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ORIGINAL PAPER

Nocardia zapadnayensis sp. nov., isolated from soil

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Abstract A novel Gram-stain positive, rod-shaped, non-motile and mycolic acid containing strain, FMN18^{T}, isolated from soil, was characterised using a polyphasic approach. The organism showed a combination of morphological, biochemical, physiological and chemotaxonomic properties that were consistent with its classification in the genus *Nocardia* and it formed a phyletic line in the *Nocardia* 16S rRNA gene tree. The cell wall contained *meso*-diaminopimelic acid (type IV) and whole cell sugars were galactose, glucose, arabinose and ribose. The predominant menaquinone was MK-8($H_{4\omega\text{-cyclo}}$). The major phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. Major fatty acids are $C_{16:0}$, 10-methyl $C_{18:0}$ (TBSA), $C_{18:1}$*cis*9 and $C_{16:1}$*trans*9. These chemotaxonomic traits are in good agreement with those known for representatives of the genus *Nocardia*. The phylogenetic analysis based on the 16S rRNA gene sequence of strain FMN18^{T} showed it to be closely related to *Nocardia grenadensis* GW5-5797^{T} (99.2 %), *Nocardia speluncae* N2-11^{T} (99.1 %), *Nocardia jinanensis* 04-5195^{T} (99.0 %) and *Nocardia rhamnosiphila* 202GMO^{T} (98.3 %). The phylogenetic analysis based on the *gyr*B gene sequence of strain FMN18^{T} showed it to be closely related to *N. rhamnosiphila* 202GMO^{T} (99.0 %), *N. grenadensis* DSM 45869^{T} (96.6 %), *N. jinanensis* DSM 45048^{T} (93.1 %), *N. carnea* IFM 0237^{T} (89.7 %) and *N. speluncae* DSM 45078^{T} (89.1 %). A combination of DNA–DNA hybridization results and phenotypic properties demonstrated that strain FMN18^{T} was clearly distinguished from all closely related *Nocardia* species. It is proposed that the organism be classified as representing

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a novel species of the genus *Nocardia*, for which the name *Nocardia zapadnayensis* (type strain FMN18^T = DSM 45872^T = KCTC 29234^T) is proposed.

Keywords *Nocardia zapadnayensis* · Polyphasic taxonomy · 16S rRNA gene

Introduction

The genus *Nocardia,* established by Trevisan (1889), is a member of the family *Nocardiaceae*, suborder *Corynebacterineae* (Stackebrandt et al. 1997). Members of the genus are aerobic, Gram-stain positive, weakly acid-fast, non-motile, mycolic acid-containing actinomycetes that form part of the soil microflora and play a role in the turnover of organic matter (Orchard 1981; Maldonado et al. 2000). Many *Nocardia* species have also been shown to be agents of human and animal diseases, such as *Nocardia nova*, *Nocardia asteroides*, *Nocardia farcinica*, *Nocardia concava, Nocardia amikacinitolerans* and *Nocardia vulneris* (Wallace et al. 1991; Schaal and Lee 1992; Kageyama et al. 2005; Ezeoke et al. 2013; Lasker et al. 2014). Members of some of the novel *Nocardia* species produce secondary metabolites, such as nocardicin, brasilicardin-A, tubelactomisin-A and amicoumacin B (Aoki et al. 1976; Komaki et al. 1999; Igarashi et al. 2000; Sun et al. 2009). The genus *Nocardia* has had a long and complex taxonomic history, but a comprehensive summary of the genus has been given by Goodfellow and Maldonado (2012).

In an effort to discover novel actinomycetes from divers habitats, a number of strains were found to have morphological properties typical of *Nocardia*. In the present study, the taxonomic position of the *Nocardia*-like strain FMN18^T is clarified by using a polyphasic taxonomic approach. Based on phenotypic and genotypic evidence, it is proposed that strain FMN18^T represents a novel species of the genus *Nocardia*, for which the name *Nocardia zapadnayensis* sp. nov. is proposed.

