Relationships of *Frankia* Isolates Based on Deoxyribonucleic Acid Homology Studies

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A molecular approach was used to examine the genetic relatedness of 19 *Frankia* isolates by measuring the extent of deoxyribonucleic acid-deoxyribonucleic acid homology and the fidelity of hybrid duplex molecules. The *Frankia* isolates examined were divided into two groups based on the results of hybridization tests. The members of genogroup 1, consisting of isolates from *Alnus*, *Myrica*, and *Comptonia* host plants, exhibited high levels of homology (67.4 to 94.1%) with strain Ar14, an isolate from *Alnus rubra*. Isolates from *Elaeagnus*, *Ceanothus*, *Purshia*, and *Casuarina* and isolate Air12 from *Alnus* did not exhibit significant homology (less than 39%) with strain Ar14. None of the strains showed a high degree of homology with strain Eu11a (less than 33%), an isolate from *Elaeagnus umbellata*. Among the isolates not belonging to genogroup 1, subgroupings seemed to exist, as evidenced by a very high level of homology (97%) between two isolates from *Casuarina*, strains D11 and the G2, but a low level of homology (27%) between other strains and strain G2. Thermal stability studies of the hybrid deoxyribonucleic acids which showed high levels of homology revealed an average mismatch of 3%, whereas the low-homology duplexes exhibited about 5% mismatch. The genome molecular weights of two probe *Frankia* strains, strains Ar14 and Eu11a, were 8.3×10^9 and 6.0×10^9 , respectively.

Since the first confirmed isolation of a nitrogen-fixing *Frankia* endophyte from *Comptonia* root nodules (11), more than 50 strains from 20 different plant species have been isolated (2). The availability of increasing numbers of isolates has made it possible to examine various aspects of these endophytes grown in vitro.

Morphologically, there appear to be no fixed distinctive differences among the isolates other than pigmentation and the occurrence of vesicles in culture. The size, shape, and number of the sporangia, as well as the overall density of the colonies, depend largely on the culture conditions (7, 16). Biochemical studies (18) also have demonstrated a uniformity among *Frankia* isolates in terms of cell wall composition (type III) and phospholipid pattern (type I), although their cell sugar patterns vary widely. Based on serological (3), physiological (19), and cross-inoculation studies (5, 7, 16), the isolates can be divided into at least two groups. Generally, the isolates from *Alnus*, *Myrica*, and *Comptonia* belong to the same group (serogroup I, physiological group B), whereas the isolates from *Elaeagnus*, *Ceanothus*, *Purshia*, and *Casuarina* form a separate group (serogroup II, physiological group A).

Despite these studies, there are no consistently useful taxonomic criteria for proper classification of *Frankia* isolates. Accordingly, the principal purpose of this study was to use a molecular approach to examine the genetic relatedness of *Frankia* isolates. This approach was based on the base composition of deoxyribonucleic acid (DNA) (1), the degree of DNA-DNA hybridization, and the thermal stability of the hybrid DNA duplexes.

MATERIALS AND METHODS

Organisms and culture conditions. The 19 strains used in this study, their sources, and the media on which they were grown are shown in Table 1. The methods used to culture and harvest cells have been described previously (1).

Extraction and in vitro labeling of DNA. DNA was extracted as described by An et al. (1), and DNAs to be used for probes were labeled in vitro by nick translation (25),

using ³H-labeled deoxycytidine triphosphate (Amersham Corp.). The other reagents used for labeling were obtained in the form of a nick-translation kit (kit NEX-004E; New England Nuclear Corp.) and were used according to the directions of the supplier. A typical 30-μl reaction mixture consisted of 0.6 nM ³H-labeled deoxycytidine triphosphate, 500 ng of DNA, 4 μl of a deoxyribonucleoside triphosphate mixture (lacking deoxycytidine triphosphate), 2 μl of DNA polymerase I, and 2 μl of deoxyribonuclease I. After 2 h of incubation at 15°C, the reaction was stopped by adding 3 μl of 200 mM ethylenediaminetetraacetate. To separate labeled DNA from unreacted nucleotides, the entire reaction mixture was layered onto the top of a 1-ml bed of Sephadex G-50 in a 3-ml plastic syringe, and chromatography was carried out by washing the column with elution buffer [0.25 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane, pH 7.9, 0.5% sodium dodecyl sulfate, 2 mM ethylenediaminetetraacetate]. About 30 100-μl fractions were collected, and the radioactivity of 5 μl of each fraction was estimated by using a scintillation counter. Fractions which corresponded to the first peak of radioactivity were pooled and used as the DNA probe.

