Citreicella aestuarii sp. nov., isolated from a tidal flat

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A Gram-negative bacterium, designated AD8^T, was isolated from a tidal flat of the Taean coast in South Korea. Cells were strictly aerobic, motile, ovoid rods or rods, with two lateral flagella on one side. Strain AD8^T was catalase- and oxidase-positive and able to oxidize thiosulfate and reduce nitrate to nitrite. Growth of strain AD8^T was observed at 15–40 °C (optimum 25–30 °C), at pH 5.0–9.5 (optimum pH 6.0–7.5) and with 1.0–11.0 % (w/v) NaCl (optimum 2.0–4.0 %). The predominant fatty acids were C_{18:1}ω7c and C_{19:0} cyclo ω8c. The G + C content of the genomic DNA was 67.5 mol% and the major quinone was Q-10. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain AD8^T belonged to the genus *Citreicella* and was most closely related to *Citreicella marina* CK-13-6^T within the class *Alphaproteobacteria* (96.9 % 16S rRNA gene sequence similarity). On the basis of phenotypic, chemotaxonomic and phylogenetic analysis, strain AD8^T represents a novel species within the genus *Citreicella*, for which the name *Citreicella aestuarii* sp. nov. is proposed. The type strain is AD8^T (=KACC 13699^T =DSM 22011^T).

The genus *Citreicella*, a member of the family *Rhodobacteraceae* in the *Alphaproteobacteria*, was proposed by Sorokin *et al.* (2005). Currently, the genus contains only two species, *Citreicella thiooxidans* (Sorokin *et al.*, 2005; Sorokin *et al.*, 2006) and *Citreicella marina* (Lai *et al.*, 2010), which were isolated from marine environments: an oxygen-sulfide interface of the Black Sea and a deep-sea sediment of the South-west Indian Ocean Ridge, respectively. Both species are moderately halophilic and have the ability to oxidize thiosulfate to sulfate. Coastal seashores are valuable biological resources as they contain a diversity of micro-organisms and marine animals and play important roles in the restoration of ecosystems. Therefore, efforts have been made in our laboratory to isolate and characterize members of the bacterial community from the coastal seashores of the Yellow Sea in South Korea (Kim *et al.*, 2010a, b; Jin *et al.*, 2011; Jung *et al.*, 2011; Lee *et al.*, 2011). Of note for this study, *Yangia pacifica* was isolated from a coastal sediment of the East China Sea.

Strain AD8^T was isolated from a surface tidal flat (depth <3 cm) of the Taean coast (36° 49' 55.04" N 126° 10' 04.68" E) of the Yellow Sea, South Korea, during a low tide

in December 2008, using a previously described procedure with some modifications (Kim *et al.*, 2008). Briefly, the sample was serially diluted in marine broth (MB; Difco), spread on marine agar 2216 (MA; Difco) and incubated at 25 °C for 5 days. PCR amplification from randomly selected colonies was performed using primers 27f and 1492r (Lane, 1991) as described previously (Lu *et al.*, 2006). The resulting 16S rRNA gene sequences were compared with GenBank entries using BLASTN searches (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the search results were used as a guide to classify the colonies. A novel strain belonging to the genus *Citreicella*, designated strain AD8^T, was selected for further characterization. The isolate was routinely grown aerobically on MA at 30 °C for 3 days, except where indicated otherwise, and stored at –80 °C in MB supplemented with 10 % (v/v) glycerol. *C. thiooxidans* DSM 10146^T, *C. marina* LMG 25230^T and *Y. pacifica* JCM 12573^T were purchased from DSMZ (Germany), BCCM/LMG (Belgium) and JCM (Japan), respectively, and used as reference strains for phenotypic characterization and fatty acid analysis.

The 16S rRNA gene amplicon of strain AD8^T was purified and ligated into the pCR2.1 vector using a TOPO cloning kit (Invitrogen), according to the manufacturer's instructions, and sequenced with the M13 reverse and T7 primers of the TOPO cloning kit. The almost-complete 16S rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AD8^T is FJ230833.

A supplementary figure is available with the online version of this paper.

