Donghicola xiamenensis sp. nov., a marine bacterium isolated from seawater of the Taiwan Strait in China

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A Gram-negative, ovoid rod-shaped bacterium that lacked bacteriochlorophyll *a*, designated strain Y-2^T, was isolated from seawater of the Taiwan Strait, China. The strain was oxidase- and catalase-positive, and reduced nitrate to nitrite. Optimal growth occurred at around pH 7.0 and 25 °C in the presence of 1–3% (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Y-2^T belonged to the class *Alphaproteobacteria* and formed a coherent cluster with *Donghicola eburneus* SW-277^T (96.2% sequence similarity). The major whole-cell fatty acid was C_{18:1}ω7c (75.42%). Other characteristic fatty acids were C_{18:0} (9.16%) and C_{16:0} (7.32%). The DNA G+C content of strain Y-2^T was 62.4 mol%. Therefore, based on phenotypic properties, phylogeny and genomic data, strain Y-2^T should be placed in the genus *Donghicola* as representing a novel species, for which the name *Donghicola xiamenensis* sp. nov. is proposed. The type strain is Y-2^T (=MCCC 1A00107^T=LMG 24574^T=CGMCC 1.7081^T).

The genus *Donghicola* was proposed by Yoon *et al.* (2007). The only recognized species is *Donghicola eburneus*, which was isolated from water of the East Sea, Korea. In a recent study on crude oil pollution, we isolated some strains that play important roles in oil degradation. A comparative 16S rRNA gene sequence analysis indicated that strain Y-2^T fell within the genus *Donghicola* and formed a coherent cluster with *Donghicola eburneus* SW-277^T (96.2% sequence similarity). Accordingly, in this paper we present data for strain Y-2^T obtained using a polyphasic taxonomic approach, to confirm this proposal.

Strain Y-2^T was isolated from oil-contaminated surface water of the Taiwan Strait, China, in November 2004. The water sample was enriched in artificial seawater medium (ASM) by using crude oil (Quanzhou Oil Refinery, China) as the sole carbon source. The ASM used for enrichment contained (l⁻¹ distilled water): 10.0 g crude oil, 24.0 g NaCl, 7.0 g MgSO₄·7H₂O, 1.0 g NH₄NO₃, 0.7 g KCl, 2.0 g KH₂PO₄, 3.0 g Na₂HPO₄ and 10 ml trace element solution, pH 7.4. The trace element solution contained (l⁻¹ distilled water): 2.0 mg CaCl₂, 50.0 mg FeCl₃·6H₂O, 0.5 mg CuSO₄, 0.5 mg MnCl₂·4H₂O and 10.0 mg ZnSO₄·7H₂O (Liu & Shao, 2005). The enrichment was incubated in

250 ml flasks at 28 °C, at 200 r.p.m. and was transferred to fresh medium every 5 days. This was repeated six times. After enrichment, the mixture was diluted decimally (10⁴–10⁷) and aliquots (0.1 ml) were spread on SLB plates. The SLB medium (1 l) was composed of 0.5 l ASM and 0.5 l HLB. HLB comprised Luria–Bertani (LB) medium (Sambrook *et al.*, 1989), modified by the addition of 30.0 g NaCl l⁻¹. Plates were incubated at 28 °C for 3 days. Individual colonies on the plates were picked and purified by successive streaking on HLB plates. Isolate Y-2^T, which was shown to be an important member of the enrichment consortium, was used in the present study. SLB was used to separate Y-2^T from other strains in the enrichment. HLB was used for routine cultivation of the isolates and most of the phenotypic tests. Unless otherwise stated, assays were carried out using cells grown in HLB.

For genotypic characterization, DNA was prepared according to the method of Ausubel *et al.* (1995). The 16S rRNA gene was amplified by PCR using the primers: 16SF (positions 8–27, *Escherichia coli* numbering; 5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (positions 1512–1493, 5'-ACGGCTACCTTGTTACGACT-3'). PCR was performed in a T3 thermal cycler (Biometra). The PCR products were purified using an EZNA Cycle-Pure kit (Omega), ligated into pMD18-T (Takara) and then transformed into *E. coli* DH5a (Takara). Sequencing was carried out on a model 3730 automated DNA sequencer with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The 16S rRNA gene sequences of related taxa

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Y-2^T is DQ120728.

Transmission electron micrographs of negatively stained cells of strain Y-2^T and a table of the cellular fatty acid contents of strain Y-2^T, *Donghicola eburneus* SW-277^T and *Salipiger mucosus* A3^T are available as supplementary material with the online version of this paper.

were obtained from GenBank. Phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987) in DNAMAN (version 5.1; Lynnon Biosoft), with bootstrap values based on 1000 replications.

