Nocardioides astragali sp. nov., isolated from a nodule of wild Astragalus chrysopterus in northwestern China

Lin Xu · Yong Zhang · Chongyang Li · Xiaoqin Wang · Jinrong Liu · Ville-Petri Friman

Received: 29 October 2017 / Accepted: 18 January 2018 / Published online: 25 January 2018
© The Author(s) 2018. This article is an open access publication

Abstract A Gram-positive, non-motile, rod-shaped bacterial strain, designated HH06T, was isolated from a nodule of Astragalus chrysopterus in northwestern China. Phylogenetic analysis of the 16S rRNA gene sequence showed that the strain is closely related to Nocardioides alpinus Cr7-14T and Nocardioides furvisabuli DSM 18445T with 98.5 and 98.1% similarity, respectively. Growth was observed at 4–28°C in R2A medium (optimum at 25°C), at 10–30°C in YMA and LB medium (optimum in both at 28°C) and at pH 5.0–10.0 in R2A medium (optimum at pH 7.0–8.0). The cell wall peptidoglycan was found to contain LL-diaminopimelic acid as the principal diamino acid and MK-8(H4) was identified as the predominant menaquinone. The major polar lipids were identified as phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, two unidentified glycolipids and two unidentified polar lipids. The major fatty acids were identified as iso-C16:0 (32.8%) and C18:1ω9c (15.1%). The DNA G+C content of strain HH06T was determined to be 71.4 mol%. Based on phenotypic, chemotaxonomic, phylogenetic properties and DNA–DNA relatedness, it is concluded that strain HH06T represents a novel species of the genus Nocardioides, for which the name Nocardioides astragali sp. nov. is proposed. The type strain is HH06T (= CGMCC 4.7327T = NBRC 112322T).

Keywords Astragalus chrysopterus · Nocardioides · Novel species · Symbiosis

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10482-018-1020-1) contains supplementary material, which is available to authorized users.

L. Xu · Y. Zhang · X. Wang
Key Laboratory of Hexi Corridor Resources Utilization, Hexi University, Zhangye 734000, Gansu, People’s Republic of China

L. Xu · Y. Zhang
Institute of Agricultural and Biological Technology, Hexi University, Zhangye 734000, Gansu, People’s Republic of China

L. Xu · V.-P. Friman
Department of Biology, University of York, York Y010 5DD, UK
e-mail: ville.friman@york.ac.uk

L. Xu
State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, People’s Republic of China

C. Li · J. Liu
College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730020, People’s Republic of China
Introduction

The genus *Nocardioides*, with *Nocardioides albus* as the type strain, was first proposed by Prauser (1976). At present, the genus contains more than 90 species with validly published names (http://www.bacterio.net/nocardioides.html). Strains of this genus have been isolated from various sources such as soils (Sultanpuram et al. 2015; Sun et al. 2014; Lee and Seong 2014; Srinivasan et al. 2014; Liu et al. 2015), plant rhizosphere (Tuo et al. 2015; Xu et al. 2016; Kämpfer et al. 2016; Glaeser et al. 2014), marine and lake environments (Wang et al. 2016; Deng et al. 2015; Fan et al. 2014; Zhang et al. 2014; Cho et al. 2013a, b) as well as from within animals and plants (Lin et al. 2015).

During a study of the diversity of rhizobial endophytes of wild leguminous plants in July 2015, a strain designated HH06T was isolated from a root nodule of *Astragalus chrysopterus*. Based on phylogenetic analysis, strain HH06T shows around 98% 16S rRNA gene sequence similarity to several members of the genus *Nocardioides*. The taxonomic position of this strain is reported in this paper. Polyphasic taxonomic analyses showed that the strain HH06T is distinct from previously described species of *Nocardioides*, and thus, represents a novel species of this genus, for which the name *Nocardioides astragali* sp. nov. is proposed.

Materials and methods

Organisms, maintenance and cultural conditions

Strain HH06T was collected from *A. chrysopterus* in Rouge mountain, Zhangye, China (2880 m; 38°25’58”N, 101°15’06”E) and was isolated on YMA agar plates by using the serial dilution method described by Xu et al. (2014). The YMA medium was prepared according to the instructions from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (http://www.dsm.de/microorganisms/medium). Plates were incubated at 28 °C for 6 days before isolation of single bacterial colonies, one of which was subsequently selected and cryopreserved at −80 °C as a suspension in TY (DSMZ) medium supplemented with 30% (w/v) glycerol.