Table 1. Differential phenotypic characteristics of strain AD8^T and its closest phylogenetic neighbours

Strains: 1, *Citireicella aestuarii* sp. nov. AD8^T (this study); 2, *C. marina* LMG 25230^T (Lai *et al.*, 2010); 3, *C. thiooxidans* DSM 10146^T (Sorokin *et al.*, 2005); 4, *Yangia pacifica* JCM 12573^T (Dai *et al.*, 2006). All strains are positive for catalase, oxidase, alkaline phosphatase*, leucine arylamidase*, hydrolysis of aesculin* and assimilation of D-glucose*, D-mannitol* and potassium gluconate*. All strains are negative for Gram-staining, indole production*, hydrolysis of gelatin* and Tween 80*, assimilation of capric acid*, arginine dihydrolase*, β-glucosidase*, N-acetyl-β-glucosaminidase*, α-mannosidase* and sporulation*. OR, Ovoid rods; R, rods; +, positive; (+), weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4
Morphology	OR, R	R	OR	R
Motility	+	–	–	+
NaCl for growth (%)				
Range	1–10	1–12	0.5–6.0	1–10
Optimum	2–4	2–7	2	5
Temperature for growth (°C)				
Range	15–40	4–38	8–35	22–40
Optimum	25–30	25–28	25–28	37
Hydrolysis of starch	–	–	+	+
Oxidation of thiosulfate	+	+	+	ND
API 20 NE*				
Nitrate reduction	+	–	–	+
Glucose acidification	+	–	–	+
Urease	–	+	–	–
Assimilation of:				
Adipic acid	+	–	+	(+)
Malic acid	(+)	+	–	+
Trisodium citrate	–	+	+	–
Phenylacetic acid	–	–	+	(+)
API ZYM*				
Esterase (C4)	–	+	+	+
Esterase lipase (C8)	–	+	+	+
Lipase (C14)	–	–	(+)	–
Cystine arylamidase	–	(+)	+	+
Trypsin	+	–	+	(+)
α-Chymotrypsin	(+)	–	(+)	–
α-Galactosidase	(+)	–	+	(+)
DNA G + C content (mol%)	67.5	67.5	67.5–69.2	63.3

*Data were taken from this study.

sequence (1384 nt) was compared with sequences available in GenBank using BLAST to determine an approximate phylogenetic affiliation. Sequence similarity values between the isolate and related taxa were evaluated using the Identify program of Eztaxon server (version 2.1; available at <http://147.47.212.35:8080/>; Chun *et al.*, 2007) and aligned using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic trees using the neighbour-joining and maximum-parsimony algorithms were reconstructed and evaluated by bootstrap analysis based on 1000 resampled datasets using PHYLIP (version 3.6; Felsenstein, 2002). Maximum-likelihood analysis with

bootstrap values was performed using RAxML-HPC on ABE (version 7.2.6; Stamatakis *et al.*, 2005) of the Cyberinfrastructure for Phylogenetic Research project (CIPRES, www.phylo.org) at the San Diego Supercomputer Center.

Comparative analysis of 16S rRNA gene sequences showed that strain AD8^T was most closely related to *C. marina* CK-I3-6^T and *C. thiooxidans* CHLG 1^T, with which it shared 96.9 and 95.4% 16S rRNA gene sequence similarity, respectively. These values are below the threshold generally accepted for species delineation (Rosselló-Mora & Amann, 2001). The neighbour-joining phylogenetic analysis indicated that strain AD8^T formed a cluster with *C. marina* CK-I3-6^T, with a bootstrap value of 83%, and this was confirmed using the maximum-likelihood and maximum-parsimony methods (Fig. 1).

