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CHAPTER 1.1.11

The Families Frankiaceae, Geodermatophilaceae, Acidothermaceae and Sporichthyaceae

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Introduction

The four families, Frankiaceae, Geodermatophilaceae, Acidothermaceae and Sporichthyaceae, embrace bacteria that have been poorly studied for several years, mostly owing to their slow growth rate and fastidious growth requirements. Apart from this common trait, the four families have little in common, which is why they had been considered unrelated until a new hierarchic classification system was proposed (Stackebrandt et al., 1997). These families, together with Microsphaeraceae, are today placed in the suborder Frankineae. The best known of these, the family Frankiaceae, comprises nitrogen-fixing bacteria that infect the roots of a number of woody dicotyledonous plants and induce the synthesis of nodules, studied for many years since their description 135 years ago (Woronin, 1866). The first confirmed isolation in pure culture of a *Frankia* strain was reported only in 1978 (Callaham et al., 1978) despite almost a century of unsuccessful efforts. The other bacteria were isolated from various environments such as soil (Geodermatophilaceae), thermal springs (Acidothermaceae), or compost (Sporichthyaceae). *Geodermatophilus* was grouped together with *Dermatophilus* in the family Dermatophilaceae until 16S rDNA studies revealed their phylogenetic unrelatedness (Stackebrandt et al., 1983). Only *Geodermatophilus* was considered morphologically related enough to be grouped together with *Frankia* in the family Frankiaceae. It was only later, with the advent of molecular techniques, that all these families were determined to be relatives of *Frankia*.

Phylogeny

The first phylogenetic study made on members of these four families was based partly on 16S reverse sequencing and mostly on 16S rRNA oligonucleotides sequencing (Hahn et al., 1989). The main conclusion of this work was that *Geodermatophilus* (Luedemann, 1968) and *Blastococcus* isolated from the Baltic Sea (Ahrens and Moll, 1970) were close relatives of *Frankia*, but the study also confirmed earlier work that showed the genus *Dermatophilus* formed a separate lineage, even though this genus shared a rare morphological feature (i.e., having hyphae dividing in more than one plane to produce multilocular sporangia).

From then on, several (more than 310 in April 2002) 16S rRNA genes have been deposited in sequence databanks and a comprehensive phylogenetic study was made (Normand et al., 1996), showing strains in genus *Frankia* could be grouped into four clusters (Fig. 1) that correspond roughly to host specificities: cluster 1 comprises strains infective on *Alnus* and also strains infective on *Casuarina*; cluster 2 comprises strains not cultivated in pure culture but present in the root nodules of members of plant families Rosaceae, Datiscaceae, and Coriariaceae; cluster 3 comprises strains infective on Eleagnaceae and *Gymnostoma*; and cluster 4 comprises a number of diverse strains isolated from different host plants but unable to fulfill Koch's postulates and also a few strains that infect *Alnus* but not efficiently (Fig. 1). The largest distance in the 16S rRNA gene between any two *Frankia* strains was around 4% (Normand et al., 1996).

As expected, given the unusual ecological niche and the particular morphological features, all *Frankia* strains formed a coherent cluster with *Geodermatophilus* and *Blastococcus*, which are close phylogenetic neighbors. Surprisingly, *Frankia* had even closer relatives sharing hardly any morphological features with it: *Sporichthya polymorpha* isolated from compost (Rainey et al., 1993b) and *Acidothermus cellulolyticus* isolated from hot springs (Rainey and Stackebrandt, 1993a). The distances between *Geodermatophilus* and the different *Frankia* 16S rRNA sequences are 5.3–7.2%, while the distances between *Acidothermus* and the *Frankia* sequences are 4.8–6.5% (Normand et al., 1996).

These findings were found sufficiently surprising to warrant a new study. The strains were