*Nocardioides alpinus* Cr7-14T and *Nocardioides furvisabuli* DSM 18445T were obtained from the China General Microbiological Culture Collection Center (CGMCC) and cultured under the same conditions as the reference strains.

Phylogenetic analysis and molecular studies

The phylogenetic position of the isolate was determined by 16S rRNA gene sequence analyses. The total DNA of the novel isolate and two closely related reference strains of the genus *Nocardioides* (*N. alpinus* Cr7-14T and *N. furvisabuli* DSM 18445T) was extracted by using the method by Marmur (1961). Amplification of the 16S rRNA gene was performed with universal primers P1/P6 as described previously (Wang et al. 1999). It is possible to obtain genes associated with rhizobial symbiosis from bacteria isolated from nodules. Therefore, we tested the presence of two important symbiosis genes: *nodA* (acyltransferase) and *nifH* (nitrogenase reductase) as described by Xu et al. (2013).

The almost complete 16S rRNA gene sequence of the novel strain was used for calculating relatedness with its phylogenetic neighbours by using the EzTaxon-e server version 2.1 (http://www.ezbiocloud.net; Yoon et al. 2016). The phylogenetic analyses based on 16S rRNA sequences of the novel and reference strains belonging to the genus *Nocardioides* were performed by using the software package phylowin (Galtier et al. 1996); multiple alignments were performed by using the CLUSTAL X program (version 1.64b) (Thompson et al. 1997). The phylogenetic tree was constructed by using neighbour-joining methods (Fitch 1971; Saitou and Nei 1987) with the Jukes-Cantor parameter calculation model. The robustness of the topology of the phylogenetic trees was evaluated by bootstrap analyses based on 1000 resamplings (Felsenstein 1985).

The G+C content of DNA was measured by using the thermal denaturation method described by Marmur and Doty (1962) with *Escherichia coli* K-12 DNA as a standard. The DNA-DNA relatedness was determined by using the spectrophotometric method of De Ley et al. (1970).
Morphological, physiological and biochemical analysis

Strain HH06T was cultivated for 6 days at 25 °C on R2A agar for morphological observation by scanning electron microscopy (Quanta 200; FEI). Gram-staining was performed by using a previously published staining method (Smibert 1994) for cells grown on R2A agar at 25 °C. The growth range and optimum were determined in R2A broth after 6 days of incubation at 4, 10, 20, 25, 30, 37, 40 and 45 °C. The pH range and optimum were determined at pH of 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10 and 11; KH₂PO₄/HCl, KH₂PO₄/K₂HPO₄ and K₂HPO₄/NaOH buffer systems were used to maintain the desired pH. Tolerance to NaCl was examined in R2A broth containing 0–5% of NaCl (w/v, at intervals of 0.5%). Physiological and biochemical properties and enzyme activities were tested by using API 20 NE (identification of Gram negative non-Enterobacteriaceae) and API 50 CH (performance of carbohydrate metabolism) kits (bioMérieux) according to the manufacturers’ instructions. Indole production, reactions in the methyl red, Voges-Proskauer tests, hydrolysis of starch, gelatin, Tween 80, activities of catalase, urease, oxidase, reduction of nitrate and nitrite, and hydrogen sulphide production from cysteine were also determined as described by Smibert (1994). The chitinase, lipase, coagulase and amylase activity were tested as described by Cappuccino and Sherman (1998).

