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Nocardia lijiangensis sp. nov., a novel actinomycete strain isolated from soil in China

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Abstract

A novel actinomycete strain YIM 33378^T was isolated from a soil sample collected from Lijiang, Yunnan Province, China. Based on the results of phenotypic and genotypic characteristics, strain YIM 33378^T should be assigned to a new species of the genus *Nocardia*, for which the name *Nocardia lijiangensis* sp. nov. is proposed. The type strain is YIM 33378^T (= CCTCC AA 204005^T = KCTC 19028^T).

The GenBank accession number for the sequence reported in this paper is AY779043.

Keywords: *Nocardia lijiangensis* sp.nov.; Polyphasic taxonomy; 16S rRNA

Introduction

The application of chemotaxonomic, numerical phenetic and molecular systematic methods promoted a radical reappraisal of the genus *Nocardia* [8,9,24]. The improved classification provides a sound framework for the recognition of additional species [51]. Members of the genus form extensively branched hyphae. The substrate hyphae fragment into rod-shaped, non-motile elements. Aerial hyphae are usually formed but are sometimes only visible microscopically [10,14,15]. Nocardiae are also characterized by a number of chemical markers, including the presence of *meso*-DAP, arabinose and galactose, mycolic acids and DNA G+C content of 64–72% [7,10]. Currently the genes *Nocardia* encompasses more than 50 species with validly published names. Much of the emphasis in nocardial systematics has focused on the causal agents of actinomycetoma and nocardiosis [7,8,31]. Little is known about nocardial species diversity, functional activities and commercial value in natural habitats [19,28,34–36,43,46]. In the course of our screening program for new antibiotics, several actinomycete strains, which contained both type I and type II polyketide bio-synthesis pathway genes, were isolated from soil samples collected from Yunnan province, China [50].

Methods

Micro-organisms and culture conditions

Strain YIM 33378^T was isolated, using the dilution plating method, from a soil sample collected from Lijiang, Yunnan Province, the west of China. The

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medium used for selective isolation was HV agar [16] and incubated at 28 °C for about 2 weeks. The strain was maintained on ISP 2 agar or ISP 5 agar slants at 4 °C and as glycerol suspensions (20%, v/v) at −20 °C. Biomass for chemical and molecular systematic studies was obtained by growing in shake flasks (about 150 rpm.) of ISP 2 broth supplemented with the vitamin mixture of HV medium [16] at 28 °C for 1 week.

Phenotypic characteristics

Cultural characteristics were determined after 2 weeks at 28 °C by methods used in the International *Streptomyces* Project (ISP) [39] except for modified Sauton's agar [33]. Morphological properties were examined by light microscopy (Olympus microscope BH-2) and scanning electron-microscopy with a JEOL model JSM 5600LV. A range of phenotypic properties were examined using standard procedures [6,45]. In addition, acid production from carbohydrates was carried out using media and methods described by Gordon et al. [13]. The utilization of sole carbon and sole carbon/nitrogen sources was investigated after Gordon and Mihm [15] and Tsukamura [42]. Resistance to lysozyme was determined by the method of Gordon et al. [13]. Tolerance to temperature (10, 27, 30, 37, 45 °C), sodium chloride (4%, 7%, 10%, 13%) and phenol (0.1%, 0.2%, 0.5%, 1.0%) was tested using modified Bennett's agar [45]. Resistance to antibiotics was examined using amikacin (30 μg), aureomycin (30 μg), ciprofloxacin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin sulfate (10 μg), kanamycin (15 μg), netilmicin (10 μg), novobiocin (30 μg), oleandomycin (10 μg), penicillin G (10U), polymyxin B (300U), streptomycin sulfate (10 μg), terramycin (30 μg), tetracycline (30 μg), tobramycin sulfate (10 μg) and vancomycin (10 μg) disks [11] with glucose-yeast extract agar [15] as the basal medium. The results were recorded following incubation at 28 °C for up to 14 days. Color determination was done with color chips from the ISCC-NBS COLOR CHARTS standard samples no. 2106 [21]. Gram [5] and Ziehl-Neelsen [12] preparations were also observed by light microscopy.

Chemotaxonomy

The amino acid and sugar analysis of whole cell hydrolysates followed procedures described by Stanek and Roberts [40]. Phospholipid analysis was carried out as described by Lechevalier et al. [27]. The acid methanolysis procedure was used to detect mycolic acids [32]. Menaquinones were determined using the procedures of Collins et al. [2]. Biomass for the quantitative fatty acid analysis was prepared by scraping growth from TSB agar plates [trypticase soy broth (BBL), 3% (w/v); Bacto agar (Difco), 1.5% (w/v)] that had been incubated for 3 days at 28 °C. The fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system [20,38].

