Citricoccus zhacaiensis sp. nov., isolated from a bioreactor for saline wastewater treatment

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A Gram-positive, neutrophilic, non-motile and non-spore-forming actinobacterium, strain FS24^T, was isolated from a bioreactor treating salt-containing wastewater. This isolate grew in the presence of 0–15 % (w/v) NaCl and at 10-37 °C. The optimum NaCl concentration for growth of FS24^T was 5 % (w/v) at 37 °C or 1 % (w/v) at 25 °C. Chemotaxonomic analysis revealed MK-9(H_2) as the predominant menaquinone and the major cellular polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, four unknown glycolipids, two unknown phospholipids and an unknown lipid. The major fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The genomic DNA G+C content was 66.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain FS24^T clustered with members of the genus *Citricoccus*, exhibiting high sequence similarity to the 16S rRNA gene sequences of the type strains of *Citricoccus alkalitolerans* (98.9 %) and *Citricoccus muralis* (98.8 %), respectively. The DNA–DNA relatedness values of strain FS24^T to *C. alkalitolerans* DSM 15665^T and *C. muralis* DSM 14442^T were 54 and 39 %, respectively. On the basis of phenotypic and genotypic data, strain FS24^T represents a novel species of the genus *Citricoccus*, for which the name *Citricoccus zhacaiensis* sp. nov. is proposed. The type strain is FS24^T (=CGMCC 1.7064^T =JCM 15136^T).

The genus *Citricoccus* was proposed by Altenburger *et al.* (2002a) and contains two species at the time of writing, *Citricoccus muralis* and *Citricoccus alkalitolerans* (Li *et al.*, 2005). The members of the genus are Gram-positive cocci and have the following chemotaxonomic characteristics: MK-9(H_2) as the predominant menaquinone, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and several unknown lipids as the major polar lipids, and anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$ as the major fatty acids (Altenburger *et al.*, 2002a; Li *et al.*, 2005).

Although the type strains of *C. muralis* and *C. alkalitolerans* displayed more than 99.5 % 16S rRNA gene sequence similarity, the DNA–DNA relatedness value between them (56 %; Li *et al.*, 2005) was lower than the threshold value of 70 % for species delineation (Wayne *et al.*, 1987). Besides, they showed some striking phenotypic differences, such as the type strain of *C. alkalitolerans* being alkalitolerant with optimum growth at pH 8.0–9.0, while that of *C. muralis* prefers neutral environments.

Abbreviations: DPG, diphosphatidylglycerol; GL, unknown glycolipid; L, unknown polar lipid; MK, menaquinone; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FS24^T is EU305672.

A micrograph showing cells of strain FS24^T and the polar lipid profile of strain FS24^T are available with the online version of this paper.

Strain FS24^T was isolated from the water-sludge mixture of a bioreactor treating saline wastewater generated from pickled vegetable production. A 100 µl sample of the liquid mixture was spread on autoclaved wastewater-agar plates. The wastewater used was adjusted to pH 7.0 with NaOH and autoclaved at 121 °C for 30 min beforehand. Plates were incubated aerobically at 30 °C for 5 days. Single colonies were picked up and purified by repeated restreaking on ZC medium. The ZC medium was designed according to the chemical composition analysis of the wastewater and contained (per litre distilled water) 10.0 g NaCl, 1.0 g KCl, 2.0 g $MgCl_2.6H_2O$, 2.0 g Casamino acids (Difco, Becton Dickinson) and 5.0 g Bacto yeast extract (Becton Dickinson), pH 7.5.

The optimum temperature for growth was determined in ZC broth at 4, 10, 20, 25, 30, 37 and 42 °C. The optimal pH for growth was tested at pH 5–10 (at intervals of 1.0 pH unit) in ZC broth using the following buffers at a

concentration of 25 mM: MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0). The salt range for growth was determined in ZC broth with 0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v) NaCl at pH 7.0. Both the pH and salt range tests were performed at 25 °C and 37 °C. Anaerobic growth was tested in an atmosphere of N_2 at 30 °C on ZC agar for 7 days. Cell morphology and motility were examined under an Olympus BX40 optical microscope and a JEOL JEM-1200EX transmission electron microscope.

