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Thioclava arenosa sp. nov., isolated from sea sand

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Abstract

A Gram-staining-negative, non-spore-forming, non-motile, rod-shaped, facultatively anaerobe bacterial strain, designated CAU 1312^T, was isolated from sea sand of Eurwangri beach, South Korea. The strain's taxonomic position was investigated using a polyphasic approach. CAU 1312^T grew at temperatures from 20 to 40 °C, in the range of pH 6.0–9.0 and at salinities from 1–4 % (w/v). The results of phylogenetic analysis based on the 16S rRNA gene sequence revealed that CAU 1312^T represented a member of the genus *Thioclava* and was most closely related to *Thioclava atlantica* 13D2W-2^T (similarity 96.53 %). The strain contained Q-10 as the predominant menaquinone and summed feature 8 ($C_{18:1}\omega7c/\omega6c$) as the major fatty acid. The polar lipids of CAU 1312^T consisted of phosphatidylethanolamine, phosphatidylglycerol, two aminophospholipids, a phosphoglycolipid, and two unidentified phospholipids. The DNA G+C content was 64.7 mol%. On the basis of phenotypic and chemotaxonomic properties and phylogenetic inference, CAU 1312^T is considered to represent a novel species of the genus *Thioclava*, for which the name *Thioclava arenosa* sp. nov. is proposed. The type strain is CAU 1312^T(=KCTC 52190^T=NBRC 111989^T).

The genus *Thioclava*, a member of the family *Rhodobacteraceae* was first described by Sorokin *et al.* [1]. At the time of writing, the genus *Thioclava* comprises four species with validly published names, *Thioclavs pacifica* [1], *Thioclava dalianensis* [2], *Thioclava atlantica* [3], and *Thioclava indica* [4]. All members of this genus have been isolated from diverse marine environments. In the course of the screening of bacteria from sea shore, strain CAU 1312^T was isolated from a sea sand sample collected from Eurwangri beach (37° 26′ 55.8″ N 126° 22′ 15.7″ E), Incheon in the Republic of Korea. The purpose of the study was to establish the taxonomic position of this bacterial strain by using a polyphasic approach that included the determination of phenotypic and chemotaxonomic properties and phylogenetic inference based on 16S rRNA gene sequences.

Selective isolation of CAU 1312^T was done according to the protocol of Gordon and Mihm [5] by the standard dilution plate technique. The appropriate dilutions were spread on marine agar 2216 (MA; Difco) plates and incubated under aerobic conditions at 30 °C for 7 days. A single colony of CAU 1312^T was sub-cultured on MA at 30 °C for 5 days. The strain was maintained at −80 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol. *T. pacifica* DSM 10166^T and *T. dalianensis* DSM 29618^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany), while *T. atlantica* LMG 27145^T and *T. indica* KCTC 33533^T were obtained from the Belgian Co-Ordinated Collection of Microorganisms (BCCM/LMG) and the Korean Collection for Type Cultures (KCTC; Jeongeup, Korea), respectively. These strains were used as reference strains in biochemical characterization and fatty acid analysis.

Genomic DNA was extracted according to the method of Marmur [6] and the 16S rRNA gene was amplified by PCR using 27F/1525R universal primers [7]. The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing Kit and an automatic DNA sequencer (model 3730; Applied Biosystems). The similarity of the 16S rRNA gene sequences and sequences for related taxa were obtained by EzTaxon-e – EzBioCloud.net (www.ezbiocloud.net). The sequence data were aligned with those for a member of the genus *Thioclava* using CLUSTAL-X 2.1 software [8]. Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes and Cantor [9]. Phylogenetic trees were reconstructed using the neighbour-joining [10], least-squares [11] and maximum-likelihood [12] algorithms in the PHYLIP package [13]. Tree topology was evaluated by the bootstrap resampling method [14] with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. The mol% G+C content of the genomic DNA was determined using HPLC by the method of Tamaoka and Komagata [15].

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1312^T is KU671051.

Two supplementary figures are available with the online Supplementary Material.

