Deoxyribonucleic Acid Relatedness among Members of the Genus *Frankia*

M. P. FERNANDEZ,^{1*} H. MEUGNIER,² P. A. D. GRIMONT,³ AND R. BARDIN¹

Laboratoire d'Ecologie microbienne, Unité de Recherche Associée au Centre National de la Recherche Scientifique 697, Université Lyon 1, F-69622 Villeurbanne Cedex, France¹; Centre National des Staphylocoques, Laboratoire de Bactériologie, Faculté de Médecine Alexis Carrel, F-69372 Lyon Cédex 8, France²; and Institut National de la Santé et de la Recherche Médicale Unité 199, Unité des Entérobactéries, Institut Pasteur, F-75724 Paris Cédex 15, France³

The diversity among 43 isolates of the genus *Frankia* was studied by determining levels of deoxyribonucleic acid relatedness (S1 nuclease method) and DNA base compositions. The guanine + cytosine contents ranged from 66 to 75 mol%. At least nine genomic species were differentiated, including three genomic species among strains compatible with members of the genus *Alnus*, five genomic species among strains compatible with members of the family Elaeagnaceae, and one genomic species among strains compatible with members of the genus *Casuarina*. Genomic species 1, which contained proposed type strain Cp11 and nine strains that were compatible with members of the genus *Alnus* and were 64 to 97% related to strain ACoN24d, is *Frankia alni*. Four genomic species contained single strains that were 0 to 30% related to the other genomic species. Typical strains isolated from members of the genus *Casuarina* were found to be very homogeneous (69 to 100% related to strain ORS020606) and clearly separated from atypical strains. The nine genomic species delineated in this study cannot be named since no phenotypic tests are available for identification.

The N₂-fixing *Frankia*-actinorhizal plant symbiosis has been reported to occur in more than 194 species of woody dicotyledons belonging to 24 genera and eight diverse families (4, 37). The members of the genus *Frankia*, which was proposed by Becking (7) as the only genus of the family *Frankiaceae* (*Actinomycetales*), are recognized primarily on the basis of the symbiotic capacity of the endophytes and their unique morphology in root nodule tissues. Recent studies in which 16S ribosomal ribonucleic acid sequencing (17) confirmed morphological similarities (23) led to a proposal to include the genus *Geodermatophilus* in the *Frankiaceae*.

Various attempts have been made to delineate groups within the genus *Frankia*. Since no isolates were available, the first attempt was based on studies of inoculation compatibility groups in which crushed nodule inocula were used. Thus, 10 species were described, with *Frankia alni* as type species (8). Since the first confirmed isolation by Callaham et al. (12), hundreds of strains have been obtained from various host plants, allowing the use of more accurate methods of characterization. Thus genus *Frankia* is now relatively well described as a group of symbiotic, filamentous, gram-positive, sporangium-forming, vesicle-forming actinomycetes (28). Other characteristics, such as type III cell wall composition (30), type I phospholipids (29, 31), deoxyribonucleic acid (DNA) base ratios ranging from 68 to 72 mol% (2), and the presence of 2-O-methyl-D-mannose (33), seem to occur commonly in strains of *Frankia*. Using pure cultures as inocula, Baker (3) redefined the host specificity groups described previously. He found four overlapping groups, which demonstrated the inadequacy of the previous taxonomic system (8). The three major groups contain strains that are compatible with members of the genus *Alnus*, strains that are compatible with members of the family Elaeagnaceae, and strains that are compatible with members of the genus *Casuarina*. The first two groups have been separated by various studies in which physiological, immuno-

chemical, and genomic characteristics have been used (1, 6, 41; P. Simonet, P. Normand, A. Hirsh, and A. D. L. Akkermans, in P. M. Gresshoff, ed., *The Molecular Biology of Symbiotic Nitrogen Fixation*, in press). Few data are available concerning the later group of strains compatible with members of the genus *Casuarina* due to the small number of strains available.

The low growth rate of strains of *Frankia* and difficulties in the isolation of high-molecular-weight DNA (Simonet, Normand, Hirsh, and Akkermans, in press) have limited genomic relatedness studies. Using DNA base composition and DNA-DNA hybridization methods, An et al. (1, 2) described a genomic group within the strains compatible with members of the genus *Alnus* and showed a high degree of heterogeneity among the strains compatible with members of the Elaeagnaceae. DNA guanine-plus-cytosine (G+C) contents, the homogeneity of which did not allow An et al. to delineate groups, ranged from 68 to 72 mol%.

