Frankia asymbiotica sp. nov., a non-infective actinobacterium isolated from Morella californica root nodule

Imen Nouioui, Abdellatif Gueddou, Faten Ghodbane-Gtari, Manfred Rhode, Maher Gtari and Hans-Peter Klenk

Abstract

The taxonomic status of strain M16386T, a nitrogen-fixing but non-nodulating isolate from Morella californica, was established on the basis of a polyphasic approach. The strain grows as branched hyphae, with vesicles and non-motile productive multilocular sporangia. It metabolizes short fatty acids, TCA cycle intermediates and carbohydrates as carbon sources, and fixes nitrogen in the absence of combined nitrogen source in the growth media. Chemotaxonomic traits of strain M16386T are consistent with its affiliation to the genus Frankia. The characteristic diamino acid in the cell wall is meso-diaminopimelic acid. Strain M16386T contains phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, glycoprophospholipid and phospholipid as polar lipids; MK-9(H6) and MK-9(H8) as the predominant menaquinones; iso-C16:0 and C17:ω8c as major fatty acids; and galactose, glucose, mannose, rhamnose and ribose as whole-cell sugars. Strain M16386T showed 98.2 % 16S rRNA gene sequence similarity with its closest phylogenetic neighbour, Frankia inefficax DSM 45817T. Based on these results, strain M16386T (=DSM 100626T=CECT 9040T) is designated the type strain of a novel species of the genus Frankia, for which the name Frankia asymbiotica sp. nov. is proposed.

Soil actinobacteria of the genus Frankia can establish symbiotic relationships with a broad range of dicotyledonous host plants designated actinorhizal plants [1]. Frankia strains are able to fulfill Koch’s postulates (capable of nodulating plant roots and fixing nitrogen) and can be defined by their behaviour in culture, morphology and by mode of their infection [2]. These typical Frankia strains are scattered in several species and fall into three different phylogenetic clusters: (i) cluster 1 includes Frankia alni, Frankia casuarinae [3] and relatives; (ii) cluster 2 contains Frankia coriariae and relatives; and (iii) Frankia discariae [4], Frankia elaeagni [3] and relatives make up cluster 3. An additional cluster 4 associates atypical Frankia strains which fail to fulfill one or more of Koch’s postulates by their inability to nodulate and/or to fix nitrogen. Among this broad cluster, only one species, Frankia inefficax, was described to induce ineffective, non-nitrogen-fixing root nodules on its actinorhizal host plants [5]. An atypical strain, M16386T [6], able to fix nitrogen but unable to nodulate its host plant origin and any of the tested actinorhizal plants, is here described as the type strain of a novel species of the genus Frankia.

Frankia strain M16386T was isolated from Morella californica in 1986 (Westport-Legget, CA) [6]. This strain was kindly provided by ARS USDA bacterial collection as NRRL B-16386. To avoid any confusion with the no longer availability of Frankia strains in the ARS USDA bacterial collection, the strain was designated M16386. Strain M16386T is maintained in the same conditions as for the other type strains of species of the genus Frankia described by Nouioui et al. [3–5, 7]. Chemotaxonomic and physiological traits were determined from four-week-old cultures grown, without shaking, in Basic Propionate (BAP) medium [8] at 28°C. Freeze-dried cells and wet biomass were used for chemotaxonomic and phenotypic tests, respectively. Biochemical features of strain M16386T were examined using GENIII MicroPlates in an Omnilog device (Biolog) as described by Nouioui et al. [3]. A duplicate of all the analyses was carried out. Type strains of all species of the genus Frankia, F. alni (DSM 45986T), F. casuarinae (DSM 45818T), F. coriariae (DSM 100624T), F. elaeagni (DSM 46783T), F. discariae (DSM 46785T) and F. inefficax (DSM 45817T), were included for phenotypic tests and cellular fatty acid analyses in this study. A scanning electron microscope (FE-SEM)
Merlin; Zeiss) was used for the morphological description of strain M16386T.

Strain M16386T grew as unpigmented colonies with extremely branched vegetative hyphae, multicellular sporangia and ovate to round vesicles. Good growth of the tested strain was observed in BAP medium at pH 6.3–6.8 and after 4 weeks of incubation at 28 °C. Strain M16386T has several phenotypic features that distinguish it from the type strains of other species of the genus Frankia as shown in Table 1. Strain M16386T was able to use acetic acid, cellobiose, α and β-hydroxybutyric acid and α-ketobutyric acid, unlike its nearest phylogenetic neighbour, F. ineflicax DSM 45817T (Table 1). Using scanning electron microscopy, strain M16386T showed the three typical cellular structures of the genus Frankia: hyphae, sporangia and vesicles (Fig. 1).

