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Kribbella yunnanensis sp. nov., *Kribbella alba* sp. nov., two novel species of genus *Kribbella* isolated from soils in Yunnan, China

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Summary

Two actinomycete strains, designated YIM 30006^T and YIM 31075^T, were isolated from soil samples in Yunnan, China and subjected to a polyphasic taxonomic study. Morphological and chemotaxonomic analysis revealed that the two isolates should be consistent with the nocardioform actinomycetes. Comparative 16S rDNA sequences confirmed that the two unknown isolates to be members of the genus *Kribbella*. Based on the results of phenotypic characteristics, phylogenetic studies and DNA–DNA hybridization results, strains YIM 30006^T and YIM 31075^T should be classified as two novel species of the genus *Kribbella*, for which the names *Kribbella yunnanensis* sp. nov. and *Kribbella alba* sp. nov. are proposed. The type strains for them are YIM 30006^T (= CCTCC AA001019^T = DSM 15499^T) and YIM 31075^T (= CCTCC AA 001020^T = DSM 15500^T), respectively.

The 16S rDNA sequences of strains YIM 30006^T, YIM 31075^T have been deposited in GenBank under the accession numbers AY 082061 and AY 082062, respectively.

Keywords: *Kribbella yunnanensis* sp. nov.; *Kribbella alba* sp.nov.; 16s rDNA

Introduction

The genus *Kribbella* was first proposed by Park et al. [17] as the reclassification results of two strains '*Nocardioides fulvus*' IFO 14399 and *Nocardioides* sp. ATCC 39419, and at present the genus comprises the following six species, *Kribbella flavida* [17], *Kribbella sandramycini* [17], *Kribbella koreensis* [21], *Kribbella antibiotica* [12], *Kribbella solani* [22] and *Kribbella jejuensis* [22].

Some novel nocardioform actinomycete strains were isolated from soil samples collected from Yunnan Province of China during our project for screening bioactive substance. Recently, the name *K. antibiotica* [12] has been proposed for one of these isolates. The aim of the present investigation was to determine the taxonomy position of another two nocardioform strains using a polyphasic taxonomic approach. Based on the following reported findings, we propose the two isolates to be two novel species of the genus *Kribbella*, *Kribbella yunnanensis* sp. nov. for strain YIM 30006^T(= CCTCC

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AA001019^T = DSM 15499^T) and *Kribbella alba* sp. nov. for strain YIM 31075^T (= CCTCC AA 001020^T = DSM 15500^T), respectively.

Methods

Micro-organisms and culture conditions

Strains YIM 30006^{T}and YIM 31075^T were isolated on glycerol–asparagine agar [20] (ISP5 medium) and incubated at 28 °C for about 2 weeks. The strains were maintained on ISP2 and ISP5 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 Medium2 broth at 28 °C for 1 week.

Phenotypic characteristics

Cultural characteristics were determined after 4 weeks at 28 °C by methods used in the International *Streptomyces* Project (ISP) [20]. Morphological properties were examined by light microscopy (Olympus microscope BH-2) and scanning electron microscopy with a JEOL model JSM5600LV. Media and procedures used for determination of physiological features and carbon source utilization were those described by Shirling and Gottlieb [20] and Locci [13]. Color determination was done with color chips from the ISCC-NBS COLOR CHARTS Standard Samples No 2106 [6].

Chemotaxonomy

The amino acid and sugar analysis of cell wall hydrolysates followed procedures described by Stanek and Roberts [24]. Polar lipids were extracted, examined by two-dimensional thin layer chromatograph and identified using published procedures [16]. Menaquinones were isolated using the methods of Minnikin et al. [16] and separated by HPLC [9,10]. Whole-cell fatty acid profile was determined on TSA using the MIDI (Microbial Identification) system as described by Sasser [19].