Chemotaxonomic characterisation

Cellular fatty acid profiles were determined for strains grown on R2A agar (Difco) for 48 h at 25 °C. Fatty acid methyl esters were extracted and prepared by following the standard protocol of the Microbial Identification System (Microbial ID; MIDI). Extracts were analysed by using a Hewlett Packard model HP6890 gas chromatograph equipped with a flame-ionization detector, an automatic sampler, an integrator and a computer, as recommended by the manufacturer. To determine the main isoprenoid quinone, which is an essential component of electron transfer system in the plasma membrane of prokaryotes, strain HH06T and the two reference strains were grown on R2A medium for 6 days at 25 °C with shaking (170 rpm). Extraction and menaquinone assay was performed according to the HPLC method described by Zhang et al. (2003) and Komagata and Suzuki (1988). Briefly, the strains were lyophilised and extracted in methanol. Lipoquinones were analysed by using reversed-phase HPLC and a chromatographic column Dionoisil C18 (200 mm × 4.6 mm, i.d. 5 µm), with 300 ml methanol and 700 ml anhydrous ethanol as the mobile phase. The bacterial biomass for the chemotaxonomic characterisation was obtained from 3-day old cultures grown on medium R2A at 25 °C. The isomer type of the cell wall diaminopimelic acid was analysed as described previously (Staneck and Roberts 1974). Polar lipid profiles were analysed by following the method described by Minnikin et al. (1975). Individual phospholipids were identified by using several spray reagents (Embley and Wait 1994) and through co-migration with authentic standards (Sigma).

Results and discussion

Phylogenetic analysis and molecular studies

Based on the 16S rRNA gene sequence analysis, strain HH06T is phylogenetically closely related to members of the genus Nocardioides. The isolate was found to be closely related to N. alpinus Cr7-14T and N. furvisabuli DSM 18445T with 98.5 and 98.1% sequence similarities, respectively (Fig. 1), and this was supported by the phylogenetic tree calculated using the maximum parsimony method from Jukes-Cantor distance matrices of the sequences (Supplementary Fig. 1). However, no amplification of nodA and nifH gene products were observed despite several attempts. Based on these measurements, the G+C content for the strain HH06T was 71.4 mol% (Table 1), while the DNA-DNA relatedness with the two other reference strains was less than 37 ± 1.2% (SD).

Morphological, physiological and biochemical analysis

Strain HH06T was observed to form tiny convex, smooth, glossy and cream coloured colonies after 6 days growth at 25 °C in R2A medium. Cells were observed to be Gram-positive, rod-shaped (Supplementary Fig. 2) and able to grow between 4 and 28 °C in R2A medium (optimum at 25 °C,) and 10–30 °C in
YMA and LB medium (optimum at 28 °C), at pH range of 5.0–10.0 (optimum at pH 7.0–8.0) and at NaCl concentrations lower than 3%. In API tests, strain HH06 T was found to be able to utilise D-fructose, gentiobiose, sucrose, malate, citrate, pyruvic acid sodium, ribose, L-arabinose, D-trehalose, D-galactose, D-xylose, D-glucose, D-sorbitol, inositol, mannitol, D-mannose, amygdalin, maltose and sucrose. However, strain HH06 T was found to be unable to utilise L-tyrosine, glycerol, asparagine, erythritol, arginine, rhamnose, D-adonitol, xylitol, mannopyranose, D-melezitose, potassium gluconate, L-xylose, L-fucose, D-fucose, sorbose, lactose, D-tagatose or D-melibiose. Additional physiological and biochemical differences between the novel isolate and the two reference strains are provided in the species description and in Table 1.

Chemotaxonomic characterisation

The main cellular fatty acids of strain HH06 T were identified as iso-C_{16:0} (32.8%) and C_{18:1}ω9c (15.1%) and a full fatty acid profile comparison of strain HH06 T and two reference strains is given in Table 2. The predominant menaquinone of strain HH06 T was identified as MK-8(H4). Strain HH06 T was found to contain LL-diaminopimelic acid as the diagnostic cell wall diamino acid. Phosphatidylglycerol,
phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, two unidentified glycolipids and two unidentified polar lipids were detected in the polar lipid profile of the strain HH06\(^T\) (two-dimensional TLCs showed in Supplementary Fig. 3). These chemotaxonomic characteristics are consistent with the classification of most strains in the genus *Nocardioides*.

Based on phenotypic, chemotaxonomic, phylogenetic properties and DNA-DNA relatedness, it is concluded that strain HH06\(^T\) represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides astragali* sp. nov. is proposed. The Digital Protologue database (Rosselló-Móra et al. 2017) TaxoNumber for strain HH06\(^T\) is TA00381.