DNA G+C content determination

The chromosomal DNA for genomic DNA G+C content analysis was extracted as described by Marmur [30]. The DNA G+C base content of strain YIM 33378^T was determined by the thermal denaturation method [29].

Extraction of genomic DNA and amplification of 16S rRNA gene

Genomic DNA extraction and PCR amplification of 16S rRNA gene from strain YIM 33378^T were carried out using procedures described by Xu et al. [49]. Multiple alignments with sequences of a broad selection of *actinobacteria* and calculations of levels of sequences similarity were carried out using CLUSTAL X [41]. A phylogenetic tree was reconstructed using neighbor-joining method of Saitou and Nei [37] from K_{nuc} values [22,23]. The topology of the phylogenetic tree was evaluated by bootstrap resampling method of Felsenstein [4] with 1000 replicates. *Gordonia bronchialis* (sequence accession no. X79287) was used as an outgroup.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of strain YIM 33378^T determined in this study has been deposited in GenBank under the accession number AY779043. The accession numbers of the reference strains, which are closely related to strain YIM 33378^T, are indicated in Fig. 1.

Results

Morphological observations

Morphological observation of a 14-day-old culture of strain YIM 33378^T grown on modified Bennett's agar revealed it had the typical characteristics of genus *Nocardia* (Fig. 1). Strain YIM 33378^T formed extensively branched substrate hyphae which fragment into rod-shaped, non-motile elements; Aerial mycelium consisting of hyphae that fragmented into short to elongated rod-like elements.

Cultural characteristics

Strain YIM 33378^T is aerobic, Gram-positive, slightly acid-alcohol-fast. As shown in Table 1, the isolate developed well on several media including ISP 2, ISP 5, modified Bennett's agar, Potato agar. It showed moderate growth on ISP 4, ISP 3, Czapek's agar, modified Sauton's agar, nutrient agar. YIM 33378^T developed white to yellow white aerial hyphae on all media tested, especially on modified Bennett's agar. No diffusible pigments were produced.

Physiological and biochemical characteristics

Strain YIM 33378^T utilized arabinose, cellobiose, fructose, galactose, glucose, inositol, lactose, maltose, mannose, melibiose, melicitose, raffinose, ribose, sorbinose, sucrose, xylose, adonitol, dulcitol, salicin, xylitol, glycerol, oxalate (weak) and dextrin, but not arabitol, malonate, tartrate. Acid is not formed from these carbon sources tested. L-alanine, L-asparagine, L-cysteine (weak), L-histidine, L-proline and L-valine, are used as sole nitrogen sources, but not acetamide, L-arginine, glycine, L-glutamic acid, L-hydroxyproline, L-lysine, L-methionine, phenylalanine, L-threonine and L-tryptophane. Amygdalin, chitin and keratin are hydrolysed, Tweens 20 and Tweens 80 are degraded, but not allantoin, cellulose, DNA, glucosamine and starch. Tests for gelatin, melanin production, milk coagulation and peptonization and H_2S production are negative, but for resistance to KCN is positive. Growth between 28 and 37 °C, from pH 7 to pH 9 and in the presence of phenol at 0.1%, but not in the presence of sodium chloride. Resistant to lysozyme, kanamycin, nalidixic acid, novobiocin (weak), oleandomycin (weak), penicillin G, polymyxin B and vancomycin (weak), but sensitive to amikacin, aureomycin (weak), chloramphenicol, erythromycin, gentamicin sulfate, netilmicin, streptomycin, terramycin, tetracycline and tobramycin.

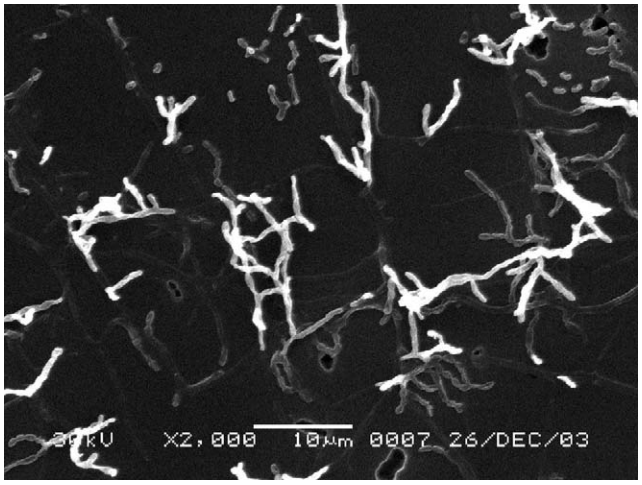

Fig. 1. Scanning electron micrograph of strain YIM 33378^T grown on modified Bennett's agar medium for 14 d at 28 °C.