Carbon source utilization and acid production tests were performed using the medium described by Kämpfer *et al.* (1991). Oxidase and catalase activity, H_2S production, hydrolysis of casein, starch, Tweens 20 and 80, tyrosine and urea, indole production and phenylalanine deamination were tested in ZC medium according to the methods given by Mata *et al.* (2002). Additional enzyme activities were determined by using API ZYM test kits (bioMérieux) according to the manufacturer's instructions. Sensitivity to antimicrobial agents was determined in ZC broth containing each antimicrobial agent at 50 mg l^{-1} for at least 2 days.

Genomic DNA was obtained by using the method described by Marmur (1961). The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). PCR products were cloned into the pMD19-T vector (TaKaRa) and then sequenced to determine the almost complete sequence of the 16S rRNA gene. Phylogenetic affiliation to sequences available in GenBank was determined by using the BLAST program (Altschul *et al.*, 1990). Sequence data were aligned by using CLUSTAL_X (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed by using the neighbour-joining method with the MEGA 4 program (Tamura *et al.*, 2007). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Menaquinones were isolated by using the methods of Collins (1985) and analysed by HPLC. Polar lipids were extracted and examined by two-dimensional thin layer chromatography and were identified by using published procedures (Kamekura & Kates, 1988; Xin *et al.*, 2000). Fatty acid methyl esters obtained from cells grown on ZC agar for 36 h at 30 °C were analysed by using GC/MS (Kuykendall *et al.*, 1988); the results were compared with the database of fatty acids in the MIDI Sherlock Microbial Identification system (MIDI). Cell wall extracts were prepared according to Kawamoto *et al.* (1981) and the qualitative analyses of amino acids in peptidoglycan hydrolysates were carried out by using HPLC as described by Janssen *et al.* (1986). Polyamine analysis was carried out as described by Altenburger *et al.* (1997).

The DNA G+C content was determined by using HPLC according to Mesbah *et al.* (1989). DNA–DNA hybridizations were performed by using the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983) by using a Beckman DU 800 spectrophotometer.

Cells of strain FS24^T were aerobic, Gram-positive, non-motile and ellipsoidal, 0.6 × 0.5 μm in size. Flagella were not observed (Supplementary Fig. S1, available in IJSEM Online). Colonies were yellow-greenish, smooth, circular, convex, opaque and 1–2 mm in diameter after 2 days of incubation at 30 °C on ZC agar. Temperature and pH ranges for growth were 10–37 °C and 6.0–9.0, respectively. Unlike *C. alkalitolerans* DSM 15665^T, no growth was observed at initial pH greater than 10.0. Strain FS24^T grew at 25 °C or 37 °C when NaCl concentrations were 0 to 15 % (w/v), but the optimum NaCl concentration for growth differed with temperature. Optimum conditions for growth in ZC broth were 1 or 5 % (w/v) NaCl at 25 or 37 °C, respectively. Strain FS24^T was able to grow on tryptone soy agar (Oxoid), PYES agar (Altenburger *et al.*, 2002b) and CasMM agar (Altenburger *et al.*, 1996) under aerobic conditions, but no growth was observed under anaerobic conditions.

The major fatty acids (greater than 1 %) of strain FS24^T were anteiso-$C_{15:0}$ (74.1 %), anteiso-$C_{17:0}$ (16.5 %), iso-$C_{15:0}$ (5.5 %) and iso-$C_{16:0}$ (1.7 %). MK-9(H_2) was the predominant menaquinone with moderate amounts of MK-7(H_2) and MK-8(H_2). The major cellular polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, four unknown glycolipids, two unknown phospholipids and an unknown lipid (Supplementary Fig. S2). The peptidoglycan of strain FS24^T contained Ala, Gly, Glu and Lys in a molar ratio of 1.0 : 0.5 : 1.3 : 1.0. Spermidine was the major polyamine component along with various amounts of 1, 3-diaminopropane, putrescine, cadaverine and spermine. The quinone system composition, polar lipid composition, major fatty acid profile, peptidoglycan and polyamine patterns of strain FS24^T were in accordance with the description of the genus *Citricoccus*.

