Citreicella marina sp. nov., isolated from deep-sea sediment

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A taxonomic study was carried out on a novel strain, designated CK-I3-6^T, which was isolated from deep-sea sediment of the south-west Indian Ocean Ridge. Cells were Gram-reaction-negative, oxidase- and catalase-positive, rod-shaped and non-motile. Growth was observed at 4–38 °C and in 1–12 % (w/v) NaCl. Cells were able to degrade gelatin and oxidize thiosulfate but did not reduce nitrate. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain CK-I3-6^T belonged to the genus *Citreicella* with a sequence similarity of 97.3 % to *Citreicella thiooxidans* CHLG 1^T, while similarities with other taxa were <95.7 %. DNA–DNA hybridization showed that strain CK-I3-6^T and *C. thiooxidans* CHLG 1^T showed a low DNA–DNA relatedness (48 ± 3 %). The principal fatty acids were $C_{16:0}$ (7.8 %), $C_{18:1}\omega7c$ (66.6 %), summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; 6.3 %) and $C_{19:0}\omega8c$ cyclo (10.0 %). The chromosomal DNA G+C content was 67.5 mol%. On the basis of the combined genotypic and phenotypic data, strain CK-I3-6^T represents a novel species of the genus *Citreicella*, for which the name *Citreicella marina* sp. nov. is proposed. The type strain is CK-I3-6^T (=CCTCC AB 209064^T =LMG 25230^T =MCCC 1A03060^T).

In an attempt to investigate arsenite-resistant bacteria from deep-sea sediment of the south-west Indian Ocean Ridge, many bacterial strains were isolated and characterized taxonomically (Chen & Shao, 2009). This study focused on one of these isolates, designated strain CK-I3-6^T, which was isolated from sediment enriched by arsenite. Comparative 16S rRNA gene sequence analysis indicated that strain CK-I3-6^T was a member of the genus *Citreicella*, which belongs to the family *Rhodobacteraceae* of the class *Alphaproteobacteria*. The genus *Citreicella* was proposed by Sorokin *et al.* (2005) and, at the time of writing, included only one species, *Citreicella thiooxidans*. The aim of the present work was to determine the exact taxonomic position of strain CK-I3-6^T by using a polyphasic approach.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified by PCR using primers that have been described previously (Liu & Shao, 2005). Sequences of related taxa were obtained from the GenBank database. Multiple sequence alignment was performed using DNAMAN software (version 5.1; Lynnon Biosoft) and phylogenetic analysis was performed using MEGA version 4 (Tamura *et al.*, 2007). Distances were calculated according to the Kimura two-parameter model and phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992, 1993) methods with bootstrap values based on 1000 replications. Results obtained using the minimum-evolution approach (data not shown) were similar to those obtained using the neighbour-joining method.

The nearly full-length 16S rRNA gene sequence (1425 bp) of strain CK-I3-6^T was determined. The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) showed that strain CK-I3-6^T and *C. thiooxidans* CHLG 1^T formed an independent monophyletic cluster with a 16S rRNA gene sequence similarity of 97.3 %, while type strains of other species shared sequence similarities <95.7 % with strain CK-I3-6^T. This high similarity strongly confirmed that strain CK-I3-6^T belonged to the genus *Citreicella*.

DNA–DNA hybridization experiments were performed with genomic DNA from strain CK-I3-6^T and *C. thiooxidans* CHLG 1^T by using a previously described method (Liu & Shao, 2005) and a DIG High Prime DNA Labelling and Detection Starter kit II (Roche). The hybridization temperature was 42 °C and the formamide concentration was 50 %. The results showed that strain

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CK-I3-6^T is EU928765.

One supplementary figure and one supplementary table are available with the online version of this paper.

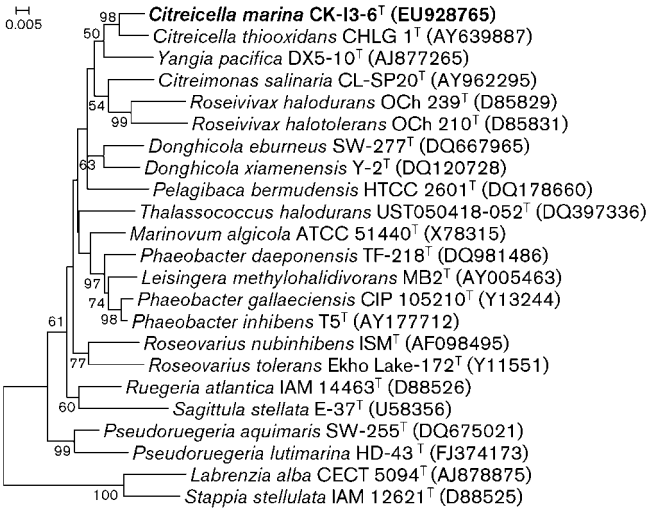

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain CK-I3-6^T and representatives of related taxa. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.005 substitutions per nucleotide position (K_{nuc}).

