Tropicimonas arenosa sp. nov., isolated from marine sand

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A Gram-staining-negative, aerobic, non-motile, rod-shaped bacterial strain, designated CAU 1062^T, was isolated from marine sand in Jeju island, Republic of Korea and its taxonomic position was investigated using a polyphasic approach. CAU 1062^T grew optimally at 30 °C and pH 8 in the presence of 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that CAU 1062^T formed a distinct lineage within the genus *Tropicimonas* and was the most closely related to *Tropicimonas sediminicola* M97^T (similarity 96.11 %). The strain had ubiquinone-10 (Q-10) as the predominant respiratory quinone and $C_{18:1}\omega7c$ as the major cellular fatty acid. The polar lipid pattern of CAU 1062^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an aminolipid, six phospholipids and five lipids. The DNA G+C content was 65.7 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic data, CAU 1062^T represents a novel species of the genus *Tropicimonas*, for which the name *Tropicimonas arenosa* sp. nov. is proposed. The type strain is CAU 1062^T (=KCTC 52178^T=NBRC 111995^T).

The genus *Tropicimonas*, of the class *Alphaproteobacteria*, was proposed by Harwati *et al.* (2009). Currently, this genus comprises three species with validly published names (Parte, 2014; http://www.bacterio.net/palleronia.html); *Tropicimonas isoalkanivorans* (Harwati *et al.*, 2009), *Tropicimonas aquimaris* (Oh *et al.*, 2012), and *Tropicimonas sediminicola* (Shin *et al.*, 2012). All members of the genus *Tropicimonas* have been isolated from marine environments; *T. isoalkanivorans* and *T. aquimaris* were from seawater (Harwati *et al.*, 2009; Oh *et al.*, 2012) and *T. sediminicola* was from marine sediment of a cage-cultured ark clam farm (Shin *et al.*, 2012). The members of this genus are Gram-staining-negative, aerobic, non-motile and characterized by the presence of ubiquinone-10 (Q-10) as the dominant quinone. In the course of the screening of bacteria from marine environments, a bacterial strain, designated CAU 1062^T, was isolated from marine sand sample collected from Jeju island, Republic of Korea. The purpose of this study was to investigate the taxonomic position of this bacterial strain by using a polyphasic study that included the determination of phenotypic and chemotaxonomic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Isolation of CAU 1062^T was performed according to the protocol of Gordon & Mihm (1962) using marine agar 2216 (MA; Difco). The sand sample was serially diluted with sterilized 0.9 % sodium chloride solution and plated on MA plates. After incubation under aerobic conditions at 30 °C for 7 days, CAU 1062^T was purified by subculture and preserved at −80 °C in marine broth 2216 (MB; Difco). CAU 1062^T was deposited in the Korean Collection for Type Cultures (KCTC; Taejon, Republic of Korea) and the Biological Resource Center, National Institute of Technology and Evaluation (NITE) (NBRC; Chiba, Japan) as KCTC 52178^T and NBRC 111995^T, respectively. The type strains of closely related species, *T. sediminicola* M97^T, *T. aquimaris* DPG-21^T, and *T. isoalkanivorans* B51^T were used as reference strains in most analyses. *T. aquimaris* DPG-21^T (KCTC 23424^T) and *T. sediminicola* M97^T (KACC 15544^T) were obtained from the Korean Collection for Type Cultures (KCTC; Jeongeup, Korea), and *T. isoalkanivorans* B51^T (JCM 14837^T) was obtained from the Japan Collection of Microorganisms (JCM; Osaka, Japan).

Genomic DNA of CAU 1062^T was extracted and purified using a genomic DNA extraction kit (Intron). The 16S rRNA gene sequence of CAU 1062^T was amplified by PCR using the established procedures (Cho *et al.*, 2008). Sequencing of the amplified 16S rRNA gene was performed using an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple sequence alignments of the three members of the genus *Tropicimonas* with validly published names and closely related strains of the family *Rhodobacteraceae,* and calculation of sequence similarity levels were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1062^T is KU719510.