There were some differences that distinguished strain FS24^T from other species of the genus *Citricoccus*. The proportion of anteiso-$C_{15:0}$ in strain FS24^T (74.1 %) was greater than that of *C. muralis* DSM 14442^T (55.6 %), whereas the proportion of iso-$C_{16:0}$ in strain FS24^T (1.7 %) was smaller than that of *C. muralis* DSM 14442^T (8.0 %) (Altenburger *et al.*, 2002a). Physiological and biochemical properties which distinguished strain FS24^T from *C. alkalitolerans* DSM 15665^T and *C. muralis* DSM 14442^T, such as the salt and pH ranges for growth, utilization of hydrocarbons and antibiotic sensitivity, are given in Table 1.

An almost complete 16S rRNA gene sequence for strain FS24^T (1490 nt) was obtained. Analysis of this sequence revealed that the isolate was phylogenetically related to the type strains of *C. alkalitolerans* and *C. muralis*, with similarities of 98.9 and 98.8 %, respectively. The similarity between the type strains of *C. alkalitolerans* and *C. muralis* was 99.6 % (Li *et al.*, 2005). The 16S rRNA gene sequences of the three strains were quite similar; however, relative distance comparison on the phylogenetic tree indicated a closer relationship between *C. alkalitolerans* and *C. muralis* than between either of these species and strain FS24^T (Fig. 1). The DNA–DNA hybridization relatedness values of strain

Table 1. Phenotypic characteristics differentiating strain FS24^T from *Citricoccus muralis* DSM 14442^T and *Citricoccus alkalitolerans* DSM 15665^T

The following phenotypic characteristics are the same for all strains. Gram-positive, catalase-positive and oxidase-negative. Urease, tyrosinase, H_2S production and indole production are negative. Tweens 20 and 80, casein and starch are not decomposed. Nitrate is not reduced to nitrite. The following compounds are utilized as sole carbon sources: acetate, L-glutamate, gluconate, L-glutamine, isoleucine, malate, maltose, propionate, pyruvate, salicin, L-serine, succinate, sucrose, trehalose and L-valine. The following compounds are not utilized as sole carbon sources: adonitol, L-alanine, L-arginine, L-aspartate, cellobiose, L-cysteine, ethanol, formate, D-fructose, fumarate, glycerol, L-histidine, inositol, lactate, lactose, lysine, mannitol, D-mannose, melibiose, L-methionine, L-proline, raffinose, rhamnose, ribose, L-sorbitol, sorbose, xylitol or xylose. Acid production was not observed. In API ZYM tests, acid and alkaline phosphatases, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase are detected. +, Positive; −, negative; R, resistant; S, susceptible.

Characteristic	Strain FS24^T	*C. muralis* DSM 14442^T	*C. alkalitolerans* DSM 15665^T
Growth in 15 % (w/v) NaCl	+	−*	+†
Growth at 37 °C	+	−*	+†
pH range (optimum)	6.0–9.0 (7.0)	6.0–10.0 (7.0)	6.0–10 (9.0)
α-Glucosidase	+	+	−
Utilization of:			
L-Asparagine	−	+	+
Citrate	+	+	−
D-Galactose	−	+	−
Glucose	−	+	+
Glycine	−	+	−
Malonate	+	+	−
Antibiotic sensitivity			
Rifampicin	R	R	S
Streptomycin	R	R	S
DNA G+C content (mol%)	66	68*	63.8†

*Data from: Altenburger *et al.* (2002a).
†Data from: Li *et al.* (2005).

FS24^T with *C. muralis* DSM 14442^T and *C. alkalitolerans* DSM 15665^T were 39.3 % (standard deviation 4.0 %) and 53.9 % (standard deviation 5.0 %), respectively. These values were based on five replicates and were lower than the threshold value of 70 % for species delineation (Wayne *et al.*, 1987).

