Phylogenetic Relationships among *Frankia* Genomic Species Determined by Use of Amplified 16S rDNA Sequences

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Actinomycetes of the genus *Frankia* establish a nitrogen-fixing symbiosis with a large number of woody dicotyledonous plants. Hundreds of strains isolated from various actinorhizal plants growing in different geographical areas have recently been classified into at least nine genomic species by use of the DNA-DNA hybridization technique (M. P. Fernandez, H. Meunier, P. A. D. Grimont, and R. Bardin, *Int. J. Syst. Bacteriol.* 39:424–429, 1989). A protocol based on the amplification and sequencing of 16S ribosomal DNA segments was used to classify and estimate the phylogenetic relationships among eight different genomic species. A good correlation was established between the grouping of strains according to their 16S ribosomal DNA sequence homology and that based on total DNA homology, since most genomic species could be characterized by a specific sequence. The phylogenetic tree showed that strains belonging to the *Alnus* infectivity group are closely related to strains belonging to the *Casuarina* infectivity group and that strains of these two infectivity groups are well separated from strains of the *Elaeagnus* infectivity group, which also includes atypical strains isolated from the *Casuarina* group. This phylogenetic analysis was also very efficient for classifying previously unclassified pure cultures or unisolateable strains by using total DNA extracted directly from nodules.

Slowly growing actinomycetes of the genus *Frankia* can establish a nitrogen-fixing symbiosis with a wide range of woody dicotyledonous plants (2). To date, this symbiosis has been reported in more than 194 species of plants belonging to 24 genera. Since the first isolation of a *Frankia* strain in 1978 (5), hundreds of isolates have been obtained from a number of plant species and from various geographical areas. The members of the genus *Frankia* are now taxonomically clearly distinguished from other actinobacterial genera on the basis of their host specificity, morphology (hyphae, vesicule, and sporangia), biochemistry (type III cell wall and type I phospholipids), and physiology (20). First attempts to structure the genus *Frankia* were based on infectivity groups. Baker (1) grouped strains into four infectivity groups, using pure cultures in cross-inoculation tests: strains infective on *Alnus* and *Myrica* spp., strains infective on *Casuarina* and *Myrica* spp., strains infective on *Elaeagnus* and *Myrica* spp., and strains infective only on *Elaeagnus* spp. Lalonde et al. (18) used a more complex approach that relied heavily on phenotypic characteristics, which yielded two species, *Frankia alni* and *F. elaeagni*.

It has recently been agreed that DNA reassociation is the most objective means of delineating bacterial species, and the following definition of a bacterial species was proposed: strains sharing at least 70% reassociation at optimal temperature with a divergence below 5°C belong to the same genomic species (41). This approach used on *Frankia* isolates yielded at least nine genomic species (10), with three, including *F. alni*, in the *Alnus* infectivity group, five in the *Elaeagnus* infectivity group, and one in the *Casuarina* infectivity group.

16S rRNA sequence similarity is now in general use to measure phylogenetic relationships. Relatively well conserved regions of the sequence can be used to infer natural relationships between distantly related species, whereas variable regions can be used to analyze closely related ones. Reverse transcriptase sequencing provides a rapid method for obtaining 16S rRNA sequences (19), though it frequently results in sequencing anomalies since only one strand can be determined. It also requires considerable amounts of rRNA, which may be hard to obtain with some bacteria. Application of this method is time-consuming and unsuitable for slowly growing and noncultivable microorganisms. The polymerase chain reaction (PCR) developed for amplifying a specific portion of the genome constitutes a powerful tool for overcoming these difficulties (23).

In this study, a rapid and reproducible protocol based on DNA amplification and double-strand sequencing of the resulting partial 16S ribosomal DNA (rDNA) sequences was used to estimate the phylogenetic relationships among the genomic species defined by our group (10).

MATERIALS AND METHODS

Bacterial strains. Thirty-five *Frankia* strains, most of which were found to belong to eight different genomic species described by Fernandez et al. (10), were included in this study (Table 1). Strains were grown at 28°C in FTW medium (*Alnus* infective strains) (35), FTW medium without Tween 80 (*Elaeagnus* infective strains), or BAP medium (*Casuarina* infective strains) (24).