Growth of strain AD8^T was examined on MA at 4, 10, 15, 20, 25, 30, 37, 40 and 45 °C on MA and at pH 4.5–10.0 (at intervals of 0.5 pH unit) in MB, with the pH adjusted using appropriate biological buffers (Na₂HPO₄/NaH₂PO₄, pH <8.0; Na₂CO₃/NaHCO₃, pH 8.0–11.0; and Na₂HPO₄/NaOH, pH 11.0; Gomori, 1955). The pH was checked after sterilization. Gram-staining was performed using a Gram-stain kit (bioMérieux), according to the manufacturer's instructions. Cell morphology, motility and the presence of flagella were studied using phase-contrast microscopy (Axio Lab.A1; Carl Zeiss) and transmission electron microscopy (JEM-1010; JEOL) with 2-day-old cells grown on MA as described previously (Jeon *et al.*, 2004). Growth with 0–15% (w/v) NaCl (at intervals of 0.5%) was determined using nutrient broth (NB; per litre: 3.0 g beef extract, 5.0 g peptone) made with modified artificial seawater (per litre: 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl,

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain AD8^T and related taxa. Bootstrap values (>50%) are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup. Bar, 0.01 changes per nucleotide position.

1.3 g CaCl₂). Hydrolysis of casein, Tween 80, Tween 20, tyrosine and starch was investigated on R2A agar according to the methods described elsewhere (Lányi, 1987; Smibert & Krieg, 1994). The oxidation of thiosulfate was tested in MB supplemented with 10 mM Na₂S₂O₃ · 5H₂O, as described by Chung *et al.* (2007). Negative controls were prepared with the same medium without inoculation or thiosulfate addition. After aerobic cultivation for 7 days, the concentration of sulfate, the end product of thiosulfate oxidation in MB, was quantified using ion chromatography (ICS-1000; Dionex). Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide (Smibert & Krieg, 1994). Additional enzyme activities and biochemical features were determined using API ZYM and API 20 NE kits (bioMérieux), according to the manufacturer's instructions except that inocula were prepared by suspending cells in 3% (w/v) NaCl. Incubation was at 30 °C. Anaerobic growth was assessed on MA under 4–10% CO₂ using the GasPak Plus system (BBL) at 30 °C for 20 days.

Strain AD8^T grew at 15–40 °C (optimum 25–30 °C), at pH 5.0–9.5 (optimum pH 6.0–7.5) and with 1.0–11.0% (w/v) NaCl (optimum 2.0–4.0% NaCl). Cells were Gram-stain-negative, aerobic, ovoid rods or rods (1.0–1.4 µm wide, 1.6–2.2 µm long) and motile by means of two lateral flagella on one side (Supplementary Fig. S1; available in IJSEM Online). Motility clearly differentiated strain AD8^T from other members of the genus *Citreicella* (Table 1). Anaerobic growth of strain AD8^T was not observed after 20 days at 30 °C on MA. The physiological and biochemical characteristics of strain AD8^T are given in Table 1 and the species description. Some characteristics of strain AD8^T were in accordance with those of members of the genus *Citreicella*, whereas others differed (Table 1).

Isoprenoid quinones were analysed using HPLC (LC-20A; Shimadzu) with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm; Kromasil, Akzo Nobel) as described elsewhere (Komagata & Suzuki, 1987). For analysis of fatty acid methyl esters, cells of strain AD8^T and the reference strains, *C. thiooxidans* DSM 10146^T, *C. marina* LMG 25230^T and *Y. pacifica* JCM 12573^T, were harvested from MA after incubation at 30 °C for 3 days and analysed according to the instructions of the Sherlock Microbial Identification System (MIDI). The DNA G+C content of strain AD8^T was determined using HPLC with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984).

The major respiratory lipoquinone of strain AD8^T was ubiquinone-10. The major cellular fatty acids (>5% of the total fatty acids) of strain AD8^T were C_{18:1}ω7c (56.95%), C_{19:0} cyclo ω8c (15.46%), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 11.39%) and C_{16:0} (10.22%). Although the overall fatty acid profile of strain AD8^T was similar to those of other members of the genus

Citreicella, the presence or absence of some hydroxy fatty acids clearly distinguished the strain (Table 2). The DNA G+C content of strain AD8^T was 67.5 mol%, which is within the range for the genus *Citreicella* (67.5–69.2 mol%).

Therefore, on the basis of physiological, biochemical and phylogenetic analysis, strain AD8^T should be placed into a novel species within the genus *Citreicella*, for which the name *Citreicella aestuarii* sp. nov. is proposed.