The purpose of this work was to examine the genetic diversity of the genus *Frankia* by using DNA-DNA hybridization and DNA base composition and a larger spectrum of strains isolated from various geographical areas. As a result, more than nine genomic species were found.

MATERIALS AND METHODS

Bacterial strains. The sources of the strains of *Frankia* which we studied are shown in Table 1. The strains were grown at 28°C in F medium (40) (strains belonging to the *Alnus* compatibility group), F medium without Tween 80 (strains belonging to the Elaeagnaceae compatibility group), or BAP medium (34) (strains belonging to the *Casuarina* compatibility group).

DNA extraction. Previously described methods were used for the extraction (39) and purification (11) of DNA.

Determination of base composition. The chromatographic method of Gehrke et al. (22) was used to determine base composition. Tubes containing 50 µg of DNA in 250 µl of distilled water were heated at 100°C for 5 min and then plunged into ice. The denatured DNA was then hydrolyzed by adding 200 µl of 30 mM sodium acetate solution (pH 5.3),

* Corresponding author.

TABLE 1. Origins of strains of *Frankia* tested

Strain			Original host	Geographic origin	Reference
Serial no.	Registry no.	Other designation			
<i>Alnus</i> compatibility group					
1	ULF01010244	ACoN24d	<i>Alnus cordata</i>	Orléans, France	38
2	ULF0131024083	Ar24H3	<i>Alnus rubra</i>	Orléans, France	Simonet et al. ^a
3	ULF0131024152	Ar24O2	<i>Alnus rubra</i>	Orléans, France	Simonet et al. ^a
4	ULF0107024251	Ag24 ₂₅₁	<i>Alnus glutinosa</i>	Orléans, France	Unpublished data
5	HFP013003	ArI3	<i>Alnus rubra</i>	Oregon	9
6	ULQ013202204	ARgN22d	<i>Alnus rugosa</i>	Québec, Canada	35
7	ULQ0102001007	ACN1 ^{AG}	<i>Alnus crispa</i>	Québec, Canada	25
8	HFP07010701	CpI1	<i>Comptonia peregrina</i>	Massachusetts	12
9	ULF0141016011	A2J	<i>Alnus viridis</i>	Samoens, France	Unpublished data
10	ULF014102203	AV22c	<i>Alnus viridis</i>	Lautaret, France	16
11	ULF014101715	AVN17o	<i>Alnus viridis</i>	La Toussuire, France	Unpublished data
12	ULF010102340	Ac23 ₄₀	<i>Alnus cordata</i>	Saou, France	Unpublished data
13	ULF011104301	AI43 ₁	<i>Alnus incana</i>	Articol, France	Unpublished data
14	ULQ0132105009	ARgP5 ^{AG}	<i>Alnus rugosa</i>	Québec, Canada	35
15	ULF01070602	Mg60 ₂ ^{AG}	<i>Alnus glutinosa</i>	Landes, France	Unpublished data
16	ULF010102171	AcVc1	<i>Alnus cordata</i>	Corsica, France	Unpublished data
17	UGL010701	UgAg111b	<i>Alnus glutinosa</i>	Glasgow, Scotland	43
18	UGL010708	UgAg118b	<i>Alnus glutinosa</i>	Glasgow, Scotland	43
<i>Elaeagnaceae</i> compatibility group					
19	ULF130100112	Ea1 ₁₂	<i>Elaeagnus angustifolia</i>	Ecully, France	Unpublished data
20	ULF130100102	Ea1 ₂	<i>E. angustifolia</i>	Ecully, France	Unpublished data
21	ULF130100206	Ea2 ₆	<i>E. angustifolia</i>	Miribel, France	Unpublished data
22	ULF130100303	Ea3 ₃	<i>E. angustifolia</i>	St. Bonnet, France	Unpublished data
23	ULF130100501	Eacm5 ₁	Soil on <i>E. angustifolia</i>	L'Escarène, France	Unpublished data
24	ULF140102714	HR27 ₁₄	<i>Hippophaë rhamnoides</i>	Foncouverte, France	Unpublished data
25	ULQ00231058	TX31e ^{HR}	Soil on <i>H. rhamnoides</i>	Québec, Canada	10
26	ULQ130100144	EAN1pec	<i>E. angustifolia</i>	Ohio	26
27	ULF140104001	HRX401a	<i>H. rhamnoides</i>	Ornon, France	36
28	ULQ132500106	EUN1f	<i>Elaeagnus umbellata</i>	Illinois	26
29	ULF140101801	HRN18a	<i>H. rhamnoides</i>	Alps, France	32
30	ULF130105001	Ea50 ₁	<i>E. angustifolia</i>	Mions, France	Unpublished data
31	ORS060501		<i>Colletia spinosissima</i>	Argentina	21
32	GFN140101	GFN14	<i>H. rhamnoides</i>	People's Republic of China	Unpublished data
<i>Casuarinaceae</i> compatibility group					
33	ORS020606	CeD	<i>Casuarina equisetifolia</i>	Dakar, Senegal	14
34	ORS022602		<i>Allocasuarina torulosa</i>	Australia	Nazaret et al. ^b
35	ORS020607	CeF	<i>Casuarina equisetifolia</i>	Florida	14
36	HFP020203	CcI3	<i>Casuarina cunninghamiana</i>	Florida	44
37	ORS020608		<i>Casuarina equisetifolia</i>	Brazil	Nazaret et al. ^b
38	HFP022801	AlII1	<i>Allocasuarina lehmaniana</i>	Florida	45
39	ORS021001	Cj1-82	<i>Casuarina junghuniana</i>	Thailand	15
40	ORS020609		<i>Casuarina equisetifolia</i>	Madagascar	Nazaret et al. ^b
41	HFP020202	CcI2	<i>Casuarina cunninghamiana</i>	Florida	44
42	ORS020602	D11	<i>Casuarina equisetifolia</i>	Dakar, Senegal	20
43	DDB020110		<i>Casuarina</i> sp.	Hawaii	5

