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ORIGINAL PAPER

Thioclava atlantica sp. nov., isolated from deep sea sediment of the Atlantic Ocean

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Abstract A taxonomic study was carried out on strain 13D2W-2^T, which was isolated from a sulphur-oxidizing bacterial consortium, enriched by the deep-sea sediment of the Atlantic Ocean. The isolate was observed to be Gram-negative, oxidase- and catalase-positive, short rod-shaped and motile by means of a flagellum. Growth was observed at salinities from 0.5 to 12 % and at temperatures from 4 to 41 °C, and the strain found to be able to reduce nitrate but not degrade gelatin. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain 13D2W-2^T belongs to the genus *Thioclava*, with highest sequence similarity of 97.8 % to *Thioclava dalianensis* DLFJ1-1^T, followed by *Thioclava pacifica* TL 2^T (97.7 %), while the sequence similarities to other members of the genus were all below 97.0 %. The digital DNA:DNA hybridization estimated values between strain 13D2W-2^T and, respectively, *T. dalianensis* DLFJ1-1^T and *T. pacifica* TL 2^T were 22.6 ± 2.4 and 25.6 ± 2.4 %. The ANI values between strain 13D2W-2^T and *T. dalianensis* DLFJ1-1^T and *T. pacifica* TL 2^T were 78.49 and 81.91 % respectively. The principal fatty acid identified was Summed Feature 8 ($C_{18:1}\omega7c/\omega6c$) (74.38 %). The isoprenoid quinone of strain 13D2W-2^T was identified as Q10 (100 %). The major polar lipids of strain 13D2W-2^T were found to be comprised of phosphatidylethanolamine, phosphatidylglycerol, an aminophospholipid, a glycolipid and three unknown phospholipids. The G+C content of the chromosomal DNA was determined to be 65.3 mol%. The combined genotypic and phenotypic data show that strain 13D2W-2^T represents a novel species of the genus *Thioclava*, for which the name *Thioclava atlantica* sp. nov. is proposed, with the type strain 13D2W-2^T (= MCCC 1A02612^T = LMG 27145^T).

Keywords *Thioclava atlantica* sp. nov. · Atlantic Ocean · Taxonomy · Deep-sea sediment

Shaoneng Li and Qiliang Lai contributed equally to the paper.

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Abbreviations

MCCC Marine culture collection of China

Introduction

The global sulfur cycle is closely connected to the cycling of carbon, nitrogen, phosphorus and iron,

which depends on the activities of metabolically and phylogenetically diverse microorganisms, most of which reside in the ocean (Sievert et al. 2007). One of these sulfur related metabolic groups is the sulfur-oxidizing bacteria (SOB), of which marine SOB thrive in low-oxygen environments (Marshall and Morris 2013). In an attempt to investigate SOB in the deep sea environment of the Atlantic Ocean, many bacterial strains were isolated and characterized taxonomically. This study focuses on one of those isolates, designated strain 13D2W-2^T. Comparative 16S rRNA gene sequence analysis indicated that strain 13D2W-2^T belongs to the genus *Thioclava*, which belongs to the family *Rhodobacteraceae*. The genus *Thioclava* was proposed by Sorokin and colleagues (Sorokin et al. 2005) and currently includes two species *Thioclava pacifica*, the type species, and *Thioclava dalianensis* (Zhang et al. 2013). Accordingly, the aim of the present work was to determine the exact taxonomic position of strain 13D2W-2^T using polyphasic characterization.

