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Citricoccus alkalitolerans sp. nov., a novel actinobacterium isolated from a desert soil in Egypt

Wen-Jun Li,[1,2] Hua-Hong Chen,[1,3] Yu-Qin Zhang,[1] Chang-Jin Kim,[2] Dong-Jin Park,[2] Jae-Chan Lee,[2] Li-Hua Xu[1] and Cheng-Lin Jiang[1]

[1]The Key Laboratory for Microbial Resources of Ministry of Education, P. R. China, Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan, 650091, P. R. China

[2]Korea Research Institute of Bioscience and Biotechnology, 52 Oeundong, Yusong, Daejeon 305-333, Republic of Korea

[3]Department of Chemistry, Chuxiong Normal College, Chuxiong, Yunnan, 675000, P. R. China

Correspondence
Chang-Jin Kim
changjin@kribb.re.kr *or*
wjli@ynu.edu.cn

An actinobacterium, strain YIM 70010^T, which was isolated from a desert soil sample collected in Egypt, was subjected to a polyphasic taxonomy study. The organism was alkalitolerant and its optimum growth occurred at pH 8·0–9·0. The isolate contained chemotaxonomic markers that were characteristic of the genus *Citricoccus*, i.e. the peptidoglycan type Lys–Gly–Glu (variation A4α), the predominant menaquinone MK-9(H_2) and a polar lipid profile consisting of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and two unknown glycolipids. The major fatty acids were anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The G+C content of the genomic DNA was 63·8 mol%. Strain YIM 70010^T exhibited a 16S rRNA gene sequence similarity of 99·6 % and DNA–DNA relatedness value of 56 % with *Citricoccus muralis* DSM 14442^T. The phenotypic characteristics and DNA–DNA relatedness data indicate that strain YIM 70010^T can be distinguished from *C. muralis* (DSM 14442^T). Therefore, on the basis of the polyphasic taxonomic data presented, a novel species of the genus *Citricoccus*, *Citricoccus alkalitolerans* sp. nov. (type strain, YIM 70010^T=CCTCC AA 203008^T=DSM 15665^T=KCTC 19012^T) is proposed.

The genus *Citricoccus* was proposed by Altenburger *et al.* (2002a) with a single species, *Citricoccus muralis*. Its representative strain was isolated from a medieval wall painting and comprises Gram-positive, non-motile, non-spore-forming coccoid cells and the aerobic type of metabolism. In this report, we present the description of the second species of the genus, for which we propose the name *Citricoccus alkalitolerans* sp. nov.

Strain YIM 70010^T was isolated from a desert soil sample collected in eastern Egypt by using medium recommended by Sato *et al.* (1983) for isolation of alkaliphilic and alkaline-resistant micro-organisms. Sodium carbonate was sterilized separately and then added to the medium. The pH of the medium was 10–10·5; $NaHCO_3$/Na_2CO_3 buffer was used to adjust the pH. Strain YIM 70010^T was cultivated aerobically at 28 °C for 1 week. Cells for biochemical and molecular systematic analyses were grown in shake flasks (at about 150 r.p.m.) of trypticase soy broth medium (pH 8·0–9·0) at 28 °C for 1 week. Stock cultures were maintained at 4 °C using modified Sato's slants and as glycerol suspensions (20 %, v/v) at −20 °C.

Strain YIM 70010^T was grown on PYES medium (Altenburger *et al.*, 2002b) for observation of the cell and colony morphology. The growth temperature was tested at 4, 10, 20, 28, 37, 40 and 45 °C on the same medium. Motility of cells and pH and NaCl tolerance of the strain were determined as described by Altenburger *et al.* (2002a, b). Metabolic properties were determined using API Coryne and API ID 32 E test kits (bioMérieux) according to the manufacturer's instructions. Other physiological and biochemical tests were performed as described previously (Shirling & Gottlieb, 1966).

