MINIREVIEW

Taxonomy of the Genus *Frankia* (*Actinomycetales*)

MARY P. LECHEVALIER†

Waksman Institute of Microbiology, Rutgers, The State University of New Jersey,
Piscataway, New Jersey 08855-0759

Members of the genus *Frankia* have been classified in the order *Actinomycetales* on the basis of morphology, cell chemistry, and 16S rRNA sequences and catalogs. This genus, which is presently defined by morphology, cell chemistry, the ability to fix nitrogen, and infectivity for and ability to enter into symbiotic relationships with certain plant hosts, may be heterogeneous. *Frankia* species groups have been difficult to delineate by classical phenotypic methods. The recent use of DNA-DNA pairing and low-frequency restriction fragment analysis, as well as probes composed of certain sequences from the *nif* (nitrogen fixation) genes or the variable regions of 16S rRNA, has contributed to substantial progress in developing species concepts. In this review I trace the taxonomic history of the genus and outline some of the problems to be resolved in the future.

Frankiae are nitrogen-fixing actinomycete symbionts that cause the formation of perennial nodules on the roots of a botanically diverse group of bushes and small trees belonging to eight families, 25 genera, and well over 200 species. The association is referred to as "actinorhizal." A number of good recent reviews (9, 10, 15) provide an overview of the *Frankia*-plant interaction. The frankiae themselves are relative newcomers to laboratory research, and their taxonomy is still developing.

From an evolutionary standpoint, actinorhizal associations are quite old; there is fossil evidence of actinomycete-containing nodules on alders in the late Pleistocene era (5). As early as 1866 (52, 100) it was hypothesized that the endophyte is microbial, but this was proven only after the introduction of electron microscopy and the actual isolation and maintenance of a pure culture in 1978 (21).

In 1970, despite his lack of success in growing a single strain in vitro, and believing the organisms to be obligate symbionts, J. H. Becking (11) proposed that the name *Frankia* should be resurrected for these organisms. The name had been first proposed by Brunchorst in 1886 (20) to honor A. B. Frank, a Swiss microbiologist who coined the word symbiosis, but had fallen into disuse. Becking correctly discerned the actinomycetic nature of the endophytes and placed them in the family *Frankiaceae* in the order *Actinomycetales*. Following the lead of *Rhizobium* taxonomists, he created 10 species on the basis of host plant specificity, which he determined by using crushed nodules as inocula.

Following the successful propagation of the first isolate, more and more strains became available, and problems of nomenclature and taxonomy became a matter of concern, especially as Becking's host compatibility data were not always found to be correct (49). The slow growth and nutritional fastidiousness of *Frankia* isolates made it difficult to pursue classical phenotypic approaches to the taxonomy of these organisms, and molecular methods have proven to be increasingly useful. Indeed, from a methodological point of view, the current taxonomic research on *Frankia* isolates might be said to be a paradigm for microbial taxonomy in

general. In this review I trace the development and present the status of the taxonomy of the genus and try to predict what the future may hold.

Phylogeny. Unlike the leguminous plants with which rhizobia form symbiotic nitrogen-fixing associations (24), actinorhizal plants are thought to be polyphyletic in origin (65). Thus, the basis for and understanding of the *Frankia*-plant symbiosis must be sought at the molecular or biochemical level. Unlike their plant hosts, frankiae have been assumed to be a phylogenetically homogeneous group on the basis of a variety of phenotypic characteristics, including morphology, cell chemistry, and nitrogen-fixing capacity. A comparison of sequences and catalogs of certain regions of 16S rRNAs by Stackebrandt and his associates (39, 93) showed that two type "B" frankiae (see below) could be shown to belong to one of the major sublines of descent of the *Actinomycetales* and to be closely related to members of the actinomycete genus *Geodermatophilus*. These authors redescribed the family *Frankiaceae* to include both the genus *Frankia* and the genus *Geodermatophilus*. The 16S rRNA sequences of type "A" strains have yet to be determined. Other workers found that sequencing of the rRNA genes of a single *Frankia* strain showed that the 5'-to-3' order is 16S-23S-5S, which is typical of bacteria in general (71).