A nearly full-length 16S rRNA gene sequence (1424nt) of strain Y-2^T was determined. Based on the results of the phylogenetic analysis, strain Y-2^T belonged to the class *Alphaproteobacteria*, forming a robust clade with the genus *Donghicola* (Fig. 1). This topology was also found in trees generated using maximum-likelihood and maximum-parsimony algorithms. Strain Y-2^T had 16S rRNA gene sequence similarity values of 96.2 % with *D. eburneus* SW-277^T, 94.2 % with *Salipiger mucosus* A3^T (Martínez-Cánovas *et al.*, 2004), 92.4 % with *Roseivivax halodurans* OCh 239^T and 92.4 % with *Roseivivax halotolerans* OCh 210^T (Suzuki *et al.*, 1999).

General cell morphology was studied using an Olympus inverted phase microscope with 3-day-old cultures of strain Y-2^T grown on HLB agar. For electron microscopy, exponential-phase cells were harvested, suspended and absorbed on a Formvar-carbon-coated grid and stained with phosphotungstic acid. After being air-dried, the grid was examined by using an H-600 transmission electron microscope (Hitachi).

The temperature range for growth was determined between 4 and 45 °C at intervals of 5 °C. The pH tolerance range was tested by using HLB medium adjusted with NaOH and HCl to obtain a pH range from 4 to 10 in increments of 1 unit. The requirement for sodium was examined at 0, 0.05, 0.2, 1, 2, 3, 6, 8, 10, 12, 14, 16, 20 and 24 % NaCl (w/v) in LB. The optimum pH and salinity values were determined by growth experiments in HLB and at various NaCl concentrations in LB at 25 °C, and 200 r.p.m. Growth was determined by an increase in OD₆₀₀, as measured with a spectrophotometer (Ultrospec 2100; Amersham Pharmacia).

Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Hydrolysis of casein, starch, Tween 80, gelatin and urease activity were determined as described by Cowan & Steel (1965), with ASM. H₂S production was tested as described by Bruns *et al.* (2001). Reduction of nitrate was tested according to Lányi (1987). Production of bacteriochlorophyll *a* was determined by using spectrometric analysis of methanolic cell extracts (Lafay *et al.*, 1995). Cells grown in HLB at 25 °C for 24 h in the dark were harvested by centrifugation of a 50 ml culture (12000 g for

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain Y-2^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. Bar 0.05 substitutions per nucleotide position.

10 min) and resuspended in an ice-cold acetone-methanol solution (7:2, v/v) in the dark for 12 h. Other biochemical tests were carried out by using API 20NE test kits (bioMérieux), according to the manufacturer's instructions, except that the API AUX inoculating solution was supplemented with 24 g NaCl l⁻¹. Microlog GN2 plates (Biolog) were used as described by Ivanova *et al.* (1998). Tests for the utilization of various other organic substrates as sole carbon sources, at concentrations of 0.1 % (w/v), were performed in 5 ml ASM.

Cells of strain Y-2^T were Gram-negative ovoid rods that were non-spore-forming and lacked flagella. The cells were 1.0–2.0 µm in length and 0.5–1.2 µm in width (see Supplementary Fig. S1, available in IJSEM Online). The optimal growth temperature, pH and salinity values are given in the species description. Differences in physiological, biochemical and chemotaxonomic characteristics

between strain Y-2^T, *D. eburneus* SW-277^T, *S. mucosus* A3^T, *R. halodurans* OCh 239^T and *R. halotolerans* OCh 210^T are given in Table 1.

Susceptibility to antibiotics was determined as described by Shieh *et al.* (2003) on HLB agar plates by using antibiotic discs: doxycycline (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), norfloxacin (10 µg), erythromycin (15 µg), minocycline (30 µg), gentamicin (10 µg), neomycin (30 µg), polymyxin B (300U), cefalexin IV (30 µg), amikacin (30 µg), cephradine (100 µg), midcamycin (30 µg), kanamycin (30 µg), fortum (30 µg), cefuroxime (30 µg), cefazolin (30 µg), ceftriaxone (30 µg), piperacillin (100 µg), carbenicillin (100 µg), ampicillin (10 µg), oxacillin (1 µg), penicillin (10 µg), cefoperazone (75 µg), vancomycin (30 µg), furazolidone (300 µg), co-trimoxazole (75 µg) and clindamycin (2 µg). The results are given in the species description.

Table 1. Characteristics that differentiate strain Y-2^T from phylogenetically related species

Species: 1, Y-2^T (*Donghicola xiamenensis* sp. nov.); 2, *D. eburneus* SW-277^T; 3, *S. mucosus* A3^T (data from Martínez-Cánovas *et al.*, 2004); 4, *R. halodurans* OCh 239^T (Suzuki *et al.*, 1999); 5, *R. halotolerans* OCh 210^T (Suzuki *et al.*, 1999). +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5
Cell morphology	Ovoid rods	Cocci or rods	Rods	Rods	Rods
Growth at 4 °C	–	–	–	ND	ND
Growth at 37 °C	+	+	+	ND	ND
Motility	–	–	–	+	+
Pigment	Beige	–	–	Pink	Pink
Bacteriochlorophyll <i>a</i>	–	–	–	+	+
Na ⁺ requirement	+	+	+	–	–
Nitrate to nitrite	+	+	–	+	+
ONPG reaction*	–	–	–	+	+
Indole production	–	–	–	+	+
Hydrolysis of:					
Gelatin	–	–	–	–	+
Tween 80	+	+	–	–	–
Urea	+	–	+	–	–
Utilization of:					
L-Arabinose	–	+	–	ND	ND
D-Glucose	–	+	–	+	+
D-Fructose	–	+	–	ND	ND
D-Galactose	–	+	–	ND	ND
Cellobiose	–	+	–	ND	ND
D-Mannose	–	+	–	ND	ND
Acetate	ND	+	–	+	+
Citrate	–	–	–	+	+
Succinate	ND	+	–	+	+
L-Glutamate	ND	–	ND	+	+
Susceptibility to:					
Penicillin	–	+	ND	–	–
Polymyxin B	+	+	–	ND	ND
DNA G + C content (mol%)	62.4	59.7	64.5	64.4	59.7