Materials and methods

Isolation and maintenance of the organism

Strain FMN18^T was isolated from soil samples collected from Zapadnaya Southwest Forest Park, Moscow, Russia, using glucose-yeast extract-malt extract (GYME) medium (0.4 % glucose, 0.4 % yeast extract, 1.0 % malt extract, 0.2 % $CaCO_3$, 1.2 % agar) supplemented with filter sterilized cycloheximide (50 μg ml^{-1}), nalidixic acid (10 μg ml^{-1}) and rifampicin (0.5 μg ml^{-1}) after 21 days incubation at 28 °C. The strain was maintained on yeast extract-malt extract agar slants (ISP2; International *Streptomyces* Project Medium No. 2) (Shirling and Gottlieb 1966) and held into glycerol suspensions (20 %, v/v) at −20 °C.

Morphological, cultural and physiological characteristics

Morphological characteristics were observed by light microscopy (Zeiss Axio Lab A1) and scanning electron microscopy (JEOL JSM 6060 instrument) using a culture grown on ISP 2 (Shirling and Gottlieb 1966) agar at 28 °C for 30 days. Cultural characteristics were investigated on media from the International *Streptomyces* Project (ISP 2–7) (Shirling and Gottlieb 1966), modified Bennett's (MBA; Jones 1949), trypticase soy (TSA), Czapek's and nutrient agar. The degree of growth, aerial mycelium and pigmentation were recorded after 14 days of incubation at 28 °C. National Bureau of Standards (NBS) Colour Name Charts (Kelly 1964) was used for determining colour designation and names. Growth at different temperatures (4, 10, 28, 37, 45, 50 and 55 °C), and pH 4.0–12.0 (at intervals of 1.0 pH unit) was determined on ISP 2 (Shirling and Gottlieb 1966) after incubation for 14 days. Growth tests for NaCl tolerance (0–10 % NaCl; w/v) was determined on ISP 2 medium at 28 °C and pH 7.0 for 14 days. Established methods were used to determine whether the strains degraded Tween 20, 40 and 80 (Nash and Krent 1991); the remaining degradation tests were examined using methods described by Williams et al. (1983). Carbon source utilization was tested as described by Goodfellow (1971) using Stevenson's basal medium (Stevenson 1967) supplemented with a final concentration of 1.0 % (w/v) of the tested carbon sources and 0.1 % (w/v) succinic acid. Nitrogen source utilization was examined using the basal medium recommended by Williams et al. (1983) supplemented with a final concentration of 0.1 % (w/v) of the tested nitrogen sources. Antimicrobial susceptibility testing was performed using antimicrobial susceptibility test discs

(Bioanalyse) according to the manufacturer's instructions. The type strains *Nocardia grenadensis* DSM 45869^{T}, *Nocardia speluncae* DSM 45078^{T}, *Nocardia jinanensis* DSM 45048^{T}, *Nocardia rhamnosiphila* DSM 45147^{T} and *Nocardia carnea* DSM 43397^{T} were included for comparison in all tests.

Chemotaxonomic characterization

Biomass for chemotaxonomic studies was obtained by growing strain FMN18^{T} under aerobic conditions in flasks containing ISP 2 broth on a rotary shaker at 160 rpm for 14 days at 28 °C. Biomass was harvested by centrifugation, washed twice in distilled water and re-centrifuged and freeze-dried. Analyses of diaminopimelic acid in whole-cell hydrolysates and whole–cell sugars were prepared according to Lechevalier and Lechevalier (1970) and analysed by thin layer chromatography (Staneck and Roberts 1974). Polar lipids were extracted and analyzed by the method of Minnikin et al. (1984), as modified by Kroppenstedt and Goodfellow (2006). The isoprenoid quinones were extracted and purified using the method of Collins et al. (1977) and analysed by HPLC (Kroppenstedt 1982). Mycolic acids were detected with TLC as described by Minnikin et al. (1980). For the extraction of whole-cell fatty acids, cells were grown in 20 ml of Trypticase Soy Broth (TSB) at 28 °C with shaking (150 rpm). After 5 days of incubation, 5 ml of seed culture was inoculated into 50 ml of TSB. The inoculated flask was incubated as before for 5 days. After harvesting by cellulose membrane filtration (0.45 µm), wet cells (200 mg) were placed in an extraction tube. Cellular fatty acids were extracted, methylated and separated by gas chromatography using an Agilent Technologies 6890N instrument, fitted with an auto sampler and a 6783 injector, according to the Standard protocol of the Sherlock Microbial identification (MIDI) system (Sasser 1990; Kämpfer and Kroppenstedt 1996), and the fatty acid methyl ester peaks were identified and quantified using TSBA 5.0 software. The DNA G+C content of the isolate was determined following the procedure of Gonzalez and Saiz-Jimenez (2005).