CK-I3-6^T and *C. thiooxidans* CHLG 1^T showed a DNA–DNA relatedness of 48 ± 3 %, which, in accordance with the cut-off value of 70 % recommended by Wayne *et al.* (1987) for the delineation of bacterial species, was low enough to distinguish them as separate species.

Gram-reaction, activities of catalase, oxidase and lipase (Tween 80), hydrolysis of aesculin and starch, growth at a range of temperatures and pH, tolerance to NaCl, antibiotic susceptibility and general cell morphology by electron microscopy were determined as described previously (Lai *et al.*, 2009). Other biochemical tests were carried out using API 20 NE, API ZYM and API 50 CH strips (bioMérieux) according to the manufacturer's instructions, except that the NaCl concentration was adjusted to 3.0 % in all tests. The ability to oxidize thiosulfate was tested in liquid medium, according to Choi & Cho (2006) with the following modifications. Strain CK-I3-6^T was grown in medium comprising (l^{-1} distilled water): HEPES (10 g); NaCl (20 g); K_2HPO_4 (0.5 g); NH_4Cl (0.5 g); $MgCl_2.6H_2O$ (0.6 g); $CaCl_2.6H_2O$ (0.3 g); sodium acetate (20 mM); sodium thiosulfate (20 mM); and yeast extract (0.05 g). Negative controls were prepared with the same medium but without sodium thiosulfate and without incubation. After 1 week of incubation, 1 ml of each sample was centrifuged to remove the cells and 0.8 ml of the supernatant was mixed with 0.2 ml HCl (10 M) and 0.4 ml $BaCl_2$ (1 M) in a microcentrifuge tube to precipitate the $BaSO_4$. The tube was centrifuged for 3 min at 13 000 r.p.m. to sediment the precipitate. The sediment was mixed with 0.5 ml HCl (10 M) to prevent dissolution. Strain CK-I3-6^T and *C. thiooxidans* CHLG 1^T were tested in parallel for comparison, the results of which are given in the species description and Table 1. All phenotypic assays were carried out at 28 °C, except for the determination of growth at different temperatures.

Whole-cell fatty acids were extracted from cells grown on marine agar (BD) at 28 °C for 48 h, saponified and esterified and the fatty acid methyl esters were analyzed by GC according to the instructions of the MIDI system (Sasser, 1997). The fatty acid profiles of strain CK-I3-6^T and *C. thiooxidans* CHLG 1^T were determined in parallel, the results of which are provided in Supplementary Table S1, available in IJSEM Online. The major fatty acids in both strains were $C_{16:0}$, $C_{18:1}\omega7c$ and $C_{19:0}\omega8c$ cyclo, which accounted for >84 % of the total fatty acids. The fatty acid profile of *C. thiooxidans* CHLG 1^T was similar to that determined previously by Sorokin *et al.* (2005), but additional fatty acids were also detected. The two strains differed in the content of $C_{18:1}\omega7c$ and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$). As the fatty acid profiles of strain CK-I3-6^T and *C. thiooxidans* CHLG 1^T were determined under the same conditions, these differences distinguished strain CK-I3-6^T from *C. thiooxidans* CHLG 1^T.

The G+C content of the chromosomal DNA was determined according to the method described by Mesbah & Whitman (1989) using reversed-phase HPLC. The DNA G+C content of the strain CK-I3-6^T was 67.5 mol%, which is similar to that of *C. thiooxidans* (67.5–69.2 mol%).

Strain CK-I3-6^T was Gram-reaction-negative, non-pigmented, rod-shaped and non-motile (Supplementary Fig. S1), and was positive for sulfur oxidation. The differential physiological, biochemical and chemotaxonomic characteristics of strains CK-I3-6^T and *C. thiooxidans* CHLG 1^T are shown in Table 1. The high 16S rRNA gene sequence similarity between strains CK-I3-6^T and *C. thiooxidans* CHLG 1^T strongly indicated that strain CK-I3-6^T belonged to the genus *Citreicella*, but was distinct from its closest relative, *C. thiooxidans* CHLG 1^T. Based on the results of physiological and chemotaxonomic characterization, phylogenetic analyses and DNA–DNA hybridization, strain CK-I3-6^T represents a novel species of genus *Citreicella*, for which the name *Citreicella marina* sp. nov. is proposed.

