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Short communication

Genetic diversity among *Elaeagnus* compatible *Frankia* strains and sympatric-related nitrogen-fixing actinobacteria revealed by *nifH* sequence analysis

Maher Gtari[a,*], Lorenzo Brusetti[b], Abdenaceur Hassen[c], Diego Mora[b], Daniele Daffonchio[b], Abdellatif Boudabous[a]

[a]*Département de Biologie, Laboratoire Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, Campus Universitaire, 2092 Tunis, Tunisia*
[b]*Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi, via Celoria 2, 20133 Milano, Italy*
[c]*Laboratoire Eau et Environnement, Institut National de Recherche Scientifique et Technique, BP24-1082, Cité Mahrajene, Tunis, Tunisia*

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Abstract

Elaeagnus compatible *Frankia* isolates from Tunisian soil have been previously clustered with *Frankia*, colonizing *Elaeagnaceae* and *Rhamnaceae* in two different phylogenetic subgroups, while strain BMG5.6 was described as a new lineage closely related to *Frankia* and *Micromonospora* genera. In this study we further assess the diversity of captured *Frankia* and the relationship with BMG5.6-like actinobacteria, by using *nifH* gene sequences. Using PCR-RFLP screening on DNA extracted from lobe nodules, additional microsymbionts sharing BMG5.6 features have been detected proving a widespread occurrence of these actinobacteria in *Elaeagnus* root nodules. Neighbour-Joining trees of *Frankia nifH* sequences were consistent with previously published 16S rRNA and *GlnII* phylogenetic trees. Although four main clades could be discerned, actinobacterial strain BMG5.6 was clustered with *Frankia* strains isolated from *Elaeagnus*. The present study underscored the emanation of new diazotrophic taxon isolated from actinorhizal nodules occupying intermediate taxonomic position between *Frankia* and *Micromonospora*. Moreover, its aberrant position in *nifH* phylogeny should open network investigations on the natural history of nitrogen-fixing gene among actinobacteria.

Keywords: *Frankia*; Nitrogen-fixing actinobacteria; *NifH*; Phylogeny; Tunisia

Frankia genus was made up of actinobacteria distinguished by their ability to establish nitrogen-fixing actinorhizes in diverse woody dicotyledonous roots (Benson and Silvester, 1993). Phylogenetic studies of *Frankia* strains based on 16S rRNA gene, glutamine synthetase I (*GlnA*) and glutamine synthetase II (*GlnII*) sequences have generally revealed four major clades (Normand et al., 1996; Clawson et al., 2004; Cournoyer and Lavire, 1999; Gtari et al., 2004). *Frankia* strains in these clades can be distinguished on the basis of cultivability, morphology, and plant root infectivity (Benson and Silvester, 1993). Strains from clade I have not been isolated in culture, while typical *Frankia* isolates have been obtained from clades II and III. Clade II strains grow preferentially on organic acids, whereas Clade III strains grow on both organic acids and simple sugars (Benson and Silvester, 1993). Although relatively few plants have been studied, Clade II strains appear to infect their hosts by root hair infection and Clade III strains infect by either intercellular penetration or root hair infection depending on the plant (Berry et al., 1986; Bosco et al., 1992; Cournoyer et al., 1993; Miller and Baker, 1985; Racette and Torrey, 1989). Atypical (non-infective and/or non-nitrogen-fixing) *Frankia* strains obtained from actinorhizal plants such as *Ceanothus*, *Coriaria*, *Datisca*, and *Purshia* species, form a broad group and were associated to clade IV. This group was described on the basis of 16S rRNA gene sequence (Normand et al., 1996; Huguet et al., 2001). Due to the lack of solid phenotypic features of typical *Frankia* strains, the inclusion

*Corresponding author. Tel./fax: +216 70 860 553.
E-mail address: maher.gtari@fst.rnu.tn (M. Gtari).

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of this clade as member of the *Frankia* assemblage is questionable and merits further investigations. Nitrogen fixation, one of the two enigmatic points of such actinorhizal isolates, being the second infectivity, could be elucidated by using genes encoding nitrogenase complex enzymes, important feature of *Frankia* within actinobacteria. The intergenic spacer between *nifD* and *nifK* genes (IGS *nif*D-K) has provided a powerful approach to assess the diversity of *Frankia* strains in culture and directly within root nodules (Jamann et al., 1993; Rouvier et al., 1996; Lumini and Bosco, 1996; Lumini and Bosco, 1999). The nitrogenase iron protein gene, *nifH*, has been also reported to be in agreement with the phylogeny inferred from 16S rRNA gene sequences for several nitrogen-fixing microorganisms (Hennecke et al., 1985; Young, 1992; Zehr et al., 1995; Ueda et al., 1995; Borneman et al., 1996; Ohkuma et al., 1996; Achouak et al., 1999; Raymond et al., 2003). However, phylogenetic studies of *Frankia*, based on *nifH* sequences, are rare and non-exhaustive (Jeong et al., 1999; Nick et al., 1992).

