**Nocardia sungurluensis** sp. nov., isolated from soil

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A novel Gram-reaction-positive, rod-shaped, non-motile and mycolic acid-containing strain, \( \text{CR3272}\)\(^{T} \), isolated from soil, was studied using a polyphasic approach. The organism showed a combination of chemotaxonomic and morphological properties typical of the genus *Nocardia*. The cell wall contained meso-diaminopimelic acid (type IV) and whole-cell sugars were galactose, glucose, arabinose and xylose. The predominant menaquinone was MK-8(H\(_{4}\)cyc). The major phospholipids were diphasphatidylglycerol and phosphatidylinositol mannosides. Major fatty acids were \( \text{C}_{16:0}, \text{C}_{18:1}\text{cis9}, \text{C}_{18:0}\text{10-methyl (TBSA)} \) and \( \text{C}_{16:1}\text{cis9} \). The novel strain formed distinct phylogenetic line in the *Nocardia* 16S rRNA gene tree and was closely associated with *Nocardia goodfellowii* A2012\(^{T} \) (98.6 % 16S rRNA gene sequence similarity), *Nocardia alba* YIM 30243\(^{T} \) (98.5 %) and *Nocardia caishijiensis* FB829\(^{T} \) (97.9 %). However, DNA–DNA relatedness values and phenotypic data demonstrated that strain CR3272\(^{T} \) was clearly distinguished from all closely related species of the genus *Nocardia*. It is concluded that the organism be classified as representing a novel species of the genus *Nocardia*, for which the name *Nocardia sungurluensis* is proposed. The type strain is CR3272\(^{T} \) (=DSM 45714\(^{T} \)=KCTC 29094\(^{T} \)).

The genus *Nocardia* belongs to the family *Nocardiaceae* and was proposed by Trevisan (1889). Members of the genus are aerobic, Gram-reaction-positive, mycolic acid-containing actinomycetes that form extensively branched mycelia and substrate hyphae that fragment into rod-shaped, non-motile elements (Goodfellow & Lechevalier, 1989). Characteristics of the genus *Nocardia* comprising chemotaxonomic, molecular genetic and numerical phenetic methods are well-defined (Goodfellow et al., 1999). At the time of writing, the genus encompasses more than 90 species with validly published names (http://www.bacterio.net/n/nocardia.html) including the recently described *Nocardia amikacinitoleraas* (Ezeoke et al., 2013), *Nocardia goodfellowii* and *Nocardia thraciensis* (Sazak et al., 2012), *Nocardia grenadensis* (Kämpfer et al., 2012), *Nocardia rhamnosiphila* (Everest et al., 2011). Members of the genus *Nocardia* are known to be human and animal pathogens (Brown-Elliott et al., 2006; Ezeoke et al., 2013), although it is also known that they play functional roles in natural habitats, notably soil (Maldonado et al., 2000), and some species produce secondary metabolites such as nocardicin, tuberculactomicin A and brasiliocardin A (Aoki et al., 1976; Komaki et al., 1999; Igarashi et al., 2000). In the present study, the taxonomic position of the soil isolate CR3272\(^{T} \) was clarified by using a polyphasic approach.