Materials and methods

Bacterial strains, isolation, and cultivation

Sediment was sampled from the deep sea of the Atlantic Ocean in 2011 by a TV-guided grab sampler. The sampling site was DY22III-S013-TVG7 (W13.9°, S26.0°). The sediment was used for enrichment of SOB. The enrichment and isolation were conducted in DSMZ medium 1121 (MMJS medium) with modification of the NaCl concentration to be 30 g/L. The last four sterile concentrated components (Vitamin solution, $NaHCO_3$, $Na_2S_2O_3$·$5H_2O$ and sulfur) were added to the medium after the other medium components (adjusted with NaOH to be pH 6.8) were autoclaved. The microaerobic condition for sample enrichment was under a gas phase of 80 % H_2/19 % CO_2/1 % O_2 (200 kPa) in a glass bottle tightly sealed with a butyl-rubber cap at 25 °C. After successfully enrichment, the isolation of the bacterial consortium was attempted on plates of the same medium solidified with agar (1.5 % w/v) in a closed jar (Anoxomat Mart II) using a standard microaerobic condition by filling with 6 % O_2 (N_2 78 %, CO_2 10 % and H_2 6 %) according to the manufacturer's instructions. Then the strains were incubated on 216L agar medium (CH_3COONa 1.0 g, tryptone 10.0 g, yeast extract 2.0 g, sodium citrate 0. 5 g, NH_4NO_3 0.2 g, 1 L sea water, 15 g agar, pH 7.5) (Lai et al. 2009) at normal atmosphere conditions for morphological and biochemical characterization.

Phenotypic characterization

Gram reaction, oxidase, catalase and lipase (Tween 80) activities, tolerance to NaCl, the optimal growth temperature and pH, hydrolysis of aesculin and starch, general cell morphology and electron microscopy were studied as previously described (Lai et al. 2009). Antibiotic susceptibility tests were performed by the disc diffusion method as described previously (Shieh et al. 2003). Other biochemical tests were carried out using API 20NE and API ZYM strips (bioMérieux) and the Biolog GN2 MicroPlate test panel according to the manufacturer's instructions, except adjusting the NaCl concentration in all tests to 3.0 %. The oxidization of inorganic sulfur compounds (thiosulfate, sulfur and sulfide) were tested in mixotrophic growth medium supplied with acetate (2.72 g/L) and yeast extract (0.2 g/L) according to the method described previously (Sorokin et al. 2005). *T. pacifica* TL 2^T and *T. dalianensis* DLFJ1-1^T were obtained as described in our earlier study and were cultured under the same conditions (Zhang et al. 2013).

Determination of 16S rRNA gene sequence and phylogenetic analysis

Genomic DNA was extracted according to a previously described method (Ausubel et al. 2002). The 16S rRNA gene was amplified by PCR using primers that have been described previously (Liu and Shao 2005) and also extracted from the draft genome sequence. The 16S rRNA gene sequence similarity was determined using the EzTaxon-e server (Kim et al. 2012). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 5.0 (Tamura et al. 2011) with distance options according to the Kimura two-parameter model and clustering with the neighbour-joining (Saitou and Nei 1987), maximum likelihood (Felsenstein 1981), and minimum evolution (Rzhetsky and Nei 1992) methods, and supported with bootstrap values based on 1,000 replications.

Genome sequencing, G + C content, dDDH and ANI estimation

The draft genome sequences of strain 13D2W-2^T, *T. dalianensis* DLFJ1-1^T and *T. pacifica* TL 2^T were determined by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China), using Solexa paired-end (500 bp library) sequencing technology. A total of 500 Mbp clean data for each strain was generated to reach about 100-fold depth of coverage with an Illumina/Solexa Genome Analyzer IIx (Illumina, SanDiego, CA). The clean data was assembled by SOAPdenovo2 (Luo et al. 2012). The G+C content of the chromosomal DNA was determined by analysis of the draft genome sequences. The digital DNA:DNA hybridization (dDDH) estimate values between the three strains were analyzed using the genome-to-genome distance calculator (GGDC2.0) (Auch et al. 2010a, 2010b; Meier-Kolthoff et al. 2013). The average nucleotide identity (ANI) was calculated using the algorithm of Goris et al. (2007) using the EzGenome web service.

Determination of fatty acid, isoprenoid quinone, and polar lipid compositions

Fatty acids in whole cells grown on marine agar 216L medium at 28 °C for 48 h were saponified, methylated and extracted using the standard MIDI (Sherlock Microbial Identification System, version 6.0B) protocol. The fatty acids were analysed by gas chromatography (Agilent Technologies 6850) and identified using the TSBA6.0 database of the Microbial Identification System (Sasser 1990). Analysis of the respiratory quinone and polar lipid were carried out by the Identification Service of DSMZ and Dr. B. J. Tindall, DSMZ, Braunschweig, Germany.