Additional phylogenetic data were obtained from a study of 1 of the 20 or so genes which code for the enzymes involved in nitrogen fixation. The *nifH* gene, which codes for the iron protein component of nitrogenase, is very highly conserved; thus, it has been proposed that sequence data for this gene from a variety of nitrogen-fixing bacteria should be used in the construction of bacterial phylogenies (101). Interestingly, a determination of the sequences of the *nifH* genes of two frankiae (70, 74) showed that these organisms group with cyanobacteria of the genus *Anabaena* rather than with *Clostridium pasteurianum*, a gram-positive bacterium to which they are more closely related on the basis of 16S rRNA sequences. Sequencing of the *nifD* gene of one of the same isolates showed that it clustered with both the genus *Anabaena* and the genus *Klebsiella* (72).

Taxonomy of the genus. When Becking proposed the genus *Frankia* (11), he could only hypothesize that the members of this genus were actinomycetes because of his lack of success in cultivating any of them in vitro. He based his conclusion

† Present address: RR 2, Box 2235, Morrisville, VT 05661.

on the gram-positive reactions of the organisms, as well as their hyphal nature and dimensions, their lack of a nuclear membrane, and the formation of what he called "terminal swellings," which he felt had morphological affinities to actinomycete conidia. (Also called "vesicles," these structures are now known to be the site of nitrogenase activity [59].) He also described the polyhedral cells that he observed within the plant cells, which are now known to be the sporangiospores of the organisms, but he did not observe the sporangia themselves. Later (12), he published data on the cell chemistry of the endophyte cells. Unfortunately, these data were incorrect since Becking analyzed material that was highly contaminated with plant debris.

Morphology is still one of the most important criteria for determining the generic status of actinomycetes, and this is true for the genus *Frankia* as well. Frankiae growing in artificial media produce the same general morphological structures that they produce in planta, including sparsely branched hyphae, vesicles (stipitate, round to oblong cells borne laterally on the hyphae), and multilocular sporangia containing nonmotile spores. The sporangiospores are unique among actinomycetes, because they are surrounded by multiple membranous layers visible by electron microscopy (52). Vesicle formation in some strains can be induced only in nitrogen-free media; in other strains vesicles are formed under all growth conditions. In the nodules, hyphae and vesicles are the most prominent features; in some cases many sporangia are also formed. Nodules containing many sporangia are referred to as "spore positive" (Sp⁺), and the frankiae in these nodules may be more difficult to isolate and grow in vitro (47, 73). It is not known whether the Sp⁺ characteristic has taxonomic value. This is because actinomycetes vary enormously in their capacity to express morphological features and, unlike fungi, this expression is often very dependent on the nutritional or physical conditions of growth.

In recent years cell chemistry has played a prominent role in the taxonomy of actinomycetes at the generic level. The compounds that are important include the amino acids, amino sugars, and sugars present in the cell wall, cellular polysaccharides not covalently linked to the wall, fatty acids (especially those such as mycolic acids), phospholipids, and menaquinones (53). From the standpoint of cell chemistry, frankiae have both common and novel compositions (55). The cell wall composition type is type III (*meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine, and muramic acid), the simplest and most common type found in the actinomycetes. In contrast, the whole-cell sugar (WCS) patterns are both diverse and (in some cases) novel. Most isolates have a type D WCS pattern (the diagnostic constituent is xylose, but the minor arabinose component found in other actinomycetes is not present). Other strains contain fucose (type E WCS pattern), a sugar not found in the polysaccharides of other actinomycetes; some isolates contain 3-*O*-methyl-*D*-galactose (madurose) (type B WCS pattern) or simply major amounts of galactose or glucose (type C WCS pattern). Most frankiae also contain 2-*O*-methyl-mannose (63, 80). The fatty acids are generally the normal and branched-chain types typical of most actinomycetes; mycolic acids are not present. The phospholipid pattern type is type PI (phosphatidylinositol, phosphatidylinositol mannosides, and diphosphatidylglycerol). The major menaquinones include MK-9(H₄), MK-9(H₆), and MK-9(H₈) (82). The G+C content ranges from 66 to 71 mol% (as determined by the thermal denaturation method) (4).

