Oceanicola marinus sp. nov., a marine alphaproteobacterium isolated from seawater collected off Taiwan

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A short-rod-shaped, Gram-negative, non-motile bacterial strain, designated AZO-C^T, was isolated from a sample of seawater collected from the Eluanbi coast of Pingtung County in southern Taiwan and was characterized by using a polyphasic approach. Phylogenetic analyses based on 16S rRNA gene sequences showed that the strain formed a monophyletic branch at the periphery of the evolutionary radiation occupied by the genus *Oceanicola* in the order *Rhodobacterales* of the *Alphaproteobacteria*. The closest neighbours were *Oceanicola batsensis* HTCC2597^T (95.6 % similarity), *Oceanicola nanhaiensis* SS011B1-20^T (94.5 %) and *Oceanicola granulosus* HTCC2516^T (94.0 %). The predominant fatty acid was 18 : 1ω7c (49.1 %), and significant amounts of 19 : 0 cyclo (24.6 %) and 16 : 0 (14.7 %) were present. The DNA–DNA relatedness of the strain with respect to recognized species of the genus *Oceanicola* was less than 70 %. The isolate was also distinguishable from members of the genus *Oceanicola* on the basis of phenotypic and biochemical characteristics. It is evident from the genotypic, chemotaxonomic and phenotypic data, therefore, that strain AZO-C^T represents a novel species of the genus *Oceanicola*, for which the name *Oceanicola marinus* sp. nov. is proposed. The type strain is AZO-C^T (=LMG 23705^T=BCRC 17591^T).

The genus *Oceanicola*, proposed by Cho & Giovannoni (2004), belongs to the order *Rhodobacterales* of the class *Alphaproteobacteria* and currently comprises three species with validly published names: *Oceanicola granulosus*, *Oceanicola batsensis* and *Oceanicola nanhaiensis*. *O. granulosus* strains HTCC2516^T and HTCC2523 and *O. batsensis* HTCC2597^T are poly-β-hydroxybutyrate-producing marine bacteria that were isolated from the Bermuda Atlantic Time-series Study (BATS) site by means of a high-throughput culturing method based on dilution to extinction in an oligotrophic seawater-based medium (Cho & Giovannoni, 2003b). *O. nanhaiensis* SS011B1-20^T was isolated from samples of sediments from the South China Sea (Gu *et al.*, 2007). The aim of the present study was to determine the taxonomic position of an *Oceanicola*-like isolate, strain AZO-C^T, obtained from seawater collected off the coast of Taiwan.

A seawater sample was collected (by a diver) in a sterile bottle at a depth of 4–5 m off the Eluanbi coast (Pingtung County, southern Taiwan), and was transported to the laboratory within 3–4 h. Strain AZO-C^T was isolated using a standard dilution plating method on marine agar 2216 (MA; BD Difco). After incubation of the plates at 25 °C for 5 days, strain AZO-C^T was purified as single colonies. The strain was preserved at −80 °C as a 20 % (v/v) glycerol suspension in marine broth 2216 (MB; BD Difco) or by lyophilization with 20 % (w/v) skimmed milk as the cryopreservation solution. Strain AZO-C^T was the subject of a polyphasic taxonomic study.

The bacterial cells were observed by phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth to ascertain their morphology. The motility of cells was tested by using the hanging drop

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AZO-C^T is DQ822569.