Extraction of Genomic DNA and amplification of 16S rDNA

Extraction of Genomic DNA and analysis of 16S rDNA were done as described by Xu et al. [27]. Phylogenetic analysis was performed using the software packages PHYLIP [3] and MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 [11] after multiple alignment of data by CLUSTALX [25]. Distances (distance options according to the Kimura two-parameter mode) [7,8] and clustering with the neighbor-joining method [18]. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1000 resamplings [2].

Nucleotide sequence accession numbers

The 16S rDNA sequences of strains YIM 30006^T, YIM 31075^T have been deposited in GenBank under the accession numbers AY 082061 and AY 082062, respectively. The accession numbers of the reference strains, which are closely related to strains YIM 30006^T and YIM 31075^T, are indicated in Fig. 2.

DNA G + C content determination and DNA–DNA hybridizations

DNAs for the base composition and renaturation studies were prepared following the method of Marmur [14]. The G + C contents were determined using the thermal denaturation method of Marmur and Doty [15]. DNA–DNA hybridizations were carried out according to described methods [1,4,5].

Results

Morphological observations

Both strains YIM 30006^{T}and YIM 31075^T had typical morphological characteristics of genus *Kribbella* (Fig. 1). The substrate mycelium was extensively branched and often fragmented into rod-shaped elements. The aerial mycelium consisted of hyphae that fragmented into short to elongated rod-like elements.

Cultural, physiological and biochemical characteristics

As shown in Table 1, both strains YIM 30006^{T}and YIM 31075^T could grow well on most tested media, while they did not produce any diffusible pigment during incubation. The colors of their mycelium were determined by comparing the cultures with the most suitable color chips from the ISCC-NBS color charts (standard sample, no. 2106) [6]. The physiological and biochemical properties useful for distinguishing strains YIM 30006^T and YIM 31075^T from the other validly published species of genus *Kribbella* are shown in Table 2.

Chemotaxonomic characteristics

For both strains, the amino acid of cell wall was LL-DAP, the predominant menaquinone was MK-9(H_4).

But for the polar lipids, strain YIM 30006^T contained diphosphatidylglycerol, phosphatidylcholine and phosphatidylglycerol, while strain YIM 31075^T also had phosphatidylinositol. For the cell wall sugars, strain YIM 30006^T contained mannose, glucose, galactose and ribose, while strain YIM 31075^T contained only glucose, galactose and ribose. For the Cellular fatty acids, there were also some differences with each other and also with the other related type strains (Table 2).

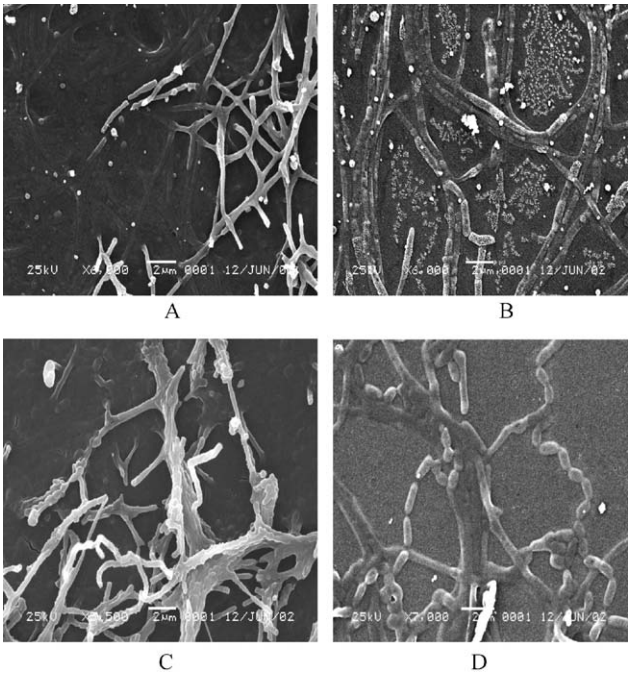

Fig. 1. Scanning electron micrographs of strain YIM 30006^T (A and B) and YIM 31075^T (C and D), grown on yeast-malt extract agar (ISP 2 medium) for 21 days at 28 °C. Bar, 2 μm.