Nitrogenase is an enzyme which is sensitive to oxygen. In

Frankia vesicles, the site of nitrogen fixation, protection against oxygen toxicity is provided by the buildup of lipid-containing layers at the surface of the vesicles at a high partial O₂ pressure (77). Freeze fracturing and sectioning have been used to demonstrate this unique feature (40, 77). A recent study (16) has demonstrated that major amounts of bacteriohopanetetrol (C₃₅H₆₂O₄) are present in extracts of cells of frankiae grown in vitro and in endophyte-containing nodule tissue, but not in host plant tissue alone. However, it is not known whether this lipid is the lipid at the surface of the vesicles because no increase in the amount of the compound was observed in cells grown under high oxygen tensions. However, this characteristic may be a good taxonomic marker if it is shown to be unique to *Frankia* spp. among the actinomycetes.

Frankiae are microaerophilic to reluctantly aerobic. Because many strains cannot be maintained on the surfaces of solid media, they are routinely grown in liquid media. They grow very slowly, and many isolates have having doubling times of 2 to 3 days or more. A few strains have a shorter doubling time (1 day, which approaches the growth rate of some of the slowly growing actinomycete saprophytes). Although frankiae are exacting in their growth requirements, they do not need complex media to grow well. A simple salts solution supplemented with an organic acid such as pyruvate or propionate as a carbon source often is sufficient since frankiae can fix nitrogen in vitro (55). The ability to fix nitrogen is considered a marker for the genus since non-nitrogenase-producing (*nif*) isolates are rare (70, 74). Recent reports (33, 45) of nitrogen fixation by certain *Streptomyces* isolates have shown that there may be problems with this characteristic; however, there is evidence that the enzymatic system involved in the *Streptomyces* isolates differs from the system of frankiae.

Two suprageneric or subgeneric groups have been proposed (51, 54) on the basis of physiology. Type A strains are quite diverse and in many cases not very different from slowly growing saprophytic actinomycetes. These organisms are relatively aerobic, and most can be maintained on slants. They are active physiologically, grow more rapidly than type B strains (see below), and utilize a variety of monosaccharides and disaccharides with or without acid production. Many produce hydrolytic enzymes, such as pectinases, cellulases, amylases, and proteases. They do not reinfect the host plants from which they were isolated, at least under the experimental conditions that are used at the present time.

In contrast, the members of the second group (type B) grow slowly (doubling times, 2 to 7 days) and are inactive physiologically, not utilizing (with few exceptions) carbohydrates, proteins, or starch. The preferred carbon sources include organic acids or Tween compounds. When Tween 80 is added to media containing certain carbohydrates, it has a synergistic effect on growth, and the dry cell weight of the test organism exceeds the sum of the cell weights of the organism grown separately on each carbon source. The type B strains are strictly microaerophilic and cannot be maintained on slants, and they can infect the host plants from which they were isolated. Other types that differ from these two types probably exist (56).

The "constant" (relatively invariable for all taxa) regions of the 16S rRNA nucleotide sequences of actinomycetes are valuable for determining the proper placement of genera within the order *Actinomycetales* and for discerning the position of the order *Actinomycetales* in the *Prokaryotae*, while it is widely believed that the "hypervariable" regions may help in phylogenetic classification at the genus or

species level (41, 60). However, efforts to utilize the differences for *Frankia* taxonomy at the genus level have met with mixed success. For example, the sequences of one region (positions 1020 to 1042; *Escherichia coli* numbering) were found to differ when the rRNAs of effective (nitrogen-fixing) and ineffective *Frankia* strains were compared (37). With one exception, probes containing 20 to 22 nucleotides targeting this region did not cross-react with the rRNAs of the other actinomycetes tested. The organisms that did not react included representatives of the genera *Streptomyces*, *Streptoverticillium*, *Actinomyces*, *Propionibacterium*, *Pimelobacter*, *Terrabacter*, *Tsukamurella*, *Nocardiopsis*, *Caseobacter*, *Dermatophilus*, and *Geodermatophilus*. However, both types of probes (probes from effective "EFP" and ineffective "IFP" types of *Frankia*) reacted with the rRNA of *Nocardioides albus* and thus could not be considered specific for the genus *Frankia*. Furthermore, the EFP probe reacted specifically with pure rRNAs from effective *Frankia* isolates obtained from *Alnus* spp. host plants but not with RNAs from effective isolates obtained from other plant hosts or ineffective *Alnus* strains. Thus, the authors concluded that the method might have only very limited value in taxonomy (37).