DNA extraction. Total DNA from isolated *Frankia* strains was obtained as described by Simonet et al. (33). Total DNA was extracted from actinorhizal nodules for amplification and sequencing as previously described (34).

PCR amplification. Double-strand amplification was performed on part of the *rrn* gene coding for 16S rRNA by a

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TABLE 1. Origins of *Frankia* spp. strains tested

Genomic species	Registry no.	Other	Original host designation	Geographical origin	References
Strains infective on <i>Alnus</i> spp.					
<i>Frankia alni</i>	ULF01010244	ACoN24d	<i>Alnus cordata</i>	Orléans (France)	33
<i>F. alni</i>	ULF0131024083	Ar24H3	<i>A. rubra</i>	Orléans (France)	36
<i>F. alni</i>	ULF0131024152	Ar24O2	<i>A. rubra</i>	Orléans (France)	36
<i>F. alni</i>	HFP013003	ArI3	<i>A. rubra</i>	Oregon (United States)	3
<i>F. alni</i>	ULQ013202204	ARgN22d	<i>A. rugosa</i>	Québec (Canada)	27
<i>F. alni</i>	ULQ0102001007	ACN1 ^{AG}	<i>A. crispa</i>	Québec (Canada)	17
2	ULF014101715	AVN17o	<i>A. viridis</i>	La Toussuire (France)	10
2	ULF014102203	AV22c	<i>A. viridis</i>	Lautaret (France)	8
3	ULQ0132105009	ARgP5 ^{AG}	<i>A. rugosa</i>	Québec (Canada)	27
U ^a	ULF01070602	Mg602 ^{AG}	<i>A. glutinosa</i>	Landes (France)	10
U	UL000018024	TN18b ^{AC}	Soil on <i>A. crispa</i>	Québec (Canada)	38
U		Nodule	<i>A. viridis</i>	La Toussuire (France)	Unp. ^b
U	ULF0111131	AIN13a	<i>A. incana</i>	Lamure (France)	Unp.
U	ULF013102	Ar24H5	<i>A. rubra</i>	Orléans (France)	36
U	ULF0141421	AVN42a	<i>A. viridis</i>	Rivier d'Almont (France)	Unp.
U	ULF010724	AgN24IIh	<i>A. glutinosa</i>	Orléans (France)	36
U	ULF0101018	AcoI8	<i>A. cordata</i>	Orléans (France)	36
U	ULF0101015	AcoI5	<i>A. cordata</i>	Orléans (France)	36
U	ULF0101019	AcoI9	<i>A. cordata</i>	Orléans (France)	36
Strains infective on <i>Elaeagnaceae</i>					
4	ULF130100112	Ea1-12	<i>Elaeagnus angustifolia</i>	Ecully (France)	10
5	ULF140104001	HRX401a	<i>Hippophaë rhamnoides</i>	Ornon (France)	28
6	ULQ132500106	EUN1f	<i>E. umbellata</i>	Illinois (United States)	17
7	ULF140101801	HRN18a	<i>H. rhamnoides</i>	Alps (France)	22
U	ORS060501	Col	<i>Colletia spinosissima</i>	Argentina	14
U	ORS140102	CH	<i>H. rhamnoides</i>	China	Unp.
Strain noninfective on any plant					
U	DDB17020110	PtI1	<i>Purshia tridentata</i>	Wyoming (United States)	1
<i>Casuarinaceae</i> strains					
Infective on the host plant					
9	ORS020606	CeD	<i>Casuarina equisetifolia</i>	Dakar (Senegal)	6
9	HFP020203	CcI3	<i>C. cunninghamiana</i>	Florida (United States)	44
9	ORS020608	Br	<i>C. equisetifolia</i>	Brazil	25
9	HFP022801	AlI1	<i>A. lehmaniana</i>	Florida (United States)	45
9	ORS021001	Cj1-82	<i>C. junghuniana</i>	Thailand	7
9	ORS020609	M2	<i>C. equisetifolia</i>	Madagascar	25
Noninfective on the host plant					
U	HFP020202	CcI2	<i>C. cunninghamiana</i>	Florida (United States)	44
U	ORS020602	D11	<i>C. equisetifolia</i>	Dakar (Senegal)	13
U	DDB020110	CeI1	<i>Casuarina</i> sp.	Hawaii (United States)	1