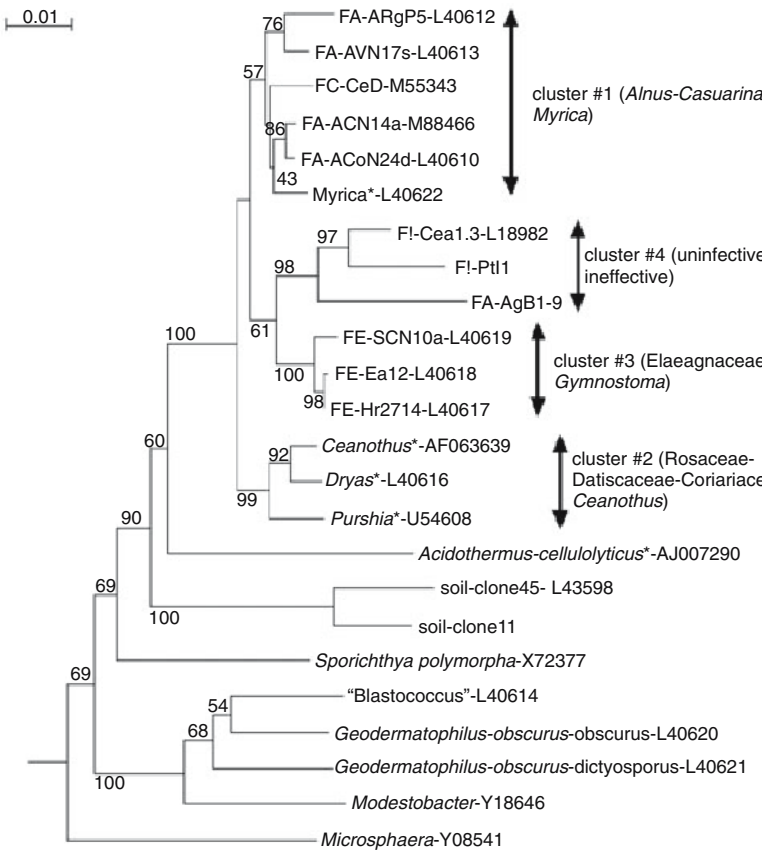

Fig. 1. Neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) of the 16S rRNA gene of representative isolates belonging to the four families: Frankiaceae, Acidothermaceae, Geodermatophilaceae and Sporicthyaceae. In the *Frankia* isolates, FA refers to strains infective on Alnus, FE to those infective on *Eleagnus*, FC to those infective on *Casuarina*, F! to uninfective strains, and the asterisk to unisolated amplificates from plant tissues. *Kineococcus* (AB007420) was used as outgroup. The numbers beside the clusters are those previously given by Normand et al. (1996). Bootstrap replicates (Felsenstein, 1985) above 50% are indicated above the nodes. Bar represents 0.01 substitution/site.

reordered from the American Type Culture Collection (ATCC), their 16S rRNA sequenced, and their *recA* genes also studied. Several errors in the 16S rRNA gene were corrected that did not modify the phylogenetic topology, but *Acidothermus* was indeed found to be closer to *Frankia* than to *Geodermatophilus* (Maréchal et al., 2000). Besides, the *recA* gene was found to confirm the 16S rRNA gene-derived phylogeny showing close proximity of *Acidothermus* to *Frankia* (Fig. 2).

Other genes have been used to study the phylogenetic relations of *Frankia* strains, such as the *nif* genes coding for nitrogenase (Navarro et al., 1997), and the proximity of the *Gymnostoma*-infective strains to cluster 3 was thereby shown; however, these genes are absent in phyletic neighbors and also in several *Frankia* strains of cluster 4. This is also the case for *gln*II that codes for the assimilatory glutamine synthetase (Cournoyer and Lavire, 1999), but its use has remained modest despite its phylogenetic potential.

Some anomalies in phylogenetic groupings were noted when 16S rDNA trees based upon partial and complete genes were compared. These anomalies concerned mainly the strains infective on *Casuarina* (cluster 1). When partial 16S rRNA sequences are used (lacking 300 nucleotides from the 5′ end), these *Casuarina*-infective *Frankia* are separated from the *Alnus*-infective *Frankia* and group close to cluster 2 (Huguet et al., 2001). This topology with the *Casuarina*-infective *Frankia* close to the *Alnus*-infective *Frankia* appears to be consistent with conclusions drawn from studies of morphology and the sequence of the 23S rRNA insertion (Hönerlage et al., 1994).

Given that the bona fide *Frankia* strains form a coherent phylogenetic cluster relative to its neighbors, a *Frankia*-specific 16S rDNA primer was identified and tested. Only one region in the 5′ part of the gene was found appropriate and was used in combination with a universal primer at the 3′ part of the gene. All *Frankia* strains tested yielded positive amplicons with pure culture DNA, while DNA from non-*Frankia* strains was not amplified. Consequently, the primer pair was used to follow *Frankia* in soil, along a chronosequence of soil colonization by *Alnus* in the Alps. Several positive amplicons, two of which originated from the *Alnus* rhizosphere, were obtained and found to belong to undescribed close relatives of *Frankia* with about 5% distances in the 16S rRNA gene (Normand and Chapelon, 1997). This finding emphasized the