Preparation of DNA mixtures for reassociation. Typically, a mixture of ³H-labeled DNA and 200 μg of unlabeled DNA (about 1:4,000, wt/wt) was sheared and purified, and the sizes of the resulting DNA fragments were determined electrophoretically by a modification of the methods described by Riggsby et al. (26). The DNA was sheared for 30 min at 45,000 rpm in a Virtis model 45K homogenizer, resulting in a fragment size of approximately 300 base pairs.

Reassociation (hybridization) and hydroxyapatite chromatography. Samples containing sheared, purified, labeled and unlabeled DNAs in 0.12 M sodium phosphate buffer (pH 6.8) were sealed in sterile siliconized 20- to 100-μl capillary tubes. Sample sizes ranged from 10 to 70 μl depending on the specific activity of the labeled DNA. To dissociate the DNAs, the capillary tubes were heated to 115°C in an ethylene glycol-water mixture for 10 min and placed immediately in a water bath set at the reassociation temperature. The reassociation temperature was 64.5°C for *Escherichia coli* DNA and 74.5°C for *Frankia* DNAs. These tempera-

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TABLE 1. *Frankia* isolates used in this study

Strain	Host plant		Source	Medium ^a
	Species or subspecies	Collection site		
ACN1 ^{AG}	<i>Alnus viridis</i> subsp. <i>crispa</i>	Quebec, Canada	M. Lalonde	T/C
AGN1g	<i>Alnus glutinosa</i>	Holland	M. Lalonde	T/C
AGN1 ^{EXO} ^{AG}	<i>Alnus glutinosa</i>		M. Lalonde	T/C
Air11	<i>Alnus incana</i> subsp. <i>rugosa</i>	Vermont	M. P. Lechevalier	T/C
Air12	<i>Alnus incana</i> subsp. <i>rugosa</i>	Vermont	M. P. Lechevalier	YC _z
ARbN4b	<i>Alnus rubra</i>	Oregon	M. Lalonde	T/C
Ar14	<i>Alnus rubra</i>	Washington	D. Baker	T/C
Avcl1	<i>Alnus viridis</i> subsp. <i>crispa</i>	Ontario, Canada	D. Baker	T/C
Ca11	<i>Ceanothus americanus</i>		M. P. Lechevalier	YC _z
Cp11	<i>Comptonia peregrina</i>	Massachusetts	D. Baker	FB
D11	<i>Casuarina equisetifolia</i>	Senegal	Y. Dommergues	YC _z
G2	<i>Casuarina equisetifolia</i>	Guadeloupe	Y. Dommergues	YC _z
Mp11	<i>Myrica pensylvanica</i>	Massachusetts	M. P. Lechevalier	T/C
Pt11	<i>Purshia tridentata</i>		D. Baker	YC _z
Eu11a	<i>Elaeagnus umbellata</i>		D. Baker	FB
EUN1fS20	<i>Elaeagnus umbellata</i>	Illinois	M. Lalonde	Qmod
EUN1wb	<i>Elaeagnus umbellata</i>	Illinois	M. Lalonde	Qmod
EAN1 ^{EA}	<i>Elaeagnus angustifolia</i>		M. Lalonde	Qmod
EAN1 ^{PEC} ^{EA}	<i>Elaeagnus angustifolia</i>	Ohio	M. Lalonde	Qmod

^a T/C, Tween-casein medium; YC_z, Czapek broth supplemented with yeast extract (5); FB, *Frankia* broth (4); Qmod, Qmod medium (16).