Chemotaxonomic characteristics

Whole-organism hydrolysates of strain YIM 33378^T were rich in *meso*-diaminopimelic acid, arabinose and galactose (cell wall chemotype IV sensu Lechevalier and Lechevalier [26]) and diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidyl inositol mannosides (phospholipid type II sensu Lechevalier et al. [25]). The menaquinones were MK-8($H_4\omega$-cycl) (95%) and MK-8(H_2) (5%). It was also characterized by the presence of mycolic acids that co-migrated (RF value around 0.47) with those from marker strains of *Nocardia*. The fatty acid profile mainly contained straight

Table 1. Cultural characteristics of strain YIM 33378^T

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract-malt extract (ISP[a] medium 2)	Good	White	Deep orange yellow
Oatmeal agar (ISP[a] medium 3)	Moderate	Yellow white	Pale yellow
Inorganic salt-starch agar (ISP[a] medium 4)	Moderate	Yellow white	Pale yellow
Glycerol-asparagine (ISP[a] medium 5)	Good	Yellow white	Light yellow
Czapek's agar	Moderate	Yellow white	Yellow white
Modified Sauton's agar	Moderate	White	Pale yellow
Modified Bennett's agar	Good	White	Pale yellow
Potato agar	Good	White	Pale orange yellow
Nutrient agar	Moderate	Yellow white	Brill yellow

Note: Diffusible pigments were not produced on any of the media listed.
Colors taken from ISCC-NBS COLOR CHARTS (standard samples no. 2106) [21].
[a]ISP, International *Streptomyces* Project [39].

chain saturated, unsaturated and 10-methyl-branched fatty acids. The fatty acid profiles contained ai-$C_{15:0}$, 5.0%; i-$C_{16:0}$, 2.4%; cis7-$C_{16:1}$, 1.6%; i-2OH-$C_{15:0}$, 6.2%; $C_{16:0}$, 26.1%; ai-$C_{17:0}$, 2.3%; cis6,9-$C_{18:2}$, 8.9%; cis9-$C_{18:1}$, 12.5%; $C_{18:0}$, 16.8%; 10-methyl $C_{18:0}$, 11.0%; cis6,9-$C_{20:2}$, 2.3%. The G + C content of genomic DNA was 65.4 mol%.

Phylogenetic analysis

Almost complete 16S rRNA gene sequence data of strain YIM 33378^T (1513 bp) was determined. BLAST search results of strain YIM 33378^T came from non-redundant GenBank + EMBL + DDBJ + PDB, and sequences have been chosen as reference sequences in which unidentified and unpublished sequences were not included. Comparison of this sequence with those of related organisms showed clearly that it contained all the signature nucleotides designated for the genus *Nocardia* [1]. The phylogenetic tree (Fig. 1) from representative strains of the related species indicated that strain YIM 33378^T should be in genus *Nocardia* and form a separate lineage together with its two closest neighbours, *N. xishanensis* CGMCC 4.1860^T [47] and *N. polyresistens* YIM 33361^T[48] (Fig. 2).