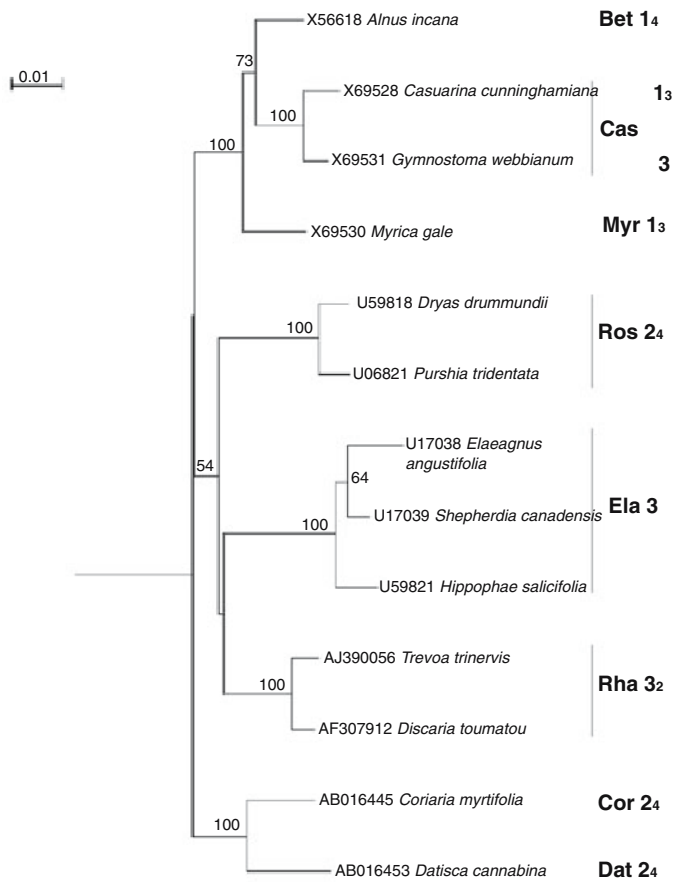

Fig. 2. Neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) of the *rbcL* gene of representative actinorhizal species belonging to the eight families: Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Elaeagnaceae, Rhamnaceae, Coriaraceae and Datisaceae. *Pinus pinea* (AB019822) was used as outgroup. The numbers beside the family abbreviations refer to the cluster (Fig. 1) to which the majority (large characters) or a minority (small characters) of *Frankia* strains belong. Bootstrap replicates (Felsenstein, 1985) above 50% are indicated above the nodes. Bar represents 0.01 substitution/site.

previous notion that the group of actinomycetes described here contains slow growers and may remain poorly defined until an appropriate screening strategy has been developed.

Some isolates are listed in Genbank as *Frankia* strains although a phylogenetic analysis shows that they do not belong to the cluster. This is true of strains G48 (accession number L11306) and L27 (M59075) described as *Frankia* strains isolated from *Podocarpus*, a plant genus belonging to the Coniferales for which nodulation by *Frankia* has been claimed (Benson et al., 1996). As this claim was later dropped, there is now a general agreement among scientists working on *Frankia* that *Podocarpus* is not in symbiosis with *Frankia*. The isolates that appear to belong to the actinomycetes genus *Micromonospora* are still wrongly labeled in Genbank.

One major phylogenetic question that arises from the study of the actinorhizal symbiosis is whether the phylogenetic trees obtained from the symbionts and the host organisms are sufficiently congruent to indicate coevolution. Indeed, the congruency between the 16S rRNA gene of the bacteria and the *rbcL* gene of the host plants is relatively convincing (Jeong et al., 1999). However, under closer scrutiny, several deviations (Fig. 2) from complete congruence are noted: 1) among the Casuarinaceae, *Gymnostoma* is indeed close to *Casuarina* yet it is nodulated by strains of cluster 3; 2) Elaeagnaceae and Rhamnaceae both belong to the Rhamnales, yet the former is nodulated by strains of cluster 3, while genus *Ceanothus* of the Rhamnaceae (Clawson et al., 1998) is nodulated by strains of cluster 2; and 3) Rosaceae that are nodulated by strains of cluster 2 are closer to the Rhamnales nodulated mainly by strains of cluster 3 than to the Coriariaceae and Datiscaceae nodulated by strains of cluster 2. Finally, *Alnus* is nodulated by strains of clusters 4 and 1, while *Myrica* is nodulated by strains of clusters 3 and 1. All these inconsistencies indicate that the evolution of *Frankia* did not proceed in a linear fashion, dominated by gradual plant evolution, but rather that other factors (such as climate or soil formation) may have played a major role.

The genus *Geodermatophilus*, which was originally described for isolates obtained from desert soils, has new isolates regularly added to it. These are obtained from various environments (soil, stones, walls, etc.) and the current tally is 33 16S rDNA entries in Genbank. *Blastococcus*, which was isolated from the Baltic Sea (Ahrens and Moll, 1970) and which has very few phenotypic

Table 1. Discriminatory features of members of Frankineae.

	Frankia	*Geodermatophilus*	*Acidothermus*	*Sporichthya*
Hyphae	+	+	–	+/–
Spore motility	–	+	–	+
Multilocular sporangia	++	+	–	–
Growth temperature °C	25–30	24–28	55	28
N_2-fixation	Most	–	–	–
Nitrate reduction	–	+/–	?	?

Symbols: +, present; –, absent; and +/–, occasionally present.

features that distinguish it from *Geodermatophilus*, may eventually be reclassified as a member of *Geodermatophilus*, inasmuch as the single known strain was placed by 16S rRNA gene analysis in the middle of the *Geodermatophilus* cluster (Fig. 1). *Modestobacter multiseptatus* isolated from polar soil (Mevs et al., 2000) is positioned at the bottom of the Geodermatophilaceae cluster.

All these families (Frankiaceae, Geodermatophilaceae, Acidothermaceae and Sporichthyaceae) are listed on the National Center for Biotechnology Information (NCBI) taxonomy page as belonging to the Frankineae suborder as defined recently (Stackebrandt et al., 1997). Other families (not treated in this chapter) of this suborder are Kineosporiaceae (*Cryptosporangium*, *Kineococcus* and *Kineosporia*) and Microsphaeraceae (*Microsphaera*).

Taxonomy

The discriminatory phenotypic features of the families Frankiaceae, Geodermatophilaceae, Acidothermaceae and Sporichthyaceae are shown in Table 1.

Frankia strains are characterized as producing branched hyphae, multilocular sporangia and nonmotile spores. Most strains will have an optimal growth temperature of 25–30°C. Most strains will grow on nitrogen-free medium, except of course those belonging to cluster 4.

