Nocardia colli sp. nov., a new pathogen isolated from a patient with primary cutaneous nocardiosis

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Abstract
A novel nocardioform strain, CICC 11023T, was isolated from a tissue biopsy of neck lesions of a patient with primary cutaneous nocardiosis and characterized to establish its taxonomic position. The morphological, biochemical, physiological and chemotaxonomic properties of strain CICC 11023T were consistent with classification in the genus Nocardia. Whole-cell hydrolysates were rich in meso-diaminopimelic acid, galactose, arabinose and fructose. Mycolic acids were present. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, one unidentified phospholipid and two unidentified lipids, and the predominant menaquinone was cyclo-MK-8 (H4, 4-cyclo). The main fatty acids (>5%) were C18:0 10-methyl (TBSA), C16:0 summed feature 4 (C16:1 trans 9/C15:0 iso 20H), C15:0 and C17:0 10-methyl. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the isolate is most closely related (>98% similarity) to the type strains Nocardia alba YIM 30243T, and phylogenetic analysis of gyrB gene sequences showed similarity (89.1–92.2%) to Nocardia vulneris NBRC 108936T, Nocardia brasiliensis IFM 0236T and Nocardia exalbida IFM 0803T. DNA–DNA hybridization results for strain CICC 11023T compared to Nocardia type strains ranged from 20.4 to 35.4%. The genome of strain CICC 11023T was 8.78 Mbp with a G+C content of 67.4 mol% overall. The average nucleotide identity (ANI) values between strain CICC 11023T and N. alba YIM 30243T were low (OrthoANI=77.47%), and the ANI values between strain CICC 11023T and N. vulneris NBRC 108936T were low (OrthoANI=83.75%). Consequently, strain CICC 11023T represents a novel Nocardia species on the basis of this polyphasic study, for which the name Nocardia colli sp. nov. is proposed. The type strain is CICC 11023T (=KCTC 39837T).

The genus Nocardia, belonging to the suborder Corynebacterineae [1], was established by Trevisan in 1889 [2] and consists of Gram-stain-positive, variably acid-fast, strictly aerobic bacteria that form filamentous, branched cells that fragment into pleomorphic, rod-shaped or coccoid elements [3]. Since Pijper and Pullinger identified Nocardia transvalensis as the pathogenic micro-organism associated with a case of mycetoma in a South African patient in 1927 [4], more and more cases of clinical Nocardia infection have been reported worldwide every year. This increased prevalence is partly due to advances in phylogenetic analyses based on 16S rRNA and partial gyrB gene sequences, allowing for the more rapid identification of nocardial isolates compared to standard phenotypic techniques [5, 6]. More than 40 species within the genus Nocardia have been reported as clinically relevant, and many of these show resistance to several classes of antimicrobials [7]. Nocardia species are widely distributed in the environment and cause a variety of suppurative and granulomatous infections of humans and animals, including cutaneous, subcutaneous, lymphocutaneous, pulmonary, cerebral or disseminated nocardiosis. Treatment of nocardiosis often requires long-term therapy with a combination of drugs [8]. In the present study, a novel strain of Nocardia was isolated from a patient with primary cutaneous nocardiosis and the
phenotypic, morphological, chemotaxonomic and molecular characteristics of strain CICC 11023T are presented.

A 36-year-old woman, who is a farmer by occupation, presented to the Department of Dermatology of the Second Affiliated Hospital of Kunming Medical University (Kunming, Yunnan Province, PR China) with a 10 year history of gradually enlarging and infiltrating painless papulo-nodular lesions of the neck and chest [9]. Two strains were isolated from an aerobic culture of the biopsied skin tissue specimens at 25°C in Sabouraud agar medium after 1 week. One of the strains showed 99.8% 16S rRNA gene sequence similarity to Staphylococcus epidermidis ATCC 14990T. S. epidermidis is part of the normal human flora, typically the skin flora, and is less commonly found in the mucosal flora [10]. The other part of the normal human flora, typically the skin flora, and/or C16ωc 9 and/or C15ω9c detected.

