Isolation of *Elaeagnus*-compatible *Frankia* from soils collected in Tunisia

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Abstract

The occurrence and diversity of *Frankia* nodulating *Elaeagnus angustifolia* in Tunisia were evaluated in 30 soils from different regions by a *Frankia*-capturing assay. Despite the absence of actinorhizal plants in 24 of the 30 soils, nodules were captured from all the samples. Eight pure strains were isolated from single colonies grown in agar medium. On the basis of 16S rRNA and GlnII sequences, seven strains were clustered with *Frankia*, colonizing *Elaeagnaceae* and *Rhamnaceae* in two different phylogenetic groups while one strain described a new lineage in the *Frankia* assemblage, indicating that *Frankia* strains genetically diverse from previously known *Elaeagnus*-infective strains are present in tunisian soils. Genomic fingerprinting determined by rep-PCR, and tDNA-PCR-SSCP, confirmed the wide genetic diversity of the strains.

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1. Introduction

*Frankia* can grow as microsymbiont in the root nodules of several woody dicotyledonous plants. Outside the plant host this bacterium is a slow growing actinobacterium, for which it is relatively difficult to establish laboratory cultures. Currently, only a few hundred *Frankia* cultures have been established and for certain recalcitrant strains the obligate symbiosis assumption remains plausible [1–3].

*Frankia* have been isolated by plant-capturing assay from rhizospheric and non rhizospheric soils hosting actinorhizal plants, as well as from soils with no history whatever of actinorhizal plants [4–6]. Such findings suggest that *Frankia* could also be widespread in areas where actinorhizal plants do not grow.

The commonly accepted relationship between *Frankia* and its host plants has led to a systematic investigation of the occurrence and diversity of *Frankia* only in continents hosting various actinorhizal flora, i.e. Australia, Europe, America and Asia. In Africa only two native actinorhizal plant genera can be found: *Alnus* in the north-African regions and *Myrica* in the south. Thus, except for studies on *Frankia* that nodulate the *Casuarinaceae* that was introduced into Africa from Australia [7–9], and the *Alnus glutinosa* native to Tunisia [8] and other countries in North Africa, there has been relatively little exploration into the possible occurrence and diversity of other *Frankia* strains in the African continent [8,10,11].

In this study the occurrence of *Elaeagnus*-compatible *Frankia* has been investigated in Tunisia in 30 different soils, most of which were devoid of *Elaeagnus* plants.
From captured nodules, eight strains have been isolated. A study of the phenotypic and genotypic diversity revealed that strains markedly differing from previously described Frankia inhabit Tunisian soils.

2. Materials and methods

2.1. Plant trapping bioassay and isolation of Frankia strains

Soil samples, taken at a depth of 10 cm, were collected in Tunisia from 30 sites characterized by different pedoclimatic conditions. Each sample was sub-divided into 10 plastic pots and mixed with sterile sand. Sand was sterilised three times (each time separated by 12 h) for 30 min at 180 °C and mixed with 1/10 (w/v) sampled soil. Fruits from Elaeagnus angustifolia were scarified for 15 min in a concentrated H2SO4 bath and, after several rinsings with sterile distilled water, the recovered seeds were further sterilized for 10 min in 30% (v/v) H2O2; the seeds were washed again with sterile distilled water and pregerminated in sterile humidified vermiculite. Three seedlings were transferred to each plastic pot and grown in a separate and dedicated room of a greenhouse at the University of Tunis in the following conditions: photoperiod 16 h, day/night temperatures 28/18 °C; after six months the plants were harvested and the roots checked for the presence of root nodules. Uninoculated plants grown in sterile sand were used as control and none of them showed nodules at the end of the experiment.