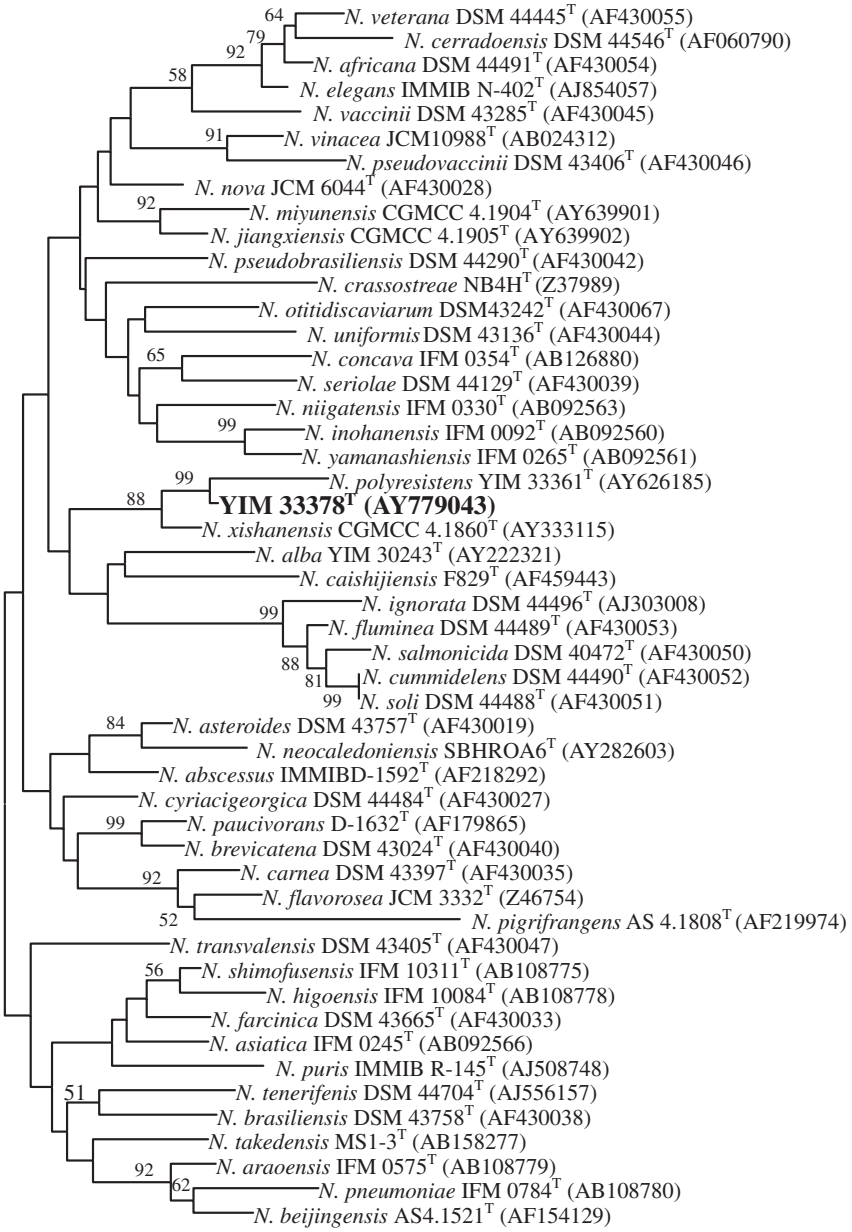

Fig. 2. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA sequences, showing the position of strain YIM 33378^T among phylogenetic neighbors. Numbers on branch nodes are bootstrap values (1000 resamplings). The sequence of *Gordonia bronchialis* DSM 43247^T (X79287) was used as root. Bar, 1% sequence divergence.

Discussion

The described above chemotaxonomic, morphological and physiological properties of strain YIM 33378 ^{T} are consistent with assignment to the genus *Nocardia* [9,10]. The values for 16S rRNA gene sequence similarity to members of the genus *Nocardia* (95.1–99.3%) support the addition of the strain to this genus. This assignment is also supported by the fact that the 16S rRNA sequence contains the signature nucleotide characteristic for the genus *Nocardia* [1]. It is evident from the 16S rRNA trees (Fig. 1) that the isolate YIM 33378 ^{T} is associated with its two closest relatives, *N. xishanensis* CGMCC 4.1860^{T} (16S rRNA accession no. AY333115, similarity value of 99.3%) [47] and *N. polyresistens* YIM 33361^{T} (16S rRNA accession no. AY626185, similarity value of 99.1%) [48], and also these three strains formed same subclade with high bootstrap values (88%).

Accordingly, comparative taxonomic studies were performed among strains YIM 33378^{T}, *N. polyresistens* YIM 33361^{T} and *N. xishanensis* CGMCC 4.1860^{T} to determine whether strain YIM 33378^{T} could be considered as a new species of the genus *Nocardia* or would be assigned to one of the two species.

DNA–DNA relatedness tests were performed among strains YIM 33378^{T}, *N. polyresistens* YIM 33361^{T} and *N. xishanensis* CGMCC 4.1860^{T} using the optical renaturation method [3,17,18], and DNA–DNA reassociation similarities between strain YIM 33378^{T} and the other two type strains, YIM 33361^{T} and CGMCC 4.1860^{T}, were with 45.4% and 28.6%, respectively. DNA–DNA relatedness provided decisive evidence that the new isolate YIM 33378^{T} and related two type strains *N. polyresistens* YIM 33361^{T}, *N. xishanensis* CGMCC 4.1860^{T}, are members of different genomic species [44].

This is also supported by differential phenotypic data, such as some carbon and nitrogen utilization, decomposition of xanthine, nitrate reduction, reaction to gentamicin sulphate, erythromycin, the major fatty acid compositions and DNA G+C content, which showed the new isolate YIM 33378^{T} was different from its two closest phylogenetic neighbours, *N. polyresistens* YIM 33361^{T} and *N. xishanensis* CGMCC 4.1860^{T} (Table 2).