In general, a >5% fatty acid content is considered to present a ‘major fatty acid’ [21]. Analyses of the fatty acids by gas–liquid chromatography revealed that the main fatty acids (>5%) of strain CICC 11023T were C18:0 10-methyl (TBSA, 30.36%), C16:0 (20.52%), summed feature 4 (C16:1 trans 9/C15:0 iso 2OH; 14.33%), C15:0 (13.01%) and C17:0 10-methyl (5.41%). The fatty acid patterns of the novel strain and the reference strains are presented in Table 1. Comparisons of the fatty acid profiles showed that all seven tested strains contained C16:0, C17:0, C18:0, C19:0ω9c, C19:0ω6c, C16:1 10-methyl (TBSA) and C16:0 99c; however, strain CICC 11023T exhibited relatively large amounts of C17:0 and C18:0 10-methyl (TBSA), and small amounts of C16:0 and C18:1ω9c. Thus, compared to the six reference strains, strain CICC 11023T showed a distinct major fatty acid pattern. The whole-cell hydrolysates were rich in meso-diaminopimelic acid, galactose, arabinose and fructose. The major polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, one unidentified phospholipid and two unidentified lipids, and the predominant menaquinone was cyclo MK-8(H4, ω9-cyclo) (86.5%). The strain also contained mycolic acid, which is characteristic of the genera Nocardia and Rhodococcus [22]. The chemotaxonomic features of strain CICC 11023T were consistent with those of members of the genus Nocardia [6].

Pigmentation, production of aerial hyphae, and morphological characteristics were observed under a light microscope (Olympus CX41) and a scanning fiber-optic electron microscope (FEI Quanta). Strain CICC 11023T was grown

Table 1. Main fatty acid compositions (>5%) of strain CICC 11023T and the type strains of related Nocardia species

| Fatty acid | 1    | 2    | 3    | 4    | 5    | 6    | 7    |
|-----------|------|------|------|------|------|------|------|
| C16:0     | 13.0 | –    | –    | –    | –    | <5   | –    |
| C16:1     | 20.5 | 35.8 | 31.4 | 24.7 | 32.9 | 40.0 | 25.9 |
| C17:0 10-methyl | 5.4 | –    | <5   | <5   | –    | –    | –    |
| C18:0     | <5   | <5   | <5   | 5.0  | <5   | 7.9  | 5.7  |
| C16:1 10-methyl (TBSA) | 30.3 | 8.1  | 15.7 | 11.7 | 6.2  | 12.9 | 8.2  |
| C18:1ω9c  | <5   | 24.0 | 11.4 | 19.5 | 18.0 | 24.0 | 17.1 |
| Summed feature 3* | –   | 13.4 | 30.8 | 18.3 | 16.0 | –    | 12.8 |
| Summed feature 4* | 14.3 | –    | –    | –    | –    | –    | –    |

*Summed features represent groups of two or three fatty acids that could not be separated by GLC using the MIDI system. Summed feature 3 contained C16:1ω7c and/or C16:1ω6c; summed feature 4 contained C16:1 trans 9 and/or C15:0 iso 2OH.
separately on Gause 1, ISP 2, ISP 3, ISP 4 and ISP 5 at 30°C for 5 days, and then examined for colour determination using colour chips from the ISCC–NBS colour charts (standard sample no. 2106). Growth at 21, 28, 37 and 45°C was measured on ISP 2 for 5 days. The pH range for growth using the buffer system described in [23] (pH 4–10 at intervals of 0.5 pH units) and the requirement for NaCl (1, 4, 7 and 10%) were determined in ISP 2 broth. Phenotypic characteristics such as Gram-staining, catalase and oxidase activity, and hydrolysis of casein, Tween 20 and 80, egg yolk and starch were examined using the methods described by Smibert and Krieg [24]. Utilization of various substrates as sole carbon sources was tested at the CICC using the GN2 MicroPlate Gram-negative identification test panel (Biolog), and the result was determined after incubation at 30°C for 24 h. Physiological and biochemical properties were further determined with API 20NE, API 20E and API ZYM strips (bioMérieux). Tests were generally performed according to the manufacturer’s instructions. The API 20NE tests were read after 24–48 h at 28°C, the API 20E tests were read after 18–24 h at 36°C, and the API ZYM tests were read after 4 h of incubation at 37°C [13].