Results and discussion

Phenotypic characteristics

Strain 13D2W-2^T was observed to be a Gram-negative, non-pigmented, short rod-shaped bacterium that was motile by means of a flagellum (see Fig S1). Moderately halophilic, 13D2W-2^T was found to be able to grow in 0.5–12 % of NaCl (optimum 3–5 %) and at 4–41 °C (optimum 28–37 °C), but not at 45 °C. The strain was found to be sensitive to ampicillin (10 μg/per disk, OXOID), carbenicillin (100), cefalexin (30), cefazolin (30), cefobid (30), cephradin (30), chloromycetin (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), minomycin (30), ofloxacin (5), oxacillin (1), penicillin G (10), piperacillin (100), polymyxin B (30IU), rifampicin (5), rocephin (30), streptomycin (10), tetracycline (30), vancomycin (30), and vibramycin (30); and resistant to clindamycin (2), Co-trimoxazole (25), lincomycin (2), norfloxacin (10 μg) and metronidazole (5). Strain 13D2W-2^T can oxidize reduced sulfur compounds such as thiosulfate, sulfur and sulfide as energy source. Sulfate was the most oxidised product from thiosulfate, while sulfite was the main product from sulfide and sulfur used by this strain after culture for 3 days.

16S rRNA gene sequence analysis

A nearly full-length 16S rRNA gene sequence (1433 nt, GenBank accession number KJ755834) of strain 13D2W-2^T was determined. As shown in Fig. 1, phylogenetic trees based on 16S rRNA gene sequences showed that strain 13D2W-2^T and the two species of the genus *Thioclava* formed an independent monophyletic cluster separated from other genera, with high a bootstrap (90 %) value support. Clustering results using the maximum likelihood and minimum-evolution approach were similar to that obtained using the neighbour joining method (Fig. S2 and S3). Strain 13D2W-2^T shared the highest 16S rRNA gene sequence similarity of 97.8 % to *T. dalianensis* DLFJ1-1^T, follow by *T. pacifica* TL 2^T (97.7 %), while the similarities to other species were all below 97.0 %.

Genome sequencing, DNA G+C content, dDDH and ANI estimation

A total of 500 Mbp clean sequence data for strains 13D2W-2^T, *T. pacifica* TL 2^T and *T. dalianensis* DLFJ1-1^T were generated to reach about 100-fold depth of coverage with an Illumina/Solexa Genome Analyzer IIx. The clean data was assembled by SOAPdenovo2 (Luo et al. 2012). The draft genome accession number for strains 13D2W-2^T, *T. pacifica* TL 2^T and *T. dalianensis* DLFJ1-1^T are AQRC00 000000, AUND00000000 and JHEH00000000 respectively.

