Donghicola eburneus gen. nov., sp. nov., isolated from seawater of the East Sea in Korea

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A Gram-negative, aerobic, slightly halophilic, non-motile and coccoid- or rod-shaped bacterial strain, SW-277^T, was isolated from seawater, Korea, and subjected to a polyphasic taxonomic study. Strain SW-277^T was catalase- and oxidase-positive and grew optimally at pH 7.0–8.0 and 37 °C in the presence of 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain SW-277^T belongs to the *Alphaproteobacteria* as part of the clade comprising the genera *Roseivivax* and *Salipiger* with a bootstrap resampling value of 72.5 %. 16S rRNA gene sequence similarity values between strain SW-277^T and members of the genera *Roseivivax* and *Salipiger* were between 93.8 and 94.7 %. Strain SW-277^T contained Q-10 as the predominant ubiquinone and C_{18:1}ω7c and C_{16:0} as the major fatty acids. The DNA G + C content was 59.7 mol%. Strain SW-277^T could be distinguished from members of the phylogenetically related genera *Salipiger* and *Roseivivax* by phenotypic differences and low 16S rRNA gene similarity values. On the basis of phenotypic, chemotaxonomic, genotypic and phylogenetic data, strain SW-277^T (=KCTC 12735^T=JCM 13604^T) should be classified as a representative of a novel species in a new genus, *Donghicola eburneus* gen. nov., sp. nov.

In the course of screening novel micro-organisms from seawater of the East Sea, Korea, many moderately halophilic or halotolerant bacteria have been isolated and characterized taxonomically (Yoon *et al.*, 2003b, 2004a, b, c). This study is focused on one of these isolates, SW-277^T. Comparative 16S rRNA gene sequence analysis indicated that strain SW-277^T forms an independent branch within the *Alphaproteobacteria*. Accordingly, the aim of the present work was to determine the exact taxonomic position of strain SW-277^T by a polyphasic characterization that included phenotypic and chemotaxonomic properties and detailed phylogenetic analysis based on 16S rRNA gene sequences.

Strain SW-277^T was isolated by the standard dilution plating technique at 25 °C on marine agar 2216 (MA; Difco). The morphological, physiological and biochemical characteristics of strain SW-277^T were investigated using routine cultivation on MA at 37 °C. Cell morphology and the presence of flagella were examined by light microscopy (Nikon E600; see Supplementary Fig. S1 in IJSEM Online) and transmission electron microscopy. For transmission electron microscopic observation, cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after air-drying with a Philips CM-20

transmission electron microscope. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and MA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. Growth at various NaCl concentrations (0.5 % and 1.0–15.0 %, w/v, at intervals of 1.0 %) was investigated in marine broth 2216 (MB; Difco) or trypticase soy broth (Difco). Growth at various temperatures (4–45 °C) was measured on MA. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965) using MA as base medium. Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea, and nitrate reduction were investigated as described previously (Lanyi, 1987) with a modification that artificial seawater was used for preparation of media. The artificial seawater contained (per l distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described previously (Bruns *et al.*, 2001). For *in vivo* pigment-absorption spectrum analysis, two strains were cultivated aerobically in the dark at 37 °C in MB and liquid *Erythromicrobium/Roseococcus* medium (Yurkov *et al.*, 1994; DSMZ medium no. 767) with the modification that D-glucose was used

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SW-277^T is DQ667965.

A light micrograph of *Donghicola eburneus* gen. nov., sp. nov. strain SW-277^T is available as supplementary data in IJSEM Online.

instead of acetate. The cultures were washed twice by centrifugation using a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by sonication with a Branson Sonifier 450. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer. Susceptibility to antibiotics was detected on MA plates using antibiotic discs with the following concentrations: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; carbenicillin, 100 µg; kanamycin, 30 µg; lincomycin, 15 µg; neomycin, 30 µg; and oleandomycin, 15 µg. Acid production from carbohydrates was determined as described by Leifson (1963) in open tubes. Utilization of substrates as sole carbon and energy sources was tested according to the method of Baumann & Baumann (1981) using supplementation with 2 % (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968). Other physiological and biochemical tests were performed with the API 20E and API ZYM systems (bioMérieux) according to the manufacturer's instructions.

