Hwanghaeicola aestuarii gen. nov., sp. nov., a moderately halophilic bacterium isolated from a tidal flat of the Yellow Sea

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A moderately halophilic Gram-staining-negative bacterium, designated strain Y26^T, was isolated from a tidal flat of Taean coast in South Korea. Cells were strictly aerobic, motile cocci with a single flagellum and showed catalase- and oxidase-positive reactions. Growth of strain Y26^T was observed at 15–35 °C (optimum 25–30 °C), pH 6.0–8.0 (optimum pH 6.5–7.5) and with 1.5–6.0% (w/v) NaCl (optimum 2.0–3.0%). The predominant fatty acids were C_{18:1}ω_{7c} (66.2%), C_{16:0} (12.4%) and C_{10:0} 3-OH (5.0%) and the G + C content of the genomic DNA was 61.0 mol%. Strain Y26^T contained ubiquinone-10 (Q-10) as the major respiratory quinone. Comparative 16S rRNA gene sequence analysis showed that strain Y26^T formed a distinct phyletic lineage from other genera within the *Roseobacter* clade of the class *Alphaproteobacteria* and was most closely related to members of the genera *Maribius*, *Maritimibacter* and *Palleronia* with 93.8–94.6% sequence similarity. On the basis of chemotaxonomic data and molecular properties, strain Y26^T represents a novel genus, *Hwanghaeicola*, within the family *Rhodobacteraceae*, for which the name *Hwanghaeicola aestuarii* gen. nov., sp. nov. is proposed. The type strain is Y26^T (=KACC 13705^T =DSM 22009^T).

Since a Gram-negative bacterium belonging to the *Roseobacter* clade within the class *Alphaproteobacteria* was first reported by Shiba (1991), many new genera of the *Roseobacter* clade have been isolated from various marine environments such as seawater, tidal flats, marine algae, and hypersaline microbial mats (Choi *et al.*, 2007; Lee *et al.*, 2007; Y.-G. Kim *et al.*, 2008; Vandecastelaere *et al.*, 2009; Wang *et al.*, 2009; Yoon *et al.*, 2009; Zheng *et al.*, 2010). The *Roseobacter* clade is known as one of the most abundant groups in marine environments and its members show diverse physiological and morphological features. The Yellow Sea coasts of the Korean peninsula consist of vast tidal flats known as getbol, which are marine habitats having valuable biological resources such as micro-organisms and marine animals. Therefore, bacteria were isolated from these tidal flats and characterized in our laboratory (Kim *et al.*, 2010). Here, we describe the taxonomic characterization of a novel genus isolated from a flat of the

Yellow Sea belonging to the *Roseobacter* clade, for which the name *Hwanghaeicola aestuarii* gen. nov., sp. nov. is proposed.

Strain Y26^T was isolated from a tidal flat of the Taean coast of the Yellow Sea, South Korea, using a previously described procedure with some modifications (J. M. Kim *et al.*, 2008). Briefly, a sediment sample was serially diluted with marine broth (MB; Difco), spread on marine agar 2216 (MA; Difco) plates and incubated at 25 °C for 5 days. Colonies were randomly selected and crude lysates containing genomic DNA from respective colonies were prepared by boiling a small amount of cell material in 100 µl 5% Chelex 100 solution (Bio-Rad) for 10 min followed by centrifugation at 15 000 *g* for 10 min. PCR amplification of 16S rRNA genes from the crude lysates was performed using the universal primers F1 (5'-AGAGTTTGATCMTGGCTCAG-3') and R13 (5'-TACG-GYTACCTTGTTACGACTT-3'), as described previously (Lu *et al.*, 2006). The amplicons were double-digested with *Hae*III and *Hha*I. Restriction fragment length polymorphism (RFLP) patterns were analysed on 2.5% MetaPhore agarose (BioWhittaker) gels and representative PCR

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Y26^T is FJ230842.

Maximum-likelihood and maximum-parsimony trees are available with the online version of this paper.

products containing distinct RFLP patterns were sequenced. The resulting 16S rRNA gene sequences were analysed using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) in GenBank. From the analysis, a novel strain belonging to the *Roseobacter* clade, designated strain Y26^T, was selected for further phenotypic and phylogenetic analysis. The strain was routinely grown aerobically on MA at 30 °C for 3 days, except where indicated otherwise. The strain was stored at -80 °C in MB supplemented with 10 % (v/v) glycerol for preservation. The type strains of related taxa, including *Maribius pelagius* B5-6^T, *Maribius salinus* CL-SP27^T, *Palleronia marismenor* LMG 22959^T and *Maritimibacter alkaliphilus* HTCC2654^T, were used as reference strains for the biochemical tests or fatty acid analysis.