^a U, undetermined species.^b Unp., unpublished data.

modification of the PCR procedure of Mullis and Faloona (23). One 20-base oligonucleotide and one 21-base oligonucleotide were used as primers. These oligonucleotides have the following sequences: primer FGPS849, 5'-GCCTTGG GAGTACGGCCGCA-3'; and primer FGPS1146', 5'-GG GGCATGATGACTTGACGTC-3'. The sequences of the primers were compared with the corresponding regions of 16S rRNA of other microorganisms to estimate their specificity and ensure specific hybridization. The amplified fragments consisted of a 325-bp double-stranded DNA fragment between primers FGPS849 and FGPS1146'. The PCR reaction was carried out in a final volume of 100 µl containing template DNA, reaction buffer (10 mM Tris-Cl [pH 8.3], 1.5 mM MgCl₂, 50 mM KCl, 10% [wt/vol] gelatin), 200 µM each deoxynucleoside triphosphate, 1 µM oligomers, and 2 U of *TaqI* DNA polymerase (Bethesda Research Laboratories). The amplification reactions were carried out for 30 cycles. The tubes were transferred manually between three heat blocks in the following sequence: denaturation of DNA at 93°C for 1 min, annealing at 57°C for 1 min, and

extension at 70°C for 1 min. To analyze the amplification products, 5 µl of the reaction mix was separated by electrophoresis on a 2% (wt/vol) NuSieve (FMC, Rockland, Maine) agarose gel.

Sequencing of DNA fragments. Before sequencing, the amplification reaction mix was passed through an Elutip-D column to remove salts and deoxynucleoside triphosphates. The sample was precipitated with ethanol and sequenced by using the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio) and the double-stranded DNA sequencing procedure described by Winship (42) for amplified DNA (325-bp fragment). The sequenced fragments covered the region corresponding to the variable domains V3 and V4 of the *Escherichia coli* 16S rRNA (43). Sequencing primers were the same as amplification primers. In each genogroup and for all strains that exhibited a specific pattern, the sequence was determined for both strands.

Alignment of sequences and cluster analysis. The phylogeny of the bacteria shown in Fig. 2 was deduced by a distance matrix method (the Kitsch program [11] of the Felsenstein-

	0		49
<i>F. alni</i>	TTGACGGGGG	CCCGCACAAG	CGGCGGAGCA TGTGGCTTAA TTCGATGCAA
Genomic species 2			
Genomic species 3			
nodule <i>A. viridis</i>			
Genomic species 4, 5			
Col			
Genomic species 6			
Genomic species 7			
CH			
D11			
Genomic species 9			
M2			
PtI1			
Ag45/Mut15			..N.....
<i>S. ambofaciens</i>			C.....
	50		99
<i>F. alni</i>	CGCGAAGAAC	CTTACCAGGG	CTTGACATGC AGGGAATCT CGTAGAGATA
Genomic species 2		T.....	C TCC.....G
Genomic species 3		T.....	C TCC.....G
nodule <i>A. viridis</i>		T.....	C T.....
Genomic species 4, 5			..A.....C T.....
Col			..A.....C T.....
Genomic species 6			..A.....C T.....
Genomic species 7			..A.....C T.....
CH			..A.....C T.....
D11			..A.....C T.....
Genomic species 9			C.....
M2			C.....
PtI1			G.....C ATC.....G
Ag45/Mut15		..A TA.....	..-.....TC TCC.....G
<i>S. ambofaciens</i>		A.....	..CC.G..AGC AT.....
	100		149
<i>F. alni</i>	CGGGGTCCGT	AAGGGTCCTG	C-ACAGGTGG TGCATGGCTG TCGTCAGCTC
Genomic species 2	G.....		
Genomic species 3	G.....		
nodule <i>A. viridis</i>			
Genomic species 4, 5	T.....T..	.G...CT...	
Col	T.....T..	.G...CT...	
Genomic species 6	T.....T..	.G...CT...	
Genomic species 7	T.....T..	.G...CT...	
CH	T.....T..	.G...CT...	
D11	T.....T..	.G...CT...	
Genomic species 9			
M2			
PtI1	GT.....T..	.G.....C..	T.G.....
Ag45/Mut15	G...A.....		T.....
<i>S. ambofaciens</i>	GT.CCC..C.	TGT....GGT	GT.....