Species description of genus *Frankia* has taken a notably long period to achieve owing to the slow growth rate of many strains, and several actinorhizal plants have not yielded isolates even able to fulfill Koch's postulates (see below). The first DNA-DNA reassociations, made on a small number of isolates, resulted in the expected conclusion that isolates belonged to more than one species (An et al., 1985). Several isolates had high levels of similarity with an *Alnus*-infective strain, several with a *Casuarina*-infective strain, and none with an *Elaeagnus*-infective strain. A more comprehensive study (Fernandez et al., 1989) was done with 35 isolates. Within the group of isolates infective on *Alnus*, one genomic species dominated numerically and was named "*Frankia alni*" by Becking (1970). Two other *Alnus*-infective genomic species emerged with four and one representatives. The group of strains infective on *Elaeagnus* comprised 5 genomic species, while the group of strains infective on *Casuarina* comprised only a single species. This is in line with the published diversity in the *nifH* restriction fragment length polymorphism (RFLP) patterns of these two groups of strains (Nazaret et al., 1989). Three other papers (Bloom et al., 1989; Akimov and Dobritsa, 1992; Lumini et al., 1996) have been published that show a profusion of genomic species defined by DNA-DNA reassociation values, but no species names have been proposed. Results of these studies agreed with the characterization of the *nif* region, done in parallel with hybridization studies.

Only one species, *Frankia alni*, was named because of the few available phenotypic characters that would circumscribe the genomic clusters identified by DNA-DNA hybridization data. This is true for both *Alnus*-infective strains and especially *Elaeagnus*-infective strains. Of these, the group of strains infective on *Casuarina* merit species status, and the strains sharing several phenotypic features (host spectrum and DNA sequence) should be named *Frankia casuarinae*. Discriminatory phenotypic features are listed below together with group-specific primers that target a hypervariable region located around position 990 in the 16S rRNA gene (Bosco et al., 1992; Table 2).

In the Geodermatophilaceae, species (and genera) have been named without DNA-DNA hybridization as recommended by the International Committee on Systematics of Prokaryotes. In genus *Geodermatophilus*, only one species has been named, *Geodermatophilus obscurus*, on the basis of phenotypic characterization, and it contains several subspecies (*amargosae*, *dictyosporus*, *obscurus*, and *utahensis*; Luedemann, 1968; Table 3).

Isolation

Even though nowadays several hundreds of *Frankia* isolates are available, isolation in pure

Table 2. Discriminant phenotypic features of groups of *Frankia* species.

	Species #1–3	Species #4–8	Species #9
Hyphae	Straight	Irregular	Straight, torulose
Pigment production	Rare	Frequent, orange	Rare
Sporangia	Large (5–50 μM)	Small (<5 μM)	Small (<5 μM)
Host	*Alnus*	*Elaeagnus*	*Casuarina*
16S rRNA signature	GGGGTCCGTAAGGGTC	GGGGTCCTTAGGGGCT	GGGGTCCGTAAGGGTC

Table 3. Discriminatory phenotypic features of the *Geodermatophilus obscurus* subspecies.

	amargosae	*dictyosporus*	*obscurus*	*utahensis*
Colony color	Black	Pink >dark brown	Black	Black
Spore motility	+/–	+	+	+
Gelatin	+/–	+	–	–
Inositol	+	–	+	+
β-Lactose	–	+	–	–
Nitrate reduction	–	+/–	+/–	+

Symbols: +, present; –, absent; and +/–, occasionally present.

culture of *Frankia* strains remains a difficult task. The isolation varies from one host plant to the other: the easiest to work with are the Elaeagnaceae and the most difficult are the Coriariaceae, Datiscaceae and Rosaceae. In these three plant families and in the genera *Allocasuarina* (Casuarinaceae) and *Ceanothus* (Rhamnaceae), no isolate is able to fulfill Koch's postulates. Though some of these isolates do belong to the genus *Frankia*, they do not fix nitrogen (Mirza et al., 1994).

Several procedures for isolating *Frankia* have been published and some, especially the historic ones, were complex with mechanical and enzymatic dissection steps (Callaham et al., 1978). Subsequent protocols were much simpler. Although time consuming, the dissection step is nevertheless considered crucial to avoid isolation of "atypical" isolates present in the outer cortical layers and unable to fulfill Koch's postulates (H. G. Diem, personal communication). The addition of activated charcoal or polyvinylpolypyrrolidone appears to increase the probability of obtaining bacterial growth because these agents remove inhibiting plant phenolic compounds (Lechevalier and Lechevalier, 1990). Different media, both liquid and with agar, have been used successfully to obtain isolates from nodule tissues of *Alnus*, *Elaeagnus*, *Casuarina*, etc., and in one instance, directly from the soil (Baker and O'Keefe, 1984). Most strains are microaerophilic, and thus agitation of liquid medium should be avoided or a soft agar overlay used in the case of solid medium. Most isolation attempts use complex media, for instance yeast dextrose agar (Baker and Torrey, 1979) or QmodB medium that contains lecithin (Lalonde and Calvert, 1979), although use of simpler media such as tap water agar has been advocated (Lechevalier and Lechevalier, 1990) to reduce the growth of competing microbes. This notion raises a crucial point relating to the purity of the cultures used, especially when a poor medium has been used in the first steps of isolation. Most strains presently in use have not been derived from single cells, and thus it is not surprising to still find slow-growing contaminants, even after years of study. Optimal temperature for growth is 28°C, although tropical strains (e.g., from Casuarinaceae) have a higher optimum temperature. Incubation proceeds for 3–4 weeks before putative *Frankia* isolates are checked under the microscope and subcultured.