Phylogenetic analysis

The lengths of the almost complete 16S rDNA sequences analyzed for strains YIM 30006^{T}and YIM 31075^T were 1493 and 1469 bp, respectively. Both strains had highest similarity values with sequences of members of genus *Kribbella* and the 100% bootstrap value clearly indicated the position of the two isolates within the coherent cluster of the genus *Kribbella*. A phylogenetic tree is shown in Fig. 2.

Phylogenetically, strain YIM 30006^T formed a subclade with the species, *K. sandramycini* KCTC 9609^T of genus *Kribbella* and they shared 99.3% sequence similarity with each other. While for strain YIM 31075^T, it formed a subclade with another species, *K. koreensis* LM 161^T, and they shared 99.1% sequence similarity. Additionally, the two isolates had higher sequence similarities (all above 97%) with the other validly published species of the genus *Kribbella*. Thus, DNA–DNA hybridizations were needed to further clarify their relatedness [23].

The DNA base compositions and DNA–DNA hybridizations

The DNA G+C contents of the two isolates YIM 30006^T and YIM 31075^T were 68.6%, 67.9%, respectively. For strain YIM 30006^T, the DNA–DNA relatedness with *K. sandramycini* KCTC 9609^T, *K. koreensis* LM 161^T, *K. flavida* KCTC 9580^T, *K. antibiotica* YIM 31530^T and the other isolate YIM

Table 1. Cultural characteristics of strains YIM 30006^T and YIM31075^T

Media	YIM 30006^T			YIM 31075^T		
	Growth	Aerial mycelium	Substrate mycelium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract-malt extract (ISP[a] medium 2)	Moderate	Pale yellow	Moderate yellow	Good	Yellow white	Soft yellow
Oatmeal agar (ISP[a] medium 3)	Moderate	White	Pale yellow	Good	White	Pale yellow
Inorganic salt-starch agar (ISP[a] medium 4)	Poor	White	Yellow white	Good	White	Pale yellow
Glycerol–asparagine (ISP[a] medium 5)	Moderate	White	Yellow white	Good	White	Yellow white
Czapek's agar	Good	White	Pale yellow	Good	Yellow white	Pale yellow
Potato agar	Moderate	Moderate yellow	Moderate yellow	Good	White	Pale yellow
Nutrient agar	Moderate	Pale yellow	Soft yellow	Moderate	Pale yellow	Soft yellow

Note: Colors taken form ISCC-NBS COLOR CHARTS (Standard Samples No 2106) [6]. No diffusible pigment production on all tested media.
[a]ISP, International *Streptomyces* Project [20].

Table 2. Comparison of phenotypic characteristics among strains YIM 30006^T, YIM 31075^T and the other six valid published species of the genus *Kribbella*