Lobes of the harvested nodules were cleaned thoroughly with sterile tap water and sterilized superficially, by shaking in 30% (v/v) H2O2 for 30 min. Fragments from individual lobes were incubated in modified DPM medium [12] containing in g l−1 of distilled water: 0.5 sodium propionate; 0.5, sodium succinate; 0.5, K2HPO4; 1, MgSO4; 0.1, CaCl2; 0.01, FeSO4; 0.0055, Na2EDTA; 0.0074, H3BO3; 0.005, MnCl2; 0.0032, ZnSO4; 0.0004, CuSO4; 0.00014, Na2MoO4; 0.000045, CoCl2; 0.000045. After sufficient mycelium growth from the culture, an aliquot of head lobe nodules was substituted with commercially purified C2H2 (20% v/v). After incubation, aliquots of head space gas were injected into a gas chromatograph (Girld, Porapak R column; flame ionization detector) to analyze the C2H2 and C2H4 amounts, and to calculate the acetylene reduction activity [14]. Growth was evaluated by determining the total protein content using the Bradford method [15] or by visual inspection of the cultures.

2.2. Plant reinfeciton experiments and acetylene reduction assay

The authenticity of the isolates as true Frankia strains was confirmed by re-infection experiments of axenic Elaeagnus angustifolia seedlings grown in glass tubes containing a sterile mix of sand and vermiculite (1/1), watered with 1/10 strength Hoagland’s solution [13] and maintained in controlled environment (photoperiod 16 h, day/night temperatures 28/18 °C); the four-week old plants were inoculated with washed Frankia culture equivalent to 10 μg of protein.

The capacity of the strains to fix atmospheric nitrogen was tested by the acetylene reduction assay using a nitrogen free DPM liquid medium. The internal atmosphere of the serum bottles containing the isolates or lobe nodules was substituted with commercially purified C2H2 (20% v/v). After incubation, aliquots of head space gas were injected into a gas chromatograph (Girld, Porapak R column; flame ionization detector) to analyze the C2H2 and C2H4 amounts, and to calculate the acetylene reduction activity [14]. Growth was evaluated by determining the total protein content using the Bradford method [15] or by visual inspection of the cultures.

2.3. Genotypic characterization of the strains

DNA extraction was performed on one month-old liquid cultures. Colonies in one to five ml of culture were forced several through a 0.7 × 30 mm sterile needle to homogenize the mycelium and centrifuged. The resulting pellet was washed twice with sterile distilled water, incubated for 30 min in DNA extracting buffer (100 mmol l−1 Tris–HCl, pH 8; 20 mmol l−1 EDTA, pH 8.2; 1.4 M NaCl, and 2% w/v cetyl trimethyl ammonium bromide (CTAB), chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 50 μl TE (10 mmol l−1 Tris–HCl, pH 8; 20 mmol l−1 EDTA, pH 8.2).

For PCR amplifications all the reactions were run on a Perkin–Elmer 2400 geneAmp PCR thermocycler. PCR amplifications of 16S rRNA gene and glnII were performed using the following primers: S-D-Bact0008-a-S-20 and S-D-Bact-1495-a-A-20 [16], and FGgs19 and FGgs417’ [17]. For 16S rRNA gene and glnII PCR the same reaction conditions were used: 100 μl final reaction volume containing 10 ng genomic DNA, 1× Taq polymerase buffer (Amersham Pharmacia Biotech, Milan, Italy), 1.5 mmol l−1 MgCl2, 0.1 μM each dNTP, 0.2 μM each primers and 2 U Taq DNA polymerase. The thermal program consisted of three min at 95 °C followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and
72 °C for 45 s. Repetitive element polymorphism-PCR (rep-PCR) was performed in 25 μl final volume using 50 ng genomic DNA, 1× Taq polymerase buffer, 2.5 mmol l⁻¹ MgCl₂, 0.5 μM each dNTP, 0.5 μM BOX-AIR primer [18], 0.04 U μl⁻¹ Taq DNA polymerase and 5% (v/v) DMSO, and subjected to a thermal program: 95 °C for 5 min, 35 cycles consisting of 1 min at 94 °C, 1 min at 45 °C and 2 min at 72 °C, tDNA-PCR was performed in 50 μl final volume containing 10 ng genomic DNA, 1× Taq polymerase buffer, 1.5 mmol l⁻¹ MgCl₂, 1 μM each dNTP, 1 μM of primers T5A and T5B [19] and 1.25 U Taq DNA polymerase. A hot-start protocol was performed before running the thermal program: 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min.