FIG. 1. Aligned sequences of the 13 different 16S rDNA patterns obtained for the 35 *Frankia* strains studied. Base positions in the sequences are numbered consecutively, and nucleotide 0 corresponds to nucleotide 921 in the standard *E. coli* 16S rRNA numbering (4). The Ag45/mut15 sequence (15) and *S. ambofaciens* sequence (29) were added for comparison. Seven gaps were introduced to allow the best alignment with the *S. ambofaciens* sequence. Strains AcoN24d, Ar24O2, Ar24H3, Ar24H5, ARgN22d, ACN1^{AG}, AgN241lh, Aco15, Aco18, Aco19, Mg602^{AG}, and TN18b^{AC} have the same pattern as the species *F. alni*; strains AV22c, AIN13a, and AVN42a have the same pattern as genomic species 2. HRX401a and Ce11 have the same pattern as genomic species 4 and 5. The atypical strain Cc12 has the same pattern as genomic species 9. Only the nucleotides that differ from those of *F. alni* are shown (identities are denoted by dots, and deletions are marked by hyphens). Nucleotide positions that could not be identified are denoted by N.

Phylip package) (9). Briefly, sequences were first aligned to bring homologous sequence positions into correspondence. For each pair of sequences, the evolutionary distance (average number of nucleotide substitutions per sequence position) was estimated as $-3/4 \ln(1-4L/3)$, where L is the observed fractional sequence difference (16). The sequences obtained were compared with the 16S rRNA sequences of *Frankia* strain Ag45/mut15 (15) and *Streptomyces ambofaciens* (29). Seven gaps were introduced to obtain the best alignment with the 16S rRNA sequence of *S. ambofaciens* (Fig. 1). Other treeing algorithms used were Fitch of the Felsenstein-Phylip package (11), UPGMA (37), modified UPGMA (21), and Neighbor-Joining (31).

RESULTS

Amplification from chromosomal DNA. Chromosomal DNA of *Frankia* spp., extracted from pure cultures or nodules, was used as source of amplifiable material. The

amplified DNA was, in all cases, a single product of the expected size, 325 bp between primers FGPS849 and FGPS1146'.

Sequencing and comparison of amplified 16S rDNA. A partial 16S rDNA sequence of 268 bp was obtained for all 35 strains studied (Fig. 1). This sequence covered region 921 to 1189 according to the *E. coli* numbering system (4). Sequences were aligned with the published 16S rRNA of *Frankia* strain Ag45/mut15, isolated from *Alnus glutinosa* (15), and with the actinomycete *S. ambofaciens* (29). Comparison of the aligned sequences showed that some *Frankia* strains have exactly the same pattern. The 35 sequences were grouped into 13 different sequence patterns (Fig. 1). In each group of strains sharing the same pattern, at least one sequence was determined in the two directions. Small but significant differences were found between the 13 patterns. Among the 268 nucleotides of this partial sequence, there were 24 variable positions distributed in two variable regions corresponding to the variable domains V3 and V4 in the