Table 1. Differential characteristics of strains CK-I3-6^T and *Citreicella thiooxidans* CHLG 1^T

Strains: 1, strain CK-I3-6^T; 2, *Citreicella thiooxidans* CHLG 1^T. All data from this study unless indicated. Tests in the API 20 NE, API ZYM and API 50 CH systems and tests for catalase, oxidase, tolerance to NaCl and antibiotic susceptibility were performed in parallel with both strains. In the API 20NE system, both strains were positive for D-glucose fermentation, urease and β-galactosidase activities and utilization of D-glucose, maltose, D-mannitol, D-mannose, L-arabinose, malic acid, potassium gluconate and trisodium citrate but negative for nitrate reduction, indole production and utilization of capric acid. In the API ZYM system, both strains were positive for alkaline phosphatase, leucine aminopeptidase and valine aminopeptidase activities and weakly positive for cystine aminopeptidase, esterase (C4), lipase (C14), α-chymotrypsin and β-glucosidase activities but negative for *N*-acetyl-β-glucosaminidase, trypsin, α-fucosidase, α- and β-galactosidase, α-mannosidase and β-glucuronidase activities. In the API 50 CH test system, both strains were positive for D-arabinose, cellobiose, D-fructose, D-fucose, D-glucose, D-lyxose, maltose, D-mannitol, D-mannose, D-ribose, aesculin ferric citrate and L-xylose utilization but negative for starch, amygdalin, arbutin, melezitose, raffinose, D-tagatose, turanose, dulcitol, gentiobiose, glycogen, *myo*-inositol, inulin, L-arabitol, L-sorbose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, *N*-acetylglucosamine and potassium 2-ketogluconate utilization. Both strains were sensitive to (μg per disc; Oxoid) ampicillin (10), carbenicillin (100), cefazolin (30), cefobid (30), chloromycetin (30), ciprofloxacin (5), erythromycin (15), kanamycin (30), minomycin (30), norfloxacin (10), ofloxacin (5), piperacillin (100), rifampicin (5), rocephin (30), streptomycin (10) and vibramycin (30) but resistant to clindamycin (2), Co-trimoxazole (25), lincomycin (2), metronidazole (5) and oxacillin (1). +, Positive; W, weakly positive; −, negative; ND, no data.

Characteristic	1	2
Temperature range for growth (optimal) (°C)	4–38 (25–28)	8–35 (25–28)*
pH range for growth (optimal)	6–9 (6–7)	6.5–8.5 (7.5–7.8)*
NaCl range for growth (optimal) (%, w/v)	1–12 (2–7)	0.5–16 (2)*
Susceptible to (μg unless stated otherwise):		
Cefalexin (30), cephradin (30), penicillin G (10) and polymyxin B (30 IU)	−	+
Gentamicin (10), neomycin (10) and vancomycin (30)	+	−
API 20 NE		
Arginine dihydrolase	−	+
Gelatin hydrolysis	+	−
β-Glucosidase (aesculin hydrolysis)	W	+
Adipic acid, phenylacetic acid	−	+
N-Acetylglucosamine	+	W
API ZYM		
Acid phosphatase	−	+
Naphthol-AS-BI-phosphoamidase	W	−
α-Glucosidase	W	+
Esterase lipase (C8)	+	W
API 50 CH		
D-Adonitol, D-arabitol, D-galactose, lactose, melibiose, D-sorbitol, D-xylose, glycerol, L-arabinose, L-fucose, L-rhamnose and methyl β-D-xylopyranoside	+	−
Sucrose, trehalose, erythritol, potassium 5-ketogluconate, potassium gluconate, salicin and xylitol	−	+
DNA G+C content (mol%)	67.5	67.5–69.2*

*Data from Sorokin *et al.* (2005) for *Citreicella thiooxidans* strains CHLG 1^T and CHLG 2.

Description of *Citreicella marina* sp. nov.

Citreicella marina (ma.ri′na. L. fem. adj. *marina* of the sea, marine).

Cells are Gram-reaction-negative, rod-shaped, 0.9–1 × 1.4–1.6 μm and non-motile. Oxidase- and catalase-positive. Tests are positive for gelatinase, β-glucosidase (weak), β-galactosidase and urease activities, D-glucose fermentation (weak) and sulfur oxidation but negative for nitrate reduction, indole production, starch hydrolysis and lipase (Tween 80) and arginine dihydrolase activities. Cells form smooth, grey colonies with regular edges that are 2–3 mm in diameter, non-pigmented and slightly raised in the centre when grown on marine agar plates at 28 °C for 72 h. Moderately halophilic. Grows in 1–12 % NaCl (optimum 2–7 %), at pH 6–9 (optimum 6–7) and at 4–38 °C (optimum 25–28 °C) but not at 39 °C. The principal fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$, summed

feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and $C_{19:0}\omega8c$ cyclo. Table 1 shows the characteristics that distinguish strain CK-I3-6^T from *C. thiooxidans* CHLG 1^T, including the results of antimicrobial susceptibility tests and tests using API 20 NE, API ZYM and API 50 CH strips.

The type strain, CK-I3-6^T (=CCTCC AB 209064^T =LMG 25230^T =MCCC 1A03060^T), was isolated from deep-sea sediment of the south-west Indian Ocean Ridge. The DNA G+C content of the type strain is 67.5 mol%.

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