tures were chosen to obtain optimum reassociation rates according to the criteria of Wetmur and Davidson (30). To measure reassociation kinetics, the capillary tubes were removed from the water bath at different times (at C_0t values of 0.05 to 200 mol · s/liter) and transferred immediately to a dry ice-ethanol bath. The contents of each capillary tube were diluted into 1 ml of 0.12 M sodium phosphate buffer (pH 6.8) containing 50 µg of calf thymus DNA per ml and frozen for analysis later. These latter procedures were designed to discourage further reassociation in subsequent manipulations during hydroxyapatite fractionation. For hybridization reactions, reassociation was stopped at a C_0t value of 100 mol · s/liter, at which point more than 80% of the *Frankia* probe DNA had become reassociated (Fig. 1). To distinguish unreacted, single-stranded DNA (ssDNA) from reacted, double-stranded DNA (dsDNA), hydroxyapatite chromatography was carried out as described previously (26), and the data were analyzed by the computer program developed by Pearson et al. (24). The results of each hybridization test were normalized to the extent of the

homologous reaction (77% for strain Ar14 and 76% for strain Eu11a). Self-reassociation of labeled DNA (the extent of reassociation for a reaction mixture consisting of only labeled DNA) was less than 1% (data not shown).

Window diagram for thermal elution studies. The reliability of the hydroxyapatite technique for assessing the thermal stability of DNA-DNA duplexes depends strongly on the choice of the ionic environment and temperature range at which the studies are carried out. In order to determine the proper criteria for thermal elution studies, a partial window diagram was constructed by using the protocol of Martinson and Wagenar (21). Thermal chromatography of strain Ar14 DNA was carried out by using several different concentrations of potassium phosphate buffer (range, 20 to 100 mM) made by mixing equimolar amounts of monobasic and dibasic potassium phosphate in 50% (vol/vol) formamide. For each buffer system, successive thermal elutions were carried out at intervals of 3°C from 40 to 95°C. Hydroxyapatite chromatography was performed as described above. The fractions eluted at each temperature were collected and assayed for radioactivity. The thermal elution temperature [$T_{m(e)}$] for each concentration of potassium phosphate buffer was calculated from a graphic representation by interpolation of the temperature at which 50% of the radioactivity was eluted from the column. A partial window diagram was obtained by plotting $T_{m(e)}$ on a linear scale against concentration of potassium phosphate buffer on a logarithmic scale (22).

RESULTS

Reassociation of *Frankia* DNA. To measure the genome size of *Frankia*, as well as to define the reaction system for hybridization tests, reassociation kinetics experiments were carried out by using DNAs from two representative strains, strains Ar14 and Eu11a. The reassociation data are plotted in Fig. 1, in which the percentage of DNA not bound to hydroxyapatite is plotted as a function of the logarithm of C_0t (10). Data points for each DNA are distributed along the lines, which are the best-fit second-order kinetics curves for each data set generated by the computer program (24). Although substantial amounts of total DNA did not partici-

FIG. 1. Reassociation kinetics of DNAs from two frankiae, strains Ar14 and Eu11a, and *E. coli* assayed by hydroxyapatite chromatography. The lines represent the best fits to the data generated by the computer program described by Pearson et al. (24).

TABLE 2. Reassociation kinetics data and genome molecular weights

Strain	Fraction of DNA reassociated ^a	K_{obs} (liter/mol · s) ^b	K_{pure} (liter/mol · s) ^c	Pure $C_{0t_{1/2}}$ (mol · s/liter) ^d	Root mean square error (%) ^e	Genome mol wt ($\times 10^9$) ^f
Ar14	0.774	0.112	0.144	6.94	1.74	8.3
Eu11a	0.729	0.141	0.194	5.15	1.57	6.0
<i>E. coli</i>	0.967	0.309	0.313	3.13	3.47	2.8 ^g

^a Determined by using unconstrained one-component conditions. The number of data points was 17 for strain Ar14, 18 for strain Eu11a, and 19 for *E. coli*.

^b Observed rate constant.

^c Pure rate constant (corrected values for the relative fraction of the total DNA reacted).

^d $C_{0t_{1/2}}$ for pure DNA; calculated from $1/K_{pure}$.

^e Root mean square deviation of the data points from the curve.

^f Calculated from the following equation: genome (*Frankia*) = genome (*E. coli*) $\times$ (pure $C_{0t_{1/2}}$ for *Frankia*/pure $C_{0t_{1/2}}$ for *E. coli*).

^g Data from reference 20.

pate in reassociation, the reactions were essentially completed between C_{0t} values of 1 and 100 mol · s/liter.