Geodermatophilus was first isolated from desert and forest soils (Luedemann, 1968) on a dilute medium containing yeast extract 0.1%, glucose 0.1%, soluble starch 0.1%, $CaCO_3$ 0.1% and 1.5% agar (to prevent overgrowth of fungi and bacteria following incubation at 28°C for 2–3 weeks). After microscopic observation, colonies were subcultured on richer medium (yeast extract, 0.5%; mixture of amines, 0.5%; glucose, 1%; soluble starch, 2%; $CaCO_3$, 0.1%; and 1.5% agar). A recently described technique (Hayakawa et al., 2000), based on the fact that *Geodermatophilus* has zoospores that are motile by means of terminal flagella, includes a gentle centrifugation step that sediments nonmotile actinomycetes prior to plating on a medium selective for actinomycetes. A recent paper (Mevs et al., 2000) describes the isolation from Antarctic soils on an oligotrophic medium of a bacterium designated "*Modestobacter multiseptatus*, sp. nov., gen. nov.," a close relative of *Geodermatophilus*. Isolates belonging to genus *Geodermatophilus* are now regularly described from dry soils and also from rock surfaces (Eppard et al., 1996; Urzi et al., 2001).

Acidothermus cellulolyticus is available as a single strain (ATCC 43068^T), although three strains were isolated initially during the screening program aimed at exploiting the natural microbial biodiversity in Yellowstone National Park (Mohagheghi et al., 1986). To obtain microbial isolates that could efficiently convert biomass into alcohol for fuel, samples of mud and decaying wood were inoculated in liquid medium containing cellulose as sole carbon source and incubated at 55°C and pH 5.2. No other isolate has been described, even though the procedure appears straightforward and the potential benefits are important.

Isolation of *Sporichthya polymorpha* was first reported in 1968 (Lechevalier et al., 1968) from soil samples using a dilute medium (tap water or one-tenth strength Czapek agar). This organism is considered a rare actinomycete as only five isolates have been obtained over 20 years. Recently, on the basis of the fact that *Sporichthya* also has motile zoospores, the same gentle centrifugation technique followed by plating on selective medium previously used for the isolation of *Geodermatophilus* was used and found successful for *Sporichthya* (Hayakawa et al., 2000). Suzuki (1999) used the same technique together with a highly selective medium (humic acid-vitamin agar [HVA] that contains soil humic acid as the sole source of carbon and nitrogen supplemented with gellan gum and calcium chloride to stimulate formation of spores and aerial mycelium) and reported numerous isolates of *Sporichthya*.

Preservation and Cultivation

Most *Frankia* strains are microaerophilic, grow slowly, and have an optimal growth temperature in the range of 25 to 33°C (Lechevalier and Lechevalier, 1990). This is why *Frankia* strains are routinely cultivated in liquid media. For particular purposes such as germination of spores to obtain single cell isolates (Prin et al., 1991), regeneration of protoplasts (Normand et al., 1987; Tisa and Ensign, 1987), or to assess resistance to metals (Richards et al., 2002), cells are in general embedded in a soft (0.8%) agar overlay maintained at 42°C before plating. For routine purposes, several media are used, including those already described for isolation, the defined propionate medium (DPM; Baker and O'Keefe, 1984) or Biotin-Ammonium-Propionate medium (BAP; Murry et al., 1984).

The most frequent preservation method of *Frankia* strains is maintenance in rich liquid medium with infrequent (once or twice per year) subcultures. Some comprehensive lyophilization programs were dropped when reports indicated that reculture of such lyophilized material was often problematic. Skim milk suspension that appears to contain some inhibitory compounds should be avoided, and instead, bacteria in spent medium should be used (Lechevalier and Lechevalier, 1990). CpI1, the first strain that was deposited in the American Type Culture Collection (ATCC) and seemingly kept as lyophilized material, was found not to be viable and is now absent from the list of available strains. The only strain listed as available from the ATCC is AvcI1, a cluster 1 *Alnus*-infective strain (Baker et al., 1980), available freeze-dried.

Frankia spores are not able to withstand high temperatures. The highest reported temperature (55°C) is only slightly higher than the highest temperature (52°C) tolerated by hyphae (Lalonde and Calvert, 1979). Some strains isolated from *Casuarina* produce particular hyphae called "torulose hyphae," which have thickened walls, are produced in old cultures, and can be subcultured (Diem and Dommergues, 1985). Diazo-vesicles also have the potential to differentiate into hyphae (Schultz and Benson, 1989).

Geodermatophilus is relatively easy to grow at 28°C on solid rich medium (e.g., 0.5% yeast extract, 0.5% amine mixture, 1% glucose, 2% soluble starch, 0.1% $CaCO_3$, and 1.5% agar; Luedemann, 1968) or on TYB (tryptone, yeast extract, glucose and NaCl at pH 7; Ishiguro and Wolfe, 1970). Carbohydrate utilization in *Geodermatophilus* isolates is in general broad: L-arabinose, D-galactose, D-glucose, D-levulose, D-mannitol, sucrose and D-xylose (Luedemann, 1968).