All the PCR products except the tDNA-PCR products were electrophoresed on 1.5% (w/v) agarose gel in TBE buffer (89 mmol l⁻¹ Tris, 89 mmol l⁻¹ borate, 2 mmol l⁻¹ EDTA) [20]. The tDNA-PCR products were mixed with a gel loading solution (400 g l⁻¹ sucrose, 0.1M EDTA, 0.5% w/v SDS), denatured at 94 °C for 15 min and immediately cooled in an ice-bath [19]. Five to ten μl were electrophoresed in 6% polyacrylamide gel (Amersham Pharmacia Biotech, Milan, Italy) containing 10% v/v glycerol. Agarose and polyacrylamide gels were ethidium bromide stained and photographed under UV light.

The 16S rRNA gene and glnII PCR products were purified from PCR reaction mixtures using the QIAquick PCR purification Kit (Qiagen, Milan, Italy), according to manufacturer instructions. The sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Monza, Italy), and underwent fragment separation in an ABI Prism™ 310 DNA sequencing as previously described [18].

The nucleotide sequences of 16S rRNA gene and glnII aminoacid sequences were aligned using Clustalw (http://clustalw.genome.ad.jp) and compared to reference sequences from isolated and unisolated Frankia in order to have a representative range of infectivity groups. Phylogenetic trees were achieved using TRECON for Windows software version 1.2 [21] and the neighbor-joining algorithm [22]. Bootstrap values were determined from 100 replicates [23]. For 16S rRNA gene sequences, the similarity matrix with closest neighbors was calculated using RDP utilities (Ribosomal Database Project II: http://rdp.cme.msu.edu/html).

2.4. Nucleotide sequence Accession Numbers

The EMBL Accession Numbers for the 16S rRNA gene and glnII sequences of Frankia strains BMG5.2, BMG5.3, BMG5.4, BMG5.5, BMG5.6, BMG5.10, BMG5.11, BMG5.12 determined in this study are AJ549319-AJ549326 and AJ545038-AJ545045, respectively.

3. Results

3.1. Occurrence of Frankia in Tunisian soils

The occurrence of E. angustifolia-compatible Frankia was studied in 30 different soils from different regions in Tunisia, in the areas of Rimel, Jdaida, Tamra, Tazarka, Kairouan, Sfax, Sidi Bouzid, Tozeur and Gafsa.

Plant trapping bioassay using surface-sterilized E. angustifolia seeds revealed that all 30 sampled soils led to nodule development in the plant root. However only six of the soils had E. angustifolia plant growing, all the others being totally devoid of actinorhizal plants. A preliminary investigation into the nature of the nodules revealed 90 of them, out of 150, to be positive by acetylene reduction assay, yielding DNA amplifiable with nifD-K primers specific for Frankia; this indicates that the roots were effectively colonized by the actinobacterium (data not shown).

The nodules recovered from the different soils were surface-sterilized and dissected into 582 nodule lobes that were then inoculated in DPM medium. After three to six weeks of incubation mycelia outgrowth from the small lobe pieces appeared on 1.4% of the dissected lobes and 27% with respect to the soils tested. The remaining lobes did not yield any culture. Microscopic examination of the growing culture showed typical Frankia morphology (data not shown). To isolate the strain, single colonies in the liquid medium were homogenized, being passed through a sterile needle, and then plated onto agarized medium. After several weeks small single colonies appeared. These colonies were picked and used to inoculate sterile DPM medium. In this way eight pure cultures were obtained and named BMG5.2, BMG5.3, BMG5.4, BMG5.5, BMG5.6, BMG5.10, BMG5.11 and BMG5.12. All the strains showed diazovesicles, especially when grown without any nitrogen source in the medium, and all were able to fix nitrogen in the nodules, as evaluated by acetylene reduction assay.

The ability of the strains to fix atmospheric nitrogen was tested by acetylene reduction assay. All the strains were able to reduce acetylene both in pure culture and in nodule, although efficiency varied among the strains. In the pure culture assay conditions, strains BMG5.2, BMG5.4, BMG5.5, BMG5.6, BMG5.10 and BMG5.12 showed an acetylene reduction activity (ARA) higher than 200 nmoles h⁻¹ mg⁻¹. Strain BMG5.11 showed ARA values around 120 nmoles h⁻¹ mg⁻¹, while strain BMG5.3 had relatively low ARA values, below 100 nmoles h⁻¹ mg⁻¹.

