Nonomuraea muscovyensis sp. nov., isolated from soil

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A novel actinomycete, strain FMN03T, was isolated from a soil sample collected from Yuga Zapadnaya South-West Forest Park, Moscow, Russia. The isolate had chemical and morphological properties typical of members of the genus Nonomuraea and formed a distinct 16S rRNA gene subclade with the type strains Nonomuraea roseoviolacea subsp. carminata DSM 45913T and Nonomuraea roseoviolacea subsp. roseoviolacea DSM 14098T. The organism formed extensively branched substrate and aerial hyphae, which generated spiral chains of spores with smooth surfaces. The cell wall contained meso-diaminopimelic acid and the whole cell sugars were glucose, galactose and trace amounts of madurose, mannose and xylene. The polar lipids were phosphatidylethanolamine, hydroxyphosphatidylethanolamine, four unidentified phospholipids, four unidentified glycolipids and one unidentified lipid. The predominant menaquinone was MK-9(H4). The major fatty acids were iso-C16:0 2-OH, C17:0 10-methyl, C17:1 cis9 and iso-C16:0. Analyses of its morphological, physiological and biochemical characteristics, together with DNA–DNA relatedness data, confirmed that strain FMN03T is a representative of a novel species of the genus Nonomuraea, which is distinct from closely related reference strains. Strain FMN03T (=DSM 45913T =KCTC 29233T) is proposed as the type strain of a novel species, for which the name Nonomuraea muscovyensis sp. nov. is proposed.

The genus Nonomuria was originally proposed by Zhang et al. (1998) as a member of the family Streptosporangiaceae, but subsequently the spelling was corrected to Nonomuraea by Chiba et al., (1999). The genus is characterized chemo-taxonomically by the presence of meso-diaminopimelic acid in the cell wall, madurose as a characteristic sugar in whole-cell hydrolysates, and di-, tetra- and hexa-hydrogenated menaquinones with nine isoprene units as the predominant ubiquinones with nine isoprene units as the predominant menaquinone (Nonomura & Ohara, 1971; Zhang et al., 1998). The genus comprises 32 species with validly published names and two subspecies, all identified on the basis of a polyphasic approach (http://www.bacterio.net/index.html); most of these have been described in the last decade. The type species of the genus is Nonomuraea pusilla (Nonomura & Ohara, 1971; Zhang et al., 1998). As part of a programme to discover actinomycetes from diverse habitats an aerobic actinomycete strain, FMN03T, was isolated. In this study, we performed a polyphasic taxonomic investigation of this strain, and propose that strain FMN03T is assigned to a novel species of the genus Nonomuraea.

Strain FMN03T was isolated from soil collected from the Yuga Zapadnaya South-West Forest Park, Moscow, Russia. The soil sample was passed through a 2 mm mesh sieve, air-dried at room temperature for 14 days, and triturated using a sterile pestle. One gram of soil was suspended in 9 ml sterile strength Ringer’s solution (Merck) and shaken for 10 min in a pre-warmed water bath (60 °C for 20 min). These 10⁻¹ dilutions were serially diluted down with Ringer’s solution to dilutions of 10⁻⁴. The diluted soil suspension was inoculated on modified tryptone-yeast extract-glucose (TYG) with vitamin agar (Bowers & Hucker, 1935), supplemented with filter-sterilized cycloheximide (50 μg ml⁻¹), nalidixic acid (10 μg ml⁻¹) and rifampicin (0.5 μg ml⁻¹), and incubated at 28 °C for 21 days. The strain was isolated as a pure culture and maintained on yeast extract-malt extract agar.

*These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of FMN03T is JN896617.

Three supplementary figures and one supplementary table are available with the online version of this paper.
ag 16S rRNA genes were amplified using the universal primers, 27f \( (5'\text{-AGAGTTTGATCMTGGCTCAG-3'}) \) (Lane, 1991) and 1525r \( (5'\text{-AAGAGTTTGATCMTGGCTCAG-3'}) \) (Lane, 1991). The almost-complete 16S rRNA gene sequence of the novel strain (1495 nt) was determined and aligned with corresponding sequences of representative type strains of the genus Nonomuraea (retrieved from the EzTaxon-e server; Kim et al., 2012) by using CLUSTAL W in MEGA5 (Tamura et al., 2011). Phylogenetic analysis was carried out using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. Evolutionary distances were calculated using the model of Jukes & Cantor (1969). The topography of the reconstructed trees was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