The kinetic parameters derived from these data are summarized in Table 2, along with the calculated genome sizes. Pure rate constant values of 0.194 and 0.144 were used to calculate the genome size of each *Frankia* isolate based on a $C_{0t_{1/2}}$ value of 3.13 mol · s/liter for *E. coli*. The calculated genome molecular weights were 6.0×10^9 and 8.3×10^9 for strains Eu11a and Ar14, respectively, based on a genome molecular weight of 2.8×10^9 for *E. coli* (20). The differences between these two values are largely attributable to the last few data points in Fig. 1. Since the data points are largely overlapping over most of the course of reassociation, we do not attach any significance to the difference in measured genome molecular weights.

DNA-DNA hybridization. To measure the genetic similarities among the 19 *Frankia* strains, DNA-DNA hybridization

TABLE 3. Hybridization of the DNAs of *Frankia* isolates

Strain ^a	% Hybridization with:	
	Strain Ar14	Strain Eu11a
Genogroup 1^b		
Ar14(= DDB 010210)	100 (77) ^c	
ARbN4b	94 $\pm$ 4	20
ACN1 ^{AG}	90 $\pm$ 7	30
AGN1g	81 $\pm$ 5	
Cp11	81 $\pm$ 2	14
AGN1 ^{EXO} ^{AG}	79 $\pm$ 7	
MP11(= LLR 162001)	72 $\pm$ 5	
Air11(= LLR 01321)	72 $\pm$ 1	
Avcl1(= DDB 010110)	67 $\pm$ 5	
Strains not in genogroup 1^b		
Eu11a(= DDB 130110)	30	100(76) ^c
Ca11(= LLR 03011)	26	33
G2(= ORS 020604)	36	27
D11(= ORS 020602)	29	22
Pt11(= DDB 170110)	36	19
EAN ^{PEC} ^{EA}	39	15
EUN1fS20	18	13
Air12(= LLR 01322)	27	10
EAN1 ^{EA}	21	
EUN1wb	28	

^a New designations are given in parentheses (D. Baker and M. P. Lechevalier, personal communication).

^b Genogroup 1 strains showed more than 65% homology with strain Ar14, and all other strains showed less than 40% homology.

^c Values normalized to homologous reactions.

TABLE 4. DNA-DNA hybridization among some strains not in genogroup 1

Strain	Host plant genus	% hybridization with: ^a		
		Strain Eu11a	Strain Ca11	Strain G2
Eu11a	<i>Elaeagnus</i>	100		
Ca11	<i>Ceanothus</i>	33	100	
G2	<i>Casuarina</i>	27	47	100
D11	<i>Casuarina</i>	22	60	97
Pt11	<i>Purshia</i>	19	38	27

^a Values normalized to homologous reactions.

tests were carried out by using labeled strain Ar14 and Eu11a DNAs as probes. As Table 3 shows, *Frankia* strains could be divided into two major groups based on DNA-DNA homology data. Genogroup 1 strains had high levels of homology with strain Ar14 (range, 67 to 94%; average, 80%). On the other hand, the remaining strains had low levels of homology with strain Ar14 (range, 18 to 39%; mean, 29%). When strain Eu11a DNA was used as a probe, however, none of the other *Frankia* strains was found to have a high degree of homology. The highest homology value obtained with this probe was less than 33%. As a group, these strains exhibited slightly less homology with strain Eu11a than genogroup 1 strains did.

Based on these results, genogroup 1 strains seemed to be very closely related to each other and to form a homogeneous group, whereas the remainder of the strains were not closely related to either strain Eu11a or strain Ar14. To test for the possibility of homology groups other than genogroup 1, two additional strains, strains Ca11 and G2, were used to prepare DNA probes for hybridization tests. As Table 4 shows, when strain Ca11 DNA was used as a probe, two isolates from *Casuarina* showed moderate degrees of homology (mean, 54%), whereas strain Pt11 showed a low degree of homology (38%). When strain G2 DNA was used as a probe, strain D11 showed very high homology (97%), but strain Pt11 exhibited only a low level of homology (27%). Thus, strains G2 and D11 were assigned to genogroup 2.

Window diagram and thermal elution studies. In order to lower the $T_{m(e)}$ of duplex DNA, organic solvents (formamide

FIG. 2. Window diagram of strain Ar14 DNA obtained by using potassium phosphate buffer (concentration range 20 to 100 mM) made in 50% formamide. The $T_{m(e)}$ was plotted for each concentration of buffer on a logarithmic scale.