^a Simonet, Thi Le, Moiroud, and Bardin, in press.^b Nazaret et al., in press.

5 µl of 20 mM zinc sulfate, and 10 µl of P1 nuclease (Boehringer GmbH, Mannheim, Federal Republic of Germany) and incubating the tubes for 3 h at 37°C. The chromatographic system used for the separation and quantitation of nucleotides was the following: C₁₈ reversed-phase column (Société Française de Chromatographie); solvent, 0.6 M NH₄H₂PO₄, pH 4.25; flow rate, 1.5 ml/min; absorption measured at 260 nm.

DNA-DNA hybridizations. Native DNA was labeled in vitro by nick translation with tritium-labeled nucleotides (Amersham International, Amersham, England). The procedure used for the hybridization experiments (S1 nuclease-trichloroacetic acid method) has been described previously

(13, 24). The reassociation temperature was 75°C. DNA-DNA hybridization tests were carried out by using labeled DNAs from *Frankia* spp. strains ACoN24d, AV22c, ARgP5^{AG}, Ea1₁₂, TX31e^{HR}, EUN1f, HRN18a, Ea50₁, and ORS020606 (serial numbers 1, 10, 14, 19, 25, 28, 29, 30, and 33, respectively) as probes.

Thermal stability of reassociated DNAs. The temperature (*T_m*) at which 50% of the reassociated DNA became hydrolyzable by the S1 nuclease was determined as described previously (13), with slight modifications. After DNA hybridization was completed (16 h at 75°C), the hybridization mixture was diluted 1:2 (final NaCl concentration, 0.21 M) with distilled water to bring the *T_m* below the boiling

temperature of the mixture. The hybridization mixture was incubated in a glycerol bath, and the temperature was increased in 5°C steps from 75 to 105°C. At each temperature step, a sample was withdrawn, mixed with S1 buffer, and treated with S1 nuclease at 60°C (24). In addition, a sample withdrawn at 75°C was mixed with S1 buffer without addition of S1 nuclease. This served to determine the radioactivity of unhydrolyzed DNA (24).

The value called ΔT_m is the difference between the T_m of the heteroduplex (in the heterologous reaction) and the T_m of the homoduplex (in the homologous reaction).

RESULTS

The G+C contents of the strains ranged from 66.0 to 75 mol% (Table 2).

Unstandardized reassociation values for homoduplexes ranged from 75 to 100%. The levels of reassociation in control tubes containing only labeled DNAs ranged from 6 to 11%. The T_m values for homoduplexes (in 0.2 M NaCl) ranged from 83.5 to 96.7°C.