### Description of *Nocardioides astragali* sp. nov.

*Nocardioides astragali* (as.tra'ga.li. N.L. gen. n astragali of Astragalus) a genus of leguminous plants,
Cells are Gram-stain positive, short rods, 0.3–0.6 by 0.6–1.1 μm, (occasionally 1.2–2.2 μm in length) after 6 days of growth at 25 °C on R2A agar. Substrate and aerial mycelia are not observed, and colonies on R2A agar are round, convex, glossy with entire margins, cream white, with diameter is 0.1–0.3 cm after 6 days growth at 25 °C. Cells grow better on R2A (optimum at 25 °C) than LB (optimum at 28 °C) or YMA (optimum at 28 °C) media. Growth occurs between 4 and 28 °C in R2A medium, between 10 and 30 °C in YMA and LB media, between pH of 5.0–10.0 (optimum at pH 7.0–8.0) and with NaCl concentrations of 0–3% (w/v). Cells are positive for oxidase, catalase activity, hydrolysis of cellulose, starch, Tweens 80, β-glucosidase and N-acetyl-β-glucosaminidase and negative for nitrate reduction, urease production and milk peptonisation. The cell wall peptidoglycan contains LL-diaminopimelic acid; MK-8(H4) is the predominant menaquinone. The main cellular fatty acids are iso-C16:0 and C18:1 x9c. Phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine are present as the main polar lipids. The G+C content of the type strain is 71.4 mol%.

The type strain HH06T (= CGMCC 4.7327T = NBRC 112322T) was isolated from nodules of Astragalus chrysopterus in Zhangye, China. The GenBank accession number for the 16S rRNA gene sequence of strain HH06T is KU358689.

Acknowledgements We are grateful to Dr. Yuguang Zhou and Lei Song for deposition of the strains in the culture collections.

Funding Author A has received research Grants from National Science Foundation of China (31360004) and project of Education Department of Gansu Province (2014A-107).

Compliance with ethical standards

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

Ethical statement No specific ethical or institutional permits were required to conduct sampling and the experimental studies did not involve endangered or protected species.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

Cappuccino JG, Sherman N (1998) Microbiology-A laboratory manual, 5th edn. Benjamin Cummings Science Publishing, California

Cho Y, Jang GI, Cho BC (2013a) Nocardioides marinquinilus sp. nov., isolated from coastal seawater. Int J Syst Evol Microbiol 63:2594–2599

Cho Y, Jang GI, Hwang CY, Kim EH, Cho BC (2013b) Nocardioides salisbiostaticola sp. nov., isolated from biofilm formed in coastal seawater. Int J Syst Evol Microbiol 63:3800–3806

De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 1:133–142

Deng S, Chang X, Zhang Y, Ren L, Jiang F, Qu Z, Peng F (2015) Nocardioides antarciticus sp. nov., isolated from marine sediment. Int J Syst Evol Microbiol 65:2615–2621

Embley TM, Wait R (1994) Structural lipids of eubacteria. In: Goodfellow M, O’Donnell (eds) Chemical methods in prokaryotic systematics. Wiley, Chichester, pp 121–161

Fan X, Qiao Y, Gao X, Zhang XH (2014) Nocardioides pacificus sp. nov., isolated from deep sub-seafloor sediment. Int J Syst Evol Microbiol 64:2217–2222

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fitch WM (1971) Toward defining the course of evolution: minimum change for a specific tree topology. Syst Zool 20:406–416

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12:543–548

Glaeser SP, McInroy JA, Busse HJ, Kämpfer P (2014) Nocardioideszeae sp. nov., an endophyte actinobacterium of Zea mays. Int J Syst Evol Microbiol 64:2491–2496

Kämpfer P, Glaeser SP, McInroy JA, Busse HJ (2016) Nocardioides zeicaulis sp. nov., isolated from the stem of Zea mays. Int J Syst Evol Microbiol 66:1869–1874

Lee SD, Seong CN (2014) Nocardioides echinoideorum sp. nov., isolated from soil of a cactus. Int J Syst Evol Microbiol 65:1953–1958

Lee SD (2007) Nocardioides furvisabuli sp. nov., isolated from a cactus. Int J Syst Evol Microbiol 60:1611–1615

Komagata K, Suzuki KI (1988) 4 Lipid and cell-wall analysis in prokaryotic systematics. Methods Microbiol 19:161–207

Lee SD, Seong CN (2014) Nocardioides opuntiae sp. nov., isolated from the stem of a cactus. Int J Syst Evol Microbiol 65:2094–2099