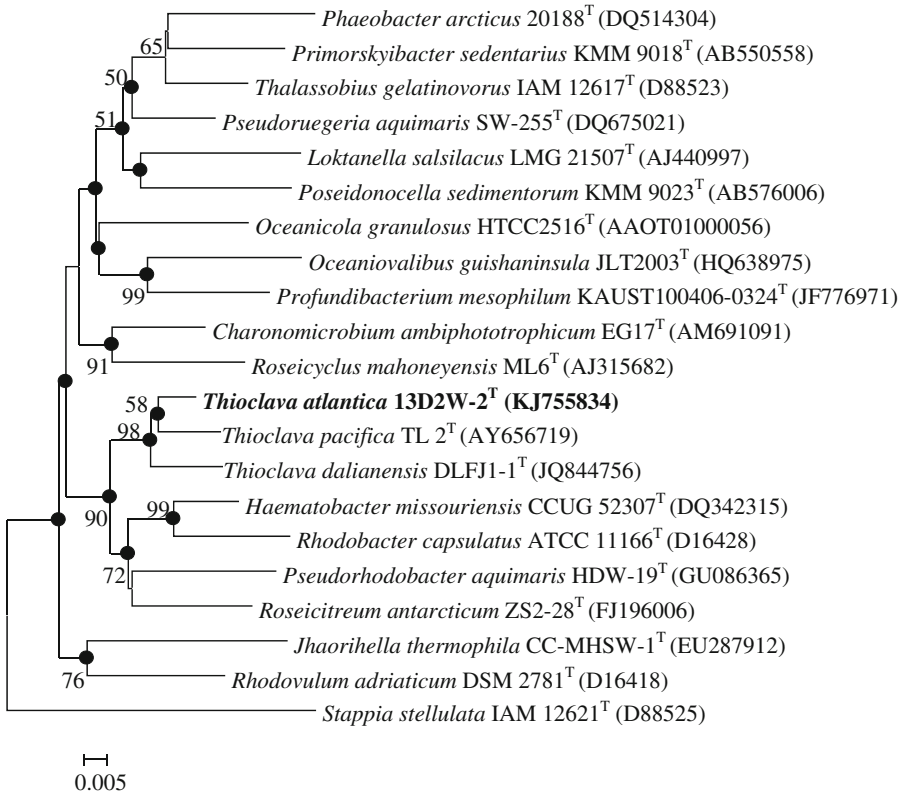

Fig. 1 Neighbour-joining tree showing the phylogenetic positions of strain 13D2W-2^T and representatives of some other related taxa based on 16S rRNA gene sequences. *Filled circles* indicate nodes that were also recovered in maximum-likelihood and minimum evolution trees based on the same sequences. Bootstrap values (expressed as percentages of 1,000 replications) are shown at branch points. *Bar*, 0.005 nucleotide substitution rate (K_{nuc}) units. *Stappia stellulata* IAM 12621^T (D88525) as used as the outgroup

The chromosomal DNA G+C content of strain 13D2W-2^T was determined to be 65.3 mol%, which is close to the values of the other two species of genus *Thioclava* (62.5–63.9 mol%). The estimated dDDH values between strain 13D2W-2^T and, respectively, *T. dalianensis* DLFJ1-1^T and *T. pacifica* TL 2^T were 22.60 ± 2.36 and 25.60 ± 2.41 %, values which are below the standard criteria (70 %) for delineation of bacterial species (Wayne et al. 1987). The ANI values between strain 13D2W-2^T and the related type species *T. dalianensis* DLFJ1-1^T and *T. pacifica* TL 2^T were 78.49 and 81.91 %, respectively, which are below standard ANI criteria for species identity (95–96 %; Richter and Rossello-Mora 2009). These data confirm that strain 13D2W-2^T is a novel species of the genus *Thioclava*.

A complete sulfur oxidation gene cluster (*soxTRSVWXYZABCD*) was found to present in the genomes of strain 13D2W-2^T and *T. Pacifica* TL 2^T but not in that of *T. dalianensis* DLFJ1-1^T. This is in accordance with their features i.e. that strain 13D2W-2^T and *T. pacifica* TL 2^T can oxidize inorganic sulfur compounds, but *T. dalianensis* DLFJ1-1^T cannot (Zhang et al. 2013). A Form I ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*cbbL*) was also found in the draft genome sequences of strain 13D2W-2^T and *T. pacifica* TL 2^T, but not in that of *T. dalianensis* DLFJ1-1^T.

Chemotaxonomic characteristics

The fatty acid profile of 13D2W-2^T was obtained at the same time as the data was obtained for the two reference species of the genus *Thioclava* during our earlier study of *T. dalianensis* (Zhang et al. 2013). The results for the three strains are shown in Table 1, which shows that the three profiles are qualitatively similar. The major fatty acids identified in strain 13D2W-2^T were Summed Feature 8 ($C_{18:1}\omega7c/\omega6c$ as defined by MIDI). The isoprenoid quinone detected in strain 13D2W-2^T was Q10 (100 %), as is the case with the two species of the genus *Thioclava* Query.

The major polar lipids of strain 13D2W-2^T were found to comprise phosphatidylethanolamine, phosphatidylglycerol, an aminophospholipid, a glycolipid and three unknown phospholipids, as shown in Fig. S4. This profile is the same as that obtained for *T. dalianensis* DLFJ1-1^T (Zhang et al. 2013).