Acidothermus is more tedious to work with mainly because of evaporation problems connected with its optimal growth above 55°C. Good growth occurs in low phosphate basal salts medium (Mohagheghi et al., 1986) containing yeast extract and cellulose. Different optimum temperatures have been reported but the average was 58°C, while the optimum pH was 5.0.

Sporichthya is grown at 26°C in standard yeast malt extract agar from Difco (Lechevalier et al., 1968).

Geodermatophilus, *Acidothermus* and *Sporichthya* are available from the ATCC in freeze-dried form.

Physiology

Nitrogen Fixation, Partial Pressure of Oxygen, and Vesicles

In contrast to most *Rhizobium* strains, nitrogen fixation has been found to occur not only in actinorhizal nodules but also in pure cultures of *Frankia* in the absence of fixed nitrogen. Growth

without fixed nitrogen, however, is much slower and is associated with the synthesis of diazovesicles (specialized cells with thickened walls that provide a barrier to the diffusion of oxygen). It was even found that nitrogen fixation, which is a reductive process inhibited by molecular oxygen, could occur at normal oxygen pressure but also at hyperbaric oxygen concentration (Harris and Silvester, 1992). Increasing partial oxygen pressure results in increased thickness of the vesicles' cell wall and the compound involved was identified as hopanoid lipids (Berry et al., 1993). Hopanoid lipids, which behave like cholesterol as membrane hardeners, are present at such high concentrations in *Zymomonas mobilis* that cells are able to grow in up to 10% ethanol and in *Alicyclobacillus acidocaldarius* that cells are able to grow in acidic thermal springs (Poralla et al., 1980). Hopanoid lipids are also present at concentrations of 15% in *Acidothermus cellulolyticus* (Maréchal et al., 2000).

In *Frankia* cells, two types of glutamine synthetases (GSs) incorporate ammonium into glutamic acid to yield glutamine (Schultz and Benson, 1990). Both GSI (heat-stable, dodecameric, constitutive, and coded by *glnA*) and GSII (heat-labile, octameric, inducible by N-starvation, and coded by *glnB*) are present in vesicles at levels similar to those detected in vegetative hyphae from N_2-fixing cultures (Rochefort and Benson, 1990). However, glutamate synthase, glutamate dehydrogenase, and alanine dehydrogenase activities, all of which are involved in subsequent steps of ammonium assimilation, are restricted to the vegetative hyphae. Thus, diazovesicles apparently lack a complete pathway for assimilating ammonia beyond the glutamine stage.

Effect of Phenolic Compounds

In actinorhizal nodules, *Frankia* growth is restricted to the central part of the thickened cortex and it has been hypothesized that the plant controls the growth of its symbiont by preventing its access to some parts of the nodule. Given that root nodules contain high amounts of tannins (which can be described as phenolic), the effect of phenolic compounds on the growth and morphology was investigated and it was found that some compounds repressed sporulation (coumaric, ferulic, and *trans*-cinnamic acids), induced vesicle formation (benzoic and hydroxybenzoic acids), or repressed growth (coumaric acid; Perradin et al., 1983).

Superoxide Dismutase

Normal aerobic metabolism produces molecular oxygen (O_2), which is toxic to cell constituents in general and to the 4Fe-4S cluster containing enzymes in particular. Nitrogenase is one of the enzymes in the 4Fe-4S cluster and thus is particularly sensitive to oxygen radicals. Enzymes capable of metabolizing this compound, the superoxide dismutases (Sods), belong to two types: the manganese-iron Sods and the copper-zinc Sods. Given the sensitivity of nitrogenase to O_2, this enzyme has been looked for in numerous nitrogen-fixers and indeed detected in actinorhizal nodules (Alskog and Huss-Danell, 1997) and in pure cultures, where its level was among the highest reported in prokaryotes (Steele and Stowers, 1986). Using a two-dimensional (2D)-gel electrophoresis approach, the effect of plant exudates on *Frankia* in pure culture was investigated. Among the five spots overexpressed in response to *Alnus glutinosa* root exudates, one spot was found to be an iron Sod (Hammad et al., 2001).

Carbon/Nitrogen Exchange in Nodules

Frankia strains can grow on a variety of carbon sources, relatively well on small molecular weight compounds such as acetate or propionate, and also on malate, pyruvate and succinate, and particularly cluster 1 strains can grow on Tween 80. In contrast, growth on sugars is poor. Sugars are added to growth media to stimulate growth (and thus increase detection) of contaminants. ^{14}C-labeling experiments with cluster 1 strains, by far the most studied strains, showed that propionate metabolism proceeds via active transport followed by activation with coenzyme A, carboxylation to methyl-malonyl CoA and racemization to succinyl-CoA, which then can be processed in the tricarboxylic acid cycle (Stowers et al., 1986). Glycogen and trehalose have been identified as major storage compounds in *Frankia* (Lopez et al., 1984), with their level correlated negatively with the energy-demanding nitrogen fixation activity (Fontaine et al., 1984).

Hydrogenase

In contrast to leguminous nodules, actinorhizal nodules emit little or no hydrogen, a by-product of nitrogenase activity. The presence of an uptake hydrogenase, the enzyme involved in the recycling of the energy-rich gas, has been demonstrated in nodules (Benson et al., 1980) and in pure cultures (Lindblad and Sellstedt, 1989; Murry and Lopez, 1989). The enzyme's large subunit was located in the membrane by immunogold staining (Mattsson and Sellstedt, 2000) and its activity correlated with nitrogenase activity.