FIG. 3. Thermal elution profiles of strain Arl4 dsDNA and ssDNA with 60 mM potassium phosphate buffer made in 50% formamide.

and urea, among others) can be used either as a buffer system for hybridization (23) or as an elution buffer (29). The latter method was chosen because a reduced reassociation rate in the organic solvent system would require that the whole reaction system be recharacterized (13). To determine the thermal elution conditions under which elution of the duplex from the hydroxyapatite column really corresponded to strand separation of the duplex, a partial window diagram (Fig. 2) was constructed as described above. Only those concentrations giving a vertical line in Fig. 2 (namely, from 50 to 80 mM) could be used for thermal elution studies, and 60 mM was chosen. Under these conditions, 50% of the homologous strain Arl4 duplex was eluted at 66°C.

The ability of the new elution buffer to distinguish ssDNA from dsDNA was tested by using strain Arl4 DNA. As shown in Fig. 3, there was no clear-cut temperature at which the two could be distinguished since ssDNA was not eluted completely until 75°C, whereas dsDNA was eluted over a broad range (from 60 to 80°C). However, 60°C was chosen to elute ssDNA with the new buffer system, because only 5% of the dsDNA eluted at that temperature and more than 80% of the ssDNA eluted. At 65°C, more than 90% of the ssDNA eluted, but about 40% of the dsDNA eluted at that temperature. Thus, 60°C appeared to be the optimum temperature for separating ssDNA and dsDNA in this system.

$T_{m(e)}$ values were obtained graphically by plotting the cumulative percentage of DNA eluted at each elution temperature on a linear scale and obtaining the midpoint of the transition from the plot. The following three representative duplexes are shown to demonstrate their thermal elution profiles: strain Arl4 $\times$ strain Arl4, representing a homologous duplex; strain Arl4 $\times$ strain ACN1^{AG}, representing a high-homology duplex; and strain Arl4 $\times$ strain D11, representing a low-homology duplex. As shown in Fig. 4, where the ordinate is plotted on a linear scale, the elution profiles of the homologous and high-homology duplexes were sigmoidal, indicating little mismatch in their duplexes; $T_{m(e)}$ values were obtained easily from these graphs. On the other hand,

low-homology duplexes produced more complex elution profiles, suggesting a higher degree of mismatch in these duplexes; $T_{m(e)}$ values were not easily obtained from the graph. $T_{m(e)}$ values were also obtained from the elution profile plotted on normal probability paper (15), on which data points for homologous and high-homology duplexes fell on a straight line, whereas points for low-homology duplexes fell on two different lines (data not shown). $T_{m(e)}$ values obtained by these two methods did not differ significantly from each other (data not shown).

Table 5 summarizes the results of our thermal elution studies. The $T_{m(e)}$ values for homologous duplexes were 67.8°C for strain Arl4 DNA and 66.2°C for strain Eu11a DNA. The differences in $T_{m(e)}$ values between homologous and heterologous reactions [$\Delta T_{m(e)}$] are also shown. $\Delta T_{m(e)}$ is closely correlated with mismatch. The mean $\Delta T_{m(e)}$ value of high-homology DNA duplexes (homology more than 60%) was 3.2°C, whereas that of low-homology DNA duplexes (homology less than 40%) was 4.5°C, indicating 3 and 5% mismatches in the duplexes, respectively. According to a widely used convention, a 1°C decrease in thermal stability is estimated to reflect a 1% mismatch in a duplex (9). In Fig. 5, another presentation of thermal elution is shown in which the percentage of DNA eluted at each elution temperature is plotted on a linear scale instead of as cumulative percentages, as in Fig. 4. The temperature for peak elution of each duplex fell within the range from 63.5 to 67.5°C, despite wide differences in the homology values.

DISCUSSION

As a first step in the characterization of the *Frankia* genome, the base compositions of the DNAs of 12 *Frankia* isolates were reported (1). The base compositions of seven additional isolates have now been measured by the thermal denaturation method (data not shown). The guanine-plus-cytosine contents for these additional strains ranged from 66.6 to 68.3 mol%. These values are lower than the previ-

FIG. 4. Thermal elution curves of duplexes formed by strains Arl4, ACN1^{AG}, and D11 with strain Arl4 at 74.5°C on a linear plot.

ously reported values of 68.4 to 72.1 mol%, which are above the midpoint of the range of base compositions for members of the order *Actinomycetales* (53 to 78 mol%) (12).