Characteristics	YIM 30006^T	*K. sandramycini*[a] KCTC 9609T	YIM 31075^T	*K. koreensis*[a] LM 161^T	*K. flavida*[a] KCTC 9580^T	*K. antibiotica* YIM 31530^T	*K. solani*[a] KACC 20196^T	*K. jejuensis*[a] KACC 20266^T
Gelation liquefaction	–	+	+	+	–	+	ND	+
Starch hydrolysis	+	–	+	–	–	+	–	–
Nitrate reduction	–	–	–	+	+	–	–	–
Melanin production	w	–	+	+	+	+	–	–
Carbon utilization								
Lactose	+	–	+	+	+	+	+	+
Galactose	–	+	+	+	w	+	ND	ND
Mannose	+	–	+	+	+	+	+	ND
Arabinose	+	+	+	+	–	+	+	+
Xylose	+	+	+	+	–	+	+	+
Raffinose	+	+	+	+	–	+	+	+
Trisodium citrate	+	–	–	+	+	+	ND	ND
Sodium oxalate	+	–	+	+	+	+	ND	ND
Chemical characteristics								
Sugars	man, glu gal, rib	man, glu gal	glu, gal rib	man, glu gal, rib	man, glu gal	glu, xyl rib	man, glu gal, rib	man, glu gal, rib
Polar lipid(s)	DPG, PC PG	PC	DPG, PC PG, PI	DPG, PC PG, PI	PC	DPG, PC PG, PI	DPG, PC PI	DPG, PC PI
Major fatty acids (>5%)	i-$C_{15:0}$;	i-$C_{14:0}$;	i-$C_{15:0}$;	i-$C_{15:0}$;	i-$C_{15:0}$;	i-$C_{14:0}$;	i-$C_{14:0}$;	i-$C_{14:0}$;
	i-$C_{14:0}$; ai-$C_{15:0}$; i-$C_{16:0}$; i-$C_{17:0}$;	i-$C_{15:0}$; ai-$C_{15:0}$; i-$C_{16:0}$; i-$C_{17:0}$;	ai-$C_{15:0}$; i-$C_{16:0}$; ai-$C_{17:0}$; i-$C_{17:1\ \omega9c}$;	ai-$C_{15:0}$; i-$C_{16:0}$; ai-$C_{17:0}$; i-$C_{17:0}$;	ai-$C_{15:0}$; i-$C_{16:0}$; ai-$C_{17:0}$; $C_{16:0}$	i-$C_{15:0}$; ai-$C_{15:0}$; i-$C_{16:0}$;	i-$C_{15:0}$; ai-$C_{15:0}$; i-$C_{16:0}$	i-$C_{15:0}$; ai-$C_{15:0}$; i-$C_{16:0}$; $C_{16:0}$ 9-methyl; i-$C_{16:0}$ 2OH
	i-$C_{17:1\ \omega9c}$	$C_{16:0}$	$C_{16:0}$	$C_{16:0}$				
G+C mol%	68.6	68.3	67.9	71.3	70	67	69	68

Abbreviations: +, positive reaction; –, negative reaction; w, weakly positive reaction; ND, not done.

Note: The amino acid of cell wall and menaqunione composition of all tested strains are *meso*-DAP (diaminopimelic acid) and MK-9(H_4); Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; glc, glucose; man, mannose; gal, galactose; rib, ribose;

[a]Some data taken from this study or Park et al. [17], Sohn et al. [21] and Song et al. [22].