Of the 43 strains tested, 34 fell into nine discrete DNA relatedness groups (genomic species) (Table 2). The remaining nine strains were not studied further and thus may represent one or more additional groups. Genomic species 1 contained nine strains that were compatible with members of the genus *Alnus* and were 60 to 97% related to strain 1, with low levels of divergence (ΔT_m , less than 3°C). Four other strains compatible with members of the genus *Alnus* formed a tight genomic species (genomic species 2) that was more than 80% related to strain 10. Genomic species 4 contained six strains that were 69 to 100% related to strain 19, with ΔT_m values less than 2.7°C. Three strains compatible with members of the Elaeagnaceae formed genomic species 5. These strains were more than 79% related to strain 25 and yielded low relative binding ratios (less than 49%) and high ΔT_m values (7°C) with genomic species 4. Genomic species 9 contained eight strains belonging to the *Casuarina* compatibility group. These strains were 69 to 100% related to strain 33, with ΔT_m values less than 4.3°C.

A total of 13 strains did not belong to any of the genomic species described above. Four of them (strains 14, 28, 29, and 30) were used as sources of reference DNA and were 0 to 30% related to the five genomic groups described above; thus, these four strains should be considered different genomic species (designated genomic species 3, 6, 7, and 8, respectively). The nine remaining strains were not studied further.

DISCUSSION

The G+C contents of DNAs have been reported to range from 68 to 72 mol% in the genus *Frankia* (2). In this study we found a wider range of G+C values, 66 to 75 mol%. Nevertheless, delineation of groups by using this criterion is not possible. The only exception could be genomic species 5, which included the strains with the lowest G+C contents.

The single species concept in the genus *Frankia* is untenable since at least nine genomic species can be delineated. The nine strains which fell into none of the delineated genomic species might represent one or more new genomic species. We did not attempt to resolve all genomic species since the cost and effort are disproportionate with the results when the genomic species contain single strains.

***Alnus* compatibility group.** At least three genomic species were found among the strains compatible with members of

the genus *Alnus*. Genomic species 1 corresponds to genomic group 1 of An et al. (1), which was represented in this study by strains Cp11 and ACN1^{AG}. In *Bergey's Manual of Determinative Bacteriology*, 8th ed., Becking (8) defined *F. alni* on the basis of its ability to nodulate members of the genus *Alnus* and *Frankia brunchorstii* on the basis of its ability to nodulate members of the genera *Myrica* and *Comptonia*. This proposal is inconsistent with our results and those of An et al. (1) since strain Mp11 (1) and strain 15 from *Myrica* sp. and strain 8 from *Comptonia* sp. fell into genomic species 1. Five strains of genomic species 1 were shown to have the same malate dehydrogenase, peptidase, and 6-phosphogluconate dehydrogenase electromorphs (electromorphs 7, 5, and 4, respectively) (18) and identical protein electrophoretic patterns (19). Lalonde et al. (27) have designated strain Cp11 (strain 8) as the type strain of *F. alni*. We support this proposal, and, as a consequence, our genomic species 1 corresponds to *F. alni*, which is presently the only valid species in the genus (type species).

Genomic species 2, which contained three strains isolated from *Alnus* species in France, has not been described previously.

Genomic species 3 contained a single strain, strain ArgP5^{AG}, which was found to produce specific malate dehydrogenase, peptidase, and 6-phosphogluconate dehydrogenase electromorphs (electromorphs 2, 1, and 1, respectively) (18). Our DNA data do not support the proposal of Lalonde et al. (27) that strain ArgP5^{AG} represents a subspecies of *F. alni* ("*Frankia alni* subsp. *vandijkii*") since it was only 2% related to genomic species 1.

Among the strains compatible with members of the genus *Alnus*, neither the original host genus nor the geographical origin seems to be related to the degree of genetic relatedness. For example, French strains isolated from *Alnus viridis* were expected to be closely related to each other because of the restricted area of the host in the Alps, but one of these strains belongs to genomic species 1, and the two other strains belong to genomic species 2. Strains 1, 2, and 3 of genomic species 1 have been isolated from the same alder stand but from different *Alnus* species. It may be asked whether these strains are really different or whether they represent four isolates of the same strain. These organisms were compared by using different molecular characterization techniques (P. Simonet, N. Thi Le, A. Moiroud, and R. Bardin, Plant Soil, in press) and were shown to be closely related but separable by these methods. Consequently, in this case, host genotype does not seem to take a prominent part in the sorting of the strains from the soil since different *Alnus* species selected the same *Frankia* genomic species from the soil. Nevertheless, it remains to be demonstrated whether this result is correlated with a real homogeneity of the *Frankia* population in this soil. Other explanations could be the better competitiveness of this genomic species for nodulation or for growth in pure culture after isolation.