Lin SY, Wen CZ, Hameed A, Liu YC, Hsu YH, Shen FT, Young CC (2015) Nocardioides echinoideorum sp. nov., isolated from sea urchins (Tripneustes gratilla). Int J Syst Evol Microbiol 65:1953–1958
Liu Q, Liu HC, Zhang JL, Zhou YG, Xin YH (2015) *Nocardioides glacialisoli* sp. nov., isolated from a glacier. Int J Syst Evol Microbiol 65:4845–4849

Marmur J (1961) A procedure for the isolation of DNA from microorganisms. J Mol Biol 3:208–218

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Minnikin DE, Alshamaony L, Goodfellow M (1975) Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. Microbiology 88:200–204

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Smibert RM (1994) Phenotypic characterization. Methods for general and molecular bacteriology. American Society for Microbiology, Washington DC, pp 607–654

Srinivasan S, Lee SS, Lee JJ, Kim MK (2014) *Nocardioides solii* sp. nov., a bacterium isolated from a mountain soil. Antonie Van Leeuwenhoek 106:271–278

Staneck JL, Roberts GD (1974) Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl Microbiol 28:226–231

Sultanpuram VR, Mothe T, Mohammed F (2015) *Streptomyces alkalithermotolerans* sp. nov., a novel alkaliphilic and thermotolerant actinomycete isolated from a soda lake. Antonie Van Leeuwenhoek 107:337–344

Sun LN, Zhang J, Gong FF, Wang X, Hu G, Li SP, Hong Q (2014) *Nocardioides solii* sp. nov., a carbendazim-degrading bacterium isolated from soil under the long-term application of carbendazim. Int J Syst Evol Microbiol 64:2047–2052

Tan ZY, Xu XD, Wang ET, Gao JL, Martinez-Romero E, Chen WX (1997) Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. Int J Syst Evol Microbiol 47:874–879

Thompson JD, Gibson TJ, Plewniak F, Jeannougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 25:4876–4882

Tuo L, Dong YP, Habden X, Liu JM, Guo L, Liu XF, Zhang YQ (2015) *Nocardioides deserti* sp. nov., an actinobacterium isolated from desert soil. Int J Syst Evol Microbiol 65:1604–1610

Wang ET, Van Berkum P, Sui XH, Beyene D, Chen WX, Martínez-Romero E (1999) Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. Int J Syst Evol Microbiol 49:51–65

Wang L, Li J, Zhang G (2016) *Nocardioides rotundus* sp. nov., isolated from deep seawater. Int J Syst Evol Microbiol 66:1932–1936

Xu L, Zhang Y, Deng ZS, Zhao L, Wei XL, Wei GH (2013) *Rhizobium qilianshanense* sp. nov., a novel species isolated from root nodule of *Oxytropis ochrocephala* Bunge in China. Antonie Van Leeuwenhoek 103:559–565

Xu L, Zhang Y, Wang L, Chen W, Wei G (2014) Diversity of endophytic bacteria associated with nodules of two independent legumes at different altitudes of the Qilian Mountains in China. Syst Appl Microbiol 37:457–465

Xu H, Zhang S, Cheng J, Asem MD, Zhang MY, Manikprabhu D, Zhang YX (2016) *Nocardioides ginkgobiloba* sp. nov., an endophytic actinobacterium isolated from the root of the living fossil *Ginkgo biloba*. Int J Syst Evol Microbiol 66:2013–2018

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2016) Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. Int J Syst Evol Microbiol. https://doi.org/10.1099/ijsem.0.001755

Zhang YJ, Yuan QP, Liang H (2003) The biosynthesis of coenzyme Q10 in *Bullera pseudoalba*. Microbiology 30:65–69

Zhang DC, Schumann P, Redzic M, Zhou YG, Liu HC, Schinner F, Margesin R (2012) *Nocardioides alpinus* sp. nov., a psychrophilic actinomycete isolated from alpine glacier cryoconite. Int J Syst Evol Microbiol 62:445–450

Zhang DF, Zhong JM, Zhang XM, Jiang Z, Zhou EM, Tian XP, Zhang S, Li WJ (2014) *Nocardioides nanhaiensis* sp. nov., an actinobacterium isolated from a marine sediment sample. Int J Syst Evol Microbiol 64:2718–2722