method. The Spot Test flagella stain (BD Difco) was used to stain any flagella that might be present. A Gram-stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction of strain AZO-C^T. Poly-β-hydroxybutyrate granule accumulation was observed under light microscopy after staining of the cells with Sudan black. Colony morphology was examined using a stereoscopic microscope (SMZ 800; Nikon). The pH range for growth was examined in MB with the appropriate biological buffers (pH 4–10, using increments of 0.5 pH units) (Chung *et al.*, 1995). Tolerance of various NaCl concentrations was tested in nutrient broth prepared according to the formula of the Difco medium, except that the NaCl concentration was altered as required (0, 0.5 and 1.0–10 %, w/v, using increments of 1.0 %). Growth at various temperatures (4–45 °C) was measured in MB. Cellular growth was determined by measuring the turbidity (OD_{600}) of cultures grown at various pH values, NaCl concentrations and temperatures. Anaerobic cultivation was performed on MA, using the Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chen *et al.*, 2001). The 16S rRNA gene sequences of strain AZO-C^T were obtained using a DNA sequencer (ABI Prism 310; Applied Biosystems) and the DNA sequences were then assembled using the Fragment Assembly System program from the Wisconsin package version 9.1 (GCG, 1995). An almost-complete 16S rRNA gene sequence (1396 nt) of the strain was compared against 16S rRNA gene sequences available from the Ribosomal Database Project and GenBank databases. Multiple sequence alignment including strain AZO-C^T and its closest relatives was performed using BioEdit software (Hall, 1999) and MEGA, version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm by using the Jukes & Cantor (1969) distance model; bootstrap analysis for the neighbour-joining tree was performed on the basis of 1000 resamplings. A comparison of the 16S rRNA gene sequence of strain AZO-C^T with those of members of genera of the order *Rhodobacterales* in the *Alphaproteobacteria* showed that the strain fell within the evolutionary radiation occupied by the genus *Oceanicola* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain AZO-C^T formed a coherent clade with *O. batsensis* HTCC2597^T and *O. nanhaiensis* SS011B1-20^T, and this clade had moderate bootstrap support (>70 %). The relationship between strain AZO-C^T and *O. granulosus* HTCC2516^T, however, did not have robust bootstrap support (<50 %). Similar topology was obtained in phylogenetic trees generated with the maximum-parsimony and maximum-likelihood algorithms (data not shown). According to the pairwise sequence comparisons, strain AZO-C^T was most closely related to *O. batsensis* HTCC2597^T (95.6 % similarity), followed by *O. nanhaiensis* SS011B1-20^T (94.5 % similarity) and *O. granulosus* HTCC2516^T (94.0 % similarity). The levels of 16S rRNA gene sequence similarity between strain AZO-C^T and other species with validly published names within the order *Rhodobacterales* of the *Alphaproteobacteria* were below 94 %.

Whole-genome DNA–DNA relatedness levels were determined using photobiotin-labelled probes in triplicate, as described previously by Ezaki *et al.* (1989). *O. batsensis* HTCC2597^T and *O. granulosus* HTCC2516^T showed only 25.6 ± 5.1 % and 7.2 ± 2.8 % DNA–DNA relatedness, respectively, with respect to strain AZO-C^T. These DNA–DNA relatedness values were significantly lower than the level accepted as demarcating species (Wayne *et al.*, 1987). On the basis of these DNA–DNA relatedness data, isolate AZO-C^T warrants separate species status within the genus *Oceanicola*.

The DNA G+C content of strain AZO-C^T was estimated, in duplicate, as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by means of HPLC. The DNA G+C content of strain AZO-C^T was found to be 70.9 ± 1.0 mol% and was within the range previously reported for *Oceanicola* species (64.7–71.5 mol%). Biomass of AZO-C^T was obtained after growing the strain on MA at 30 °C for 3 days. Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI) (Sasser, 1990). The predominant fatty acid constituents of strain AZO-C^T were 18 : 1ω7*c* (49.1 %), 19 : 0 cyclo (24.6 %) and 16 : 0 (14.7 %) (Table 1). The fatty acid profile of the strain was in good agreement with those of members of the genus *Oceanicola* (Cho & Giovannoni, 2004; Gu *et al.*, 2007).

The phenotypic and biochemical characteristics of strain AZO-C^T were examined according to Cho & Giovannoni (2003a, b). Additional biochemical tests were performed using the Biolog GN2, API ZYM (bioMérieux) and API 20NE (bioMérieux) microtest systems according to the methods outlined by the manufacturers. The presence of flexirubin, carotenoid and bacteriochlorophyll *a* pigments was investigated as described by Reichenbach (1992), Richards (1994) and Schmidt *et al.* (1994). Sensitivity to antibiotics was examined after spreading cells (0.5 McFarland standard) on MA and applying antibiotic discs containing the following: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 μg), streptomycin (10 μg) or tetracycline (30 μg). The effects of the antibiotics on cell growth were assessed after 3 days incubation, and susceptibility was scored on the basis of the distance from the edge of the clear zone to the disc. The cells were scored as 'susceptible' if the distance was greater than 3 mm, as 'moderately susceptible' if the distance was 1–3 mm and as 'resistant' if the distance was less than 1 mm. Detailed results from the phenotypic and biochemical analyses of strain AZO-C^T are provided in Table 2 and in the