Elaeagnaceae compatibility group. In *Bergey's Manual*, 8th ed., Becking (8) proposed a single species ("*Frankia elaeagnii*") for strains that nodulate members of the family Elaeagnaceae (the genera *Elaeagnus*, *Hippophaë*, and *Shepherdia*). Lalonde et al. (27) agreed with this proposal. However, this proposal is not supported by DNA relatedness data. Recent results obtained by using various characterization methods (1, 36, 41; Simonet, Normand, Hirsh, and Akkermans, in press) have indicated that the group of strains compatible with members of the Elaeagnaceae is a highly heterogeneous group. Our results showed that at least two genomic species containing six and three strains and three

TABLE 2. Levels of DNA relatedness among strains of *Frankia* species

Source of unlabeled DNA (strain)	G+C content (mol%)	% Reassociation at 75°C with labeled DNA from:								
		Strain 1	Strain 10	Strain 14	Strain 19	Strain 25	Strain 28	Strain 29	Strain 30	Strain 33
<i>Alnus</i> compatibility group										
Genomic species 1										
1	74	100 ^a	13	22	7	— ^b	0	6	0	—
2	75	81 (0) ^c	—	—	—	—	0	—	—	—
3	71	97	—	—	—	—	—	—	—	3
4	72	60 (2.7)	—	—	—	—	—	—	—	—
5	71	82	—	—	—	—	—	—	—	—
6	—	77	—	32	—	—	—	—	—	—
7	73	87 (0.5)	—	—	—	—	—	—	—	—
8	72	81	—	—	—	8	0	—	—	—
9	75	64 (3)	—	18	—	—	—	—	—	—
Genomic species 2										
10	71	1	100	13	—	0	0	—	0	8
11	70	2	89	—	—	—	—	—	—	—
12	71	—	80	—	—	—	—	—	—	—
13	72	7	110	21	—	—	0	—	—	0
Genomic species 3										
14	70	2	8	100	2	—	—	0	—	—
Not yet classified										
15	—	53	—	—	—	—	—	—	—	—
16	—	34	5	—	—	—	—	—	—	—
17	—	6	16	11	—	—	—	—	—	—
18	73	8	33	19	—	—	—	—	—	—
<i>Elaeagnaceae</i> compatibility group										
Genomic species 4										
19	70	—	—	—	100	—	12	16	—	—
20	73	0	0	—	71 (2.7)	—	0	—	—	0
21	—	—	—	—	81	—	—	—	—	—
22	71	—	—	—	69 (0.7)	—	2	20	—	0
23	71	—	—	—	85	—	—	—	—	—
24	75	4	6	—	103	—	2	22	—	0
Genomic species 5										
25	—	—	—	—	—	100	—	0	—	—
26	66	—	—	—	38	81	—	—	0	—
27	67	—	—	—	49 (7.0)	79	0	4	—	—
Genomic species 6										
28	70	0	—	0	—	4	100	3	—	—
Genomic species 7										
29	72	—	—	—	—	—	—	100	—	—
Genomic species 8										
30	—	—	—	—	19	—	12	16	100	—
Not yet classified										
31	—	0	—	—	—	—	0	0	—	—
32	—	—	—	—	6	9	1	2	53	—
<i>Casuarinaceae</i> compatibility group										
Genomic species 9										
33	71	1	—	—	—	—	0	—	—	100
34	69	—	2	—	3	—	—	1	0	83
35	—	—	—	—	—	—	—	—	—	87
36	69	—	—	—	—	—	—	—	—	107
37	70	—	—	0	—	0	—	—	—	88
38	70	—	—	—	—	—	—	—	—	80
39	69	—	—	—	—	—	—	0	—	79 (4.3)
40	70	—	—	—	—	—	—	—	—	69 (3)
Not yet classified										
41	71	—	—	—	0	—	—	0	—	38 (10)
42	72	—	0	—	0	—	—	0	—	0
43	73	—	—	—	30	—	0	8	—	0

^a Level of relatedness of 75°C.^b —, No data.^c The numbers in parentheses are ΔT_m values (in degrees Celsius).