On the basis of phenotypic, chemotaxonomic and phylogenetic data that distinguish the isolate from other species of the genus *Citricoccus*, we suggest that strain FS24^T represents a novel species of the genus *Citricoccus*, for which the name *Citricoccus zhacaiensis* sp. nov. is proposed.

Description of *Citricoccus zhacaiensis* sp. nov.

Citricoccus zhacaiensis (zha.ca.i.en′sis. N.L. masc. adj. *zhacaiensis* pertaining to zhacai, the Chinese name for preserved vegetables).

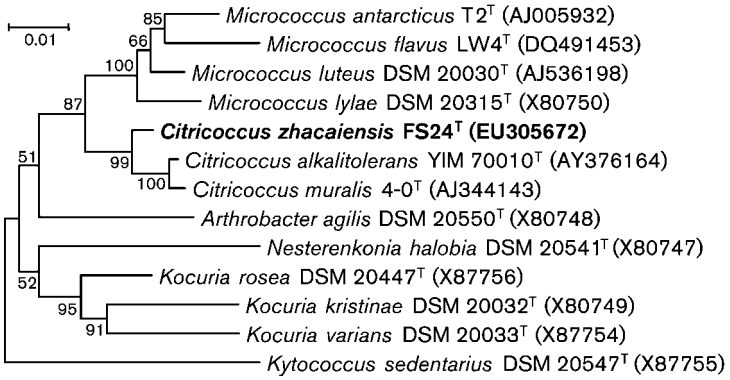

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain FS24^T and related taxa. The dendrogram was reconstructed by using the neighbour-joining method. Evolutionary distances were calculated according to the algorithm of the Kimura two-parameter model. Bootstrap values (based on 1000 replicates) greater than 50 % are shown at nodes. Bar, 0.01 sequence dissimilarity per nucleotide position.

Gram-positive, non-spore-forming and aerobic. Cells are non-motile and ellipsoidal, approximately 0.6×0.5 μm in size. Colonies are yellow-greenish, smooth, circular, convex, opaque and 1–2 mm in diameter. The temperature and pH range for growth are 10–37 °C (optimum 25–35 °C) and pH 6.0–9.0 (optimum pH 7.0). Grows in the presence of 0–15 % (w/v) NaCl, optimum at 1 % (w/v) below 25 °C or 5 % (w/v) below 37 °C. Catalase-positive and oxidase-negative. Casein, starch, Tweens 20 and 80 and urea are not decomposed. Nitrate reduction, H_2S production, phenylalanine deamination, indole production and tyrosinase activity are negative. The following constitutive enzyme activities are detected in API ZYM tests: acid and alkaline phosphatases, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α-glucosidase, lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase. β-Fucosidase, α- and β-galactosidases, β-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase and α-mannosidase are not observed. Chemo-organotrophic. The following compounds are utilized as sole carbon sources: acetate, citrate, L-glutamate, gluconate, L-glutamine, isoleucine, malate, malonate, maltose, propionate, pyruvate, salicin, L-serine, succinate, sucrose, trehalose and L-valine. The following compounds are not utilized as sole carbon sources: adonitol, L-alanine, L-arabinose, L-arginine, L-asparagine, L-aspartate, cellobiose, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, glucose, glycerol, glycine, L-histidine, inositol, lactate, lactose, lysine, mannitol, D-mannose, melibiose, L-methionine, L-proline, raffinose, rhamnose, ribose, L-sorbitol, sorbose, starch, xylitol or xylose. Acid production was not observed. Susceptible to cefotaxime, chloramphenicol, erythromycin, neomycin, novobiocin, polymyxin B and tetracycline, but not to kanamycin, nalidixic acid, nitrofurantoin, nystatin, rifampicin or streptomycin. The predominant menaquinone is MK-9(H_2). The major cellular polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, four unknown glycolipids, two unknown phospholipids and an unknown lipid. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The cell wall amino acids are alanine, glycine, glutamic acid and lysine. Spermidine is predominant in the polyamine pattern. The DNA G+C content of the type strain is 66.0 mol%.

The type strain, FS24^T (=CGMCC 1.7064^T =JCM 15136^T), was isolated from a bioreactor treating wastewater generated from preserved vegetable production.

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