	150		199
<i>F. alni</i>	GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTCGTCCT		
Genomic species 2			
Genomic species 3			
nodule			
Genomic species 4, 5			T.....
Col			T.....
Genomic species 6			T.....
Genomic species 7			T.....
CH			T.....
D11			T.....
Genomic species 9			
M2			
PtI1			
Ag45/Mut15			
<i>S. ambofaciens</i>			T.....C
	200		249
<i>F. alni</i>	ATGTTGCCAG CG--AGTTAT GTC---GGG GACTCATAGG AGACTGCCGG		
Genomic species 2	A.....		
Genomic species 3	CGA.....		
nodule <i>A. viridis</i>			
Genomic species 4, 5	C.....		
Col	C.G.....	G.....	
Genomic species 6	C.N.....	G.....	
Genomic species 7	C.ATA.....	G.....	
CH	C.C.....	G.....	
D11	C.....	G.....	
Genomic species 9	CG.....		
M2	N.CG.....		
PtI1			
Ag45/Mut15	C.....	N.....	
<i>S. ambofaciens</i>	G.....AAGCCC.TC	GGTGTT.....CG.....C.....	
	250		274
<i>F. alni</i>	GGTCAACTCG GAGGAAGGTG GGGAT		
Genomic species 2			
Genomic species 3			
nodule <i>A. viridis</i>			
Genomic species 4, 5			
Col			
Genomic species 6			
Genomic species 7			
CH			
D11			
Genomic species 9			
M2			
PtI1			
Ag45/Mut15		N.....	
<i>S. ambofaciens</i>		C.....	

FIG. 1—Continued.

homologous *E. coli* sequence (43). Seventeen informative sites could be distinguished in the V3 region, and seven could be identified in the V4 region.

The grouping of strains according to their 16S rDNA sequence homologies is in line with the classification obtained by total DNA-DNA hybridization; strains belonging to the same genomic species share the same 16S rDNA sequence, whereas strains belonging to different genomic species exhibited different sequences. However, there was one exception among genomic species defined within the *Elaeagnus* infectivity group. Strains belonging to genomic species 4 and 5 all exhibited the same sequence. Furthermore, the 16S rDNA sequences determined among *Elaeagnus* infective strains did not vary in as many positions as those determined among *Alnus* infective strains.

Our study also included unclassified strains. Some strains, such as PtI1, Col, CH, and D11 and a strain called nodule *A. viridis* had specific sequences not previously found among strains of the eight different genomic species. On the other hand, some strains had sequences identical to those previously found among the eight genomic species. For example, strains Ar24H5, AgN24IIh, AcoI5, AcoI8, AcoI9, Mg602^{AG}, and TN18b^{AC} had a sequence identical to that characterizing members of the species *F. alni*, and strains AIN13a and

AVN42a showed a sequence identical to that of strains belonging to genomic species 2.

Nucleotide distance tree. Figure 2 shows the rooted tree obtained by the Fitch-Margoliash contemporary tips method (11). Two major subdivisions may be distinguished, one subdivision containing strains of the *Elaeagnus* infectivity group and the other containing strains from both the *Alnus* and *Casuarina* groups.

The species *F. alni* and strain called nodule *A. viridis* branched closer to strains in the *Casuarina* group than to the other genomic species in the *Alnus* group (genomic species 2 and 3) whatever the treeing method used. Another unexpected finding was that the atypical strains isolated from *Casuarina* nodules and infective on *Hippophaë* spp., such as D11, CeI1, and CcI2, branched either with the *Elaeagnus* group (D11, close to genomic species 6, and CeI1, close to genomic species 4) or with the *Casuarina* group (CcI2). These two features were confirmed by parsimony analysis (results not shown).

The phylogenetic tree also showed that strain PtI1, isolated from a nodule of *Purshia tridentata*, branched far apart from all other *Frankia* strains studied (Fig. 2), though this situation varied with the treeing algorithm used (results not shown).

FIG. 2. Fitch-Margoliash method with contemporary tips on 268 bases in the 16S rDNA gene (coordinates 921 to 1189 of *E. coli*) of 35 *Frankia* strains. The scale bar corresponds to one substitution per 100 sequence positions. Gen. sp., genomic species.