Morphological characteristics of strain CICC 11023 T presented typical properties of the genus Nocardia. Strain CICC 11023 T was aerobic, Gram-stain-positive, non-motile, with modified acid alcohol-positive actinomycetes forming extensively branched grey-white substrate mycelium and aerial mycelium with fragments that appeared as short coliform bodies under scanning electronic microscopy (0.5×0.7–0.9 μm in diameter). When grown on Gause 1, ISP 3 and ISP 5 media at 30°C for 5 days, the surface of colonies appeared as a velvet powder, with a grey aerial mycelium and substrate mycelium, and a grey spore head. When grown on ISP 2 at 30°C for 5 days, the colonies had a corrugated surface, with a white aerial mycelium, light brown substrate mycelium and a white spore head. Culture inserts on ISP 2 at 30°C for 5 days showed formation of short spore chains, a spore chain flex and mycelium breaking into a rod-like curved body after 8 days. Growth was weak on ISP 4 at 30°C for 5 days. No soluble pigments were found on any medium. Strain CICC 11023 T grew at 21, 28 and 37°C, but not at 45°C. Positive reactions were observed for milk peptonization, catalase, aesculin, urease and cellulose; negative reactions were observed for milk coagulation, nitrate reduction, gelatin liquefaction, tyrosinase, hydrolysis of starch, casein, xanthine, hypoxanthine and production of H₂S. Strain CICC 11023 T utilized L-arabinose, rhamnose, d-fructose, salicin, d-xylene, inositol, lactose, melibiose, d-glucose, raffinose, sucrose, d-mannitol, maltose, trehalose and arabinose as sole carbon sources. The main differential characteristics between strain CICC 11023 T and closely related Nocardia species are presented in Table 2.

Antibiotic sensitivity analysis of strain CICC 11023 T was performed using Etest (bioMérieux) to determine the minimal inhibitory concentration values for some antibiotics according to the manufacturer instructions. Sulfonamides have been the mainstay of antimicrobial therapy for human nocardiosis [25]. Thus, the patient was treated with oral Co-SMZ (containing 0.4 g sulfamethoxazole and 0.08 g trimethoprim; two tablets/time, three times/day, twice the first dose) for 8 weeks and achieved very good improvement with this treatment: the nocardiosis resolved 6 months after the administration of Co-SMZ [9]. No recurrence of the infection was observed for approximately 3 years. Although the majority of these infections can be treated with sulfonamides, there are in vitro differences noted in the antimicrobial susceptibility among different cases of Nocardia. The drug susceptibility testing showed that strain CICC 11023 T was susceptible to levofloxacin, ofloxacin, tobramycin and clindamycin, and was resistant to fosfomycin, imipenem, vancomycin and erythromycin.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene from strain CICC 11023 T were performed as described previously [26]. The program Clustal X was used to conduct multiple alignments with sequences of the most closely related Actinobacteria strains and for calculations of sequence similarity [27]. Phylogenetic trees were reconstructed using the neighbour-joining [28], maximum-parsimony [29] and maximum-likelihood [30] algorithms in MEGA version 4.0 [31]. The stability of the clades in the trees was appraised using a bootstrap value with 1000 replications [32]. The 16S rRNA gene sequence (1506 bp) of strain CICC 11023 T was determined. Phylogenetic analysis showed that strain CICC 11023 T was most closely related to members of the genus Nocardia, and sequence similarity calculations obtained by pairwise comparisons indicated that the closest relatives of strain CICC 11023 T were Nocardia niniae OFN 02.72 T (98.4%), Nocardia iowensis UI 122540 T (98.3%) and Nocardia alba YIM 30243 T (98.1%; Fig. 1). The gyrB gene sequence for strain CICC 11023 T (1094 bp) was also determined and analysed according to the methods reported by Takeda et al. [5]. The closest phylogenetic neighbours were Nocardia vulneris NBRC 108936 T (92.2%), Nocardia brasiliensis IFM 0236 T (91.8%) and Nocardia exalbida IFM 0803 T (89.1%; Fig. 2). The Nocardia type species were clustered based on gyrB sequence similarity values of 93.5% and above [5]. Therefore, N. niniae OFN 02.72 T, N. iowensis UI 122540 T, N. alba YIM 30243 T, N. vulneris NBRC 108936 T, N. brasiliensis IFM 0236 T and N. exalbida IFM 0803 T as reference strains were used for phenotypic comparisons and DNA–DNA hybridization (DDH) tests.