*ONPG, o-Nitrophenyl β-D-galactopyranoside.

Cellular fatty acid analysis was carried out as described by Mrozik *et al.* (2004). Analysis of the fatty acid methyl esters was performed using a GC-MS (QP2010; Shimadzu) equipped with a RTX-5MS column. The fatty acid profiles of strain Y-2^T, *D. eburneus* SW-277^T and *S. mucosus* A3^T are shown in Supplementary Table S1 in IJSEM Online. The major fatty acids of strain Y-2^T and *D. eburneus* SW-277^T were C_{18:1}ω7c (75.42 %), C_{18:0} (9.16 %) and C_{16:0} (7.32 %) and accounted for >84 % of the total fatty acids, whereas they accounted for >92 % of the total fatty acids of *S. mucosus* A3^T. Based on data from Martínez-Cánovas *et al.* (2004), it was determined that *R. halodurans* OCh 239^T and *R. halotolerans* OCh 210^T also contained C_{18:1} as a major fatty acid.

The G + C content of the genomic DNA of strain Y-2^T was determined by HPLC after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984; Mesbah & Whitman, 1989). The DNA G + C content of strain Y-2^T was 62.4 %, a value that lies within a 5 % range of that for the genus *Donghicola*. DNA–DNA hybridization between strain Y-2^T and its nearest neighbours was not attempted as strains that differ by >3.0 % at the 16S rRNA gene level are unlikely to exhibit >70 % DNA–DNA relatedness at the whole-genome level (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001). A DNA–DNA hybridization value of 70 % is accepted for species delineation (Wayne *et al.*, 1987). The 16S rRNA gene sequence similarities between strain Y-2^T and the reference type strains were below 96.2 %, indicating that strain Y-2^T represents a novel species. In addition, strains Y-2^T and SW-277^T could be differentiated based on phenotypic characteristics, i.e. urease and utilization of carbon sources, suggesting that strains Y-2^T and SW-277^T represent separate species.

In conclusion, based on the phenotypic and chemotaxonomic characteristics and phylogeny based on 16S rRNA gene sequence analysis, strain Y-2^T should be categorized as representing a novel species of the genus *Donghicola*, for which the name *Donghicola xiamenensis* sp. nov. is proposed.

Description of *Donghicola xiamenensis* sp. nov.

Donghicola xiamenensis (xi.a.men.en'sis. N.L. masc. adj. *xiamenensis* pertaining to Xiamen).

Cells are Gram-negative, ovoid rods (1.0–2.0 µm in length and 0.5–1.2 µm in width), non-spore-forming and lack flagella. Colonies are beige, smooth, opaque, circular and convex with entire margins and 2.0–2.5 mm in diameter after 3 days cultivation at 28 °C on HLB. Cells do not contain bacteriochlorophyll *a*. Grows at temperatures ranging from 18 to 37 °C. Optimal growth occurs at 25 °C and at around pH 7.0. No growth occurs in the absence of NaCl or in the presence of >12 % NaCl. Optimum concentration of NaCl for growth is 1–3 % (w/v). Catalase- and oxidase-positive. Tween 80 is hydrolysed, but gelatin, starch and arginine are not. Nitrate is reduced to nitrite. Among the 95 carbon sources in the

Biolog GN2 system, positive reactions were observed for glycogen, L-arabinose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, glycyl L-glutamic acid, L-histidine, L-proline, L-phenylalanine, L-pyroglutamic acid, urocanic acid, phenethylamine and 2,3-butanediol. Susceptible to doxycycline, ofloxacin, ciprofloxacin, chloramphenicol, tetracycline, norfloxacin, erythromycin, minocycline, gentamicin, neomycin, polymyxin B, cefalexin IV, amikacin, cephradine, midecamycin, kanamycin, fortum, cefuroxime, cefazolin and ceftriaxone. Major fatty acids are C_{18:1}ω7c, C_{18:0} and C_{16:0}. The DNA G + C content of the type strain is 62.4 mol%.

The type strain, Y-2^T (=MCCC 1A00107^T=LMG 24574^T=CGMCC 1.7081^T), was isolated from seawater collected near Haitian Dock of Xiamen island at the west bank of the Taiwan Strait, China, in 2004.

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