The purpose of the DNA reassociation kinetics experiments was to measure genome sizes based on kinetic complexity. The mean genome molecular weight of two *Frankia* isolates was 7.1×10^9 , or about 2.5 times the molecular weight of the *E. coli* genome. This value is in the higher range of the known genome molecular weights of other actinomycetes (2.0×10^9 to 7.0×10^9) (8), but smaller than the genome molecular weights of yeasts which have genomes about three times bigger than the *E. coli* genome and near the lower limit for eucaryotic fungal genomes (17).

Genetic relatedness among *Frankia* isolates was investigated by measuring the quality of hybrid duplexes, as well as the extent of hybrid formation. *Frankia* isolates were divided into two major groups on the basis of our results. Based on their homology values with strain ArI4 DNA, the genogroup 1 strains exhibited high levels of homology (average, 80%), whereas the 10 additional strains tested showed low levels of homology (less than 29%). The choice of strains for use in preparing DNA probes was arbitrary; strain ArI4 proved to be useful for this purpose, but strain EuI1a did not turn out to be a good choice, in the sense that none of the isolates exhibited more than 33% homology with strain EuI1a. With new probe strains, strains CaI1 and G2, at least three additional homology groups were identified. Complete characterization of the levels of DNA relatedness in this group will undoubtedly require the use of several additional probe strains.

The relationship among *Frankia* isolates revealed by this study is consistent with, but goes beyond, that suggested by other studies. Based on the unique morphology of their sporangia and vesicles and their common cell wall type (type III) and phospholipid pattern (type PI), all of the isolates

FIG. 5. Thermal elution profiles of duplexes formed by strains ArI4, ACN1^{AG}, and D11 with strain ArI4 at 74.5°C.

examined belong to a single genus, *Frankia*. This conclusion was supported by the small differences (5.5 mol%) in the guanine-plus-cytosine contents of their DNAs. Two major groups which had been suggested by serological, physiological, and cross-inoculation studies were also identified based on differences in DNA homology values (80 versus 29% with strain ArI4). So far, physiological groupings correspond to genogroupings, although it remains to be seen whether the reverse is true, because five strains from genogroup I and

TABLE 5. Thermal elution study of the hybrids formed at 74.5°C

Strain	Hybridization with strain ArI4		Hybridization with strain EuI1a	
	$T_{m(e)}$ (°C) ^a	$\Delta T_{m(e)}$ (°C) ^b	$T_{m(e)}$ (°C) ^a	$\Delta T_{m(e)}$ (°C) ^b
Genogroup 1				
ArI4	67.8		64.5	1.7
ARbN4b	65.5	2.3	63.5	2.7
AirI1	65.3	2.5		
ACN1 ^{AG}	65.7	2.1		
AvcI1	65.3	2.5		
AGN1g	64.7	3.1		
AGN1 ^{EXO} ^{AG}	64.0	3.8		
MPI1	63.4	4.4		
CpI1	62.7	5.1	60.8	5.4
Strains not in genogroup 1				
EuI1a	64.7	3.1	66.2	
AirI2	62.4	5.4	60.5	5.7
CaI1	62.2	5.6	64.6	1.6
D11	62.4	5.4	59.6	6.6
PtI1	60.7	7.1	58.8	7.4
G2	64.0	3.8	60.2	6.0
EAN1 ^{EA}	66.5	1.3		
EAN1 ^{PEC} ^{EA}	64.2	3.6		
EUN1fS20	61.4	6.4		
EUN1wb	65.2	2.6		

^a $T_{m(e)}$ was the temperature at which one-half of the DNA bound to hydroxyapatite was eluted.

^b $\Delta T_{m(e)}$ was the difference between the $T_{m(e)}$ for the homologous reaction and the $T_{m(e)}$ for the heterologous reaction.