Table 1 Cellular fatty acid content of strain 13D2W-2^T and related members of the genus *Thioclava*

Fatty acid	1	2	3
$C_{16:0}$	5.58	2.02	7.97
$C_{18:0}$	2.95	2.64	3.85
$C_{10:0}$3OH	2.86	3.35	3.29
$C_{12:0}$3OH	ND	ND	1.71
$C_{18:0}$3OH	2.51	4.75	4.53
$C_{17:0}$ cyclo	tr	ND	tr
$C_{19:0}$ $\omega 8c$ cyclo	3.11	6.42	49.04
$C_{19:0}$ 10-methyl	tr	1.09	tr
$C_{18:1}\omega 9c$	tr	ND	tr
$C_{18:1}\omega 7c$ 11-methyl	tr	tr	1.15
Summed feature 3[a]	2.79	tr	tr
Summed feature 8[a]	74.38	76.73	22.61

Strains 1, 13D2W-2^T; 2, *T. pacifica* TL 2^T (Zhang et al. 2013); 3, *T. dalianensis* DLFJ1-1^T (Zhang et al. 2013). The data for 13D2W-2^T was obtained under the same conditions at the same time during our earlier study (Zhang et al. 2013). Values are percentages of total fatty acids; *tr* trace amount (<1 %). *ND* not detected

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

Taxonomic conclusion

The high 16S similarity and the results of phenotypic analysis and chemotaxonomic studies presented above support the view that strain 13D2W-2^T should be assigned to the genus *Thioclava*. However, strain 13D2W-2^T can be differentiated from the two current species of the genus *Thioclava* with the low values of dDDH (22.60–25.60 %) and ANI (78.49–81.91 %) and the different characteristics given in Table 2. On the basis of data described above, strain 13D2W-2^T should be placed into a new species of genus *Thioclava*, for which a name *Thioclava atlantica* sp. nov. is proposed.

Description of *Thioclava atlantica* sp. nov.

Thioclava atlantica (at.lan'ti.ca. L. fem. adj. *atlantica* referring to the Atlantic Ocean, where the strain was isolated).

Cells are Gram-negative short rod-shaped, 0.7–0.8 μm wide and 1.2–2.0 μm long, motile by means of a flagellum. Positive for oxidase, catalase, β-glucosidase (aesculin hydrolysis), urease, beta-galactosidase, D-glucose fermentation and nitrate reduction, but negative for indole production, gelatinase and arginine dihydrolase. Can oxidize reduced sulfur compounds such as thiosulfate, sulfur and sulfide as energy source. On 216L agar plates, forms smooth grey-white colonies with regular edges and of 2–3 mm in diameter after 72 h incubation at 28 °C, non-pigmented and slightly raised in the center. Moderately halophilic, grows in 0.5–12 % of NaCl (optimum 3–5 %) and at 4–41 °C (optimum 28–37 °C), but not at 45 °C. The principal fatty acid is Summed Feature 8 ($C_{18:1}\omega 7c/\omega 6c$). The only quinone is Q10. The polar lipids are comprised of phosphatidylethanolamine, phosphatidylglycerol, an aminophospholipid, a glycolipid and three unknown phospholipids. API ZYM test strip results indicate the type strain is positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, valine aminopeptidase, α-glucosidase, β-glucosidase; weakly positive for cystine aminopeptidase, lipase (C14), naphtol-AS-Bl-phosphoamidase, trypsin, β-glucuronidase; and negative for *N*-acetyl-β-glucosaminidase, α-chymotrypsin, α-fucosidase, α-galactosidase, α-mannosidase, β-galactosidase. According to API 20NE test, can utilize D-glucose, D-maltose, D-mannitol, D-mannose, L-arabinose, malic acid, potassium gluconate and trisodium citrate, but not adipic acid, capric acid, *N*-acetyl-glucosamine or phenylacetic acid. Of the 95 substrates in the Biolog GN2 system, the type strain is positive for D-alanine, D-fructose, D-melibiose, glucose-1-phosphate, glycerol, Hydroxy-L-proline, L-alanine, L-asparagine, L-fucose, L-glutamic acid, L-histidine, malonic acid, sebacic acid, succinic acid, β-methyl-D-glucoside; weakly positive for acetic acid, D,L-α-glycerol phosphate, D-cellobiose, itaconic acid, L-aspartic acid, N-acetyl-D-galactosamine, p-Hydroxy phenylacetic acid and uridine. The G+C content of the DNA of the type strain is 65.3 mol%. Table 2 shows the characteristics used to distinguish the type strain from the other two members of the genus.