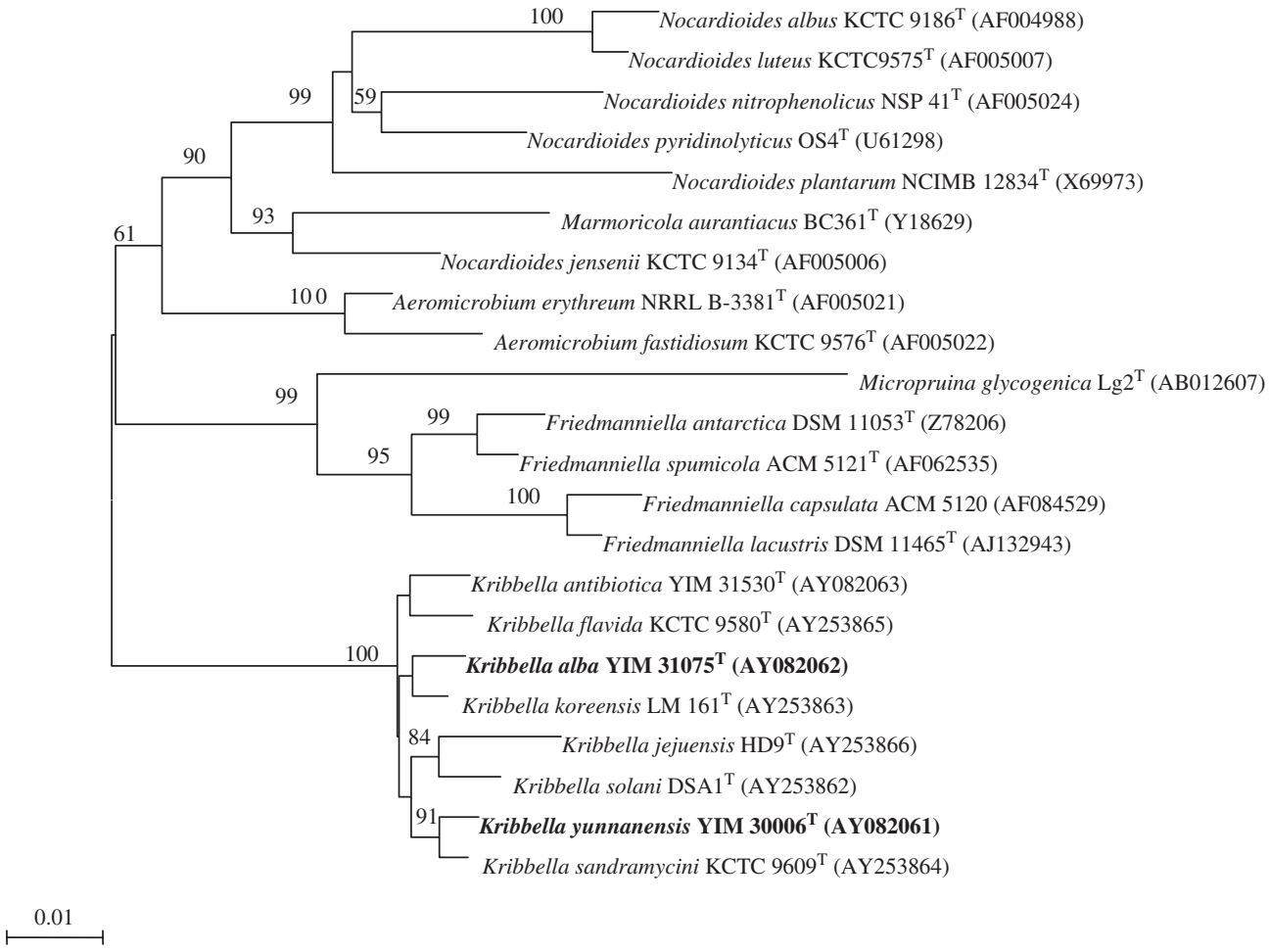

Fig. 2. Phylogenetic tree (based on 16S rDNA sequences) showing the position of strains YIM 30006^T and YIM 31075^T among phylogenetic neighbors. Numbers on branch nodes are bootstrap values (1000 resamplings). The sequence of *Streptosporangium roseum* DSM 43021^T (X89947) was used as root. Bar, 1% sequence divergence.

31075, were 61.2%, 14.4%, 33.3%, 14.4% and 53.8%, respectively. While for strain YIM 31075^T, the DNA–DNA relatedness with its closest neighbor, *K. koreensis* LM 161^{T}was 53.8%, with *K. sandramycini* KCTC 9609^T(18.4%), *K. flavida* KCTC 9580^T(35.4%) and *K. antibiotica* YIM 31530^T (35.4%). And all these results showed lower the threshold value of 70%, indicating that two isolates may represent two novel species in accordance with the recommendations of the committee on reconciliation of approaches to bacterial systematics [26].

Taxonomic conclusions

The nocardioform morphology, chemotaxonomic characteristics and 16S rDNA sequences analysis showed that the two isolates YIM 30006^T and YIM 31075^T to be members of the genus *Kribbella*. As seen the differences of their phenotypic characteristics (Table 2) among the two isolates and the other six validly published species of the genus *Kribbella*. For example, the two isolates and the six related type strains had different reaction for gelation liquefaction, starch hydrolysis, nitrate reduction and melanin production. Additionally, they have different chemical compositions for their cell wall sugars, polar lipids, fatty acids and G+C mol% content. All above data together with the low DNA–DNA similarity values (<70%) with their closest neighbors demonstrate that the two isolates under discussions are representatives of two new species of the genus *Kribbella*. We propose the name *K. yunnanensis* for strain YIM 30006^T (= CCTCC AA001019^T = DSM 15499^T), and the name *K. alba* for strain YIM 31075^T(= CCTCC AA001020^T = DSM 15500^T).