TABLE 6. Present status of *Frankia* groupings

Strain	Host genus	Host compatibility group ^a	Sero-group ^a	Physiological group ^b	Genogroup ^c
AirI1	<i>Alnus</i>	1	I	B	1
CpI1	<i>Comptonia</i>	1	I	B	1
MpI1	<i>Myrica</i>	1	I	B	1
ACN1 ^{AG}	<i>Alnus</i>	1		B	1
ArI4	<i>Alnus</i>	1	I		1
AvcI1	<i>Alnus</i>	1	I		1
ARbN4b	<i>Alnus</i>	1			1
AGN1g	<i>Alnus</i>	1			1
AGN1 ^{EXO} ^{AG}	<i>Alnus</i>				1
G2	<i>Casuarina</i>		II	A	2
D11	<i>Casuarina</i>		II	A	2
AirI2	<i>Alnus</i>	1	II	A	N
EuI1a	<i>Elaeagnus</i>	2	II	A	N
PtI1	<i>Purshia</i>	2	II	A	N
CaI1	<i>Ceanothus</i>		II	A	N
EAN1 ^{PEC} ^{EA}	<i>Elaeagnus</i>				N
EAN1 ^{EA}	<i>Elaeagnus</i>				N
EUN1wb	<i>Elaeagnus</i>				N
EUN1fS20	<i>Elaeagnus</i>				N

^a See reference 2.

^b See reference 19.

^c Genogroup 1 strains have high levels of homology with strain ArI4. N, No genogroup assigned.

four strains not homologous with strain Arl4 have not been tested by physiological criteria (Table 6). Our data also provide insight into the relationships among the isolates, not only within each group but also between groups, which were not obtained by other studies. According to the Becking system of classification (6), which was based largely on cross-inoculation studies with crushed nodule inocula, isolates belonging to genogroup I would have been classified as *Frankia alni* or *Frankia brunnhorstii*, depending on the host genera. However, we found high levels of homology (more than 80%) among the isolates from both host plants, and other workers have demonstrated that cross-inoculation within this group does occur, suggesting that they should be considered a single species. Moreover, one strain from *Alnus*, strain Arl12, which also would have been given the name *F. alni*, is at most distantly related to another isolate from the same species, strain Arl11. Strains belonging to genogroup 1 and all of the other strains tested are quite different from each other, having less than 39% homology. Finally, at least three subgroups [subgroups G2(D11), Pt11, and Eu11a] were identified among the isolates outside genogroup 1 by DNA homology tests in which three probe strains from three different host genera were used (Table 4).

Thermal elution studies to measure the quality of the hybrid duplexes revealed interesting results. Despite wide differences in their DNA homology values (80 versus 25%), the difference in $\Delta T_{m(e)}$ was very small (3.2 versus 4.5°C). In some taxonomic groups, there is a good correlation between percent homology and the thermal stability of duplexes, with about 1°C decrease in $T_{m(e)}$ corresponding to a 5% difference in interspecies homology value (14). Thus, the small difference in $\Delta T_{m(e)}$ which we observed indicated that duplexes bound to the hydroxyapatite column were quite stable regardless of the overall homology values. Additionally, peak elution of all of the hybrid duplexes occurred within a small range ($\pm 2^\circ\text{C}$). The fact that hybrid duplexes between strains belonging to genogroup 1 had a small $\Delta T_{m(e)}$ value indicates that there has been relatively little divergence in the entire genomes of these strains. The small $\Delta T_{m(e)}$ value of hybrid duplexes among all other strains indicates that although the largest portions of the genomes exhibit a great deal of divergence, small portions of the genome are highly conserved among all of the strains. The nature of the conserved section of the genomes (approximately 10%) is not known. Genes for ribosomal ribonucleic acids are known to be very conserved among different bacteria (28). Genes for nitrogen fixation activity are also known to be conserved among nitrogen-fixing organisms (27). It should be helpful in the characterization of the conserved segment of the *Frankia* genome to elute bound duplexes and compare DNA homology or to probe *Frankia* genomes with cloned genes for ribosomal ribonucleic acid, for nitrogen fixation, and for other conserved functions.

It is evident from this study that the genetic diversity among frankiae is large indeed. The use of four probe strains revealed the presence of at least four possible genogroups. For practical purposes, DNA homology data should be correlated with other biological properties that can be detected more easily. Unfortunately, it is not yet possible to make such a correlation because only limited biological characterization has been accomplished on the strains used in this study. The potential for using DNA homology data as a means of grouping isolates has been demonstrated here, and as more isolates become available for study it is likely that this approach will provide the basis for a comprehensive framework for the classification of *Frankia*.

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