The G+C content was determined using the method of Mesbah et al. [33] and was found to be 65.6 mol%. DDH experiments were carried out at the CGMCC using dot-blot hybridization and a simple fluorometric method based on thermal denaturation temperatures [34] to evaluate the DNA–DNA relatedness between strain CICC 11023 T and its most closely related species: N. niniae OFN 02.72 T (35.4%), N. iowensis UI 122540 T (20.4%), N. alba YIM 30243 T (25%), N. vulneris NBRC 108936 T (21.6%), N. brasiliensis IFM 0236 T (22.2%) and N. exalbida IFM 0803 T (23.3%). In accordance with the recommended threshold value of 70% DNA–DNA relatedness for species delineation [35], strain CICC 11023 T represents a species distinct from N. niniae OFN 02.72 T, N. iowensis UI 122540 T, N. alba YIM 30243 T, N. vulneris NBRC 108936 T, N. brasiliensis IFM 0236 T and N. exalbida IFM 0803 T.
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The extracted genomic DNA of strain CICC 11023ᵀ was sequenced by combining Illumina HiSeq at the CGMCC. An Illumina library with an insert size of about 400 bp was prepared from 500 ng of DNA using the TruSeq DNA Sample Prep Kit according to the manufacturer’s instructions. Genes were predicted within the completed genomic sequence using Glimmer software 3.02 [36]. tRNA genes were predicted using tRNAscan-SE 1.3.1 [37], and rRNA genes were identified using RNAmmer 1.2 [38]. The protein sequence of the predicted gene was BLASTP-aligned with the Nr, Swiss-prot, string and GO databases, respectively (BLAST 2.2.28+), thereby obtaining annotation information for the predicted gene. Konstantinidis and Tiedje [39] proposed that the 70% DDH standard seen as a pragmatic cut-off value for the delineation of species corresponds to 94% average nucleotide identity (ANI) value in the definition of prokaryotic species. The orthologous ANI algorithm used the usearch program [40]. The final genome of strain CICC 11023ᵀ comprised 48 scaffolds with a total size of 8.78 Mb and a G+C content of 67.4 mol% overall, 68.05 mol% for the gene regions and 62.84 mol% for the intergenic regions. The assembled contigs were annotated with the ncbi Prokaryotic Genome Annotation Pipeline pipeline [41], yielding a total of 9563 coding genes. General features of the genome of strain CICC

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---------------|---|---|---|---|---|---|---|
| Growth at 37°C | + | + | + | − | + | + | + |
| Growth at 45°C | − | − | + | − | − | − | − |
| Milk coagulation | − | − | − | − | − | − | − |
| Milk peptonization | + | − | − | − | − | − | − |
| Carbon utilization: | | | | | | | |
| Glucose | + | + | + | + | + | + | + |
| Mannitol | + | − | − | + | + | w | + |
| Inositol | + | − | − | + | − | − | − |
| Arabinose | + | + | − | − | − | − | − |
| Maltose | + | w | + | + | − | + | + |
| Galactose | + | + | − | − | + | + | w |
| Raffinose | + | − | − | − | − | − | − |
| Rhamnose | w | − | − | + | − | − | − |
| Sorbitol | w | − | − | − | − | − | − |
| Decomposition: | | | | | | | |
| Adenine | + | + | − | − | − | − | − |
| Casein | − | − | − | − | − | − | − |
| Tyrosine | − | − | + | − | + | + | + |
| Xanthine | − | − | + | − | − | − | + |
| Hypoxanthine | − | + | + | − | + | + | + |
| Uric acid | + | + | + | − | − | − | − |
| Aesculin | + | − | − | + | − | − | − |
| Polar lipids* | DPG, PE, uPL, ul1, ul2 | DPG, PE, PI, PIM | DPG, PE, PI, PIM | DPG, PE, PI, PIM | DPG, PE, PI, PIM | DPG, PE, PI, PIM | DPG, PE, PI, PIM |
| DNA G+C content (mol%) | 65.6 | 67.6 | 70.5 | 72 | 68.4 | 69.6 | 68 |

*DPG, diphosphatidylglycerol; GL, glycolipid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositolmannoside; uL, unidentified lipid; uPL, unidentified phospholipid.

Table 2. Differential phenotypic characteristics between strain CICC 11023ᵀ and closely related Nocardia species