In conclusion, the genotypic and phenotypic data showed that strain YIM 33378^{T} forms a novel species of the genus *Nocardia*, for which we propose the name *Nocardia lijiangensis* sp. nov.

Description of *Nocardia lijiangensis* sp. nov.

N. lijiangensis (li.jiang.en'sis. N.L.adj. lijiangensis pertaining to the city in Yunnan Province in the south of China where the sample was collected). Aerobic, Gram-positive, catalase-positive and slightly acid-alcohol-fast. Aerial mycelium is white to pale-yellow. Diffusible pigments are not formed. Arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melicitose, melibiose, raffinose, ribose, sorbinose, sucrose, xylose, adonitol, glycerol, inositol, dulcitol, xylitol, salicin, oxalate (weak) and dextrin are utilized as sole carbon and energy sources, but not rhamnose, trehalose, arabitol, sorbitol, acetate,

Table 2. Some phenotypic characteristics that separate strain YIM 33378^{T} from *N. polyresistens* YIM 33361^{T}, *N. xishanensis* CGMCC 4.1860^{T}, the two closest type strains of genus *Nocardia*

Character	*N. lijiangensis* YIM 33378^{T}	*N. polyresistens* YIM 33361^{T}	*N. xishanensis* CGMCC 4.1860^{T}
Carbon and nitrogen utilization			
Oxalate	w	−	−
L-cysteine	w	−	ND
L-proline	+	w	+
L-tyrosine	+	w	ND
Growth at 3% NaCl	−	−	+
Decomposition of xanthine	+	+	−
Nitrate is reduced	−	−	+
Reaction to			
Gentamicin sulphate	Sensitive	Resistant	Resistant
Erythromycin	Sensitive	Resistant	Sensitive
The major fatty acid compositions (%)			
$C_{16:0}$	26.1	22.1	33.1
$C_{18:0}$	16.8	21.2	7.6
cis-9-$C_{18:1}$	12.5	10.1	32.7
10-methyl-$C_{18:0}$	11.0	10.5	11.3
G+C mol%	65.4	65.6	68.8

Data were taken from this and previous studies [47,48]. +, Positive; −, negative; w, weak reaction; ND, no data.

citrate, malonate, tartrate. Acid is not formed from these carbon sources tested. L-alanine, L-asparagine, L-cysteine (weak), L-histidine, L-proline, L-tyrosine and L-valine are used as sole nitrogen sources, but not acetamide, L-arginine, L-glutamic acid, glycine, L-hydroxyproline, L-lysine, L-methionine, phenylalanine, L-threonine and L-tryptophane. Amygdalin, chitin, hypoxanthine, keratin, urea and xanthine are hydrolysed, Tweens 20 and Tweens 80 are degraded, but not aesculin, adenine, allantoin, cellulose, DNA, glucosamine and starch. Tests for gelatin, melanin production, milk coagulation and peptonization, nitrate reduction and H_2S production are negative, but resistance to KCN is positive. Growth between 28 and 37 °C, from pH 7 to pH 9 and in the presence of phenol at 0.1%, but not in the presence of sodium chloride. Resistant to lysozyme, ciprofloxacin, kanamycin, nalidixic acid, novobiocin (weak), oleandomycin (weak), penicillin G, polymyxin B and vancomycin (weak), but sensitive to amikacin, aureomycin, chloramphenicol, erythromycin, gentamicin sulfate, netilmicin, streptomycin, terramycin, tetracycline and tobramycin. The cell wall of strain YIM 33378^T contains *meso*-diaminopimelic acid. Whole-cell sugars are galactose and arabinose. MK-8($H_4\omega$-cycl) is the major menaquinone and minor amount of MK-8(H_2) is also present. The phospholipids are diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidyl inositol mannosides. The major cellular fatty acids are: ai-$C_{15:0}$, 5.0%; i-$C_{16:0}$, 2.4%; i-2OH-$C_{15:0}$, 6.2%; $C_{16:0}$, 26.1%; ai-$C_{17:0}$, 2.3%; cis6,9- $C_{18:2}$, 8.9%; cis9-$C_{18:1}$, 12.5%; $C_{18:0}$, 16.8%; 10-methyl $C_{18:0}$, 11.0%; cis6,9-$C_{20:2}$, 2.3%. The G+C content of genomic DNA was 65.4 mol%. It was isolated from soil in Lijiang, Yunnan Province, China. The type strain is strain YIM 33378^T (= CCTCC AA 204005^T = KCTC 19028^T).

Acknowledgments

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