other genomic species containing single strains can be differentiated. Our experimental work was finished when Lalonde et al. (27) proposed a type strain for "*F. elaeagnii*." Since this strain was not included in our study, we have no

way of knowing which of our genomic species (if any) corresponds to "*F. elaeagnii*." As observed for the *Alnus* compatibility group, there is no correlation between the original host genus and the DNA homology groups. This

result agrees with the cross-inoculation tests of Baker (3), which showed that *Elaeagnus angustifolia* and *Hippophaë rhamnoides* have exactly the same compatible strain spectrum. Genomic species seem to be more directly related to geographical origin. Strains from genomic species 4 were all isolated from France, whereas strains 25 and 26, in genomic species 5, were isolated from Canada and the United States. The close genomic relationship between strains 25 and 26 could be expected because of their identical isoenzyme and protein patterns (18, 19). Similarly, strain 28, which is markedly different from the other strains as determined by these two methods, was found in this study to constitute a separate DNA homology group. The very low level of genetic relatedness between strains 28 and 29 is in agreement with the results of a previous study conducted with *nif* gene restriction fragment length polymorphisms (Simonet, Normand, Hirsh, and Akkermans, in press).

Casuarina compatibility group. In *Bergey's Manual*, 8th ed., Becking (8) proposed the single species *Frankia casuarinae* for symbionts of *Casuarina* species. Since this proposal, most of the taxonomic studies of strains of *Frankia* have not taken into account strains that are compatible with members of the Casuarinaceae; thus, few data exist about them. Cross-inoculation tests with pure cultures isolated from *Casuarina* species nodules have shown that at least two groups can be distinguished. Some of these isolates are able to renodulate their original host plants and are called typical strains. The other isolates are not able to renodulate their original hosts but can infect plants belonging to the Elaeagnaceae; these are called atypical strains. In this study, eight typical strains and three atypical strains were tested. Our results showed that the eight typical strains are closely related to each other and constitute a single DNA homology group. Although few strains have been tested, they should be representative of strains compatible with members of the genus *Casuarina* because of their widely diverse origin (Table 1). This homogeneity could be explained by the fact that all casuarinas originate from Australia and the South Pacific Islands and have been disseminated worldwide (44). The homogeneity of this group has also been demonstrated by *nif* gene restriction fragment length polymorphisms, which separated the typical group from the atypical group (S. Nazaret, P. Simonet, P. Normand, and R. Bardin, Plant Soil, in press). The three atypical strains do not belong to the same DNA homology group, confirming the wide differences reported when *nif* gene restriction fragment length polymorphism patterns were used (Nazaret et al., in press). Several hypotheses have been proposed concerning the heterogeneity of atypical strains and their strong divergence from the typical group. Presently, these organisms are generally considered to be co-infective strains that are present in nodules with typical strains. The position of strain 41 is not clear; it was 38% related to genomic species 9 (Table 2), had the same isoenzyme pattern as three typical strains (data not published), and was very similar to the typical group strains when *nif* gene restriction fragment length polymorphism patterns were used (Nazaret et al., in press).

The taxonomic position of the strains of *Frankia* isolated from members of the genus *Myrica* should be of major interest since these strains are compatible with host genera belonging to the three major inoculation groups (3) (*Alnus*, Elaeagnaceae, and *Casuarina* compatibility groups). Generally, strains from this group are not easy to isolate, and their growth rates are slow. In this study only strain 15 originated from a member of the genus *Myrica* (obtained from *Alnus glutinosa* inoculated with crushed nodules from *Myrica*

gale). This organism was 53% related to genomic species 1, but the poor efficiency of DNA extraction for this strain did not allow us to confirm this intermediate result with a ΔT_m study.

It should be noted that the available isolates of *Frankia* are certainly not representative of the real diversity within the genus *Frankia*. For example, in this study we took into account only 7 of the 35 *Alnus* species. Moreover, isolation attempts have remained unsuccessful with a number of host plants, and few methods are available to perform isolation of non-nodulating (saprophytic) strains of *Frankia* species from the soil. Therefore, a major bias is introduced in the subsampling. A bacterial species is now defined as a DNA relatedness group (genomic species) which can be identified by phenotypic tests (42). Among the nine genomic species of the genus *Frankia*, genomic species 1 is *F. alni*. Unfortunately, the phenotypic data available are not comprehensive enough to separate these genomic species. In accordance with the recommendations published by a group of experts (42), these genomic species cannot be named at this time.

ACKNOWLEDGMENTS

We thank F. Grimont and J. Freney for professional advice, A. Moiroud for strains, and J. Haurat and E. Ageron for technical assistance.