Two supplementary figures are available with the online Supplementary Material.

made using the CLUSTAL-X 2.1 software (Larkin *et al.*, 2007) and EzTaxon-e–EzBioCloud.net (http://www.ezbiocloud.net/). Evolutionary distance matrices were created by the neighbour-joining method (Jukes & Cantor, 1969). Phylogenetic trees were generated by using the neighbor-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms in the PHYLIP package (Felsenstein, 1989). The tree topology was assessed by the bootstrap resampling method (1000 replications) (Felsenstein, 1985) with neighbor-joining dataset based on the CONSENSE and SEQBOOT programs from the PHYLIP package. The mol% G+C content of the genomic DNA was tested using HPLC following the method of Tamaoka & Komagata (1984).

The almost complete 16S rRNA gene sequence of CAU 1062^T (1406 bp) was compared with the available reference sequences in the GenBank database (access June 2016). Phylogenetic analysis among the strains and members of closely related other genera of the family *Rhodobacteraceae* was performed and the results indicated that the strain clustered with the members of the genus *Tropicimonas*. The neighbour-joining tree is shown in Fig. 1. Pairwise analysis showed that the most closely related species were *T. sediminicola* M97^T, *T. aquimaris* DPG-21^T and *T. isoalkanivorans* B51^T with 16S rRNA gene sequence similarities of 96.11, 95.83 and 94.75 %, respectively. The G+C content of the DNA of CAU 1062^T was 65.7 mol%, which was in accordance with the values of the reference species; 69.6 % (*T. sediminicola* M97^T), 69.6 % (*T. aquimaris* DPG-21^T) and 66.5 % (*T. isoalkanivorans* B51^T).

CAU 1062^T and the three reference strains of species of the genus *Tropicimonas* were cultivated on MA at 30 °C to determine all morphological, biochemical, and physiological characteristics (Harwati *et al.*, 2009), except for spore formation, which was assessed on nutrient sporulation medium (Nicholson & Setlow, 1990). Cell morphology was examined by light microscopy (model DM 1000; Leica). Transmission electron microscopy (TEM, Model JEM 1010, JEOL) was used to detect the presence of flagella of cells from an exponentially growing culture. To confirm of the flagella, a small drop of bacterial suspension was placed on a grid and excess water was removed by filter. The cells on the grid were negatively stained with 1 % (w/v) phosphotungstic acid. After being air-dried to remove stain solution, the image was viewed and photographed using TEM. Gram staining was conducted using the bioMérieux Gram staining kit. Gliding motility was assessed as described by Bowman (2000). The temperature range for growth of CAU 1287^T on MA at 4, 10, 20, 25, 30, 32, 35 37, 45 and 55 °C in an aerobic incubator (model MIR-253; Sanyo) and in an anaerobic chamber (model Bactron; Sheldon) was determined by measuring the turbidity of the broth after 72 h. Growth was determined at 30 °C on MB adjusted to pH 4.5–11.0 at 0.5 pH unit intervals. The pH values of <6, 6–9 and >9 were obtained by using sodium acetate–acetic acid, Tris–HCl and Na_2CO_3 buffers, respectively. Growth in the absence of NaCl and in the presence of 0–15.0 % (w/v) NaCl (at increments of 1 %) was tested as described by Rodriquez-Valera *et al.* (1981) at 30 °C in MB prepared according to the formula of the Difco medium, except that NaCl was excluded and that 0.45 % (w/v) $MgCl_2.6H_2O$ and 0.06 % (w/v) KCl was added (Kim *et al.*, 2016). Catalase activity was investigated by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was investigated from the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Hydrolysis of casein, gelatin and aesculin and nitrate reduction were determined according to the methods of Smibert & Krieg (1994). Acid production from carbohydrates and other biochemical and physiological features were investigated using the API 50CH systems (bioMérieux) at 30 °C. Additionally, enzyme activities were examined using the API 20E, API 20NE and API ZYM kits at 30, 30 and 37 °C, respectively, according to the manufacturer's instructions. Results were read after 24, 48 and 72 h of incubation. Antibiotic susceptibility was determined on MA plates using Sensi-Disc susceptibility test discs (BD Diagnostics) containing the following (μg per disc unless otherwise stated): amoxicillin (20), ampicillin (10), carbenicillin (100), cefoxitin (30), cephalothin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), penicillin (10 U), nalidixic acid (30), polymyxin B (300 U), rifampin (5), streptomycin (10), tetracycline (30), tobramycin (10), and trimethoprim/sulfamethoxazole (1.25/23.75).