DISCUSSION

In our experiment, a partial region of the *Frankia* 16S rDNA sequence was determined to estimate intragenetic relationships between different representatives of the genus *Frankia*. This segment which was recently shown to be highly variable in *Frankia* 16S rRNA (15) contains a sufficient number of informative sites among the 268 nucleotides determined to allow grouping of 35 strains according to their sequence homologies and to estimate the evolutionary affinities of eight different genomic species of *Frankia*. The rapid determination of these 35 sequences was greatly facilitated by the use of PCR amplification and double-stranded DNA sequencing by the Winship method (42). Specific primers allow the amplification and sequencing of *Frankia* 16S rDNA sequences obtained from total DNA extracted from either pure cultures or nodules. After alignment and comparison of these sequences, we found that *Frankia* strains belonging to the same genomic species shared similar sequences while strains belonging to different genomic species exhibited distinct sequences. A single exception was observed among strains of the *Elaeagnus* infectivity group, in which genomic species 4 and 5 could not be differentiated by a specific sequence. This could be related to the high DNA-DNA homology observed between these two genomic species, which exhibited from 38 to 49% homology (10). The clustering of strains based on 16S rDNA similarity is therefore in line with that obtained by using DNA-DNA homology. Similar findings were recently reported by Devereux et al. (5a) for *Desulfovibrio* strains. The possibility of characterizing *Frankia* genomic species from specific partial 16S rDNA sequences is interesting, since synthetic specific DNA could

be designed by using the variable regions of this sequence to conduct ecological studies. The availability of 16S rDNA and *nif* probes (34) could increase the specificity and reliability required for detection and identification tests conducted on *Frankia* strains inside the nodule or in the soil.

A phylogenetic tree may be constructed by transforming the 16S rDNA sequence variations into evolutionary distance values (Fig. 2). This shows the relationships among eight different genomic species of *Frankia* strains as well as the relationships among these genomic species and unclassified strains. Two main subdivisions were revealed by this phylogenetic tree: genomic species infective on *Elaeagnus* spp. and genomic species infective either on *Alnus* or *Casuarina* spp. According to 16S rDNA homologies, strains in the *Elaeagnus* infectivity group show less diversity than those in the *Alnus* infectivity group, since different genomic species sometimes had the same sequence. Furthermore, sequences did not vary in as many positions as in the *Alnus* group. Until now, the *Elaeagnus* group was considered to be more heterogeneous than the *Alnus* group because of its phenotypic features (fatty acid [32] and isoenzyme patterns [12], for example). This phylogenetic analysis reveals that genomic species in the *Elaeagnus* group are closer relatives than those in the *Alnus* group, suggesting that they diverged later than the *Alnus* infectivity group. This may be due to the later appearance of members of the family *Elaeagnaceae* (26). However, this is only a tentative hypothesis since the 16S rDNA segment sequenced was short and may not be truly representative of all the 16S rDNAs and consequently of the whole genome. It should be emphasized that our sample of *Alnus* strains was more comprehensive, with

divergent strains such as strains Ag45/mut 15 and AVN17o, which were not examined in the isoenzyme or fatty acid studies.

In the second subdivision, *F. alni* was found to branch closer to the genomic species 9, containing *Casuarina* infective strains, than to genomic species 2 and 3, made up of strains from the *Alnus* group. This is quite surprising but was confirmed by parsimony analysis (30) in which 9 of 10 informative sites relating *F. alni* and genomic species 2 and 9 ($P = 0.011$ that such grouping is random) showed *F. alni* to be closer to genomic species 9 than to genomic species 2. However, fatty acid profiles (32), isozyme patterns (12), and sugar analysis (38) always showed large differences between strains of *F. alni* and strains of genomic species 2 and 3, infective on *Alnus* spp.

Atypical strains isolated from *Casuarina* root nodules are unable to reinfect their original host, although they can nodulate members of the family *Elaeagnaceae*. One hypothesis proposed to explain the isolation of either typical or atypical strains is the coexistence of two strains in the nodule, one infective on *Casuarina* spp. and the other infective on *Elaeagnus* spp. (25). Such a hypothesis was reinforced by the large divergence between typical and atypical strains revealed by restriction fragment length polymorphism studies with *nif* (25) and *rrn* (unpublished data) genes. Furthermore, DNA-DNA hybridization showed that atypical strains do not belong to the same genomic species (genomic species 9) as typical strains. Our phylogenetic analysis strengthened the coinfection hypothesis, since atypical strains D11 and Ce11 were found to branch closer to *Elaeagnus* infective strains.