DNA preparation, amplification and determination of 16S rRNA and gyrB gene sequence

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out following Chun and Goodfellow (1995). The almost complete (1503 bp) 16S rRNA gene sequence of strain FMN18^{T} was determined using an ABI PRISM 3730 XL automatic sequencer. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al. 2012). Multiple alignments with sequences from closely related strains were performed by using the CLUSTAL W program in MEGA5.0 (Tamura et al. 2011). Phylogenetic trees were constructed with the neighbour-joining (Saitou and Nei 1987) (Fig. 1), maximum parsimony (Kluge and Farris 1969) and maximum-likelihood (Felsenstein 1981) algorithms in MEGA5.0 (Tamura et al. 2011). Evolutionary distances were calculated using the model of Jukes and Cantor (1969). Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein 1985) based on 1000 resamplings.

The methods for genomic DNA extraction and PCR mediated amplification of the 1221 bp *gyr*B gene fragment, primers and the sequencing protocol were described previously by Takeda et al. (2010). Phylogenetic analysis was conducted using MEGA5.0 software with bootstrap percentages based on 1000 replicates as previously described (Tamura et al. 2011).

DNA–DNA hybridization

DNA–DNA relatedness tests between strain FMN18^{T} and its most related type species of *N. grenadensis* DSM 45869^{T}, *N. speluncae* DSM 45078^{T} and *N. jinanensis* DSM 45048^{T} were performed. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization (DDH) was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/visual spectrophotometer equipped with a Peltier-thermostated 666 multi cell changer and a temperature controller with in situ temperature probe (Varian).

Results and discussion

Morphological observation of a 14-day culture of strain FMN18^{T} grown on ISP 2 medium revealed that it had