Cell biomass for respiratory lipoquinone analysis and DNA extraction was obtained from cells that were cultivated for 2 days in MB at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination of RNA. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003a). Respiratory lipoquinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For cellular fatty acid analysis, cell mass of strain SW-277^T was harvested from MA plates after cultivation for 3 days at 37 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

Morphological, cultural, physiological and biochemical characteristics of strain SW-277^T are given in the genus and species descriptions (see later) or are shown in Table 1. The almost complete 16S rRNA gene sequence of strain SW-277^T determined in this study comprised 1416 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree based on the neighbour-joining algorithm, strain SW-277^T joined the clade comprising two *Roseivivax* species and *Salipiger mucosus* of the *Alphaproteobacteria* with a bootstrap resampling value of 72.5 % (Fig. 1). This tree topology

Table 1. Differential characteristics of *Donghicola eburneus* gen. nov., sp. nov. and related taxa

Species: 1, *Donghicola eburneus* gen. nov., sp. nov.; 2, *S. mucosus* (data from Martínez-Cánovas *et al.*, 2004); 3, *R. halodurans* (data from Suzuki *et al.*, 1999); 4, *R. halotolerans* (data from Suzuki *et al.*, 1999). ND, Not determined. All species are positive for catalase, oxidase and susceptibility to chloramphenicol and streptomycin (not determined for *S. mucosus*). All species are negative for anaerobic growth, Voges–Proskauer test, hydrolysis of starch and susceptibility to tetracycline (not determined for *S. mucosus*).

Characteristic	1	2	3	4
Cell morphology	Cocci or rods	Rods	Rods	Rods
Motility	–	–	+	+
Pigment	–	–	Pink	Pink
Bacteriochlorophyll <i>a</i>	–	–	+	+
Na ⁺ requirement	+	+	–	–
Nitrate to nitrite	+	–	+	+
ONPG reaction	–	–	+	+
Indole production	–	–	+	+
Hydrolysis of:				
Gelatin	–	–	–	+
Tween 80	+	–	–	–
Urea	–	+	–	–
Acid production from:				
L-Arabinose	+	ND	+	–
D-Cellobiose	+	–	ND	ND
D-Fructose	+	–	+	+
D-Galactose	+	–	+	–
D-Glucose	+	–	+	+
Lactose	+	–	+	–
Maltose	+	–	+	–
D-Mannose	+	–	ND	ND
D-Ribose	–	ND	+	–
Sucrose	+	–	+	–
D-Xylose	–	ND	–	+
D-Mannitol	+	–	ND	ND
D-Sorbitol	+	–	ND	ND
Utilization of:				
L-Arabinose	+	–	ND	ND
D-Glucose	+	–	+	+
D-Fructose	+	–	ND	ND
D-Galactose	+	–	ND	ND
D-Cellobiose	+	–	ND	ND
D-Mannose	+	–	ND	ND
Acetate	+	–	+	+
Citrate	–	–	+	+
Succinate	+	–	+	+
L-Glutamate	–	ND	+	+
Susceptibility to:				
Penicillin	+	ND	–	–
Polymyxin B	+	–	ND	ND
DNA G+C content (mol%)	59.7	64.5	64.4	59.7

was found in trees generated with the maximum-likelihood and maximum-parsimony algorithms. Strain SW-277^T exhibited 16S rRNA gene sequence similarity values of

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Donghicola eburneus* gen. nov., sp. nov. SW-277^T and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points. *Stappia stellulata* IAM 12621^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

94.6% to the type strain of *S. mucosus* and 93.8 and 94.7% to the type strains of *Roseivivax halodurans* and *Roseivivax halotolerans*, respectively. The fatty acid profile of strain SW-277^T comprised (>0.5% of total fatty acids): unsaturated fatty acids C_{18:1}ω7c (61.6%) and C_{18:1}ω9c (0.8%); straight-chain fatty acids C_{16:0} (13.6%), C_{18:0} (9.2%), C_{14:0} (1.4%) and C_{17:0} (1.3%); 11-methyl fatty acid C_{18:1}ω7c (5.2%); hydroxy fatty acid C_{12:1} 3-OH (4.9%); and C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (0.9%). The predominant respiratory lipoquinone detected in strain SW-277^T was ubiquinone-10 (Q-10) at a peak area ratio of approximately 94%. The DNA G+C content of strain SW-277^T was 59.7 mol%.

Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain SW-277^T did not fall within the radiation encompassed by a recognized genus, but forms an evolutionarily distinct lineage within the *Alphaproteobacteria* (Fig. 1). The predominant ubiquinone and fatty acid profile of strain SW-277^T were similar to those of members of the phylogenetically related genera *Salipiger* and *Roseivivax* (Suzuki *et al.*, 1999; Martínez-Cánovas *et al.*, 2004). Members of many genera of the *Alphaproteobacteria* cannot be differentiated clearly by chemotaxonomic characteristics including major ubiquinone and fatty acid types (Nishimura *et al.*, 1994; Martínez-Cánovas *et al.*, 2004; Martens *et al.*, 2006). Strain SW-277^T could be differentiated from members of the genera *Salipiger* and *Roseivivax* by differences in some phenotypic properties as listed in Table 1. Low 16S rRNA gene sequence similarity values between strain SW-277^T and all other members of the *Alphaproteobacteria*, together with differential phenotypic properties, suggest that strain SW-277^T represents a novel

species in a new genus in the *Alphaproteobacteria*, for which the name *Donghicola eburneus* gen. nov., sp. nov. is proposed.

Description of *Donghicola* gen. nov.

Donghicola [Dong.hi'co.la. N.L. n. *Donghae* the Korean name of the East Sea in Korea; L. suff. *-cola* (from L. n. *incola*) a dweller, inhabitant; N.L. masc. n. *Donghicola* a dweller of the East Sea in Korea].

Cells are Gram-negative, aerobic, non-spore-forming cocci or rods. The predominant ubiquinone is Q-10. The major fatty acids are C_{18:1}ω7c and C_{16:0}. The type species is *Donghicola eburneus*.

Description of *Donghicola eburneus* sp. nov.

Donghicola eburneus (e.bur.ne'us. L. masc. adj. *eburneus* white as ivory).

Cells are Gram-negative, slightly halophilic, non-spore-forming cocci or rods (0.6–0.8 × 0.6–2.0 μm) existing singly, in pairs, in tetrads or long chains. Colonies on MA are circular to slightly irregular, slightly convex, smooth, ivory in colour and 2.5–3.5 mm in diameter after 3 days incubation at 37 °C. Growth occurs at 10 and 42 °C, but not at 4 or 43 °C. Optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.0, but not at pH 4.5. Optimal growth occurs in the presence of 2% (w/v) NaCl. Growth occurs in the presence of 11% (w/v) NaCl, but not in the absence of NaCl or in the presence of more than 12% (w/v) NaCl. Anaerobic growth does not occur on MA or on MA supplemented with nitrate. Hypoxanthine and Tweens 20,

40 and 60 are hydrolysed, but aesculin, casein, L-tyrosine and xanthine are not. H₂S is not produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. L-Malate and pyruvate are utilized as carbon and energy sources, but maltose, sucrose, D-trehalose, D-xylose, benzoate, formate and salicin are not. Acid is produced from melibiose, but not from D-melezitose, D-raffinose, L-rhamnose, D-trehalose or myo-inositol. Susceptible to ampicillin, cephalothin, gentamicin, novobiocin, carbenicillin, kanamycin, neomycin and oleandomycin, but not to lincomycin. The major fatty acids (> 10 % of total fatty acids) are C_{18:1}ω7c (61.6 %) and C_{16:0} (13.6 %). Other phenotypic characteristics are given in Table 1.

The type strain is SW-277^T (=KCTC 12735^T=JCM 13604^T), isolated from a seawater sample of Jungdongjin, the East Sea, Korea. The DNA G+C content of strain SW-277^T is 59.7 mol% (determined by HPLC).

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