell polypeptide patterns.

Finally, we urge the various groups involved in studies of *Frankia* taxonomy to agree on a common pool of strains supplied by a single laboratory as a basis for further work and to try to arrive at a consensus on an appropriate type strain for the genus.

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REFERENCES

1. Allardet-Servent, A., G. Bourg, M. Ramuz, M. Pages, M. Bellis, and G. Roizes. 1988. DNA polymorphism in strains of the genus *Brucella*. J. Bacteriol. 170:4603-4607.
2. An, S. C., W. S. Riggsby, and B. C. Mullin. 1985. Relationships

- of *Frankia* isolates based on deoxyribonucleic acid homology studies. *Int. J. Syst. Bacteriol.* **35**:140–146.
3. An, S. C., W. S. Riggsby, and B. C. Mullin. 1985. Restriction pattern analysis of genomic DNA of *Frankia* isolates. *Plant Soil* **87**:43–48.
 4. An, S. C., J. W. Wills, W. S. Riggsby, and B. C. Mullin. 1983. Deoxyribonucleic acid base composition of 12 *Frankia* isolates. *Can. J. Bot.* **61**:2859–2862.
 5. Baker, D., and J. G. Torrey. 1980. Characterization of an effective actinorhizal microsymbiont, *Frankia* sp. Avc11 (Actinomycetales). *Can. J. Microbiol.* **26**:1066–1071.
 6. Baker, D. D. 1987. Modification of catalog numbering system for *Frankia* strains and revision of strain numbers for DDB *Frankia* collection. *Actinomycetes* **20**:85–88.
 7. Baker, D. D. 1987. Relationships among pure-cultured strains of *Frankia* based on host specificity. *Physiol. Plant.* **70**:245–248.
 8. Baker, D. D. (Yale University). 1991. Personal communication.
 9. Becking, J. H. 1970. Frankiaceae fam. nov. (Actinomycetales) with one new combination and six new species of the genus *Frankia* Brunchorst 1886, 174. *Int. J. Syst. Bacteriol.* **20**:201–220.
 10. Becking, J. H. 1974. Family III. *Frankiaceae* Becking 1970, 201, p. 701–706. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*. The Williams & Wilkins Co., Baltimore.
 11. Beyazova, M. L., and M. P. Lechevalier. Unpublished data.
 12. Birch, A., A. Hausler, and R. Hutter. 1990. Genome rearrangement and genetic instability in *Streptomyces* spp. *J. Bacteriol.* **172**:4138–4142.
 13. Bloom, R. A., B. C. Mullin, and R. L. Tate III. 1989. DNA restriction patterns and DNA-DNA solution hybridization studies of *Frankia* isolates from *Myrica pensylvanica* (bayberry). *Appl. Environ. Microbiol.* **55**:2155–2160.
 14. Burggraaf, A. J. P., and W. A. Shipton. 1983. Studies on the growth of *Frankia* isolates in relation to infectivity and nitrogen fixation (acetylene reduction). *Can. J. Bot.* **61**:2774–2782.
 15. Callaham, D., P. Del Tredici, and J. G. Torrey. 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* **199**:899–902.
 16. Cramer, R., G. Hintermann, and R. Hutter. 1983. Deoxyribonucleic acid restriction endonuclease fingerprint characterization of actinomycete strains. *Int. J. Syst. Bacteriol.* **33**:652–655.
 17. Cullum, J., J. Altenbuchner, A. W. Birch, F. Flett, K. Kendall, J. Platt, and W. Piendl. 1986. DNA arrangements in *Streptomyces*, p. 35–44. In G. Szabo, S. Biro, and M. Goodfellow (ed.), *Sixth International Symposium on Actinomycetes Biology*. Akademiai Kiado, Budapest.
 18. Diem, H. G., D. Gauthier, and Y. R. Dommergues. 1982. Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. *Can. J. Microbiol.* **28**:526–530.