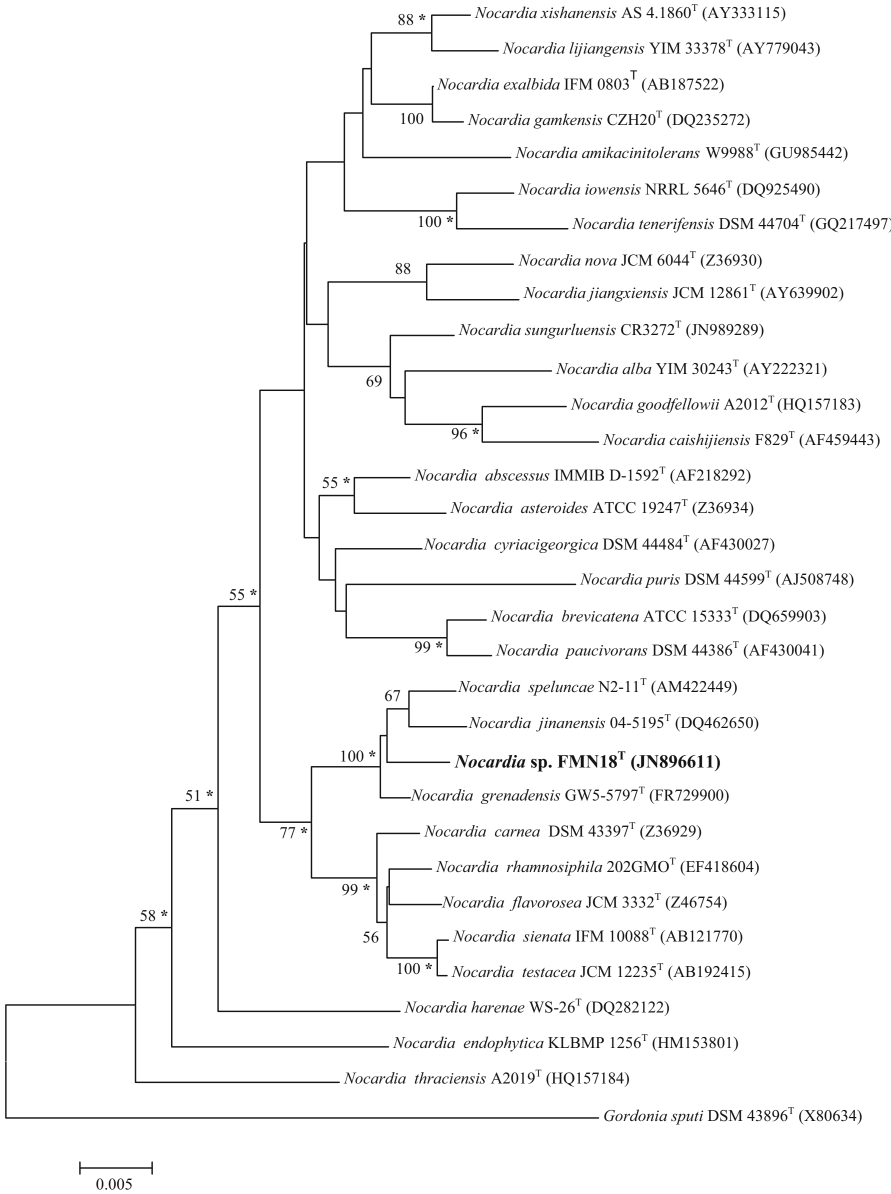

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between *Nocardia* sp. FMN18 and closely related type strains of the genus *Nocardia*

the typical characteristics of the genus *Nocardia*. Strain FMN18^T produced extensively branched substrate mycelium and aerial mycelium consisting of hyphae that fragmented into irregular rod-shaped elements (Supplementary Fig. S1). The strain grew well on ISP 2, ISP 6, modified Bennett's, nutrient and tryptic soy agar, moderate growth on ISP 3 agar, but didn't grow on ISP 4, 5, 7 and Czapek's agar (Suplementary Table S2). No diffusible pigment was detected on any tested media. Melanoid pigments were not produced on ISP 6 or ISP 7 medium. A range of phenotypic properties of strain FMN18^T and its closest phylogenetic neighbours, *N. grenadensis* DSM 45869^T, *N. speluncae* DSM 45078^T, *N. jinanensis* DSM 45048^T, *N. rhamnosiphila* DSM 45147^T and *N. carnea* DSM 43397^T were examined using well established media known to be of value in nocardial systematics (Goodfellow and Maldonado 2012) as shown in Table 1.

Table 1 Phenotypic properties of strain FMN18^T and the type strains of closely related species

Characteristics	1	2	3	4	5	6
Biochemical tests						
Allantoin hydrolysis	+	+	+	+	−	+
Nitrate reduction	+	+	+	−	+	+
Urea hydrolysis	−	+	−	−	−	−
pH tolerence						
5	+	+	−	+	+	+
9	+	−	+	+	+	−
10	−	−	+	+	+	−
11	−	−	+	−	−	−
12	−	−	+	−	−	−
Temperature						
10	+	+	+	−	+	+
NaCl (%, w/v)						
5.0	+	+	+	−	+	+
6.0	+	+	−	−	+	+
7.0	+	+	−	−	+	+
Degradation of (%, w/v)						
Adenine (0.5 %)	−	+	−	−	−	−
Casein (1 %)	−	+	+	−	−	−
Hypoxanthine (0.4 %)	−	−	−	−	−	+
Starch (1 %)	−	+	+	−	−	−
Gelatine (0.5 %)	+	+	−	−	−	+
Xylan (0.4 %)	−	+	+	−	−	+
Elastin (0.3 %)	−	+	−	−	+	+
Carbon source utilization (1.0 %, w/v)						
Adonitol	+	+	−	−	−	−
Myo-inositol	−	+	+	−	−	−
L-Arabinose	−	+	−	−	−	+
D-Cellobiose	−	+	−	−	−	−
D-Fructose	−	+	−	−	−	−
D-Ribose	−	+	+	−	+	+
D-Sorbitol	−	−	−	−	−	+
D-Galactose	+	+	−	−	+	+
D-Melezitose	+	+	−	+	−	+
D-Mannitol	−	+	+	−	−	+
D-Mannose	−	+	−	−	−	−
D-Melibiose	−	+	−	−	−	−
Dextrin	+	+	+	+	−	+
Dextran	−	−	+	−	−	−
L-Rhamnose	+	−	−	−	+	−
Maltose	−	+	+	−	−	+
Sucrose	−	+	+	−	−	−
Xylitol	+	+	−	−	−	+
Xylose	−	−	−	−	−	+