The type strain, 13D2W-2^T (= MCCC 1A02612^T = LMG 27145^T) was isolated from deep sea sediment of the Atlantic Ocean. The GenBank accession number for the 16S rRNA gene sequence of *T. atlantica* 13D2 W-2^T is KJ755834 and that of the draft genome sequence is AQRC00000000.

Table 2 Physiological characteristics of strain 13D2W-2^T and the members of the genus *Thioclava*

Characteristics	1	2	3
Temperature range (°C)	4–41	15–47	4–37
NaCl range for growth (%, w/v)	0.5–12	1–9	0.5–15
API 20NE			
Reduction of nitrate to nitrite	+	−	−
Utilization of *N*-acetyl-glucosamine, phenylacetic acid	−	−	+
Utilization of D-mannose	+	−	−
Utilization of L-arabinose, potassium gluconate	+	−	+
API ZYM			
α-galactosidase, β-galactosidase	−	−	+
α-fucosidase	−	−	w
Esterase (C4), esterase lipase (C8)	+	+	−
Valine aminopeptidase	+	+	w
Cystine aminopeptidase	w	−	−
Trypsin	w	−	w
β-glucuronidase	w	w	−
Susceptibility to antimicrobial agents			
Oxacillin (1 μg), vancomycin (30 μg)	+	−	−
Polymyxin B (30 IU)	+	+	−
Norfloxacin (10 μg)	−	+	+
Biolog GN2			
Adonitol, Cis-aconitic acid,citric acid, dextrin, D-galactonic acid lactone, D-glucuronic acid, D-psicose, D-raffinose, D-saccharic acid, D-serine, D-sorbitol, glucuronamide, inosine, L-leucine, L-phenylalanine, L-pyroglutamic acid, maltose, quinic acid, thymidine, turanose,α-Keto glutaric acid, γ-amino butyric acid	−	−	+
Bromo succinic acid, D-gluconic acid, D-mannose, D-trehalose, L-rhamnose, L-threonine, α-D-lactose	−	−	w
L-alaninamide, β-Hydroxy butyric acid	−	+	+
α-Hydroxy butyric acid	−	w	−
D,L-lactic acid,L-proline,Methyl pyruvate,succinamic acid,γ-Hydroxy butyric acid	−	w	+
Mono-methyl-succinate	−	w	w
L-histidine	+	−	−
D-alanine, D-melibiose, glucose-1-phosphate,Hydroxy-L-proline, L-alanine, L-asparagine, L-fucose, L-glutamic acid, malonic acid, sebacic acid, succinic acid, β-methyl-D-glucoside	+	−	+
Glycerol	+	w	+
D-fructose	+	w	w
D,L-α-glycerol phosphate	w	−	−
Itaconic acid, L-aspartic acid, *N*-acetyl-D-galactosamine, p-Hydroxy phenylacetic acid, uridine	w	−	+
D-cellobiose	w	−	w
Acetic acid	w	w	+
soxTRSVWXYZABCD	+	+	−
bbL	+	+	−
DNA G+C content[a] (mol%)	65.3	63.9	62.5

Strains 1, 13D2W-2^T; 2, *T. pacifica* TL 2^T (Sorokin et al. 2005, Zhang et al. 2013); 3, *T. dalianensis* DLFJ1-1^T (Zhang et al. 2013). Data of catalase, oxidase, API 20NE, API ZYM, Biolog GN2 and susceptibility were done under the same condition and the data for the two reference type strains were from our earlier study (Zhang et al. 2013). Characteristics are scored as: *w* weak; + positive; − negative

[a] data from draft genome sequence

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