LITERATURE CITED

1. An, C. S., 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.
2. An, C. S., J. H. Wills, W. S. Riggsby, and B. C. Mullin. 1983. Deoxyribonucleic acid base composition of 12 *Frankia* isolates. *Can. J. Bot.* **61**:2859-2862.
3. Baker, D. D. 1987. Relationships among pure cultured strains of *Frankia* based on host specificity. *Physiol. Plant.* **70**:245-248.
4. Baker, D. D. 1988. Opportunities for autoecological studies of *Frankia*, a symbiotic actinomycete, p. 271-276. In Y. Okami, T. Beppu, and H. Ogawara (ed.), *Biology of actinomycetes '88*. Japan Scientific Society Press, Tokyo.
5. Baker, D. 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.
6. Baker, D. D., W. L. Pengelly, and J. G. Torrey. 1981. Immunochromatological analysis of relationships among isolates of frankiae (Actinomycetales). *Int. J. Syst. Bacteriol.* **31**:138-151.
7. 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.
8. 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*, 8th ed. The Williams & Wilkins Co., Baltimore.
9. Berry, A., and J. G. Torrey. 1979. Isolation and characterization *in vivo* and *in vitro* of an actinomycetous endophyte from *Alnus rubra* Bong, p. 69-83. In J. C. Gordon, C. T. Wheeler, and D. A. Perry (ed.), *Symbiotic nitrogen fixation in the management of temperate forests*. Forest Research Laboratory, Oregon State University, Corvallis.
10. Bertrand, L. J., and M. Lalonde. 1985. *In vitro* propagation and nodulation by *Frankia* of actinorhizal Russian olive (*Elaeagnus angustifolia* L.). *Plant Soil* **87**:143-152.
11. Brenner, D. J., A. C. McWorter, J. K. Leete Knutson, and A. G. Steigerwalt. 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J. Clin. Microbiol.* **15**:1133-1140.
12. Callaham, D., P. Del Tridici, and J. G. Torrey. 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* **199**:899-902.
13. Crosa, J. M., D. J. Brenner, and S. Falkow. 1973. Use of a