Table 1 continued

Characteristics	1	2	3	4	5	6
Nitrogen source utilization (0.1 %, w/v)						
Glycine	−	−	+	+	+	+
L-Alanine	−	+	+	−	−	−
L-Histidine	−	+	−	−	+	−
L-Hydroxyproline	−	−	+	−	−	−
L-Methionine	−	−	+	+	−	−
L-Phenylalanin	−	+	−	+	−	−
L-Proline	+	+	+	−	−	−
L-Serine	+	+	−	−	+	−
L-Valine	+	+	+	−	−	−
L-Tyrosine	+	−	−	−	−	+

Strains 1, FMN18^T; 2, *N.grenadensis* DSM 45869^T; 3, *N.speluncae* DSM 45078^T; 4, *N.jinanensis* DSM 45048^T; 5, *N.rhamnosiphila* DSM 45147^T; 6, *N.carnea* DSM 43397^T. All strains were positive for hydrolysis of aesculin and arbutin, growth at pH:6.0, 7.0 and 8.0, temperatures 20, 28 and 37 °C, and 0–4 % NaCl concentrations, ability of growth at D-glucose, L-glutamin as sole carbon sources (1.0 %), alpha-iso-leucine, L-arginine, L-asparagine, L-cysteine, L-lysine as sole nitrogen sources (0.1 %). But negative for growth at pH:4.0, temperatures 4, 45, 50 and 55 °C, and 8, 9, 10 and 15 % NaCl concentrations, for degradation guanine (0.5 %), tween 20 (1 %), tween 40 (1 %), tween 80 (1 %) and xanthine (0.4 %), ability of growth at inulin, D-arabinose, L-sorbose, lactose as sole carbon sources (1.0 %) and succinic acid (0.1 %). All the data are from this study

The almost-complete 16S rRNA gene sequence (1503 bp) of strain FMN18^T determined in this study was compared with the corresponding sequences of members of the genus *Nocardia*. Based on the 16S rRNA gene sequence similarities and the neighbour-joining tree (Fig. 1), the strain forms a clade with *N. grenadensis* GW5-5797^T (99.2 %; 11 nt differences at 1432 locations), *N. speluncae* N2-11^T (99.1 %; 13 nt differences at 1407 locations) and *N. jinanensis* 04-5195T (99.0 %; 14 nt differences at 1398 locations). Lower sequence similarities (<98.3 %) were found with the type strains of all other species of the genus *Nocardia* with validly published names. Topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms (Supplementary Figs. S2, S3) were similar to the tree reconstructed by neighbour-joining analysis.

The sequence similarities between the *gyr*B gene (1221 bp) of strain FMN18^T and those of *N. rhamnosiphila* 202GMO^T, *N. grenadensis* GW5-5797^T, *N.*

testacea JCM 12235^T, *N. sienata* IFM 10088^T and *N. jinanensis* 04-5195^T were 99.0, 96.6, 95.8, 95.3 and 93.1 %, respectively. The novel strain FMN18^T showed *gyr*B sequence similarity values of <90.0 % with the type strains of other species of the genus *Nocardia* (Fig. 2).

DNA–DNA relatedness values below the 70 % threshold is accepted for the delineation of prokaryotic species (Stackebrandt et al. 2002). It has been shown that some of the *Nocardia* species share high 16S rRNA gene sequence similarities, but have low DNA–DNA relatedness values (Kageyama et al. 2004; Yassin and Brenner 2005; Kämpfer et al. 2007; Sun et al. 2009). DNA–DNA relatedness values between strain FMN18^{T}and its close phylogenetic neighbours were 34.6 ± 2.1 % with *N. gredanensis* DSM 45869^T, 4.8 ± 0.8 % with *N. speluncae* DSM 45078^T and 3.2 ± 1.0 % with *N. jinanensis* DSM 45048^T (values are mean of duplicate determinations).