The objective of this study was to obtain information about the diversity and phylogenetic relationship of *Frankia nifH* gene sequences and related nitrogen-fixing actinobacteria isolated from root nodule of *Elaeagnus* from Tunisian soils. When this study was started, no *nifH* sequences from clade IV *Frankia* of taxa had been deposited in Database (Gtari et al., 2004, EMBL accession number AJ545036).

Isolation, subcultivation and purification procedures of strains: BMG5.2, BMG5.3, BMG5.4, BMG5.5, BMG5.10, BMG5.11, BMG5.12 and BMG5.6 used in this study, were detailed elsewhere (Gtari et al., 2004). Infectivity tests of isolates were carried out on *Elaeagnus angustifolia*, *Alnus glutinosa* and *Casuarina equisetifolia* seedlings as described by Bosco et al. (1992). *NifH* gene amplification was performed on DNA extracted from cultured strains and nodule lobes (Gtari et al., 2002, 2004) by using primers described by Normand et al. (1988) and an inner set ATGGC(G/T)GCCATGGCCGAG and GTTGAT(G/A)CGGCGGCGA designed in this study to perform nested PCR. For RFLP analysis PCR products were digested with *Alu*I, *Rsa*I, *Tha*I and *Hae*III and electrophoresed in vertical 6% polyacrylamide gel. Clustering of restriction patterns was performed with MVSP software (version 3.131, Kovack Computing Service, Pentraeth, UK). Sequences were determined by cycle sequencing (Urzì et al., 2001). Partial nucleotide and predicted aminoacid sequences of *nifH* gene were analyzed using Clustalw (http://clustalw.genome.ad.jp) and phylogenetic trees were achieved using PHYLIP (Felsenstein, 1993) and TREE-PUZZLE (Strimmer and Haeseler, 1996) utilities. Bootstrap values were determined from 1000 replicates (Felstein, 1985). The *nifH* sequences were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under accession numbers from AJ545030 to AJ545037.

All seven *Frankia* strains BMG5.2, BMG5.3, BMG5.4, BMG5.5, BMG5.10, BMG5.11 and BMG5.12 were found to effectively nodulate 100% of *Elaeagnus* plantlets. Strains BMG5.2 and BMG5.12 were also found able to induce 1–5 small root nodules in some *A. glutinosa* plantlets with mean percentages of nodulated plants ranging from 20% to 30%, respectively. However, nodulated *Alnus* plantlets always remained small and yellowish. Despite initial re-nodulation experiments of *Elaeagnus* axenic seedlings with strain BMG5.6 gave nodules (Gtari et al., 2004), after repeated strain subculturing the nodulation capacity was lost. Strain BMG5.6 never showed the capacity of nodulating *Alnus* and *Casuarina* plantlets.

Using primers set designed by Normand et al. (1988), 700 bp bands were amplified from Tunisian isolates using a *nifH* specific PCR assay. For *nifH* RFLP, four restriction enzymes, *Alu*I, *Rsa*I, *Hae*III and *Tha*I, were selected on the basis of in silico digestion using DNAMAN version 5.2.2 software of *Frankia nifH* sequences available in Genbank and those of Tunisian isolates. A considerable diversity was observed among analyzed strains from *Elaeagnus* and *Alnus* compatible groups.

Similar *nifH* RFLP profiles were found with the restriction enzymes used between *Alnus* and *Elaeagnus* specificity group. The *Casuarina* infective strain CcI3 showed more divergent band patterns (Fig. 1). However, the UPGMA dendrogram obtained by PCR–RFLP analysis permitted the discrimination between the two host-specificity groups. The analysis allowed concluding that strain BMG5.6, a closely related strain to *Frankia* assemblage (Gtari et al., 2004), was associated with *Elaeagnus* compatible strains.

A nested PCR assay for the characterization of *Frankia* and related *nifH* in nodules was developed by using primers of Normand et al. (1988) as outer primers and two primers, designed in this study as inner primers. The inner primers were tested with several diazotrophic bacteria, and PCR revealed that these primers are specific for PCR amplification of *nifH* gene from *Alnus*, *Elaeagnus* compatible strains as well as the actinobacterium BMG5.6. We hence used this approach to analyze *Frankia nifH* in *Elaeagnus* root nodules developed by plant-trapping assay with soil sampled in the region of Sfax. From the soil we previously isolated strain BMG5.6 (Gtari et al., 2004). PCR amplification was performed from peeled and non-peeled root nodules (Fig. 2). Screening of 16 lobe nodules permitted the detection of PCR–RFLP band patterns typical of strain BMG5.6 in one unpeeled and three peeled lobe nodules.

Neighbor-Joining algorithm tree based on *nifH* gene nucleotide sequences showed shown four main clades (Fig. 3). A deeper clade I included uncultured endosymbionts from *Ceanothus caeruleus*, *Coriaria nepalensis* and *Datisca cannabina* with sequence identity from 81% to 95%. Clade II included two infective isolates from *Casuarina* CcI3 and INPCe16 (97.3% of identity) and the nitrogen-fixing actinobacterial isolate 7501. Strains in clade III share 93.8–98.9% of identity and were represented by the *Alnus–Myrica* infective group strains; ArI3 ACN14a and FaC1 (*Alnus* infective) and Mrp128 (*Myrica* infective).