Genetics

The genetics of *Frankia* in pure culture has been limited by the lack of a transformation system. Several attempts at genetic transformation using naked DNA (Cournoyer and Normand, 1992) or conjugation have failed. However, a promising breakthrough has been the conjugation between *Enterococcus* and *Frankia* by means of a conjugative plasmid (Myers et al., 2001[Abstract]).

Organization of the *nif* Cluster

The *nif* genes that code for the nitrogenase enzyme complex are among the most conserved bacterial genes. The organization of the gene cluster in *Frankia* has been determined by hybridization with the *nifHDK* genes of *Klebsiella pneumoniae* (Normand et al., 1988). The gene order in the core system (HDKENX) is conserved as in most systems analyzed so far.

Glutamine Synthetase Genes

Frankia strain CpI1 has two genes coding for glutamine synthetase (Hosted et al., 1993), the protein responsible for adding ammonia to glutamate to yield glutamine. The arrangement of genes appears to result from an early (a 300 million-year-old) gene duplication and is conserved in several bacteria (Kumada et al., 1993). This region (especially the *glnB* gene) is highly variable (Cournoyer and Lavire, 1999) and thus suitable for phylogenetic analysis of closely related strains. The *Frankia glnB* gene was capable of complementing an *E. coli glnA* mutant but only when transcribed from the lac promoter (Rochefort and Benson, 1990).

Proteasome

The genes coding for the extracellular protease complex called the "proteasome" have been isolated and characterized from the cluster 1 *Frankia* strain ACN14a (Pouch et al., 2000). When compared to the homologous sequences in other actinobacteria (i.e., *Rhodococcus erythropolis*, *Mycobacterium tuberculosis* and *Streptomyces coelicolor*), the structure and gene order are conserved.

Sod

As explained above, the superoxide dismutase is especially important to nitrogen-fixing microorganisms. The Sod enzyme is present in two forms, the constitutive one containing Mn as ligand and the inducible one containing Fe as ligand; the enzyme is induced by switch to nitrogen-fixing conditions (Steele and Stowers, 1986) or exposure to plant root exudates (Hammad et al., 2001). The respective gene has been isolated and found to complement *E. coli* mutants. The expression level with its own promoter was much lower than that with an *E. coli* promoter (P. Normand, in preparation), suggesting that *Frankia* promoters are not well recognized by the *E. coli* expression machinery, as was previously reported for the *glnA* gene (Rochefort and Benson, 1990).

The *shc* Gene

This gene codes for a squalene hopene cyclase, an intermediary enzyme in the anabolic pathway from acetyl-CoA to hopanetetrol, and has been characterized from several *Frankia* strains by amplification with conserved primers (Dobritsa et al., 2001). This compound is involved in protecting nitrogenase from oxygen in thick-walled specialized diazovesicles (already described).

Plasmids

Several plasmids have been detected in various strains, essentially from the cluster 1 strains infective on *Alnus*, varying in size from 8 kb (Normand et al., 1983) to 190 kb, the largest carrying a copy of the *nif* genes (Simonet et al., 1986). Three of the smaller plasmids have been sequenced to understand why transformation in both *Frankia* and in heterologous hosts has failed repeatedly and to develop efficient vectors. The 8-kb plasmids were found to have genes resembling the classic *kor* (kill over-ride) system for repression of toxic *kil* function: *par* for partition, *rep* for replication of DNA, and a replication origin (Lavire et al., 2001). A larger 22-kb plasmid (pFQ12) present in the same strains was found to have similar functions plus some genes with similarity to transfer and mobilization determinants (John et al., 2001).

Codon Usage

Codon usage is highly skewed toward –G and —C triplets, except for the underrepresented glycine GGG. This bias, which is usual for actinomycetes, is a useful signature to confirm that *Frankia* is the source of a gene (Ligon and Nakas, 1988).

Cellulase Genes of Acidothermus cellulolyticus

Acidothermus cellulolyticus was isolated using the ability to grow at high temperature on cellu-

lose as sole carbon source. The cellulase gene (U33212) was isolated and characterized as encoding a family 5 thermostable β-1,4-endoglucanase (Sakon et al., 1996). The G+C content of the region is lower (62.2 mol%) than that of *Frankia* genes known so far (66 mol% for *sodF*, 67 mol% for *recA* and *gln2*, 68 mol% for the *nifHDK* region, and 71 mol% for the proteasome region), and the highest similarity was found with a gene from *Paenibacillus polymyxa* (P23548, 63% similar), suggesting a recent lateral transfer. The gene was transformed within tobacco where it was transcribed, and the protein was targeted to the chloroplasts where it accumulated to 1.35% of soluble proteins (Dai et al., 2000).