Strain YIM 70010^T was an aerobic, Gram-positive, non-motile, non-spore-forming coccus, of about 0·5–0·8 μm in diameter (Fig. A, available as supplementary material in IJSEM Online). Colonies of strain YIM 70010^T on PYES medium were similar to those of *C. muralis* DSM 14442^T. Growth was observed at initial pH values of between 5·5 and

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 70010^T is AY376164.

An electron micrograph of a cell of strain YIM 70010^T and a dendrogram showing the relationship of the strain with its nearest phylogenetic neighbours are available as supplementary material in IJSEM Online.

12·0, with the optimum at pH 8·0–9·0. Other physiological and biochemical properties are given in Table 1 and in the species description.

The qualitative analyses of amino acids and peptides in peptidoglycan hydrolysates were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose was applied instead of paper chromatography. The N terminus of the interpeptide bridge was determined by dinitrophenylation according to Schleifer (1985). The quantitative analysis of amino acids in the total hydrolysates was done by GC and GC-MS as described by MacKenzie (1987) and Groth *et al.* (1996).

Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt *et al.*, 1981; Kroppenstedt, 1982). Polar lipids were extracted, examined by two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1984). Fatty acid analysis was performed using standard methods (Sasser, 1990) and results were compared with the database of fatty acids in the MIDI Sherlock Microbial Identification system (MIDI Inc.).

Table 1. Distinctiveness of physiological and biochemical characteristics of strain YIM 70010^T and *C. muralis* DSM 14442^T

The following phenotypic characteristics are the same for both strains. Gram-positive, non-motile, non-spore-forming coccoid cells. Catalase-positive and oxidase-negative. Urease, tyrosinase, Voges–Proskauer test, H_2S production and indole production are negative. Tweens 20 and 80, casein and starch are not decomposed. Nitrate is not reduced to nitrite. Activities for lipase and α-glucosidase are positive. Negative for ornithine decarboxylase, arginine dihydrolase, β-glucuronidase, α- and β-galactosidase, *N*-acetyl-β-glucosaminidase and β-glucosidase. The following substrates are utilized by both strains as sole carbon sources for growth: glucose, galactose, sucrose, arabinose, mannose, mannitol, maltose, starch, xylose, ribose, cellobiose, salicin, sorbitol, lactose, dextrin and lysine. *N*-Acetyl-α-glucosamine, L-alanine, β-alanine, L-histidine, L-ornithine and L-tryptophan are not utilized.

Characteristic	YIM 70010^T	DSM 14442^T
pH range for growth	5·5–12	6–10
NaCl range for growth (%)	0–15	0–10
Gelatin liquefaction	−	+
Methyl red	−	+
Lysine decarboxylase	−	+
α-Maltosidase	−	+
Carbon and nitrogen utilization		
Fructose	−	+
Amygdalin	+	−
Glycine	+	−
Arginine	−	+
G+C content (mol%)	63·8	68*

*Value for *C. muralis* was taken from Altenburger *et al.* (2002a).

The peptidoglycan of strain YIM 70010^T contained Ala, Gly, Glu and Lys in a molar ratio of 1·6 : 1·2 : 2·5 : 1·0. Labelling by using 1-fluoro-2,4-dinitrobenzene revealed that glutamic acid represents the amino terminus of the interpeptide bridge. From these results and the two-dimensional TLC peptide pattern of partial cell-wall hydrolysates (data not shown), it was concluded that the peptidoglycan type of strain YIM 70010^T was Lys–Gly–Glu, variation A4α (Schleifer & Kandler, 1972) (type A11.42 or A11.56 according to the DSMZ, 2001). Other chemotaxonomic characteristics of the strain are presented in the species description.

Genomic DNA was isolated and purified by the method of Marmur (1961). The DNA G+C base content of strain YIM 70010^T was measured as 63·8 mol% by using the thermal denaturation method (Marmur & Doty, 1962).