DNA–DNA hybridization was employed to clarify further the relatedness of strain FMN03\(^T\) to its closest neighbours (Nonomuraea salmonea DSM 43678\(^T\), Nonomuraea maheshkhalienis DSM 45163\(^T\) and Nonomuraea roseoviolacea subsp. carminata DSM 44170\(^T\)) based on 16S rRNA gene sequence similarities, and this was performed by the Identification Service at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). 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 was carried out as described by De Ley et al. (1970) [incorporating the modifications described by Huss et al. (1983)] using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 x 6 multichannel changer and a temperature controller with an in situ temperature probe (Varian).

Biomass for chemotaxonomic studies was prepared by growing strain FMN03\(^T\) in ISP 2 broth at 28 °C for 14 days on various media as described by Shirling & Gottlieb (1966); yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-extract-iron agar (ISP 6), tyrosine agar (ISP 7), tyrosine agar (ISP 7), modified Bennett’s agar (MBA; Jones, 1949), nutrient agar (NA; Difco) and trypticase soy agar (TSA; Merck). The National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1964) were used for designation of colours and names. Growth at different temperatures (4, 10, 20, 28, 30, 37, 40, 45 and 50 °C), and pH (4–11) (at intervals of 1.0 pH unit), and in the presence of NaCl (0–10 %, w/v) or other salts (at 1.0 % intervals) was determined on ISP 2. Established methods were used to determine whether the strains degraded Tween 40 and 80 (Nash & Krent, 1991); the remaining degradation tests employed the methods described by Williams et al. (1983). Carbon source utilization was tested using carbon source utilization (ISP 9) medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % (w/v) of the carbon sources tested (0.1 % for 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 nitrogen sources tested. The antimicrobial activity of strain FMN03\(^T\) in inhibiting the growth of twenty-four micro-organisms including Gram-positive and Gram-negative bacteria and fungi was observed using an overlay technique, as described by Williams et al. (1983). The test organisms were provided by the American Type Culture Collection (ATCC) and the National Center for Agricultural Utilization Research (NRRL). Spot-inoculated colonies on modified Bennett’s agar plates were inverted over 2 ml chloroform for 40 min. Killed colonies were overlaid with 5–7 ml sloppy modified Bennett’s broth (Jones, 1949) inoculated with test organisms.
Zones of inhibition were scored as positive results after 24 h at 37 °C. With the exception of those for antimicrobial activity, the type strains *N. salmonea* DSM 43678<sup>T</sup>, *N. maheshkhalensis* DSM 45163<sup>T</sup>, *N. roseoviolacea* subsp. carminata DSM 44170<sup>T</sup> and *N. roseoviolacea* subsp. roseoviolacea DSM 43144<sup>T</sup> were included for comparison in all tests. The colony morphology and micromorphological properties of isolate FMN03<sup>T</sup> were determined by examining gold-coated dehydrated specimens of 21-day-old cultures grown on ISP 2 medium at 28 °C using a scanning electron microscope (JEOL; JSM-6060).

The 1495 bp sequence corresponding to the 16S rRNA gene region of strain FMN03<sup>T</sup> was compared with sequences deposited in public databases. Comparison of the almost-complete 16S rRNA gene sequence of the isolate with corresponding sequences of phylogenetically related species with validly published names showed that it formed a branch in the 16S rRNA gene tree of the genus *Nonomuraea* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain FMN03<sup>T</sup> formed a distinct branch with the type strains *N. roseoviolacea* subsp. *roseoviolacea* NBRC 14098<sup>T</sup> and *N. roseoviolacea* subsp. *carminata* NBRC 15903<sup>T</sup>. This relationship was supported by all the tree-making algorithms used in this study (Figs S1 and S2, available in the online Supplementary Material). The highest 16S rRNA sequence similarities between the novel isolate and type strains of recognized species in the databases were: 98.07 % (28 nt differences at 1449 sites) to *N. salmonea* DSM 43678<sup>T</sup>; 97.79 % (32 nt differences at 1448 sites) to *N. maheshkhalensis* 16-5-14<sup>T</sup>; 97.75 % (33 nt differences at 1465 sites) to *Nonomuraea kuesteri* GW 14-1925<sup>T</sup>; 97.62 % (34 nt differences at 1430 sites) to *Nonomuraea turkmeniaca* DSM 43926<sup>T</sup>; 97.61 % (35 nt differences at 1466 sites) to *Nonomuraea endophytica* YIM 65601<sup>T</sup>; 97.58 % (34 nt differences at 1404 sites) to *N. roseoviolacea* subsp. *carminata* NBRC 15903<sup>T</sup>; 97.37 % (37 nt differences at 1409 sites) to *Nonomuraea angiospora* NBRC 13155<sup>T</sup>; 97.33 % (39 nt differences at 1398 sites) to *N. roseoviolacea* subsp. *roseoviolacea* NBRC 14098<sup>T</sup> (AB039959).