Ecology

Frankia strains are ubiquitous in soils. In several ecological survey programs, *Alnus*, *Myrica* and *Elaeagnus* have consistently been found to be profusely nodulated (Bond, 1976), except in some anoxic situations. Some *Allocasuarina* species have also been found to be sparsely nodulated, but this has been interpreted as an evolutionary trend of the plant towards a narrower spectrum of strains (Maggia and Bousquet, 1994) and eventual elimination of the symbiont in the drier parts of Australia (Simonet et al., 1999). Even soils from sites with no known actinorhizal plants cause nodule formation on test plants, suggesting that *Frankia* can thrive in the soil in the absence of host plants (Normand and Lalonde, 1982), which was demonstrated experimentally (Rossi, 1964). It has been suggested that the ability to thrive as saprophytes varies markedly between strains. In particular, some strains that sporulate profusely in plants have been suggested to have different ecological strategies (van Dijk, 1978) and in particular to be less saprophytic.

pH Effect on Distribution of Strains

Several soil parameters are expected to play a role on the survival of spores or on the saprophytic life of hyphae. These are texture, organic matter content, pH, chemicals, pO_2, etc. However, few of these parameters have been tested. Acidity of the soil is known to be a critical factor, acting directly on the bacterial physiology and indirectly on nutrient availability. As expected, it was found that the diversity of *Frankia* strains infective on *Elaeagnus* was reduced in an acidic soil relative to a neutral soil (Jamann et al., 1992).

Effect of Soil and Host Plant on Distribution of Strains

Actinorhizal plants are known to be pioneer plants that invade recently deforested areas following volcanic eruptions, glacier melting, forest fires, landslides, or man-made processes like deposition of mine spoils, hydrodam construction, or sand dune formation. Such processes may result in unbalanced substrate for pH or chemicals acting as selective forces on strains. Thus, it is not surprising to have differences in 50% of lethal dose (LD_{50}) for a variety of heavy metals (Richards et al., 2002). In the case of *Frankia* strains infective on *Gymnostoma* spp., this was found to result in abundance, dependent on soils and the host plant species (Navarro et al., 1999). A similar conclusion was reached in the case of *Ceanothus*-infective strains, with some strains restricted to serpentine soils (Ritchie and Myrold, 1999).

Antibiotics

Differences in antibiotic resistance were noted between strains, even closely related ones (Dobritsa, 1998), even though most differences are linked to taxonomic position. Conversely, *Frankia* has been found to synthesize unusual compounds, such as a macrocycle containing imide and orthoamide functionalities (Klika et al., 2001) and benzo[a]naphthacene quinones (Rickards, 1989).

Applications

Actinorhizal plants are pioneer species that are dominant in re-vegetating areas, following natural events such as glacier retreat or volcanic eruptions. These plants can be self-reliant in nitrogen because of their symbiosis with *Frankia*, and they are also undemanding for other nutrients, in part because of the general occurrence of symbiosis with endomycorrhizal fungi (Diem, 1996). For all these reasons, actinorhizal plants have been used for several applications where it is necessary to rapidly establish a plant cover.

The Americas and Europe

Alnus viridis subsp. *crispa* has been planted on a commercial scale in Canada (Périnet et al., 1985) to stabilize dikes in the large hydroelectric facilities of Northern Quebec and for biomass production on abandoned farmland (Prégent and Camiré, 1985) and on reclaimed oil shale sites in Alberta (Gordon and Dawson, 1979).

Interplanting is another possibility, in that the valuable but demanding tree species such as walnut are grown together with slower growing, undemanding alder or Russian olive trees which form nitrogen-rich soil (Campbell and Dawson, 1989). Furthermore, small-scale production of seabuckthorn berries (*Hippophae rhamnoides*) is recommended on marginal lands or in mountainous areas (http://www.hort.purdue.edu/newcrop/) to yield alcohol, vitamin A and C-rich juice, and a variety of jams, candies and "natural" products (http://www.genres.de/bmlfao/natber3.htm). Finally, several undemanding ornamentals plants are actinorhizals, prominent among them *Alnus cordata*, *Hippophae rhamnoides*, *Elaeagnus* spp. and *Ceanothus* spp.

Africa, Asia and Oceania

Casuarina spp., which produce dense and hard wood and have few if any diseases (Dommergues et al., 1999), are now widely used in tropical climates for planting on sand dunes and other marginal sites (Diem and Dommergues, 1990). *Casuarina* spp., especially of *Casuarina equisetifolia*, planted in Africa, India and China's seacoasts as windbreak are now commonplace (Midgley et al., 1986). For instance, up to a million hectares have thus been planted in China (Turnbull, 1983). In Egypt, *Casuarina* spp. are planted in individual fields where not only does it protect against wind but also it provides fuel wood (El-Lakany, 1990). *Gymnostoma* spp. have been used for re-vegetation of nickel mine spoils in New Caledonia (McCoy et al., 1996). *Casuarina equisetifolia* may increase the soil's nitrogen from 80 $kg \cdot ha^{-1}$ to more than 300 $kg \cdot ha^{-1}$ (Dommergues et al., 1999). *Coriaria* spp. have been planted to stabilize hilly areas in Pakistan (Chaudhary and Mirza, 1987). *Alnus nepalensis*, the first plant that appears on landslides and abandoned farmlands in Northeastern India, is used in mixed plantations with cardamom and tea and to re-vegetate degraded lands (Dommergues et al., 1999).

Conclusion

Few groups of microbes are composed of such contrasting families with ecological niches and physiological characteristics as diverse as the one described here. It is only the advent of molecular techniques that has permitted the close proximity of the thermoacidophile Acidothermaceae, the soil Geodermatophilaceae and Sporichthyaceae, and the plant symbionts Frankiaceae to be recognized. The evolutionary history of this group remains to be established and the genome sequence of *Frankia* and its neighbors will certainly be revealing.

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