Strain CR3272\(^{T} \) was isolated from soil samples collected from Sungurlu-Corum, Turkey using Stevenson’s Medium No. 3 (Tan et al., 2006) supplemented with cycloheximide (50 \( \mu \text{g mL}^{-1} \)), nystatin (50 \( \mu \text{g mL}^{-1} \)), nalidixic acid (10 \( \mu \text{g mL}^{-1} \)) and novobiocin (10 \( \mu \text{g mL}^{-1} \)), and incubated for 21 days at 28 °C. The strain was maintained on yeast extract-malt extract agar slants [International Streptomyces Project (ISP) Medium No. 2; (Shirling & Gottlieb, 1966)] and stored in glycerol suspensions (20 %, v/v) at \(-20 \text{ °C} \).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product was carried out following Chun & Goodfellow (1995). The almost-complete 16S rRNA gene sequence (1462 bp) of strain CR3272\(^{T} \) was determined using an ABI PRISM 3730 XL automatic sequencer (Applied Biosystems). The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence identities were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). CLUSTAL W software, version 1.8 (Thompson et al., 1994) was used to align the sequences of strain CR3272\(^{T} \) and related taxa retrieved from public databases. Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making algorithms from the MEGA version 5.0 program (Tamura et al., 2011), and the maximum-likelihood method (Felsenstein, 1985).
DNA–DNA relatedness values between strain CR3272\(^T\) and the related type strains *Nocardia goodfellowii* DSM 45516\(^T\), *Nocardia caishijiensis* JCM 11508\(^T\) and *Nocardia alba* DSM 44684\(^T\) were determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zelkkulturen (DSMZ), Braunschweig, 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) and incorporating the modifications described by Huss *et al.* (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

Biomass for chemotaxonomic studies was prepared by growing strain CR3272\(^T\) in ISP 2 broth at 160 r.p.m. for 10 days at 28 °C; cells were harvested by centrifugation, washed twice in distilled water, recentrifuged and freeze-dried. Whole-cell amino acids and sugars were prepared according to Lechevalier & Lechevalier (1970) and analysed by the Identification Service of the DSMZ. Respiratory quinones were extracted from 100 mg of freeze-dried cells based on the two-stage method described by Tindall *et al.* (1990a, 1990b). Respiratory quinones were separated into their different classes by thin layer chromatography on silica gel (Macherey-Nagel Art. no. 805 023), using hexane/tert-butylmethylthether (9:1, v/v) as the solvent. UV-absorbing bands corresponding to menaquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse-phase column (Macherey-Nagel, 2 mm × 125 mm, 3 μm, RP18) using methanol as the eluant; respiratory quinones were detected at 269 nm. Polar lipids were extracted and analysed by the method of Minnikin *et al.* (1984) as modified by Kroppenstedt & Goodfellow (2006). For the extraction of whole-cell fatty acids, cells were grown in 20 ml Trypticase Soy Broth (TSB) at 28 °C with shaking at 150 r.p.m. After 5 days incubation, 5 ml seed culture was inoculated into 50 ml TSB. The inoculated flask was incubated as before for 5 days. After harvesting by cellulose membrane filtration (0.45 μm), 200 mg wet cells were placed in an extraction tube. Cellular fatty acids were extracted, derivatized to their fatty acid methyl esters (FAME) and separated by the Microbial Identification System (MIDI; Microbial ID), utilizing an Agilent Technologies 6890N gas chromatograph with a G2614A autosampler and a 6783 injector (Sasser, 1999; Kämpfer & Kroppenstedt, 1996). FAME peaks were analysed using the TSBA 5.0 database. The DNA G+C content of strain CR3272\(^T\) was determined following the procedure developed by Gonzalez & Saiz-Jimenez (2003).

The morphological and physiological features of strain CR3272\(^T\) were determined in comparison with the phylogenetically closely related type strains, *N. goodfellowii* DSM 45516\(^T\), *N. caishijiensis* JCM 11508\(^T\) and *N. alba* DSM 44684\(^T\). Cultural characteristics of strain CR3272\(^T\) were determined after incubation 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 salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6), tyrosine agar (ISP 7), modified Bennett’s agar (MBA; Jones, 1949) and Czapek’s Agar (Waksman, 1967). National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1964) were used for determining colour designation and names. Growth tolerance was determined for temperatures (10–45 °C), pH (5.0–10.0) and NaCl concentrations (0–5 %; w/v) using ISP 2 media. Decomposition of various compounds was examined using glucose-yeast extract agar (GYEA) recommended by Athalye *et al.* (1985). In addition, degradation of Tween 20, 40 and 80 (1.0 %, w/v) was examined using Sierra medium (Sierra, 1957). Utilization of sole carbon sources was determined 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) propionic acids/succinic acids. 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. Colony morphology and micromorphological properties of isolate CR3272\(^T\) were determined by examining gold-coated dehydrated specimens of 14-day cultures from ISP 2 medium using a JSM 6060 (JEOL) scanning electron microscope.