Detailed phenotypic features of CAU 1062^T are listed in Table 1 and in the species description. CAU 1062^T was Gram-stain-negative, aerobic and non-motile. Cells were rod shaped approximately 0.2–0.7 μm in diameter and 2.0–3.0 μm in length. Colonies on MA were circular, pale pink and raised with entire margins after 3 days at 30 °C. Flagella were not observed (Fig. S1, available in the online Supplementary Material). CAU 1062^T grew at temperatures between 10 and 37 °C (optimum 30 °C) and at NaCl concentrations between 0 and 8 % (optimum 2 %). Visible growth occurred at pH 6.5–10.5 with the highest growth at pH 8.0. CAU 1062^T didn't hydrolyze gelatin, casein, urea and starch. CAU 1062^T was found to be susceptible to all antibiotics tested. However, phenotypic characteristics data of CAU 1062^T differed from those of closely related species, namely *T. sediminicola* M97^T by its negative reactions for inositol, glycerol, D-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, amygdalin, D-maltose, D-turanose, L-fucose, crystine arylamidase, α-glucosidase, β-glucosidase and β-galactosidase, and positive reactions for D-tagatose (API 20E, API 20NE, API 50CH and API ZYM). These characteristics are sufficient to indicate that CAU 1062^{T}represents a different species from other species of the genus *Tropicimonas*.

Chemotaxonomic properties such as fatty acids and isoprenoid quinone of CAU 1062^T were compared under the same culture conditions used in the three reference strains of species of the genus *Tropicimonas*. For fatty acid analysis, the cell mass of CAU 1062^T and the reference strains; *T. isoalkanivorans* B51^T, *T. aquimaris* DPG-21^T and *T. sediminicola* M97^T