Whole-cell hydrolysates of the soil isolate, strain FMN18^T, was found to contain *meso*-diaminopimelic acid as the cell-wall diamino acid and whole-cell sugars included arabinose, ribose, galactose and glucose (Type IV; Lechevalier and Lechevalier 1970). The predominant menaquinone of strain FMN18^T was MK-8($H_{4\omega\text{-cyclo}}$) (74.8 %); three minor unidentified short chain components (<5.0 % each) were also detected. The polar lipids were identified as diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylinositol mannosides (PIMs), an unidentified phospholipid (PL) and six unidentified glycolipids (GLs) (Supplementary Fig. S4). One dimensional TLC of whole-cell acid methanolysates of strain FMN18^T revealed the presence of mycolic acids as identified by the R_F value (0.46; Fig. S5). The major cellular fatty acids were $C_{16:0}$ (26.6 %), $C_{18:1}$*cis*9 (16.0 %), 10-methyl $C_{18:0}$ (TBSA) (16.1 %) and $C_{16:1}$-*trans*9 (11.3 %); and minor amounts of $C_{18:0}$ (8.3 %), $C_{16:1}$*cis*9 (2.8 %), $C_{20:1}$*cis*11 (3.3 %) and an unidentified fatty acid (5.7 %) were also present (Supplementary Table S1). The DNA G+C content of strain FMN18^T was determined to be 70.3 mol%. This chemotaxonomic profile is consistent with classification of isolate FMN18^T in the genus *Nocardia* (Goodfellow and Maldonado 2012).

Based on the above genotypic and phenotypic data, strain FMN18^T is considered to represent a novel species of the genus *Nocardia*, for which the name *Nocardia zapadnayensis* sp. nov. is proposed.

Description of *Nocardia zapadnayensis* sp. nov.

N. zapadnayensis (za.pad.nay.en'sis N.L. fem. adj. *zapadnayensis*, belonging to Zapadnaya Park, Moscow, Russia, source of the organism).

Aerobic, Gram-stain positive, non-acid-fast, non-motile actinomycete which forms an extensively branched, yellow-orange substrate mycelia that bear cream or gray aerial mycelia on several tested media. Aerial mycelia fragment into irregular rod-shaped elements. Diffusible pigments are not produced. Melanoid pigments are not produced on ISP6 or ISP 7 agars. Growth occurs at pH 5.0–9.0 and 10–37 °C, but not pH 4.0 or 45 °C. Optimal growth occurs at 28 °C and pH 7.0. Growth is observed in the presence of 0–7 % (w/v) NaCl. Aesculin, allantoin and arbutin hydrolysis and nitrate reduction are positive, but urea hydrolysis is negative. Gelatin is degraded, but adenine, casein, elastin, guanine, hypoxanthine, starch, tween 20, 40 and 80, xanthine and xylan are not. Adonitol, D-galactose, D-glucose, D-melezitose, dextrin, L-glutamin, L-rhamnose and xylitol are utilized as sole carbon and energy sources, but not D-arabinose, D-cellobiose, D-fructose, D-mannitol, D-mannose, D-melibiose, D-ribose, D-sorbitol, dextran, inulin, L-arabinose, L-sorbose, lactose, maltose, myo-inositol, succinic acid, sucrose or xylose. Utilizes alpha-isoleucine, L-arginine, L-asparagine, L-cysteine, L-lysine, L-proline, L-serine, L-tyrosine and L-valine as sole nitrogen sources, but not glycine, L-alanine, L-histidine, L-hydroxyproline, L-methionine or L-phenylalanine. Growth occurs in the presence of filter paper discs soaked in amoxicilin/clavulanate (30 µg), trimethoprim/sulfamethoxazole (25 µg) and penicillin (10 U), but not in amikacin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), linezolid (30 µg), tigecycline (15 µg), gentamicin (10 µg) and streptomycin (10 µg). MK-8($H_{4\omega\text{-cyclo}}$) is the major menaquinone. The phospholipids are DPG, PG, PE, PI, PIMs, an PL and six unidentified GLs. Major fatty acids are $C_{16:0}$, 10-methyl $C_{18:0}$ (TBSA), $C_{18:1}$*cis*9 and $C_{16:1}$*trans*9.