The ability of strain M16386T to fix nitrogen was assessed by acetylene reduction assay [9]. Bacterial cells were inoculated into vacutainer tubes (10 ml) containing 5 ml BAP medium without ammonium. After growth for 1 week, 10 % (v/v) of the gas volume was replaced by acetylene and incubated for 60 min at 30 °C. One millilitre of the gas volume was injected into a Girdel gas chromatograph equipped with a flame ionization detector. Strain M16386T grew on nitrogen-deficient BAP medium and was also able to reduce acetylene (201±11 nmol h−1 mg−1 protein) as soon as vesicles were formed (Fig. 1).

Seedlings of representative species of the major host plant infectivity groups [10] were used for nodulation tests of strain M16386T. Seeds of Alnus glutinosa, Casuaria glauca, Elaeagnus angustifolia, Coriaria myrtifolia and Morella californica were surface-sterilized for 15 min in 30 % (v/v) hydrogen peroxide and rinsed several times with sterile distilled water. Except for Morella californica where a cold stratification for one month at +4 °C is necessary for germination, the other seeds were immediately transferred to

### Table 1. Phenotypic and chemotaxonomic properties that distinguish strain M16386T from the type strains of species of the genus Frankia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
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<td>Colony colour</td>
<td>White</td>
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<td>Brown</td>
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<td>Carbon source</td>
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<td>Cellobiose</td>
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<td>β-Gentiobiose</td>
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<td>D-Glucose 6-phosphate</td>
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<td>D-Fructose 6-phosphate</td>
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<td>β-Hydroxybutyric acid</td>
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<td>α-Ketobutyric acid</td>
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<td>Acetoacetic acid</td>
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<td>L-Malic acid</td>
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<td>Bromosuccinic acid</td>
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<td>Growth in the presence of</td>
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<td>Acetic acid</td>
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<td>1% Sodium lactate</td>
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<td>Major fatty acids (&gt;15 %)</td>
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<td>iso-C 16:0, C 17:1, 18:1 C18:2, C 17:1, 18:2</td>
<td>iso-C 16:0, C 17:1, 18:1 C18:2, C 17:1, 18:2</td>
<td>C 16:0, C 17:1, 18:1 C18:2, C 17:1, 18:2</td>
<td>C 16:0, C 17:1, 18:1 C18:2, C 17:1, 18:2</td>
<td>C 16:0, C 17:1, 18:1 C18:2, C 17:1, 18:2</td>
<td>iso-C 16:0, C 17:1, 18:1 C18:2, C 17:1, 18:2</td>
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<tr>
<td>Host plant origin</td>
<td>Morella californica</td>
<td>Morella californica</td>
<td>Morella californica</td>
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<td>Morella californica</td>
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<td>Host plant range</td>
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<td>Genomic G+C content (mol%)</td>
<td>72.0</td>
<td>72.8</td>
<td>72.8</td>
<td>71.0</td>
<td>71.7</td>
<td>72.3</td>
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</table>
Petri dishes containing sterile filter paper moistened with sterilized tap water. After germination, seedlings were aseptically transplanted into a Magenta GA-7 box containing 100 ml Broughton and Dilworth solution [11] and grown for 1 month prior to inoculation. Fragmented hyphae from the M16386 strain culture were then applied to the root surface of tested plant species (five each) using a sterile needle.

Infectivity tests showed that strain M16386T is unable to induce nodulation on its original host of isolation Morella californica and any of the tested actinorhizal species that are known to nodulate on the original host species (Fig. 1). The failure of a single plant species (Alnus-Comptonia-Myrica HIG) and Morella pensylvanica, Myrica gale HIG also reported the failure of this strain to nodulate Morella californica, Morella cerifera, Morella pensylvanica, Myrica gale and Comptonia peregrina.