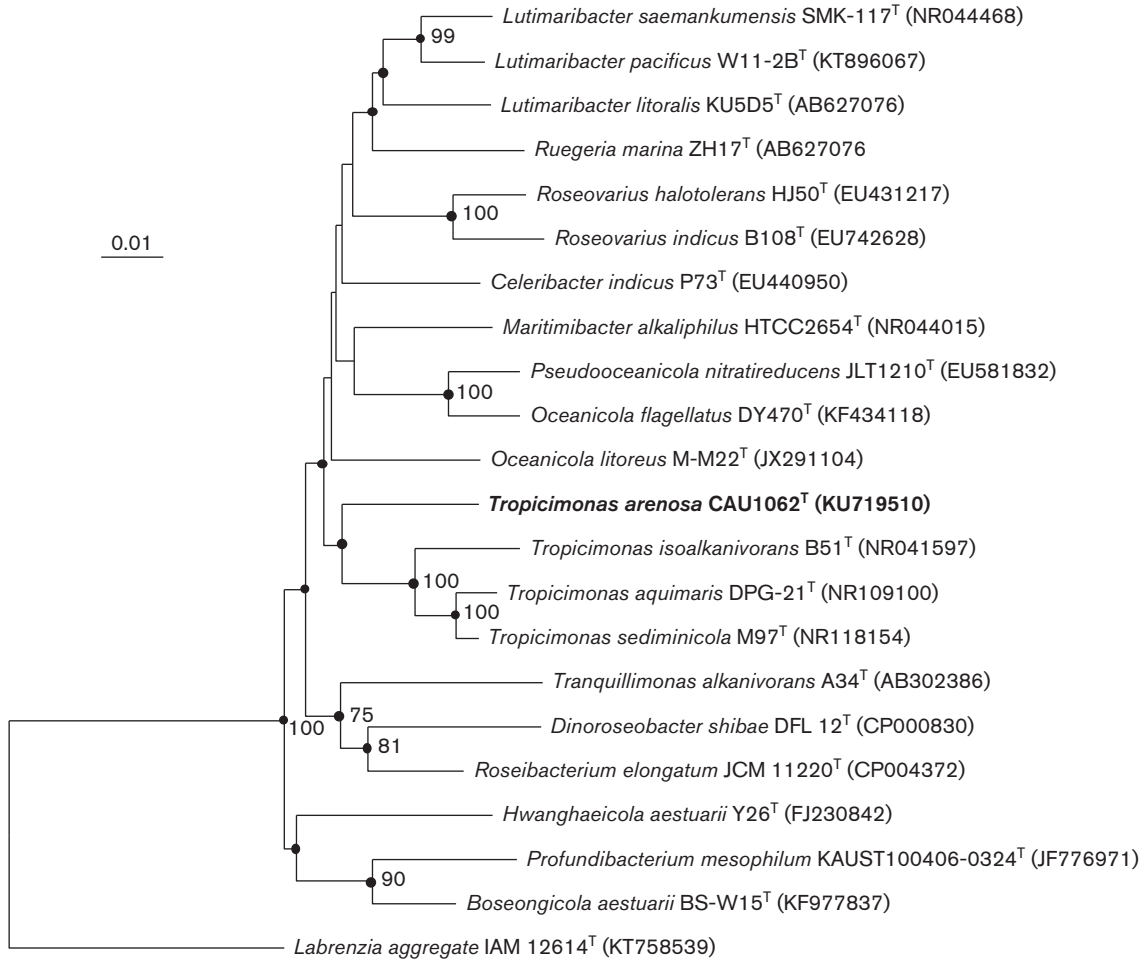

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between CAU 1062^T and the type strains of species of the genus *Tropicimonas* with validly published names. Filled circles indicate that the corresponding nodes were also recovered in the trees generated using the least-squares and maximum-likelihood algorithms. Bootstrap values support based on a neighbor-joining analysis of 1000 replicates; only values >70 % are given. Bar, 0.01 substitutions per nucleotide position. *Labrenzia aggregate* IAM 12614^T (KT758539) is used as an outgroup organism.

were harvested from MA after cultivation for 3 days at 30 °C according to a standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Fatty acid methyl esters (FAMEs) were obtained according to the protocol of Minnikin *et al.* (1980) and separated by an automated gas chromatography system (model 6890N and 7683 autosampler; Agilent). The peaks were determined using the Microbial Identification software package (MOORE library ver. 5.0; MIDI database TSBA6). The polar lipids of CAU 1062^T were extracted according to the method of Minnikin *et al.* (1984) and examined using two-dimensional thin-layer chromatography (TLC). The polar lipid profile was identified by spraying the plates with 10 % ethanolic molybdatophosphoric acid, molybdenum blue, ninhydrin, α-naphthol–sulphuric acid reagent and Dragendorff's reagent (Sigma–Aldrich). Isoprenoid quinones were extracted as described previously (Komagata & Suzuki, 1987) and analyzed by reversed-phase HPLC. The quinones were eluted with an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] using a flow rate of 1 ml min^{-1}.

The major quinone detected in CAU 1062^T was Q-10. This is in agreement with data for the three species of the genus *Tropicimonas* with validly published names, including the type species of the genus, *T. isoalkanivorans* B51^T (Harwati *et al.*, 2009; Oh *et al.*, 2012; Shin *et al.*, 2012). The polar lipids of CAU 1062^T comprised diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine as major polar lipids and an aminolipid, six phospholipids and five lipids as minor polar lipids (Fig. S2). The polar lipid profile of CAU 1062^T was similar to those of the three reference strains in that diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine are major polar lipids.

Table 1. Differential properties of CAU 1062^T and the type strains of the most closely related species of the genus *Tropicimonas*

Strains: 1, CAU 1062^T; 2, *T. sediminicola* M97^T; 3, *T. aquimaris* DPG-21^T; 4, *T. isoalkanivorans* B51^T. Data were taken from this study unless indicated. All strains were positive for hydrolysis of aesculin, for oxidase activity, 4-nitrophenyl-β-D-galactopyranoside, D-mannitol, D-ribose, D-lyxose, alkaline phosphate, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4
Colony color	Pale pink	Pale beige*	Light greyish yellow†	White‡
Motility	−	−*	−†	+‡
Temperature range (°C)	10–37	15–42*	15–40†	10–46‡
Optimum	(30)	(37*)	(30†)	(37‡)
pH range	6.5–10.5	6.0–10.0*	ND†	5.5–8.0‡
Optimum	(8)	(7–8*)	(7–8†)	(7.6‡)
NaCl (%, w/v)	2	1–2*	2†	2‡
Catalase	+	+	+	−
Hydrolysis of urea	−	+	+	+
Utilization of carbon sources:				
Glycerol	−	+	+	+
D-Arabinose	−	+	+	+
D-Xylose	−	+	+	+
D-Glucose	−	+	+	+
D-Fructose	−	+	+	+
D-Mannose	−	+	+	+
D-Mannitol	−	+	+	+
D-Maltose	−	+	+	+
D-Turanose	−	+	+	+
D-Tagatose	+	−	−	+
Enzymes:				
Cystine arylamidase	−	+	+	+
β-Galactosidase	−	+	+	+
α-Glucosidase	−	+	+	+
β-Glucosidase	−	+	+	+
DNA G+C content (mol%)	65.7	68.5*	69.6†	66.5‡

*Data taken from Shin *et al.* (2012).
†Data taken from Oh *et al.* (2012).
‡Data taken from Harwati *et al.* (2009).