A sequence in the region from position 180 to position 240 (*E. coli* numbering) was exploited as a probe by Hahn et al. (38). This probe was found to react with all 23 *Frankia* strains tested, but at higher temperatures some strains no longer hybridized, indicating that their sequences differed from the sequences of the other strains. When representatives of 22 other actinomycete genera were tested, the *Actinomadura* and *Microbispora* strains reacted positively, further indicating the lack of specificity of the probe. When the utility of other probes from other regions (positions 140 to 220, 430 to 540, and 980 to 1060) was examined, the probes were also found to be not specific at the generic level, since type A and B *Frankia* strains reacted differently. It was also found that sequences from *E. coli* positions 1 to 925 reacted differently with different *Frankia* strains.

Simonet et al. (88) tackled the problem of a genus-specific probe by using a different approach. Two different sets of primers were designed, a universal set targeted to nine nitrogen-fixing microbial genera but giving negative results with organisms that do not fix nitrogen and another set which was specific for the *nifH*-*nifD* regions of *Frankia* spp. With PCR, amplification of the DNAs of various *Frankia* strains with the two primer sets gave rise to fragments whose lengths were specific for the genus. In the same way, universal and specific primers targeted to the 16S rRNA gene region were prepared. With the specific primers the specific sizes of the fragments generated were characteristic of *Frankia* spp. and also of the closely allied genus *Geodermatophilus* and were therefore presumably specific for members of the family *Frankiaceae*. In order to separate members of the genus *Geodermatophilus* from members of the genus *Frankia*, two additional primers were designed, which took advantage of the differences in size of the sequence that separates (intergenic sequence [IGS]) the 16S and 23S rRNA genes. Following amplification, the sizes of the *Frankia* fragments that were generated centered around 561 bp and differed from the sizes of the fragments generated from *Geodermatophilus* strains and the other microorganisms tested. This promising system has yet to be tested with a large number of strains belonging to various actinomycete taxa, a difficult but feasible task.

Taxonomy of the species. Becking's proposed species (11) were inadequate because (i) his host ranges were not always

correct when they were determined by using pure strains; (ii) nodules often contain more than one endophyte strain (18, 35, 47, 67) and these strains may not be related to each other; (iii) some isolates cannot be shown to be infective for their original host under a variety of greenhouse conditions; and (iv) not all strains that infect the same host plant species are similar enough by today's standards to be placed in the same species.

The unexpected diversity of *Frankia* strains isolated from the same plant host has been amply confirmed and is based on morphological, physiological, biochemical, cell chemical, genetic, and serological differences (14, 27–29, 43, 51, 56, 57, 67, 73, 84, 92, 97, 102). Thus, simple isolation from a given plant host is not a sufficient basis for assignment to a species. Nevertheless, it has been shown by a variety of techniques, including DNA-DNA relatedness analysis, low-frequency restriction fragment analysis (LFRFA), and 16S rRNA gene sequencing (see below), that there are numerous natural groups among isolates obtained from the same host plant which may be equated with species.

Initial efforts to delineate *Frankia* species among the various isolates included the classical tests used in actinomycete classification (36, 62, 85, 99). These efforts were not successful, partly because the slow growth and metabolic inactivity of many of the organisms made these determinations difficult and because the results did not allow reliable discrimination of species (54, 82, 97). It was agreed by workers in the field that, until better criteria for species definition could be found, the use of species names would be avoided. Instead, numerical designations describing the host plant of origin and a culture collection acronym were to be used (48, 49). Subsequently, various approaches to characterization were tried in an effort to construct meaningful groups at the species level. The characteristics examined included morphological differences, such as the sporangium formation in planta discussed above (73, 95); physiology of carbohydrate uptake (51, 56); utilization of various substrates and enzyme production (30, 42, 84); serology (7, 51); host specificity (8, 19, 23, 26); isozyme patterns (13, 58); whole-cell sugar chemistry (50); polypeptide patterns (14); fatty acid contents (61, 98); restriction patterns (2, 18, 25); restriction fragment length polymorphisms (6, 28, 69); and DNA-DNA relatedness (3).