The 16S rRNA gene sequence of the strain was amplified by PCR using conserved primers close to the 3′ and 5′ ends of the gene as described previously (Cui *et al.*, 2001). Multiple alignments with sequences of actinobacteria of the family *Micrococcaceae* and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980, 1983). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

An almost-complete 16S rRNA gene sequence (1480 bp) for strain YIM 70010^T was obtained and subjected to a comparative analysis. Strain YIM 70010^T was phylogenetically most closely related to *C. muralis* DSM 14442^T, with a 16S rRNA gene sequence similarity value of 99·6 %. The phylogenetic tree (Fig. B) is available as supplementary material in IJSEM Online.

DNA–DNA hybridization between strain YIM 70010^T and *C. muralis* DSM 14442^T was carried out by applying the optical renaturation method (De Ley *et al.*, 1970; Huss *et al.*, 1983; Jahnke, 1992) under optimal hybridization conditions. The determined DNA–DNA relatedness value of 56 % (experiment repeated twice) was significantly lower than 70 %, which is considered to be the threshold value for the delineation of genomic species (Wayne *et al.*, 1987).

On the basis of phylogenetic, morphological and chemotaxonomic evidence and its physiological and biochemical distinctiveness (Table 1), it is proposed that strain YIM 70010^T be classified as *Citricoccus alkalitolerans* sp. nov.

Description of *Citricoccus alkalitolerans* sp. nov.

Citricoccus alkalitolerans (al.ka.li′to.le.rans. Arabic article *al* the; Arabic n. *qaliy* ashes of saltwort; Gr. adj. *tolerans* tolerating; N.L. part. adj. *alkalitolerans* referring to the ability of the organism to tolerate alkaline media).

Cells are aerobic, Gram-positive, non-motile, non-spore-forming cocci, of about 0·5–0·8 μm in diameter. Colonies

on PYES medium are light yellow, circular, entire, somewhat convex, opaque and approximately 1·5 mm in diameter after 24 h at 28 °C. Growth occurs between 10 and 37 °C with an optimal growth temperature of 28 °C; no growth is observed at 4 or 40 °C. Optimum growth pH and NaCl concentration are 8·0–9·0 and 0–5 %, respectively. Catalase-positive and oxidase-negative. Urease- and tyrosinase-negative. Tweens 20 and 80, casein and starch are not decomposed. H_2S production and indole production are negative. Nitrate is not reduced to nitrite. Activities for lipase and α-glucosidase are positive. Negative for ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, β-glucuronidase, α- and β-galactosidase, *N*-acetyl-β-glucosaminidase and β-glucosidase. The following substrates are utilized as sole carbon sources for growth (with no acid production): glucose, galactose, sucrose, arabinose, mannose, mannitol, maltose, starch, xylose, ribose, cellobiose, salicin, sorbitol, lactose, dextrin, amygdalin, glycine and lysine. Fructose, *N*-acetyl-α-glucosamine, L-alanine, β-alanine, L-histidine, L-ornithine, L-tryptophan and arginine are not utilized. The peptidoglycan is of the Lys–Gly–Glu type (variation A4α). The predominant menaquinone is MK-9(H_2) and the cellular polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and two unknown glycolipids. The cellular fatty acids are anteiso-$C_{15:0}$ (74·58 %), iso-$C_{15:0}$ (13·14 %), anteiso-$C_{17:0}$ (3·94 %), iso-$C_{16:0}$ (1·78 %), $C_{15:0}$ (1·69 %), iso-$C_{14:0}$ (1·14 %), iso-$C_{13:0}$ (0·3 %), iso-$C_{17:0}$ (0·85 %), anteiso-$C_{13:0}$ (0·9 %), $C_{13:0}$ (0·14 %), $C_{14:0}$ (0·68 %), $C_{13:0}$ (0·14 %) and $C_{16:0}$ (0·86 %). The DNA G + C content is 63·8 mol%.

The type strain, YIM 70010^T (= CCTCC AA 203008^T = DSM 15665^T = KCTC 19012^T), was isolated from an alkaline soil sample collected from the eastern desert of Egypt.

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