Strains unclassified by DNA-DNA hybridization (10) were included in our study. Some showed sequences similar to those found among the eight genomic species studied. We mentioned above that a good correlation exists between clustering obtained with total DNA-DNA homologies and with partial 16S rDNA homologies. This suggests that each new isolate will be found to belong to the genomic species with a similar sequence. This rapid method, which requires less DNA and consequently less biomass than the DNA-DNA hybridization technique, may be particularly useful for classifying new isolates of slowly growing bacterial species.

The unclassified strain, Pt11, isolated from *P. tridentata*, was the only isolate obtained from *Rosaceae* plants. This family comprises several nonactinorhizal plants of economic importance such as raspberry and strawberry plants. The phylogenetic tree (Fig. 2) shows that this strain stands apart from the other strains and genomic species of *Frankia*, in line with the specific characteristics of this strain. Pt11 was reported to be noninfective on its original host plant, and cross-inoculation tests with other actinorhizal plants such as those of the genera *Alnus*, *Elaeagnus*, *Myrica*, and *Casuarina* were also unsuccessful (1a). Furthermore, hybridization experiments with *nif* gene probes failed to detect these genes (unpublished data), confirming the observed inefficiency. However, treeing algorithms which do not put constraints on branch lengths (i.e., which are not based on the molecular clock hypothesis) showed that Pt11 branches within the *Alnus* group (results not shown). This highlights the fact that Pt11, like other strains infective on *Alnus* spp., has mutated more than the other *Frankia* strains and that uncorrecting algorithms tend to group slowly mutating strains together.

Our study also included an unisolatable strain designated nodule *A. viridis*. With the PCR technique, we were able to specifically amplify the 16S rDNA of this strain by using total DNA extracted from an *Alnus* nodule (with no ampli-

fication from unnodulated root tissues). This strain forms many sporangia within the nodule. This particular feature is frequently observed among *Alnus* nodules and *Myrica* nodules which are called sp+ nodules, as opposed to the sp- nodules containing few or no sporangia (2). Several attempts have been made to isolate strains from sp+ nodules (39). The few isolates obtained produced sporangia in vitro but did not induce the sp+ phenotype after reinfection of the host plant (18, 40). Some authors suggested that sporulation or lack of sporulation corresponds to different physiological responses of the same isolate to different environmental conditions (39), whereas others suggested the simultaneous presence of two genetically different strains: a few propagules of sp- strains, which are easy to isolate and have a more rapid growth rate, and sp+ strains, which are hard to isolate and have a slow growth rate. The amplified 16S rDNA that we obtained from a nodule of *A. viridis* showed a specific sequence different from those obtained with strains infective on *Alnus* spp. Phylogenetic analysis positioned this strain closer to *F. alni* than to genomic species 2 and 3. This result is quite striking since strains ARgP5^{AG} and AVN17o were both isolated from a sp+ nodule. A partial sequence with only the variable domain V3 was obtained from another sp+ nodule collected on an *A. rugosa* plant in Quebec. This strain showed a sequence similar to that of nodule *A. viridis* (results not shown). Further comparative studies of 16S rDNA sequences from a large number of sp+ nodules may help determine the phylogenetic relationships of these strains with the pure culture strains.

This study therefore made it possible not only to characterize and classify pure-cultured strains but also to characterize new strains present in a nodule at molecular level, without requiring the difficult and time-consuming isolation of the symbiont from nodules. This opens up the interesting possibility of obtaining genetic information about undescribed strains such as the symbionts of several genera in the families *Coriariaceae*, *Datisceae* and *Rosaceae* and establishing their relationships with well-known strains isolated from *Alnus*, *Elaeagnus*, or *Casuarina* root nodules.

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