These studies revealed potentially useful taxonomic techniques, but the relationships of the various groups that resulted were difficult to discern because the strains studied were often few, were from limited provenances, and differed from study to study.

Lalonde and his colleagues investigated *Frankia* isolates obtained from plants belonging to the Rhamnaceae, Myricaceae, and Elaeagnaceae growing in North America. These authors looked at polypeptide patterns (35, 86), sugar analysis data (94), fatty acid data (87), isozyme data (34), speed of nodulation (68), competitiveness with other strains (86), the ability to fix nitrogen in planta (73), plasmids (75, 91), and *nif* sequences (74). Some of the data supported the idea that the host plant of origin is an important element in the separation of *Frankia* strains into species groups. Consequently, Lalonde et al. (46) proposed a redefinition of Becking's species *Frankia alni* and *Frankia elaeagnii*. *F. alni* was described with two subspecies, "*F. alni* subsp. *pommerii*" and "*F. alni* subsp. *vandijkii*," which represent the Sp⁻ and Sp⁺ types of isolates from plants of the genus *Alnus*, respectively. The type species of the genus, *F. alni* subsp. *alni*, has yet to be described in modern terms, and a type strain has not been selected (17).

TABLE 1. Genomic *Frankia* species as determined by various DNA-DNA relatedness studies

Plant host taxon	No. of <i>Frankia</i> isolates:		No. of genomic species discerned	Reference
	Clustered at a relatedness level of >70% ^a	Ungrouped		
<i>Alnus</i>	8	2	1	3
	12	6	3	31
	16	1	5	1
Casuarinaceae	2	3	1	3
	7	3	1	31
	5	0	1	83
Elaeagnaceae	0	3	0	3
	11	2	5	31
	7	3	4	1
<i>Myrica</i>	4	11	1	18
<i>Podocarpus</i>	2	0	1	83

^a Genomic species in reference 1 were clustered at a level of relatedness of >60%.

DNA-DNA relatedness is currently believed to be one of the best methods to assess relationships among strains at the species level. The first group of workers to use this technique to study *Frankia* strains (3) found high levels of relatedness among most of the isolates obtained from *Alnus* spp. but no clear-cut patterns of relatedness among isolates obtained from *Casuarina*, *Ceanothus*, *Elaeagnus*, and *Purshia* spp. Fernandez et al. (31) identified three genomic species among 14 strains obtained from *Alnus* spp.; one of these species corresponded to "*F. alni* subsp. *pommerii*" of Lalonde et al. (46), and another corresponded to "*F. alni* subsp. *vandijkii*" of these same workers. Five genomic species were found among 12 isolates obtained from members of the Elaeagnaceae, and one genomic species comprised nine isolates obtained from members of the Casuarinaceae. Some strains were not grouped. Benson and Silvester (15) have pointed out that the finding of Fernandez et al. (31) that the type strain of "*F. alni* subsp. *vandijkii*" was related at a level of only 2% to the type strain of "*F. alni* subsp. *pommerii*" suggests that these two taxa may represent distinct species groups. Also, the DNA-DNA relatedness data summarized in Table 1 emphasize the fact that the proposal of Lalonde et al. (46) did not go far enough and that additional species need to be described.

Akimov and Dobritsa (1) have argued that groups defined by morphology in planta (Sp⁺ and Sp⁻), physiology (types A and B), serology, and whole-cell sugar patterns were not sufficiently discriminatory at the species level but were, instead, supraspecific. Whole-cell polypeptide patterns were considered infraspecific, and fatty acid and isozyme patterns were not judged to be compatible with groups based on other criteria.

Fernandez and her colleagues (32) subsequently sequenced a 274-bp region of an rRNA operon (hypervariable region E2) from various *Frankia* strains obtained from plants of the genus *Alnus*, the family Elaeagnaceae, and the family Casuarinaceae. The sequences of the isolates obtained from *Alnus* spp. representing the three genomic species discerned in the DNA-DNA relatedness study (31) differed from each other, thus confirming the separation of these organisms. However, the ribosomal sequences of several isolates obtained from members of the Elaeagnaceae were almost identical even though the DNA-DNA hybridization data

supported the division of these organisms into three genomic species. The authors concluded that the high degree of conservation of the 16S rDNA sequences in this region accounted for the lack of discrimination.