The almost complete 16S rRNA gene sequence of CAU 1312^T (1442 nt) was obtained and compared with available reference sequences in the GenBank database (accessed September 2016). The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences indicated that CAU 1312^T and the four reference strains cluster separately from other genera and that CAU 1312^T clustered with the members of the genus *Thioclava* (Fig. 1). CAU 1312^T exhibited the highest sequence similarity to *T. atlantica* 13D2W-2^T (96.53 %), followed by *T. pacifica* TL 2^T (96.46 %), *T. indica* DT23-4^T (95.26 %), and *T. dalianensis* DLFJ1-1^T (95.12 %). Moreover, the phylogenetic relatedness was found to be similar to the neighbour joining tree for the least-squares and maximum-likelihood algorithms. The DNA G+C content of CAU 1312^T was 64.7 mol%.

CAU 1312^T and the four reference strains; *T. atlantica* LMG 27145^T, *T. pacifica* DSM 10166^T, *T. dalianensis* DSM 29618^T, and *T. indica* KCTC 33533^T were cultivated on MA plates and incubated at 30 °C to examine all morphological, physiological and biochemical characteristics tests except gliding motility and NaCl tolerance. Cell morphology was examined by light (model DM 1000; Leica) and transmission electron (TEM, JEM 1010, JEOL) microscopy, using cells from an exponentially growing culture. For TEM examination, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried (Fig. S1, available in the online Supplementary Material). Gram staining was carried out using a Gram staining kit (bioMérieux) according to the manufacturer's instructions. Gliding motility was examined on a MB culture for 72 h using the hanging-drop method [16]. For observation of growth conditions, CAU 1312^T was cultured on MA and incubated at 4, 10, 20, 25, 30, 37, 40 and 45 °C in an aerobic incubator (Sanyo) and in a Bactron anaerobic chamber (Sheldon). Growth was tested at 30 °C in MB adjusted to pH 4.0–11.5 at increments of 0.5 pH units by using sodium acetate/acetic acid and Na_2CO_3 buffers. The NaCl tolerance was determined using minimal salt medium (33.9 g Na_2HPO_4 l^{-1}, 15 g KH_2PO_4 l^{-1}, 5 g NH_4Cl l^{-1}, pH 7.5) supplemented with 0–15.0 % (w/v) NaCl. Oxidase activity was evaluated with 0.1 % (w/v) tetramethyl-*p*-phenylenediamine [17]. Catalase activity was determined by observing bubble production in 3 % (v/v) H_2O_2 solution. Hydrolysis of gelatin, casein, starch and citrate were determined according to the methods of Lányi [18] and Smibert and Krieg [19]. Biochemical characterizations tests were carried out using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions. Utilization of substrates as carbon sources were tested with API 50 CH and by the conventional method as described by Yu *et al.* [20]. Antibiotic susceptibility was examined on MA at 30 °C by the disc diffusion method by using Sensi-Disc susceptibility test discs (BD BBL). The following antibiotics were examined (μg per disc unless stated otherwise): amoxicillin (20), ampicillin (10), carbenicillin (100), cefoxitin (30), cephalothin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), penicillin (10 U), polymyxin B (300 U), rifampin (5), streptomycin (10), tetracycline (30), tobramycin (10), nalidixic acid (30) and trimethoprim/sulfamethoxazole (1.25/23.75). An inhibition zone over 10 mm in diameter indicated susceptibility and the absence of inhibition zones indicated resistance.

The morphological, cultural, physiological and biochemical characteristics of CAU 1312^T and reference strains are given in Table 1 and in the species description. CAU 1312^T was Gram-stain-negative, non-motile, non-spore-forming, and facultatively anaerobic. Cells were rod shaped, approximately 0.1–0.4 μm × 1.5–3.1 μm and a flagellum was not observed (Fig. S1). CAU 1312^T can grow at 20–40 °C (optimum 30 °C) but not at 45 °C and can grow at pH 6.0–9.0 (optimum, pH 6.5) but not at pH 4.0–5.5 or 9.5–11.5 and can grow at salinities from 1–4 % (w/v) [optimum, 2 % (w/v)]. Oxidase and catalase tests are positive. CAU 1312^T hydrolyzed citrate but not gelatin, starch or casein. The phenotypic characteristics of CAU 1312^T differed from those of four closely related species, namely *T. pacifica* TL 2^T, *T. dalianensis* DLFJ1-1^T, *T. atlantica* 13D2W-2^T, and *T. indica* DT23-4^T with respect to its negative reaction for β-galactosidase and assimilation of D-mannitol, maltose and malic acid. CAU 1312^T was found to be susceptible to erythromycin, rifampin, polymyxin B, amoxicillin, carbenicillin, streptomyxin, cefoxitin, cephalothin, penicillin, gentamycin, chloramphenicol and ampicillin but resistant to nalidixic acid, tetracycline, kanamycin, trimethopin/sulfamethoxazole and tobramycin. These characteristics are sufficient to indicate that strain CAU 1312^T is distinct from other species of the genus *Thioclava*.