Description of *Kribbella yunnanensis* sp. nov.

Kribbella yunnanensis (yun.nan.en'sis. N.L. fem. adj. Yunnanensis pertaining to Yunnan, a province of

south-west China in which the sample was collected). The cells of the organism are non-motile, aerobic. The organism developed aerial hyphae on most media tested, especially Czapek's agar. The aerial mycelium consisting of hyphae that fragment into short to elongated rod-like elements. The substrate mycelium are extensively branched and often fragment into rod-shaped elements. No diffusible pigment is produced. It can utilize almost all the tested carbon sources, such as glucose, lactose, fructose, sucrose, maltose, mannose, arabinose, xylose, raffinose, rhamnose, glycerol, inositol, mannitol, sorbitol, trisodium citrate, sodium oxalate and sodium acetate, but negative for galactose. It is negative for gelatin liquefaction, growth on cellulose, H_2S production and nitrate reduction, but positive for milk coagulation, milk peptonization and starch hydrolysis, melanin production is doubt. The cell wall of strain YIM 30006^T contains LL-diaminopimelic acid. The cell wall sugars are mannose, glucose, galactose and ribose. The predominant menaquinone is MK-9(H_4). The phospholipids are diphosphatidylglycerol, phosphatidylcholine and phosphatidylglycerol. The major fatty acids are ai-$C_{15:0}$ (31.55%), i-$C_{15:0}$ (14.84%), i-$C_{16:0}$ (10.24%), i-$C_{14:0}$ (7.76%), i-$C_{17:1\ \omega\ 9c}$ (6.68%) and i-$C_{17:0}$ (6.52%). The DNA G+C content is 68.6 mol%. Optimum growth at 28 °C and pH 7.0. Isolated from soil in Yunnan Province, the west of China. The type strain is YIM 30006^T(= CCTCC AA 001019^T = DSM 15499^T).

Description of *Kribbella alba* sp. nov.

Kribbella alba ('al.ba. L. fem adj. alba white). The cells of the organism are non-motile, aerobic. The organism developed aerial hyphae on all the media tested. The color of the aerial mycelium is white on most media tested and the aerial mycelium consisting of hyphae that fragment into short to elongated rod-like elements. The substrate mycelium are extensively branched and often fragment into rod-shaped elements. No diffusible pigment is produced. It can utilize almost all the tested carbon sources, such as glucose, lactose, galactose, fructose, sucrose, maltose, mannose, arabinose, xylose, raffinose, rhamnose, glycerol, mannitol, inositol, sorbitol, sodium oxalate, sodium acetate, but negative for trisodium citrate. It is positive for gelatin liquefaction and starch hydrolysis, but negative for milk coagulation, milk peptonization, melanin production, growth on cellulose, H_2S production and nitrate reduction. The cell wall of strain YIM 31075^T contains LL-diaminopimelic acid. The cell wall sugars are glucose, ribose and galactose. The predominant menaquinone is MK-9(H_4). The phospholipids are phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The major fatty acids are ai-$C_{15:0}$ (27.90%), i-$C_{15:0}$ (9.13%), i-$C_{16:0}$ (15.27%), i-$C_{17:1\ \omega\ 9c}$ (10.43%), ai-$C_{17:0}$ (6.09%) and i-$C_{17:0}$ (4.49%). The DNA G+C content is 67.9 mol%. Optimum growth at 28 °C and pH 7.0. Isolated from soil in Yunnan Province, the west of China. The type strain is YIM 31075^T (= CCTCC AA 001020^T = DSM 15500^T).

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