- single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *J. Bacteriol.* **115**:904-911.
14. Diem, H. G., and Y. Dommergues. 1983. The isolation of *Frankia* from nodules of *Casuarina*. *Can. J. Bot.* **61**:2815-2821.
 15. Diem, H. G., D. Gauthier, and Y. Dommergues. 1983. An effective strain of *Frankia* from *Casuarina* sp. *Can. J. Bot.* **61**:2815-2821.
 16. Faure-Reynaud, M., M. A. Bonnefoy-Poirier, and A. Moiroud. 1986. Influence de pH acides sur la viabilité et l'infectivité d'isolats de *Frankia*. *Plant Soil* **96**:347-358.
 17. Fox, G. E., and E. Stackebrandt. 1987. The application of 16S rRNA cataloging and 5S rRNA sequences in bacterial systematics, p. 405-458. In R. R. Colwell and R. Grigorova (ed.), *Methods in microbiology*. Academic Press, Inc. (London), Ltd., London.
 18. Gardes, M., J. Bousquet, and M. Lalonde. 1987. Isozyme variation among 40 *Frankia* strains. *Appl. Environ. Microbiol.* **53**:1596-1603.
 19. Gardes, M., and M. Lalonde. 1987. Identification and subgrouping of *Frankia* strains using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Physiol. Plant.* **70**:237-244.
 20. Gauthier, D., H. G. Diem, and Y. Dommergues. 1981. Infectivité et effectivité des souches de *Frankia* isolées de nodules de *Casuarina equisetifolia* et d'*Hippophae rhamnoides*. *C. R. Acad. Sci.* **293**:489-491.
 21. Gauthier, D., L. Frioni, H. G. Diem, and Y. Dommergues. 1984. The *Colletia spinosissima*-*Frankia* symbiosis. *Oecol. Plant.* **5**:231-239.
 22. Gehrke, C. W., R. A. McCune, M. A. Gama Soao, M. Ehrlich, and K. C. Kuo. 1984. Quantitative reverse-phase high-performance liquid chromatography of major and modified nucleosides in DNA. *J. Chromatogr.* **301**:199-219.
 23. Goodfellow, M., and T. Cross. 1984. Classification, p. 7-164. In M. Goodfellow, M. Modarski, and S. T. Williams (ed.), *The biology of the actinomycetes*. Academic Press, Inc. (London), Ltd., London.
 24. Grimont, P. A. D., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr. Microbiol.* **4**:325-330.
 25. Lalonde, M. 1979. Techniques and observations of the nitrogen-fixing *Alnus* root nodule symbiosis, p. 421-434. In N. S. Subba Rao (ed.), *Recent advances in biological nitrogen fixation*. Oxford and IDH Publications, New Delhi, India.
 26. Lalonde, M., H. E. Calvert, and S. Pine. 1981. Isolation and use of *Frankia* strains in actinorhizae formation, p. 296-299. In A. H. Gibson and W. E. Newton (ed.), *Current perspectives in nitrogen fixation*. Australian Academy of Science, Canberra.
 27. Lalonde, M., L. Simont, J. Bousquet, and A. Séguin. 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 Fisher, Stuttgart.
 28. Lechevalier, M. P. The taxonomy of the genus *Frankia*. *Plant Soil* **78**:1-6.
 29. Lechevalier, M. P., F. Horrière, and H. Lechevalier. 1982. The biology of *Frankia* and related organisms. *Dev. Ind. Microbiol.* **23**:51-60.
 30. Lechevalier, M. P., and H. A. Lechevalier. 1979. The taxonomic position of the actinomycetic endophytes, p. 111-122. In J. C. Gordon, C. T. Wheeler, and D. A. Perry (ed.), *Symbiotic nitrogen fixation in the management of temperate forests*. Forest Research Laboratory, Oregon State University, Corvallis.
 31. Lopez, M. F., C. S. Whaling, and J. G. Torrey. 1983. The polar lipids and free sugars of *Frankia* in culture. *Can. J. Bot.* **61**:2834-2842.
 32. Moiroud, A., and M. Faure-Reynaud. 1983. Influence de quelques herbicides à large spectre sur la croissance et l'infectivité de cultures pures de *Frankia*. *Plant Soil* **74**:133-136.
 33. Mort, A., P. Normand, and M. Lalonde. 1983. 2-O-methyl-D-mannose, a key sugar in the taxonomy of *Frankia*. *Can. J. Microbiol.* **29**:993-1002.
 34. Murry, M. A., M. S. Fontaine, and J. G. Torrey. 1984. Growth kinetics and nitrogenase induction in *Frankia* sp. HFP Ar13 grown in batch culture. *Plant Soil* **78**:61-78.
 35. Normand, P., and M. Lalonde. 1986. The genetics of actinorhizal *Frankia*: a review. *Plant Soil* **90**:429-453.
 36. Normand, P., P. Simonet, and R. Bardin. 1988. Conservation of *nif* sequences in *Frankia*. *Mol. Gen. Genet.* **213**:238-246.
 37. Silvester, W. B., O. Balboa, and J. A. Martinez. 1985. Nodulation and nitrogen fixation in members of the Rhamnaceae (*Colletia*, *Retanilla*, *Talguenea* and *Trevoa*) growing in the Chilean matorral. *Symbiosis* **1**:29-38.
 38. Simonet, P., A. Capellano, E. Navarro, R. Bardin, and A. Moiroud. 1984. An improved method for lysis of *Frankia* with achromopeptidase allows detection of new plasmids. *Can. J. Microbiol.* **30**:1292-1295.
 39. Simonet, P., J. Haurat, P. Normand, R. Bardin, and A. Moiroud. 1986. Localisation of *nif* genes on a large plasmid in *Frankia* sp. strain ULQ0132105009. *Mol. Gen. Genet.* **204**:492-495.
 40. Simonet, P., P. Normand, A. Moiroud, and M. Lalonde. 1985. Restriction enzyme digestion patterns of *Frankia* plasmids. *Plant Soil* **87**:49-60.
 41. St. Laurent, L., J. Bousquet, L. Simon, and M. Lalonde. 1987. Separation of various *Frankia* strains in the *Alnus* and *Elaeagnus* host specificity groups using sugar analysis. *Can. J. Microbiol.* **33**:764-772.
 42. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst. Bacteriol.* **37**:463-464.
 43. Wheeler, C. T., J. E. Hooker, A. Crowe, and A. M. M. Berrie. 1986. The improvement and utilization in forestry of nitrogen fixation by actinorhizal plants with special reference to *Alnus* in Scotland. *Plant Soil* **90**:393-406.
 44. Zhang, Z., M. F. Lopez, and J. G. Torrey. 1984. A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. *Plant Soil* **78**:79-90.
 45. Zhang, Z., and J. G. Torrey. 1985. Studies of an effective strain of *Frankia* from *Allocasuarina lehmaniana* of the Casuarinaceae. *Plant Soil* **87**:1-16.