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on almost-complete 16S rRNA gene sequences (1495 nt) showing the relationship between strain FMN03<sup>T</sup> and recognized species of the genus *Nonomuraea*. *Amycolatopsis orientalis* NBRC 12806<sup>T</sup> was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support (%); only values ≥50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per site.
differences at 1463 sites) to *Nonomuraea maritima* FXJ7.203T; 97.30 % (38 nt differences at 1410 sites) to *Nonomuraea helvata* NBRC 14681T; 97.27 % (40 nt differences at 1464 sites) to *Nonomuraea jabiensis* A4036T; and 97.04 % (43 nt differences at 1451 sites) to *N. roseoviolacea* subsp. *roseoviolacea* NBRC 14098T.

Strain FMN03T showed DNA–DNA relatedness values of 19.9 ± 3.5 % to *N. salmonea* DSM 43678T, 17.4 ± 0.5 % to *N. maheshkhiakensis* DSM 45163T and 26.3 ± 5 % to *N. roseoviolacea* subsp. *carminata* DSM 44170T (based on a mean of duplicate determinations in 2 × SSC and 10 % (v/v) formamide at 70 °C); these are well below the 70 % threshold value for the definition of a bacterial genomic species (Wayne *et al.*., 1987; Stackebrandt & Goebel, 1994). It has been pointed out that 16S rRNA gene nucleotide similarities within the range 97.6–99.4 % have been recorded for members of several species of the genus *Nonomuraea* with validly published names that exhibit low DNA–DNA relatedness values (Fischer *et al.*., 1983; Poschner *et al.*., 1985; Tamura *et al.*, 2000; Stackebrandt *et al.*, 2001; Quintana *et al.*, 2003; Kämpfer *et al.*, 2005). For this reason DNA–DNA relatedness experiments were not carried out with strain FMN03T and the remaining related type species of the genus *Nonomuraea*.

Strain FMN03T contained meso-diaminopimelic acid (cell wall type III; Lechevalier & Lechevalier, 1970) as the cell-wall diamino acid and the whole-cell sugars were glucose, galactose (major components), mannose, madurose and xylose (Type B; madurose was the diagnostic sugar). The polar lipids were phosphatidylethanolamine, hydroxyphosphatidylethanolamine, four unidentified phospholipids, four unidentified glycolipids and one unidentified lipid (Fig S3). The predominant menaquinone of strain FMN03T was MK-9(H4) (71.0 %); MK-9(H2) (10.0 %), MK-9(H6) (9.0 %), MK-9 (5.0 %) and MK-9(H8) (1.0 %) were also detected. The major cellular fatty acids were iso-C16 : 02-OH (19.0 %), C17 : 0 10-methyl (16.4 %), C17 : 1 cis9 (14.0 %) and iso-C16 : 0 (13.4 %). The comparative cellular fatty acid compositions of strain FMN03T, *N. salmonea* DSM 43678T, *N. maheshkhiakensis* DSM 45163T, *N. roseoviolacea* subsp. *carminata* DSM 44170T and *N. roseoviolacea* subsp. *roseoviolacea* DSM 43144T are shown in Table S1. The DNA G+C content of strain FMN03T was 69.6 mol%.