3.2. Phylogenetic affiliation of the strains based on 16S rRNA gene sequencing

The phylogenetic affiliation of Tunisian Frankia was determined by sequencing about 1307 nucleotide
positions of the 16S rRNA gene (Fig. 1). Of the sequenced strains seven were found to be grouped in two branches of the tree, including Frankia colonizing Elaeagnaceae and Rhamnaceae. The strains BMG5.2, BMG5.5, and BMG5.12 were clustered (homology between 99.0% and 99.7%) with Frankia colonizing Trevoa (Rhamnaceae) and Shepherdia (Elaeagnaceae), while strains BMG5.3, BMG5.4, BMG5.10 and BMG5.11 were related to (homology between 99.7% and 100.0%) Frankia colonizing Colletia (Rhamnaceae), Hippophae (Elaeagnaceae) and Elaeagnus (Elaeagnaceae).

The eighth strain, BMG5.6 described a new lineage in the Frankia assemblage with an intermediate position between the group of atypical Frankia strains MgI5, Cea1.3, Cea5.1 and M16386 [24,25] and the assemblage including Eleagnaceae–Rhamnaceae infective strains, Alnus Myrica Casuarina infective strains and a series of uncultured nodule microsymbiontes (Fig. 1). The highest 16S rRNA gene homology shared by strain BMG5.6 was with Eleagnaceae–Rhamnaceae infective strains being in the range 96.1–96.4% (96.4% with strain HR27-14 and an uncultured nodule microsymbiontes from Colletia, AF063640). The sequence homology between the Tunisian strains ranged between 95.3% and 99.8%.

3.3. Analysis of the genetic diversity of the strains by sequencing a protein coding gene and by genomic fingerprinting

To further study the genetic diversity of the strains the sequences of 110 aa deduced from 330 bp sequences of glnII gene encoding glutamine synthetase II [26], were used to reconstruct a phylogenetic tree, including all the corresponding Frankia sequences available in public databases (Fig. 2). The reconstructed tree divided the Tunisian Frankia strains into three groups: one included the isolates BMG5.2, BMG5.5 and BMG5.12 clustering with the Eleagnaceae–infective strains HRN18a and EUN1f already described by Cournoyer and Lavire [26]; a second newly Eleagnaceae–infective group made up by BMG5.3, BMG5.4, BMG5.10 and BMG5.11 isolates. Strain BMG5.6 (typical strains) was clustered with the atypical strain PtI1.

Fig. 1. Phylogenetic tree based on 1307 bp of the 16S rRNA gene obtained by neighbor-joining algorithm. Strains isolated in Tunisia are boxed. The arrow indicate strain BMG5.6. Nucleotide sequence accession numbers are indicated in parentheses. Bootstrap values determined from 100 replicates are indicated. Atypical strains lack of infectivity and/or effectivity on their original host plant.
The strains were also analyzed by rep-PCR and tDNA-PCR-single strand conformation polymorphism (SSCP, [19]) fingerprinting (Fig. 3). The rep-PCR patterns yielded bands from 80 to about 3000 bp (Fig. 3 A). Pattern complexity ranged from 3 to 19 bands. With respect to other Frankia strains isolated from the same host [27–29] or from other plant host [30], the Tunisian isolates were found to have relatively complex rep-PCR patterns. High variability was also observed in the tDNA-PCR-SSCP patterns (Fig. 3B).

4. Discussion

There is a lack of studies on the occurrence and diversity of Frankia in African soils, including those in...
north African regions. The present study on the occurrence and diversity of *Frankia* in Tunisian soils is an attempt to address this issue. Only one actinorhizal plant is native to Tunisia, *Alnus glutinosa* in the northern regions of the country [8], and there has been little diffusion of imported actinorhizal plants, namely *Casuarinaceae* [8,31] and *Elaeagnaceae*. A few *E. angustifolia* plants grow in the north (Bizert) and in the south around the oasis. To the best of our knowledge no historical documentation about the introduction of this species is available.