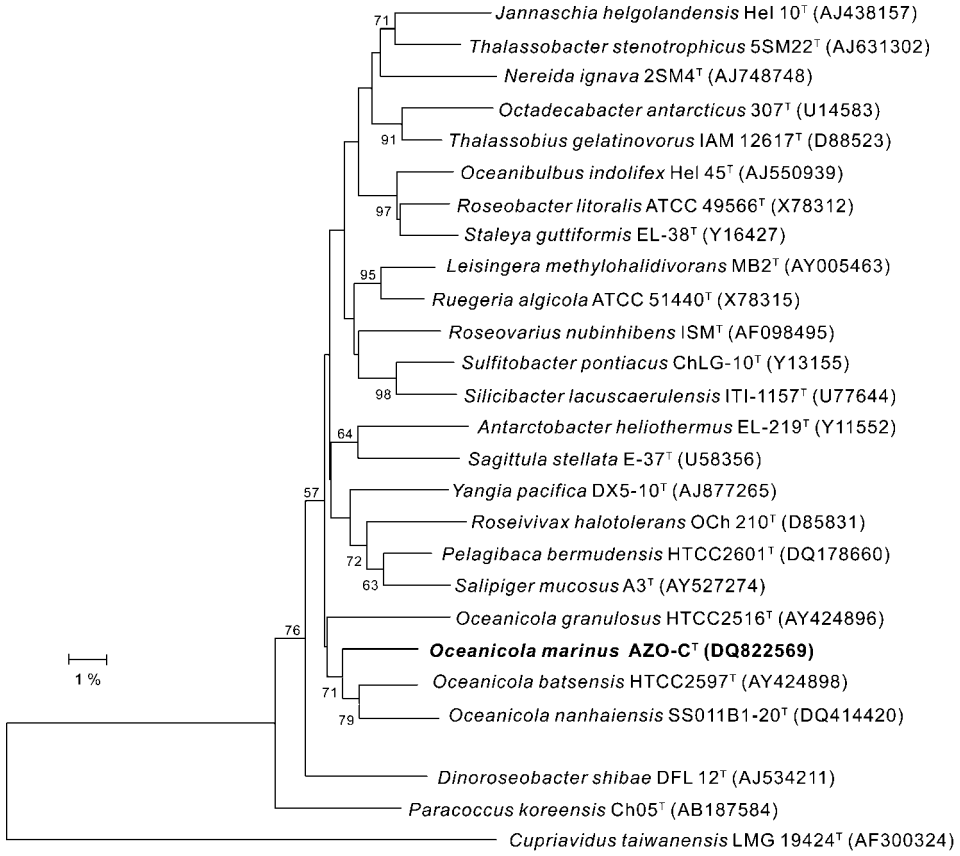

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences and constructed using the neighbour-joining algorithm with the distance model of Jukes & Cantor (1969), showing the phylogenetic position of strain AZO-C^T within the genus *Oceanicola*. GenBank accession numbers are shown in parentheses. Bootstrap percentages (based on 1000 resampled datasets) above 50 % are shown at the nodes. *Cupriavidus taiwanensis* LMG 19424^T was used as an outgroup. Bar, 1 % sequence dissimilarity per nucleotide position.

species description. Phenotypic characteristics that serve to differentiate strain AZO-C^T from the type strains of the genus *Oceanicola* are presented in Table 2.

On the basis of the 16S rRNA gene sequence comparisons, strain AZO-C^T occupies a distinct position within the genus *Oceanicola*. This genotypic insight was supported by the unique combination of chemotaxonomic characteristics (Tables 1 and 2) and biochemical traits (Table 2) of the strain. It is clear from the genotypic and phenotypic data that strain AZO-C^T represents a novel species within the genus *Oceanicola*. The name *Oceanicola marinus* sp. nov. is proposed for this taxon.

Description of *Oceanicola marinus* sp. nov.

Oceanicola marinus (ma′ri.nus. L. masc. adj. *marinus* of the sea, marine, referring to the isolation of the type strain from seawater).

Cells are Gram-negative (according to Gram staining and the KOH test), non-motile, non-spore-forming, short rods that are 0.5 μm wide and 0.9–1.0 μm long. Colonies on MA are cream–white in colour, circular and convex with entire edges. Colonies are approximately 0.8–2.0 mm in diameter on MA after 72 h incubation at 25 °C. Growth occurs at 4–42 °C, 2–8 % NaCl and pH 6–9. Optimal growth occurs at 28–35 °C, 3–5 % NaCl and pH 7.0. Cells are facultatively anaerobic. Neither carotenoid/flexirubin pigments nor bacteriochlorophyll *a* is found. Positive for the following characteristics: indole production, aesculin hydrolysis, oxidase, catalase, urease, alkaline phosphatase, esterase (C4), lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Negative for the following characteristics: nitrate reduction, glucose fermentation, arginine dihydrolase, gelatin hydrolysis, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The following carbon substrates (Biolog GN2) are oxidized: dextrin, adonitol, arabinose, arabitol, cellobiose, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, L-rhamnose,

Table 1. Fatty acid compositions (%) of strain AZO-C^T and type strains of related *Oceanicola* species

Strains: 1, AZO-C^T; 2, *O. batsensis* HTCC2597^T; 3, *O. granulosus* HTCC2516^T; 4, *O. nanhaiensis* SS011B1-20^T. Data for reference strains were obtained from Cho & Giovannoni (2004) and Gu *et al.* (2007). Strain AZO-C^T was cultivated for 72 h on MA at 30 °C, i.e. the conditions described by Cho & Giovannoni (2004) and Gu *et al.* (2007).