The type strain, FMN18^T (=DSM 45872^T = KCTC 29234^T) was isolated from soil in Zapadnaya

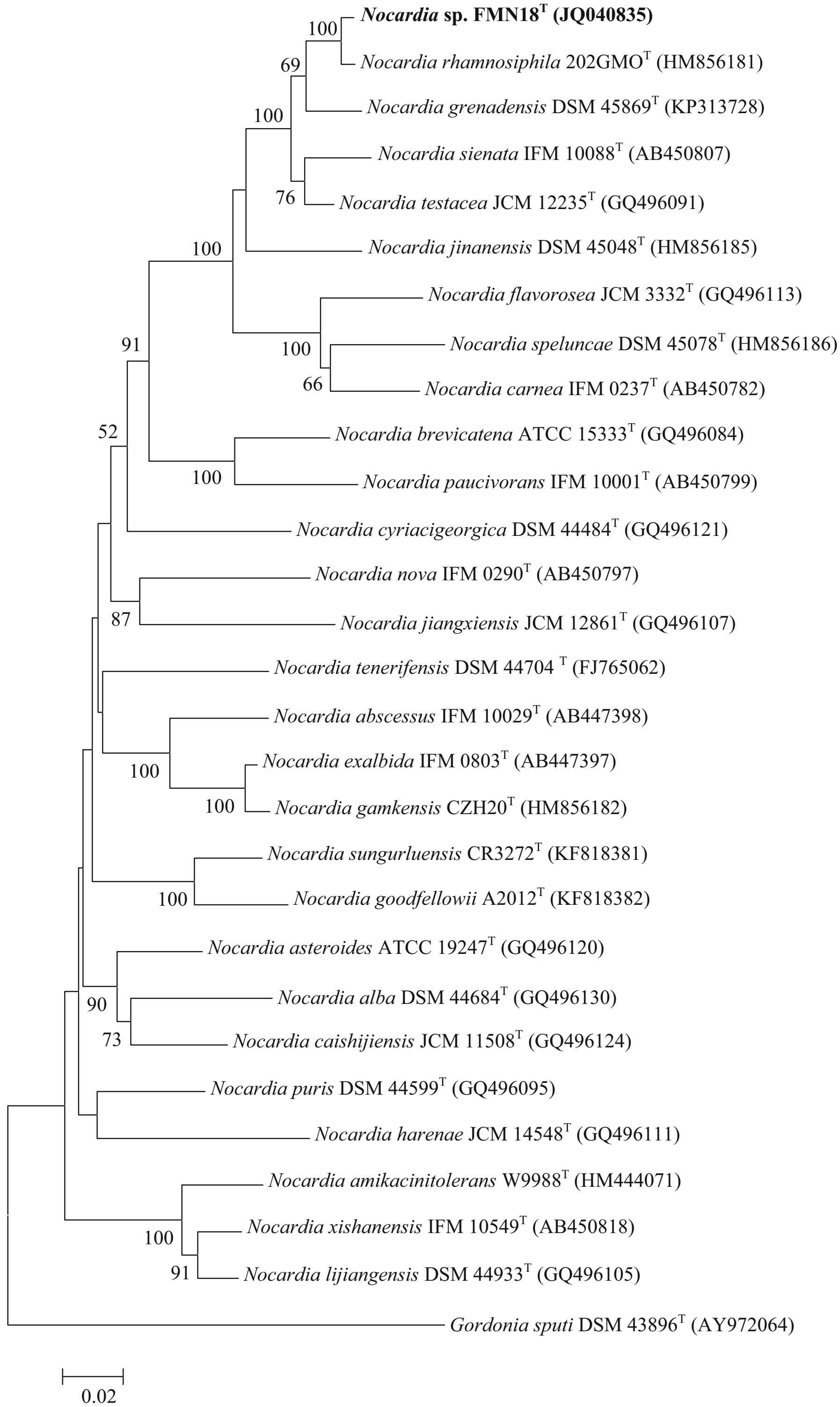

Fig. 2 Neighbour-joining phylogenetic tree based on 1221-bp gyrB gene sequences of strain FMN18^T within the genus *Nocardia*. Bootstrap percentages based on 1000 replicates are shown; values ≥50 % are shown. *Bar* 0.02 substitutions per nucleotide position

Southwest Forest Park, Moscow, Russia. The G+C content of the genomic DNA of the type strain is 70.3 mol%.

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