 19. Dobritsa, S. V. 1985. Restriction analysis of the *Frankia* spp. genome. *FEBS Microbiol. Lett.* **29**:123–128.
 20. Dobritsa, S. V., and O. S. Stupar. 1989. Genetic heterogeneity among *Frankia* isolates from root nodules of individual actinorhizal plants. *FEMS Microbiol. Lett.* **58**:287–292.
 21. Fernandez, M. P., H. Meugnier, P. A. D. Grimont, and R. Bardin. 1989. Deoxyribonucleic acid relatedness among members of the genus *Frankia*. *Int. J. Syst. Bacteriol.* **39**:424–429.
 22. Gardes, M., J. Bousquet, and M. Lalonde. 1987. Isozyme variation among 40 *Frankia* strains. *Appl. Environ. Microbiol.* **54**:1596–1603.
 23. Gardes, M., and M. Lalonde. 1987. Identification and subgrouping of *Frankia* strains using sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Physiol. Plant.* **70**:237–244.
 24. Lalonde, M., L. Simon, J. Bousquet, and A. Seguin. 1988. Advances in the taxonomy of *Frankia*: recognition of species *alni* and *elaeagni* and novel subspecies *pommerii* and *vandijkii*, p. 671–680. In H. Bothe, F. J. de Bruijn, and W. E. Newton (ed.), *Nitrogen fixation: hundred years after*. Gustav Fischer Verlag, Stuttgart, Germany.
 25. Lapage, S. P., P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, and W. A. Clark (ed.). 1975. International code of nomenclature of bacteria. 1976 Revision. American Society for Microbiology, Washington, D.C.
 26. Lechevalier, M. P. 1983. Cataloging *Frankia* strains. *Can. J. Bot.* **61**:2964–2967.
 27. Lechevalier, M. P. 1984. The taxonomy of the genus *Frankia*. *Plant Soil* **78**:1–6.
 28. Lechevalier, M. P. 1986. Catalog of *Frankia* strains. *Actinomycetes* **19**:131–162.
 29. Lechevalier, M. P., D. P. Labeda, and J.-S. Ruan. 1987. Studies of *Frankia* sp. LLR 02022 from *Casuarina cunninghamiana* and its mutant LLR 02023. *Physiol. Plant.* **70**:249–254.
 30. Lechevalier, M. P., and H. A. Lechevalier. 1989. Genus *Frankia* Brunchorst 1886, 174^{AL}, p. 2410–2417. In S. T. Williams, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 4. The Williams & Wilkins Co., Baltimore.
 31. Lechevalier, M. P., and H. A. Lechevalier. 1990. Systematics, isolation and culture of *Frankia*, p. 35–60. In C. R. Schwintzer and J. D. Tjepkema (ed.), *The biology of Frankia and actinorhizal plants*. Academic Press, San Diego, Calif.
 32. Mansour, S. R., A. Dewedar, and J. G. Torrey. 1990. Isolation, culture and behavior of *Frankia* strain HFP Cg14 from root nodules of *Casuarina glauca*. *Bot. Gaz.* **151**:490–496.
 33. Normand, P., P. Simonet, and R. Bardin. 1988. Conservation of *nif* sequences in *Frankia*. *Mol. Gen. Genet.* **213**:238–246.
 34. Shi, Y., J. Ruan, J. Zakrzewska-Czerwinska, and M. Mordarski. 1991. DNA homology of some *Frankia* strains. *Actinomycetes* **24**:86–88.
 35. Simon, L., S. Jabaji-Hare, J. Bousquet, and M. Lalonde. 1989. Confirmation of *Frankia* species using cellular fatty acid analysis. *Syst. Appl. Microbiol.* **11**:229–235.
 36. Simonet, P., P. Normand, A. Molroux, and M. Lalonde. 1985. Restriction enzyme digestion patterns of *Frankia* plasmids. *Plant Soil* **87**:49–60.
 37. Smith, C. L. (Columbia University). 1986. Personal communication.
 38. Smith, C. L., and C. R. Cantor. 1987. Purification, specific fragmentation and separation of large DNA molecules. *Methods Enzymol.* **155**:449–467.
 39. Sneath, P. H. A., and R. R. Sokal. 1973. Numerical taxonomy. W. H. Freeman and Co., San Francisco.