Fatty acid	1	2	3	4
10 : 0	–	–	0.1	0.2
10 : 0 3-OH	0.1	0.4	1.5	1.0
12 : 0	–	2.0	–	0.1
12 : 0 3-OH	2.1	–	1.6	–
12 : 1ω11*c*	–	4.9	–	–
14 : 0	0.2	1.5	–	0.3
14 : 0 3-OH	–	–	–	0.9
15 : 0	–	0.9	–	–
16 : 0	14.7	15.0	11.9	7.0
17 : 0	–	1.5	0.4	0.4
17 : 1ω8*c*	–	–	0.3	0.4
17 : 0 anteiso	–	–	0.2	–
17 : 0 cyclo	–	–	0.2	–
18 : 0	1.0	2.4	0.9	1.3
18 : 1ω7*c*	49.1	31.0	62.8	81.2
18 : 1 methyl	6.6	–	8.1	4.3
19 : 0 cyclo	24.6	40.4	10.8	1.1
20 : 2ω6,9*c*	0.2	–	–	–
Summed features*				
3	1.0	–	1.2	1.8
7	0.4	–	–	–
Total	100	100	100	100

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained 16 : 1ω7*c* and/or 15 : 0 iso 2-OH; summed feature 7 comprised an unknown fatty acid with an equivalent chain-length of 18.846, 19 : 1ω6*c* and/or 19 : 0ω10*c* cyclo.

Table 2. Comparison of phenotypic characteristics of strain AZO-C^T with those of type strains of related *Oceanicola* species

Strains: 1, AZO-C^T; 2, *O. batsensis* HTCC2597^T; 3, *O. granulosus* HTCC2516^T; 4, *O. nanhaiensis* SS011B1-20^T. Data for reference strains were obtained from Cho & Giovannoni (2004) and Gu *et al.* (2007). +, Positive; (+), weakly positive; −, negative.

Characteristic	1	2	3	4
Catalase	+	+	−	+
Urease	+	+	−	−
Nitrate reduction	−	−	−	(+)
Susceptibility to:				
Chloramphenicol	−	−	+	+
Streptomycin	−	+	−	+
Erythromycin	−	+	+	−
Gentamicin	−	+	−	+
Assimilation of:				
D-Glucose	+	−	+	+
Sucrose	+	−	+	+
D-Fructose	+	−	−	+
D-Arabinose	+	+	−	−
α-D-Lactose	−	−	+	+
D-Sorbitol	−	−	−	+
D-Trehalose	+	−	+	−
D-Cellobiose	+	−	+	+
D-Mannitol	+	+	−	+
Malonic acid	−	+	−	+
Lactic acid	+	+	−	−
Propionic acid	+	+	+	−
myo-Inositol	−	+	−	−
L-Alanine	+	−	+	−
L-Glutamic acid	+	+	+	−
DNA G+C content (mol%)	70.9	67.3	71.5	64.7

sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-proline, L-pyroglutamic acid, L-serine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine and 2-aminoethanol. Cannot oxidize α-cyclodextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, i-erythritol, L-fucose, gentiobiose, *myo*-inositol, α-D-lactose, lactulose, melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, D-sorbitol, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, alaninamide, glycyl L-glutamic acid, L-alanyl glycine, glycyl L-aspartic acid, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, D-serine, L-threonine, DL-carnitine, phenylethylamine, putrescine, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate or glucose 6-phosphate. Resistant to ampicillin, nalidixic acid and penicillin G and sensitive to chloramphenicol, erythromycin, gentamicin, kanamycin, novobiocin, rifampicin, streptomycin and tetracycline. The predominant fatty acid is 18 : 1ω7*c* and there are significant amounts of 19 : 0 cyclo and 16 : 0. The DNA G+C content is 70.9 mol%.

The type strain, AZO-C^T (= LMG 23705^T = BCRC 17591^T), was isolated from a seawater sample collected off the Eluanbi coast of Pingtung County, southern Taiwan.

Acknowledgements

W.-M. C. was supported by grants from the National Science Council, Taipei, Taiwan, Republic of China (NSC 95-2320-B-022-001-MY2 and 95-2313-B-022-001).

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