The almost-complete 16S rRNA gene sequence (1462 nt) of strain CR3272\(^T\) determined in this study was compared with the corresponding sequences of members of the genus *Nocardia*. The phylogenetic tree based on the neighbour-joining algorithm showed that strain CR3272\(^T\) formed a distinct branch from other species of the genus *Nocardia*, 1981) from the PHYLIP suite of programs (Felsenstein, 1993). Evolutionary distances were calculated using model of Jukes & Cantor (1969). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The methods for genomic DNA extraction and PCR-mediated amplification of the 1265 bp *gyrB* gene fragment, primers and the sequencing protocol were described previously by Takeda *et al.* (2010). Phylogenetic analysis was conducted using MEGA5 software with bootstrap percentages based on 1000 replicates as previously described (Tamura et al., 2011).
notably from its nearest neighbours of the genus, *N. goodfellowii* A2012<sup>T</sup>, *N. caishijiensis* F829<sup>T</sup> and *N. alba* YIM 30243<sup>T</sup> (Fig. 1). The relationship between strain CR3272<sup>T</sup> and its closest neighbours, *N. goodfellowii* A2012<sup>T</sup>, *N. caishijiensis* F829<sup>T</sup> and *N. alba* YIM 30243<sup>T</sup>, was supported by the maximum-likelihood algorithm but not a high bootstrap value, however, this relationship was not supported by maximum-parsimony or by high bootstrap values (Supplementary Figs S1 and S2). Sequence similarity of identity calculations indicated that the closest relatives of strain CR3272<sup>T</sup> were *N. goodfellowii* A2012<sup>T</sup> (98.6 %; 20 nt differences at 1462 locations), *N. alba* YIM 30243<sup>T</sup> (98.5 %; 21 nt differences at 1442 locations), *Nocardia asteroides* ATCC 19247<sup>T</sup> (98.3 %; 25 nt differences at 1455 locations), *Nocardia abscessus* IMMIB D-1592<sup>T</sup> (98.2 %; 26 nt differences at 1447 locations), *Nocardia lijiangensis* YIM 33378<sup>T</sup> (98.2 %; 27 nt differences at 1462 locations), *Nocardia cyriacigeorgica* DSM 44484<sup>T</sup> (98.2 %; 27 nt differences at 1457 locations), *Nocardia exalbida* IFM 0803<sup>T</sup> (98.1 %; 27 nt differences at 1413 locations), *Nocardia shimosuensis* IFM 10311<sup>T</sup> (98.1 %; 28 nt differences at 1462 locations), *Nocardia thailandica* IFM 10145<sup>T</sup> (98.0 %; 28 nt differences at 1430 locations), *Nocardia anaemiae* IFM 0323<sup>T</sup> (97.95 %; 30 nt differences at 1426 locations), *Nocardia neocaledoniensis* SBHR OA6<sup>T</sup> (97.91 %; 30 nt differences at 1437 locations) and *N. caishijiensis* F829<sup>T</sup> (97.90 %; 30 nt differences at 1426 locations). Lower sequence similarities (<97.90 %) were found with all other species of the genus *Nocardia* with validly published names.

The similarity of the *gyrB* gene (1265 nt) between strain CR3272<sup>T</sup> and *N. goodfellowii* A2012<sup>T</sup>, *N. asteroides* ATCC 19247<sup>T</sup>, *N. neocaledoniensis* DSM 44717<sup>T</sup>, *N. thailandica* JCM 12356<sup>T</sup> and *N. niwae* W9241<sup>T</sup> was 95.07 %, 90.86 %, 90.62 %, 90.62 % and 90.22 %, respectively. The novel strain showed *gyrB* sequence similarity values of <90.0 %

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**Fig. 1.** Neighbour-joining phylogenetic tree based on a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain CR3272<sup>T</sup> and closely related species of the genus *Nocardia*. Numbers at nodes indicate the levels of bootstrap support (% of 1000 replications); only values ≥50 % are shown. GenBank accession numbers are given in parentheses. *Streptomyces somaliensis* DSM 40738<sup>T</sup> was used as an outgroup. Bar, 0.01 substitutions per site.
with the type strains of other species of the genus Nocardia (Fig. S3).