For analysis of fatty acids of whole cells, cell masses of CAU 1312^T and the four reference strains were harvested from MA after cultivation for 3 days at 30 °C, pH 6.5, with 2 % NaCl and the cells were harvested at exponential phase. The physiological age of the biomass harvested for fatty acid analysis was standardized by observing growth development during incubation of the cultures and choosing the moment of harvesting according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Cellular fatty acid methyl esters (FAMEs) were obtained according to the methods of Minnikin *et al.* [21] and separated using a 6890 N automated gas chromatography system (Agilent). Peaks were identified by using the Microbial Identification software package (MOORE library ver. 5.0; MIDI database TSBA6). Major quinones were extracted according to the protocol of Komagata and Suzuki [22] and analyzed by HPLC. The major quinones were eluted by an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] using a flow rate of 1 ml min^{-1}. The polar lipids of CAU 1312^T were separated by using a TLC method (two-dimensional thin-layer chromatography) according to the protocol of Minnikin *et al.* [23]. The plates were sprayed with 10 % ethanolic molybdatophosphoric acid, molybdenum blue, ninhydrin, α-naphthol/sulphuric acid reagent, and Dragendoff reagent (Sigma-Aldrich).

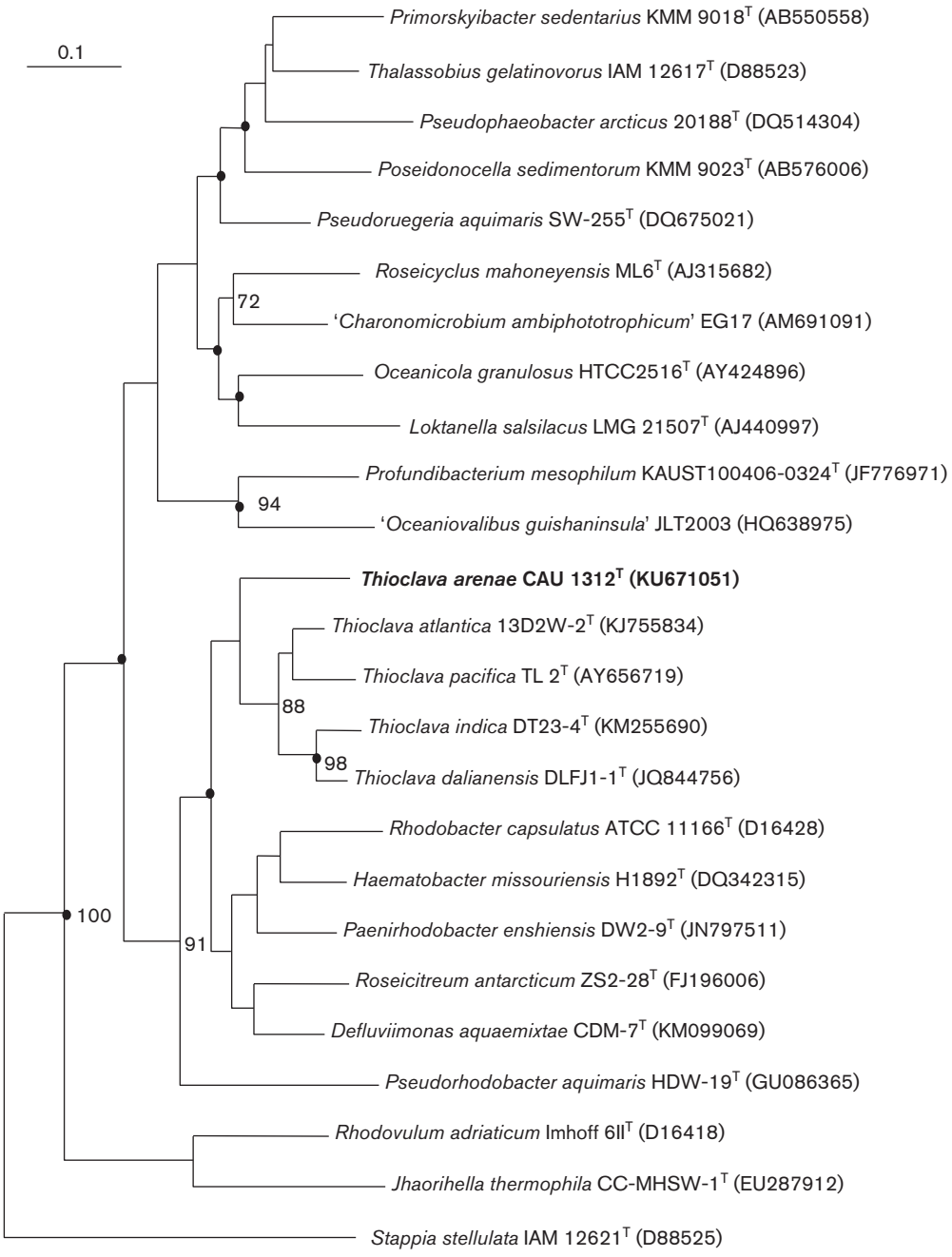

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of CAU 1312^T and representatives of some other related taxa based on 16S rRNA gene sequences. Filled circles indicate nodes that were also recovered in maximum-likelihood and least squares algorithms. Bootstrap values >70 % are shown based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 0.1 substitutions per nucleotide position. *Stappia stellulata* IAM 12621^T (D88525) was used as an outgroup organism.