The morphological characteristics of strain FMN03T were consistent with those of members of the genus *Nonomuraea*. Strain FMN03T showed good growth on modified Bennett’s, ISP 2–5 and ISP 7, and moderate growth on tryptic soy, Czapek’s, nutrient and ISP 6 media. Aerial hyphae were light orange on ISP 2, yellowish-brown on ISP 3, pale orange-yellow on ISP 4, pale pink on ISP 6, and light yellow-pink on ISP 7. The colour of the substrate mycelium was yellowish-brown to brownish-orange. Diffusible pigments were not produced. Melanoid pigments were not produced on ISP 6 or ISP 7 media. Strain FMN03T formed extensively branched substrate and aerial hyphae that often formed spiral chains of spores with smooth surfaces (Fig. 2). The physiological properties that distinguish strain FMN03T from closely related species of the genus *Nonomuraea* are presented in Table 1.

It is evident that strain FMN03T can be distinguished from its close phylogenetic relatives *N. salmonea* DSM 43678T, *N. maheshkhiakensis* DSM 45163T, *N. roseoviolacea* subsp. *carminata* DSM 44170T and *N. roseoviolacea* subsp. *roseoviolacea* DSM 43144T based on 16S rRNA gene sequence analysis, DNA–DNA relatedness and phenotypic data. It is, therefore, proposed that the micro-organism should be recognized as a representative of a novel species, with the name *Nonomuraea muscovyensis* sp. nov.

**Fig. 2.** Scanning electron micrographs of strain FMN03T grown on yeast extract-malt extract agar (ISP 2) at 28 °C for 21 days. Bar, 10 μm in main micrograph and 1 μm in the inset micrograph.

**Description of *Nonomuraea muscovyensis* sp. nov.**

*Nonomuraea muscovyensis* (mus.co.vy.en’sis. N.L. fem. adj. muscovyensis of or belonging to Moscow, Russia, the source of the type strain).

Aerobic, Gram-reaction-positive, non-motile actinomycete, which forms extensively branched, brownish-orange substrate mycelia that bear light-orange aerial hyphae on yeast extract-malt extract agar. Aerial mycelia bear spiral chains of spores with smooth surfaces. No diffusible pigment or melanin is detected on any media tested. Growth occurs at pH 6.0–11, and at 28–40 °C. It has been pointed out that 16S rRNA gene nucleotide similarities within the range 97.6–99.4 % have been recorded for members of several species of the genus *Nonomuraea* with validly published names that exhibit low DNA–DNA relatedness values (Fischer *et al.*., 1983; Poschner *et al.*., 1985; Tamura *et al.*, 2000; Stackebrandt *et al.*, 2001; Quintana *et al.*, 2003; Kämpfer *et al.*, 2005). For this reason DNA–DNA relatedness experiments were not carried out with strain FMN03T and the remaining related type species of the genus *Nonomuraea*.

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Table 1. Phenotypic properties of strains FMN03<sup>T</sup> and closely related type strains

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<td>Xylose</td>
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L-glutamic acid, maltose, myo-inositol, xyitol or succinic acid. Utilizes L-arginine, L-cysteine, L-serine, L-threonine and L-asparagine as sole nitrogen sources, but not α-isoleucine, α-phenylalanine, glycine, L-alanine, L-histidine, hydroxy-L-proline, L-methionine, L-phenylalanine, L-proline, L-valine or L-tyrosine. Antimicrobial activity is shown against Bacillus subtilis NRRL B-209 and Enterobacter aerogenes NRRL B-427, but not against Bacillus cereus NRRL B-3711, Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC 29213 or Micrococcus luteus NRRL B-1018. The predominant menaquinone is MK-9(H<sub>4</sub>), with minor amounts of MK-9(H<sub>2</sub>), MK-9(H<sub>6</sub>), MK-9 and MK-9 (H<sub>8</sub>) also detected. The polar lipid profile contains phosphatidylethanolamine, hydroxyphosphatidylethanolamine, four unidentified phospholipids, four unidentified glycolipids and one unidentified lipid. Major fatty acids are iso-C<sub>16:0</sub>, 2-OH, C<sub>17:0</sub> 10-methyl, C<sub>17:1</sub> cis 9 and iso-C<sub>16:0</sub>.

The type strain, FMN03<sup>T</sup> (=DSM 45913<sup>T</sup>=KCTC 29233<sup>T</sup>), was isolated from soil from the Yuga Zapadnaya South-West Forest Park, Moscow, Russia. The G+C content of the genomic DNA of the type strain is 69.6 mol%.

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