This study carried out a survey on the occurrence of *Elaeagnaceae*-compatible *Frankia* over a relatively wide area of Tunisia, covering almost four degrees of latitude (from 37°14'N in Rimel to 33°52'N in Tozeur) and more than three degrees of longitude (7°48'E in Tozeur to 10°60'E in Tazarka). *Frankia* was detected in all the analyzed soil samples, despite the absence of the host plant in all but seven sampling sites. This result is interesting in that for the other actinorhizal plants recorded in Tunisia, namely the native *Alnus glutinosa* and the imported species of *Casuarinaceae*, plant-trapping bioassay has never detected root colonization outside alder stands or *Casuarinaceae* experimental arboreta [8].

On dissecting 582 nodule lobes we found, from eight lobes deriving from eight of the 30 soils studied, eight isolates displaying the typical *Frankia* phenotype. All the isolates were shown to be true *Elaeagnus* microsymbiontes, fulfilling the Koch postulates, as shown by the acetylene-reduction activity in the root nodules of *E. angustifolia* seedlings, and their purity was assured by purification from single colonies grown in agar media. The strains showed different ARA activities as well as relatively variable phenotypes for all the examined parameters, indicating relatively high *Frankia* diversity in Tunisian soil. The observed diversity is in agreement with the wide pedoclimatic variability of the sampling sites.

The tree topology obtained from 16S rRNA sequences of tunisian *Frankia* strains and sequences in public databases was coherent with that of Huguet et al. [25] and indicated that one of the tunisian isolates, strain BMG5.6, is a new and different strain among *Frankia* colonizing *Elaeagnaceae* and *Rhamnaceae*. Until now actinorhizal bacteria colonizing these families have been shown, in most cases, to be phylogenetically related. An analysis of 1307 positions of the 16S rRNA gene showed that this strain differs from the closest known *Frankia* by almost 4%, indicating that it represents a new lineage in the *Frankia* assemblage. The other strains did not differ greatly on the 16S rRNA gene from already known strains nevertheless they clustered in the two different groups of the *Elaeagnaceae–Rhamnaceae* infective strains [25,32] signifying a high relative diversity.

The tree topology obtained with 16S rRNA gene sequences was confirmed by those of GlnII the glutamine synthetase. Besides two sequence groups including strains BMG5.2, BMG5.5 and BMG5.12 and strains BMG5.3, BMG5.4, BMG5.10 and BMG5.11, respectively, together with known *Elaeagnus* infective strains, strain BMG5.6 described a third branch in the tree confirming that this strain represents a new lineage in the *Frankia* assemblage, and that the diversity of *Elaeagnus*-compatible *Frankia* is wider than previously recognized. This diversity is noteworthy in view of the relatively narrow geographic distribution of the analyzed soils, and suggests that the overall diversity of *Frankia* colonizing *Elaeagnaceae* and *Rhamnaceae* is wider than previously thought [32]. In a study on the diversity of *Frankia* colonizing *Elaeagnaceae* and *Rhamnaceae*, Clawson et al. [32] detected two main types of sequences, these differing at only one position in a 378 bp fragment of the 16S rRNA gene, from nodules and isolated strains deriving from ten plant species of eight genera recovered in France (two strains from different geographic areas), USA (one nodule), New Zealand (one nodule), Argentina (one strain) and Chile (three nodules and five isolates from seven different geographic areas). Besides the two main sequences, the authors found only four other types of sequences and concluded that, despite the wide geographic distribution of the nodule analyzed, the diversity within the *Elaeagnus* group of *Frankia* was low [32].

The remarkable differences observed by rep-PCR and tDNA-PCR-SSCP fingerprinting even among strains with similar 16S rRNA gene and GlnII sequences (e.g. strains BMG5.3, BMG5.4 and BMG5.10) indicate a noteworthy genome variability, and suggest that the diversity of the *Elaeagnus*-compatible *Frankia* in Tunisia seems to be influenced by soil effect rather than by host effect. The geographic remoteness of the studied area and the absence of host plants could contribute to the emergence of local *Frankia* populations with poor migration ability in the absence of appropriate host species.

The relatively wide diversity of Tunisian *Frankia* strains opens the perspective that African soil could be an interesting reservoir for the isolation of new actinorhizal strains potential biofertilizers to counteract the progressive soil desertification which is a primary environmental problem in Northern Africa.

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**References**