The quinone of CAU 1312^T was identified as Q-10, which was similar to the phenotypes of the four reference species of genus *Thioclava*. The polar lipids were found to comprise phosphatidylethanolamine, phosphatidylglycerol, two aminophospholipids, a phosphoglycolipid and two unidentified phospholipids (Fig. S2), these polar lipids patterns are similar to those of the species of genus *Thioclava* and agree with that of the most closely related type strain *T. atlantica* 13D2W-2^T [3]. However, a significant difference was discovered for *T. indica* DT23-4^T, due to its absence of aminophospholipid [4] and this strain is different from the descriptions in all references with respect to the presented phosphoglycolipid. The major fatty acids of the novel strain were summed feature 8 ($C_{18:1}\omega6c/\omega7c$) (Table 2) and the

Table 1. Differential characteristics of CAU 1312^T from closely related species in the genus *Thioclava*

Strains: 1, CAU 1312^T; 2, *Thioclava atlantica* LMG 27145^T; 3, *Thioclava pacifica* DSM 10166^T; 4, *Thioclava dalianensis* DSM 29618^T; 5, *Thioclava indica* KCTC 33533^T. Data from this study. +, Positive; –, negative; w, weakly positive.

Characteristics	1	2	3	4	5
Motility	–	+	–	–	–
Temperature range (°C)	20–40	4–41	15–47	4–37	10–41
NaCl range (%)	1–4	0.5–12	1–9	0.5	0–18
pH range	6–9	6–8	6.5–8.5	6–9	6–10
Nitrate reduction	–	+	–	–	+
Hydrolysis of:					
Citrate	–	+	–	–	–
Aesculin	–	+	+	+	+
API 20NE					
D-glucose	+	w	–	–	–
Urease	–	+	–	–	–
β-galactosidase	–	+	+	+	+
Assimilation of					
L-arabinose	–	+	+	+	–
D-mannose	–	+	–	–	–
D-mannitol	–	+	+	+	+
N-Acetyl-glucosamine	–	–	+	+	–
Maltose	–	+	+	+	+
Potassium gluconate	–	w	+	+	–
Capric acid	–	w	–	–	–
Adipic acid	–	w	–	–	w
Malic acid	–	+	+	+	+
API ZYM					
Alkaline phosphatase	–	+	+	+	+
Esterase	+	+	+	–	+
Esterase lipase	+	+	+	–	+
Valine arylamidase	+	+	+	–	+
Crystine arylamidase	+	+	–	–	+
Trypsin	w	–	–	–	–
Napthol-AS-BI-phosphohydrolase	w	w	+	+	–
α-galactosidase	–	–	–	+	+
β-galactosidase	–	–	–	–	+
α-glucosidae	–	+	+	+	+
β-glucosidase	–	+	+	+	+
Acid production from:					
L-arabinose	+	–	–	+	–
D-xylose	–	–	–	w	–
D-galactose	–	–	–	w	–
D-glucose	–	w	–	w	–
D-ructose	–	w	w	w	–
D-mannitol	–	–	w	–	–
Aesculin ferric citrate	–	+	+	+	+
Cellobiose	–	w	w	w	–
Maltose	–	w	w	w	–
Lactose	–	–	–	w	–
Melibiose	–	–	–	w	–
D-sucrose	–	w	w	w	–
Trehalose	–	–	w	–	–
Turanose	–	w	–	–	–
D-fucose	–	w	–	+	–
L-fucose	–	–	–	–	w
D-arabitol	–	w	w	w	–
Potassium 5-ketogluconate	+	–	–	–	–
DNA G+C content (mol%)	64.7	65.3	63.9	62.5	60.3