Strains: 1, CICC 11023ᵀ; 2, Nocardia ninae OFN 02.72ᵀ; 3, Nocardia iowensis UI 122540ᵀ; 4, Nocardia alba YIM 30243ᵀ; 5, Nocardia vulneris NBRC 108936ᵀ; 6, Nocardia brasiiliensis IFM 0236ᵀ; 7, Nocardia exalbida IFM 0803ᵀ. All data were obtained in this study unless indicated otherwise. +, positive; −, negative; w, weak.
Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between Nocardia colli CICC 11023T and closely related type strains of the genus Nocardia. *Streptomyces somaliensis* DSM 40738T was used as outgroup. Bootstrap values were expressed as percentages of 1000 replications. The branching is supported by the results from the three algorithms used. Bar, 0.01 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.
11023\(^T\) are shown in Table S3. The ANI values of strain CICC 11023\(^T\) were calculated between \(N.\) \(alba\) YIM 30243\(^T\) and \(N.\) \(vulneris\) NBRC 108936\(^T\), respectively. The OrthoANIu value between CICC 11023\(^T\) and \(N.\) \(alba\) YIM 30243\(^T\) was 77.47\%, the DDH value was 25\%, and the OrthoANIu value between CICC 11023\(^T\) and \(N.\) \(vulneris\) NBRC 108936\(^T\) was 83.75\%), the DDH value was 21.6\%.

In conclusion, the morphological and chemotaxonomical characteristics and the results of phylogenetic analyses support that strain CICC 11023\(^T\) had characteristics typical of a member of the genus \(Nocardia\). The differential characteristics shown in Table 2 indicate that strain CICC 11023\(^T\) has several different phenotypic properties that allow discrimination from the closest related species of the genus \(Nocardia\), including utilization of raffinose, sorbitol, milk peptonization, and decomposition of aesculin. In addition, the cellular fatty acid analysis clearly suggested that CICC 11023\(^T\) contained relatively large amounts of \(C_{17:0}\) and \(C_{16:1}\) 10-methyl (TBSA), and small amounts of \(C_{16:0}\) and \(C_{16:0}\) 9c. The unique 16S ribosome RNA and \(gyrB\) gene sequences show relationships between \(Nocardia\) strains CICC 11023\(^T\) and closely related type strains of the genus \(Nocardia\) with low ANI values (<94\%). The OrthoANIu value between CICC 11023\(^T\) and \(N.\) \(alba\) YIM 30243\(^T\) was 77\%, and the OrthoANIu value between CICC 11023\(^T\) and \(N.\) \(vulneris\) NBRC 108936\(^T\) was 83.75\%), the DDH value was 21.6\%.

The strain shows positive reactions for milk peptonization, catalase, aesculin, urease and cellulose; negative reactions for milk coagulation, nitrate reduction, gelatin liquefaction, tyrosinase, hydrolysis of starch, casein, xanthine, hypoxanthine and production of H\(_2\)S. Strain CICC 11023\(^T\) can utilize L-arabinose, rhamnose, D-fructose, salicin, D-xylene, inositol, lactose, melibiose, D-glucose, raffinose, sucrose, D-mannitol, maltose, trehalose and arabinose as sole carbon sources. No soluble pigments are produced. The main fatty acids (>5\%) are \(C_{18:0}\) 10-methyl (TBSA), \(C_{16:0}\) summed feature 4 (\(C_{16:1}\) trans 9/\(C_{15:0}\) iso 2OH), \(C_{15:0}\) and \(C_{17:0}\) 10-methyl. The main menaquinone is cyclo MK-8 (H\(_4\), \(\omega\)-cyclo). The phospholipids are diphosphatidylglycerol, phosphatidylethanolamine, one unidentified phospholipid and two unidentified lipids. The type strain is susceptible to levofloxacin, ofloxacin, tobramycin and clindamycin; resistant to fosfomycin, imipenem, vancomycin and erythromycin. The organism is a pathogen of cutaneous infection in normal immunocompetent patients. The DNA G+C content of the type strain is 65.6 mol\%.

The strain, CICC 11023\(^T\) (=KCTC 39837\(^T\)), was isolated from aerobic culture of a biopsied skin tissue specimen from a 36-year-old female patient with primary cutaneous nocardiosis in Yunnan Province, south-west China.

**DESCRIPTION OF \(NOCARDIA COLLI\) SP. NOV.**

\(Nocardia\) \(colli\) (coll'i. L. neut. gen. n. \(colli\) of the neck).

Strain CICC 11023\(^T\) is an aerobic, Gram stain-positive, non-motile, modified acid alcohol-positive actinomycetes bacterium, which forms an extensively branched grey-white substrate mycelium and aerial mycelium with fragments forming short coli-like bodies under scanning electronic microscopy (0.5×0.7–0.9\,\mu m in diameter). The growth temperature range of strain CICC 11023\(^T\) is 21–37 \(^\circ\)C with an optimum growth temperature of 28 \(^\circ\)C. The salt tolerance is in the range of 1–4\% and the optimum growth salinity is 1\%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.
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