Table 2. Cellular fatty acid compositions (%) of CAU 1062^T and the type strains of the most closely related species of the genus *Tropicimonas*

Strains: 1, CAU 1062^T; 2, *T. sediminicola* M97^T; 3, *T. aquimaris* DPG-21^T; 4, *T. isoalkanivorans* B51^T. Data were obtained in this study. Only those fatty acids amounting to >1.0 % in all strains are shown. –, Not detected.

Fatty acids	1	2	3	4
Saturated fatty acid				
$C_{12:0}$	–	1.74	2.37	–
$C_{16:0}$	3.74	5.94	6.55	6.63
$C_{17:0}$	0.27	1.25	1.82	2.34
$C_{18:0}$	9.63	3.86	2.98	3.70
Unsaturated fatty acid				
$C_{18:1}\omega7c$	79.93	64.6	60.2	41.9
Branched fatty acid				
$C_{18:1}$ 11-methyl $\omega7c$	1.71	8.29	7.09	9.96
$C_{19:0}$ cyclo $\omega8c$	–	5.81	2.05	15.84
Hydroxyl fatty acid				
$C_{12:0}$ 3-OH	1.69	4.01	4.45	1.96
Summed Feature 7*	1.49	1.16	0.39	–

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 7 comprises an unknown fatty acid with an equivalent chain length (ECL) of 18.846 and/or $C_{19:1}\omega6c$.

The main cellular fatty acids of CAU 1062^T were $C_{18:1}\omega7c$ (79.93 %), $C_{18:0}$ (9.63 %) and $C_{16:0}$ (3.74 %). The cellular fatty acids profiles of CAU 1062^T and the reference species, which were determined using the same methods under the same conditions, are given in Table 2. The profiles are similar but there were some differences in the proportions of some fatty acids. CAU 1062^T could be distinguished from closely related species by the lower percentage of $C_{12:0}$ 3-OH, $C_{17:0}$ and $C_{18:1}$ 11-methyl $\omega7c$, and the higher percentage of $C_{18:0}$ and $C_{18:1}\omega7c$. Moreover, CAU 1062^T was distinguishable based on absence of $C_{19:0}$ cyclo $\omega8c$.

On the basis of genotypic, phylogenetic, phenotypic and chemotaxonomic data, CAU 1062^T is considered to represent a novel species of the genus *Tropicimonas*, for which the name *Tropicimonas arenosa* sp. nov. is proposed.

Description of *Tropicimonas arenosa* sp. nov.

Tropicimonas arenosa sp. nov. (a.re.no′sa. L. fem. adj. *arenosa* sandy).

Cells are Gram-stain-negative, aerobic, and rod shaped (approximately 0.2–0.7 μm×2.0–3.0 μm). Colonies on MA are pale pink, circular and raised with entire margins after 3 days of incubation at 30 °C. Growth occurs at 10–37 °C (optimum 30 °C), at pH 6.5–10.5 (optimum 8.0) and with 0–8 % (w/v) NaCl (optimum 2 %). Catalase and oxidase are present. Gelatin, starch, urea and casein are not hydrolyzed, whereas aesculin is hydrolyzed. In the API ZYM, API 20E and API 20NE systems, positive for alkaline phosphate, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphtol-AS-BI-

phosphohydrolase, 4-nitrophenyl-β-D-galactopyranoside and D-mannitol. In API 50CH, D-ribose, D-lyxose, D-tagatose, and potassium 5-ketogluconate are utilized for growth. The predominant isoprenoid quinone is Q-10. The major fatty acids are $C_{18:0}$ and $C_{18:1}\omega7c$.

The type strain CAU 1062^T (=KCTC 52178^T=NBRC 111995^T), isolated from marine sand collected from Jeju island in the Republic of Korea. The DNA G+C content of the type strain is 65.7 mol%.

Acknowledgements

This work was supported by the Chung-Ang University Research Scholarship Grant in 2016 and the project on survey of indigenous species of Korea of the National Institute of Biological Resources (NIBR) under the Ministry of Environment (MOE).

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