fatty acid profile is similar to those of members of the most closely related genus, including the type species of the genus, *T. pacifica* TL 2^T.

On the basic of the data described above, CAU 1312^T should be classified as a representative of a novel species of the genus *Thioclava*, for which the name *Thioclava arenosa* sp. nov. is proposed.

DESCRIPTION OF *THIOCLAVA ARENOSA* SP. NOV.

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

Cells are Gram-stain-negative, rod-shaped, 0.1–0.4 µm wide and 1.5–3.1 µm long, non-motile and facultatively anaerobic. Colonies on marine agar plates are circular, smooth, convex and pale pinkish after 5 days of incubation at 30 °C. Growth occurs at 20–40 °C (optimum, 30 °C), at pH 6.0–9.0 (optimum, 6.5) and with 1–4 % (w/v) NaCl (optimum, 2 %). Oxidase and catalase are positive. Casein, gelatin and citrate are not hydrolysed. Nitrate is not reduced. In assays with the API 20NE system, activity of D-glucose is present but activity of arginine dihydrolase and β-galactosidase are absent. Assimilation of D-glucose is positive but L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose and potassium gluconate are negative. In assays with the API ZYM system, activities of esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase and acid phosphatase are present, activities of trypsin and naphthol-AS-BI-phosphohydrolase are weakly present. Acid production from L-arabinose and potassium 5-ketogluconate is positive but acid is not produced from glycerol, erythritol, D-arabinose, ribose, xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, *N*-acetylglucosamine, aesculin, ferric citrate, salicin, cellobiose, maltose, lactose, melibiose, D-sucrose, trehalose, inulin, starch, fucose or potassium 2-ketogluconate. The major fatty acid is summed feature 8 ($C_{18:1}\omega6c/\omega7c$). The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, two aminophospholipids, a phosphoglycolipid and two unidentified phospholipids. The menaquinone of this species is Q-10.

The type strain CAU 1312^T (=KCTC 52190^T=NBRC 111989^T), was isolated from a sea sand collected from

Table 2. Cellular fatty acid compositions (percentages) of CAU 1312^T and the type strains of the most closely related species of the genus *Thioclava*

Strains: 1, CAU 1312^T; 2, *Thioclava atlantica* LMG 27145^T; 3, *Thioclava pacifica* DSM 10166^T; 4, *Thioclava dalianensis* DSM 29618^T; 5, *Thioclava indica* KCTC 33533^T. Data from this study. –, Not detected; TR, trace (<1.0 %).

Fatty acids	1	2	3	4	5
$C_{9:0}$	TR	–	–	–	TR
$C_{12:0}$	TR	–	–	–	–
$C_{16:0}$	6.4	1.9	3.4	6.3	2.1
$C_{17:0}$	4.6	1.3	3.2	TR	TR
$C_{18:0}$	5.9	2.7	10.0	3.8	3.7
$C_{18:0}$ 3-OH	–	2.5	2.5	2.7	2.2
$C_{10:0}$ 3-OH	2.9	2.8	–	2.7	2.7
$C_{11:0}$ 3-OH	TR	–	–	–	–
Iso-$C_{15:1}$	TR	–	–	–	TR
$C_{17:1}\omega 8c$	TR	TR	–	–	TR
Summed feature 3*	14.0	1.2	–	TR	2.7
Summed feature 8*	65.1	71.6	65.5	55.8	73.7

*Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3, $C_{16:1}\omega 6c/\omega 7c$; Summed feature 8, $C_{18:1}\omega 6c/\omega 7c$.

Eurwangri beach in Incheon, Republic of Korea. The DNA G+C content of the type strain is 64.7 mol%.

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Conflicts of interest
The authors have declared that no competing interests exist.

Ethical statement
The authors have declared that no ethical issues exist.

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