Emended description of the genus *Citreicella* Sorokin *et al.* 2006

The genus description is as given by Sorokin *et al.* (2005) with the following modifications. Cells are either non-motile short rods without flagella or motile with flagella. Thiosulfate is oxidized to sulfate. The predominant isoprenoid quinone is Q-10. The major fatty acids are C_{18:1}ω7c, C_{16:0} and C_{19:0} cyclo ω8c.

Table 2. Cellular fatty acid compositions of strain AD8^T and its closest phylogenetic neighbours

Strains: 1, *Citreicella aestuarii* sp. nov. AD8^T; 2, *C. marina* LMG 25230^T; 3, *C. thiooxidans* DSM 10146^T; 4, *Yangia pacifica* JCM 12573^T. All data were taken from this study after growth on marine agar at 30 °C for 3 days. Fatty acids accounting for <0.5% in all strains are not shown. Major components (>10.0%) are highlighted in bold. tr, Trace amount (<0.5%); –, not detected.

Fatty acid (%)	1	2	3	4
Saturated				
C _{10:0}	–	0.50	2.77	–
C _{15:0}	–	0.68	–	–
C _{16:0}	10.22	10.16	28.02	13.34
C _{17:0}	–	0.51	tr	0.74
C _{18:0}	–	–	–	0.75
Unsaturated				
C _{17:0} cyclo	–	0.66	3.3	–
C _{17:1} ω8c	–	0.60	–	–
11-Methyl C _{18:1} ω7c	2.29	0.97	–	3.31
C _{18:1} ω7c	56.95	62.31	24.84	70.42
C _{19:0} cyclo ω8c	15.46	9.86	17.98	3.95
C _{20:2} ω6,9c	1.28	–	–	–
Hydroxyl				
C _{10:0} 3-OH	2.41	–	–	–
C _{12:0} 3-OH	–	tr	4.13	2.52
C _{12:1} 3-OH	–	4.32	–	–
Summed features*				
3	11.39	7.81	17.47	4.96
7	–	tr	0.52	–

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 7 consisted of one or more of C_{19:0} cyclo ω10c, C_{19:1}ω6c and equivalent chain-length 18.846.

Description of *Citricella aestuarii* sp. nov.

Citricella aestuarii (a.es.tu.a'ri.i. L. gen. n. *aestuarii* of a tidal flat, from where the organism was isolated).

Colonies on MA are pale beige, convex, round and non-pigmented with entire margins. Cells are Gram-stain-negative, strictly aerobic rods or ovoid rods (1.0–1.4 µm wide and 1.6–2.2 µm long). Motile by means of two lateral flagella on one side of a cell. Growth occurs at 15–40 °C (optimum 25–30 °C) and pH 5.0–9.5 (optimum pH 6.0–7.5). Moderately halophilic; grows with 1.0–11.0 % (w/v) NaCl (optimum 2.0–4.0 %). Oxidase- and catalase-positive. Thiosulfate is oxidized to sulfate. Nitrate is reduced to nitrite, but nitrogen gas is not produced. With API 20 NE, positive for glucose acidification, assimilation of D-glucose, D-mannitol, gluconate and adipic acid and hydrolysis of aesculin and tyrosine, weakly positive for assimilation of N-acetylglucosamine, maltose and malic acid, but negative for indole production, arginine dihydrolase, assimilation of L-arabinose, D-mannose, capric acid, trisodium citrate and phenylacetic acid and hydrolysis of urea, gelatin, Tweens 20 and 80, casein and starch. With API ZYM, positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, weakly positive for α-chymotrypsin and α-galactosidase, but negative for esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, β-galactosidase, β-glucuronidase, α- and β-glucosidase, N-acetyl-β-glucosaminidase, α-fucosidase and α-mannose. The major isoprenoid quinone is Q-10. The major cellular fatty acids (>10 % of total) are C_{18:1ω7c}, C_{19:0} cyclo ω8c, summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH) and C_{16:0}. The DNA G+C content of the type strain is 67.5 mol% (HPLC).

The type strain is AD8^T (=KACC 13699^T =DSM 22011^T), which was isolated from a tidal flat of the Taean coast in South Korea.

Acknowledgements

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development of the Rural Development Administration (project no. J007451) and the National Research Foundation of Korea of the Korean Government (MEST), Republic of Korea (grant no. 2009-0071880).

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