DNA–DNA relatedness values between strain CR3272T and its closest phylogenetic neighbours were 37.8 ± 1.06 % with N. goodfellowii DSM 45516T, 24.4 ± 4.8 % with N. alba DSM 44684T and 23.7 ± 4.12 % with N. caishijienisi JCM 11508T (values are mean of duplicate determinations). These values are clearly well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne et al., 1987).

Strain CR3272T grew well on all of the media tested. Whitish/yellowish aerial mycelium was formed on ISP 2, ISP 3, ISP 4, ISP 5, ISP 7, Czapek’s, modified Bennett’s and nutrient agars. The colour of the substrate mycelium was orange–yellow. Diffusible pigments were not produced. Melanoid pigments were not produced on ISP 6 or ISP 7 medium. Strain CR3272T produced extensively branched, irregular rod-shaped elements (Goodfellow & Lechevalier, 1989) (Fig. 2). The physiological properties that distinguish strain CR3272T from closely related species of the genus Nocardia are listed in Table 1.

Strain CR3272T also indicated typical markers of members of the genus Nocardia. It contained meso-diaminopimelic acid as the cell-wall diamino acid and whole-cell sugars included arabinose, galactose, glucose and xylose (Type IV; Lechevalier & Lechevalier, 1970). The acyl type of strain CR3272T was N-glycated. The polar lipids included diphosthatidlyglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and an unknown glycolipid (Fig. S4). One-dimensional TLC of whole-cell acid methanolysates of strain CR3272T revealed the presence of mycolic acids as identified by its Rf value (0.41; Fig. S5). The predominant menaquinone of strain CR3272T was MK-8(H4cyc) (85 %); MK-8 (H4) (2.0 %) and two minor unidentified components were also detected. The major cellular fatty acids were C16:0 (34.2 %), C18:1cis9 (19.7 %), C18:0 10-methyl (TBSA) (19.3 %), C16:1cis9 (13.4 %); minor amounts of C18:0 (7.5 %), C14:0 (1.8 %) and C15:0 (1.4 %) were also present (Table S1). The G+C content of the DNA was 69.8 mol%.

![Fig. 2. Scanning electron micrograph of strain CR3272T grown on ISP 2 agar at 28 °C for 14 days. Bar, 1 μm.](image-url)
It is evident from the genotypic and phenotypic data that isolate CR3272T represents a novel species within the genus Nocardia, for which the name Nocardia sungurluensis sp. nov. is proposed.

**Description of Nocardia sungurluensis sp. nov.**

*Nocardia sungurluensis* (sun. gur. lu.en’sis. N.L. fem. adj. sungurluensis of or belonging to Sungurlu, Corum, Turkey, source of the organism).

Aerobic, Gram-reaction-positive, non-acid-fast, non-motile actinomycete which forms an extensively branched, orange-yellow substrate mycelia that bear white or pale yellow 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 ISP 6 or ISP 7 agars. Growth occurs at pH 6.0–10.0 and 10–37 °C, but not pH 5.0 or 45 °C. Optimal growth occurs at 28 °C and pH 7.0. Growth is observed in the presence of 0–4 % (w/v) NaCl. Aesculin, allantoin, arbutin and urea hydrolysis and nitrate reduction are positive. DNA, hypoxanithy and Tween 20 and Tween 80 are degraded, but not adonitol, cellobiose, D-sorbitol, pionic acid and succinic acid are utilized as sole carbon and energy sources, but not L-threonine, L-alanine, 416. Syst Zool minimum change for a specific tree topology. toward defining the course of evolution: an approach using the bootstrap. Evolution 39, 75–79.

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**References**