In contrast, using a specific PCR-amplified, 268-bp DNA segment of the 16S rRNA gene (*E. coli* positions 921 to 1189), Nazaret et al. (66) compared frankiae representing the nine genomic species of Fernandez et al. determined by DNA-DNA relatedness (31). With one exception, the data showed that strains belonging to the same genomic species had identical sequences and that they differed from the other genomic species. Thus, the choice of the 16S rRNA region studied is critical to the taxonomic value of the results obtained.

An evolutionary distance analysis by the same workers (66) showed that genomic species 1 of Fernandez et al. ("*F. alni* subsp. *pommerii*") branched closer to the isolates obtained from *Casuarina* spp. than to the other genomic species obtained from *Alnus* spp. This was true regardless of the analytical method used. Nazaret et al. also found that isolates obtained from *Casuarina* spp. which do not infect their original hosts but do infect *Elaeagnus* spp. branched closer to either isolates obtained from *Casuarina* spp. or isolates obtained from *Elaeagnus* spp. depending on the method of analysis. A *Frankia* isolate obtained from *Purshia tridentata* was at some distance from all of the other *Frankia* isolates, and *Streptomyces ambofaciens* was very distant from all of the *Frankia* strains studied.

Another approach to the definition of species involved restriction fragment length polymorphisms probed with sequences from the various genes coding for the nitrogenase enzyme complex (*nifAB*, *nifD*, *nifK*, and *nifH*) (67). Infective isolates obtained from *Casuarina* spp. exhibited highly homologous results regardless of the restriction enzyme used. However, noninfective strains differed from the first group and among themselves. In contrast, in a different study by other workers (1), both infective and noninfective isolates obtained from *Alnus* spp. exhibited high levels of DNA-DNA relatedness. Thus, infectivity may or may not have taxonomic value depending on the plant source of the strain. In this regard, it should be noted that *nif* probes may not always produce similar results even in closely related strains. Simonet et al. (92) reported that restriction fragment length polymorphisms of infective isolates obtained from *Alnus* spp. from the same provenance were alike when they were probed with *nifHDK*, whereas when they were probed with *nifAB*, five groups were found. Clearly, the last word has not been said on this.

The *nifD-K* DNA IGS in *Frankia* spp. is larger and more variable than the *nifH-D* IGS; thus, it was thought that the *nifD-K* IGS might be useful for distinguishing among closely related strains in PCR restriction fragment length polymorphism analyses. Using two primers composed of sequences from conserved *nifD* and *nifK* regions flanking the *nifD-K* IGS, Jamann et al. (44) found that the DNAs of *Frankia* isolates obtained from members of the Elaeagnaceae and Casuarinaceae could be amplified to yield a product of about 1,380 kb. The DNAs of isolates obtained from *Alnus* spp. were amplified only weakly or were not amplified. Depending on the stringency of the conditions, either only frankiae or both frankiae and other nitrogen-fixing organisms yielded products. When the products were digested with various restriction enzymes, *HinfI* grouped together all of the test isolates obtained from members of the Elaeagnaceae, whereas *MspI* and *SacI* resolved these organisms into the genomic species groups previously determined by DNA-

DNA pairing. Other enzymes gave strain-specific patterns. Isolates obtained from members of the Casuarinaceae were also clearly distinguished from isolates obtained from members of the Elaeagnaceae.

LFRFA has recently been used in an attempt to clarify the *Frankia* species problem (17). In this analysis, DNA was restricted by enzymes having few recognition sites in the genome of interest, and the resulting high-molecular-weight fragments were separated by pulsed-field electrophoresis. The sizes of the fragments were calculated, and the data were subjected to a numerical analysis. A total of 104 *Frankia* strains were studied, and 15 clusters at a similarity level of >75% were dominated either by isolates obtained from *Alnus* spp. and members of the Myricaceae or by isolates obtained from members of the Casuarinaceae. These LFRFA findings lend weight to the conclusions of Lalonde et al. (46), the conclusions of the DNA-DNA relatedness studies (31), and some of the 16S ribosomal DNA sequence data (66) which indicated that the host plant of origin is an important element in defining species. The results of the LFRFA study also confirmed the finding of Nazaret et al. (66) that the type strain of "*F. alni* subsp. *pommerii*" clusters at some distance from the other *Frankia* genomic species isolated from *Alnus* spp.