Chemo taxonomic markers of strain M16386T were determined using thin-layer chromatography. For this purpose, whole-cell sugars, dianamipimelic acids, isoprenoid quinones and polar lipid profile were determined following the same protocols used by Nouioui et al. [3]. Biomasses from cultures in BAP medium with and without nitrogen source were the subject for fatty acid methyl esters analyses. The extracts were prepared following the modified protocol of Miller [13] by Kuykendall et al. [14], and analysed using the same tools as described by Nouioui et al. [3]. The chemotaxonomic features of strain M16386T are coherent with its affiliation in the genus Frankia [15]. Whole-cell hydrolysates contained meso-diaminopimelic acid isomers and are rich in galactose, glucose, mannose, rhamnose and ribose, while its closest neighbour F. inef ficax additionally contains fucose. The polar lipid pattern consists of phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, phosphoglycerolipid and phospholipid. The predominant menaquinones are MK-9(H4) (56.8 %) and MK-9(H6) (24.4 %), and iso-C16:0 and C17:0 cyc were identified as the major fatty acids (>20 %) (Table S1, available in the online version of this article).

Genomic DNA was extracted from strain M16386T as described by Nouioui et al. [16]. 16S rRNA gene amplification and sequencing were realized as described by Lane [17] and Ghodhbane-Gtari et al. [18], respectively. An almost complete 16S rRNA gene sequence of strain M16386T (1492 nucleotides) was obtained and deposited in the NCBI GenBank database. 16S rRNA gene sequence similarities were calculated according to Meier-Kolthoff et al. [19] using the GGDC web server [20]. Maximum-likelihood (ML) and maximum-parsimony (MP) trees were reconstructed using the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) phylogenomics pipeline [21] with RAxML [22] and TNT [23], respectively. The MUSCLE program [24] and the X2 test as implemented in PAUP [25] were used for a multiple sequence alignment and to check the sequence for a compositional bias, respectively. For ML, rapid bootstrapping in conjunction with the autoMRE bootstrapping criterion [26] was used, while 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates were used for MP.

Strain M16386T was, with high confidence, located in a phylogenetic position close to the type strain of F. inef ficax and next to the representatives of F. elaeagni and F. discariae (Fig. 2). This relatedness is in line with the 16S rRNA gene sequence similarities which were found to be 98.2, 98.0 and 97.8 % between strain M16386T and relatives accommodated in the same subclade, F. inef ficax DSM 45817T, F. elaeagni DSM 46783T and F. discariae DSM 46785T, respectively.

Digital DNA:DNA hybridization (DDDH) between strain M16386T and the type strain of F. inef ficax, its nearest phylogenetic neighbour, was calculated using genome-to-genome distance calculator with formula 2 available at the DSMZ server (http://ggdc.dsmz.de/distcalc2.php). The dDDH was found to be 25.8 %, which is well below the 70 % threshold of delineation for wet lab DDDHs of prokaryotic species described by Wayne et al. [27].

Based on this polyphasic study, strain M16386T showed phenotypic, chemotaxonomic and genetic features distinguishable from its nearest phylogenetic neighbours. Therefore, we propose strain M16386T to be recognized as the type strain of a novel species, Frankia asymbiotica sp. nov., within the genus Frankia.

**DESCRIPTION OF FRANKIA ASYMBIOTICA SP. NOV.**

Frankia asymbiotica (a.sym.bi.o’t.i.ca. Gr. pref. a not; N.L. fem. adj. symbiotica living together; N.L. fem. adj. asymbiotica not symbiotic).

Gram-stain-positive, aerobic, heterotrophic, chemoorganotrophic and diazotrophic actinobacterium which is characterized by branched substrate hyphae, vesicles and multicellular sporangia. Unpigmented cells develop in BAP medium after incubation for 4 weeks at 28 °C. Able to use...
acetic acid, butyric acid, cellobiose, α- and β-hydroxybutyric acid, D-glucose, α-ketobutyric acid, methyl pyruvate, maltose, trehalose, sucrose, turanose, D-mannose, L-rhamnose, D-glucuronic acid and propionic acid. Growth is not inhibited by troleandomycin, rifamycin SV and aztreonam.

The chemotaxonomic profile contains meso-diaminopimelic acid isomers; galactose, glucose, mannose, rhamnose and ribose as whole-cell sugars; phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, phosphoglycolipid and phospholipid as polar lipids; MK-9(H₄) and MK-9(H₆) as predominant menaquinones (>20 %); and iso-C₁₆:₀ and C₁₇:₁ω₈c as major fatty acids (>20 %).

The type strain M16386ᵀ (=DSM 100626ᵀ=CECT 9040ᵀ) was isolated in 1986 from Morella californica located in Westport-Legget, CA [6]. The type strain is unable to nodulate its host plant of origin or any of the tested actinorhizal plant species.

Acknowledgements
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Conflicts of interest
The authors declare that there are no conflicts of interest.

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