It is evident from the results described above that knowledge of the genes that control the *Frankia*-plant relationship is probably critical to the full development of a proper species concept for the genus. The so-called *nod* genes, which are instrumental in the instigation, development, and maintenance of nodules, are central among these. Attempts to complement *nod* mutants of rhizobia with *Frankia nod* genes have met with mixed success (22, 78). Probes containing *nod* genes from *Rhizobium* or *Bradyrhizobium* isolates do not give reproducible results with *Frankia* DNA libraries. However, a pectate lyase gene probe from *Erwinia chrysanthemi* yielded evidence that there were similar DNA sequences in *Frankia* isolates (64, 81, 89). As this enzyme should be important in the infection process, it may ultimately be useful as a marker for species recognition.

Conclusions. *Frankia* taxonomy now seems on secure ground in some areas. Phylogenetically, all current data point to proper placement of the genus *Frankia* in the order *Actinomycetales*. Even the ability of *Frankia* isolates to fix nitrogen is apparently not unique but may be shared by certain *Streptomyces* species (33, 45). As indicated above, the genus *Frankia* itself is defined by a combination of morphology, cell chemistry, symbiotic capability, and nitrogenase production. It may be possible to obtain genus-specific probes based on intergenic sequences as well as probes that target nitrogen-fixing enzymes. Species are increasingly seen to be defined on the basis of host plant of origin, DNA-DNA relatedness data, and LFRFA analysis data. Workers may develop species-specific probes based on certain hypervariable regions of 16S rRNA, and recognition of specific strains based on protein patterns (86), production of various isozymes (79), restriction patterns (2, 17, 18, 27), and *rrn* (76) and *nif* (90) sequences may be possible.

What remains to be done? In the ongoing process of clarifying *Frankia* taxonomic concepts, the various groups of workers involved should agree to study a common group of strains obtained from locales and plant hosts that are as diverse as possible. It is clear that there is a high probability that a consensus concerning species definitions will grow out of molecular studies of intergenic or hypervariable 16S rRNA sequences, DNA-DNA relatedness analyses, and LFRFA, but such a consensus is difficult when each group of

workers studies a different set of strains. From the 16S rRNA sequencing and catalog data the phylogenetic position of the genus *Frankia* among the actinomycetes is clear, and the close relationship of type B frankiae to members of the genus *Geodermatophilus* has been demonstrated (39). Many other actinomycete genera still need to be positioned phylogenetically by this method, and the relationships of these organisms to the frankiae need to be made clearer. In addition, 16S rRNA sequencing of type A *Frankia* strains should also be performed. Type A strains are currently classified in the genus *Frankia* along with type B strains on the basis of morphology and ecology, but may be as different from type B strains as the genus *Rhizobium* is from the genus *Bradyrhizobium*. The LFRFA and DNA-DNA relatedness studies need to be expanded to include isolates obtained from host plants other than *Alnus* spp., *Myrica* spp. and members of the Casuarinaceae and Elaeagnaceae, where the main emphasis has been until now. As the host plant of origin appears to be a very important element in *Frankia* species delineation, as shown by hypervariable 16S rRNA sequences, DNA-DNA relatedness data, and LFRFA data, it is imperative that the effect of repeated plant passages in a given host or several different hosts on the stability of these criteria be determined.

Frankia taxonomists, like other workers dealing with difficult-to-grow organisms, are likely to have to deal with day-to-day problems of classification at the molecular, not phenotypic, level. The proposal of Wayne et al. (96) that phenotypic tests which reflect molecular reality should be developed is probably not possible for this taxon. It is well within our technological grasp to develop molecular tests which will enable us to obtain the answers that we need in a reasonable time. *Frankia* taxonomy, as stated above, is something of a model for microbial taxonomy. The constraints that microbial groups like the genus *Frankia* impose force us to develop techniques that will undoubtedly benefit all taxonomy in the future.

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