The 16S rRNA gene sequence (1350 nt) of strain Y26^T was compared with available 16S rRNA gene sequences from GenBank using the BLAST program to determine an approximate phylogenetic affiliation. Sequence similarity values between the novel isolate and related taxa were evaluated using the Nucleotide Similarity Search program (<http://147.47.212.35:8080/>; Chun *et al.*, 2007) and aligned by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in the PHYLIP software package, version 3.6 (Felsenstein, 2002). The resulting tree topology reconstructed by using the neighbour-joining method was evaluated using a bootstrap analysis based on 1000 resampled datasets with the PHYLIP package. Comparative analysis of the 16S rRNA gene sequences showed that strain Y26^T was most closely related to *Maribius pelagius* B5-6^T, *Maritimibacter alkaliphilus* HTCC2654^T and *Maribius salinus* CL-SP27^T with similarities of 94.6, 94.4 and 94.3 %, respectively. These levels of 16S rRNA gene sequence similarity are sufficient to potentially define a new genus (Rosselló-Mora & Amann, 2001). Phylogenetic analysis based on 16S rRNA gene sequences also indicated that strain Y26^T formed a distinct phyletic lineage from other related taxa (Fig. 1). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms supported the notion that there was no genus group showing a clear phylogenetic relationship with strain Y26^T within the *Roseobacter* clade (Supplementary Fig. S1, available in IJSEM Online).

Temperature and pH for growth of strain Y26^T were examined by growing the isolate at different temperatures (5–45 °C at 5 °C intervals) on MA and at different pH (5.0–10.5 at 0.5 pH unit intervals) in MB. The pH was adjusted prior to sterilization by the addition of HCl or NaOH and measured again after sterilization. Gram staining was performed using the bioMérieux Gram Stain kit according to the instructions of the manufacturer. Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was evaluated by the production of oxygen bubbles

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain Y26^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates, when greater than 50 %, at branch points. *Rhizobium vitis* NCPPB 3554^T (D14502) was used as an outgroup. Bar, 0.01 changes per nucleotide position.

in 3 % (v/v) aqueous hydrogen peroxide solution (Smibert & Krieg, 1994). Cell morphology, motility and the presence of flagella were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010, JEOL) with 2-day-old cells grown on MA as described previously (Jeon *et al.*, 2004). Requirement and tolerance of NaCl were determined using synthetic ZoBell broth (0.5 % Bacto peptone, 0.1 % yeast extract and 0.01 % ferric citrate) with modified artificial seawater [ASW; 0–15 % (w/v) NaCl at 0.5 % intervals, 5.94 g MgSO₄ · 7H₂O, 4.53 g MgCl₂ · 6H₂O, 0.64 g KCl and 1.3 g CaCl₂ per litre] (Kahng *et al.*, 2009). Ionic requirements were determined using synthetic ZoBell broth with the following combinations of salts (all w/v): (i) 3.0 % NaCl; (ii) 3.0 % NaCl, 0.6 % MgCl₂ · 6H₂O and 0.3 % MgSO₄ · 7H₂O; (iii) 3.0 % NaCl, 0.6 % MgCl₂ · 6H₂O, 0.3 % MgSO₄ · 7H₂O and 0.06 % KCl; and (iv) 3.0 % NaCl, 0.6 % MgCl₂ · 6H₂O, 0.3 % MgSO₄ · 7H₂O, 0.06 % KCl and 0.2 % CaCl₂ · 2H₂O (Choi *et al.*, 2007). Nitrate reduction was assessed according to the method of Lányi (1987). Biochemical features were characterized by using the API 20E and API 20NE kits as recommended by the manufacturer (bioMérieux) except that the kits were incubated for 3 days at 30 °C; all suspension media were supplemented with ASW containing 3.0 % (w/v) NaCl. Additional enzyme activities were determined by the API ZYM system (bioMérieux) at 30 °C based on the manufacturer's instructions. Antibiotic susceptibility tests were performed on MA in duplicate using filter-paper discs (8 mm diameter) containing the following antibiotics: ampicillin (10 µg), polymyxin B (100 U), streptomycin (50 µg), penicillin G (10 IU),

Fig. 2. Transmission electron micrograph showing the general morphology of a negatively stained cell of strain Y26^T after growth for 2 days at 30 °C on marine agar. Bar, 0.5 μm.

gentamicin (30 μg), chloramphenicol (100 μg), tetracycline (30 μg), kanamycin (30 μg), lincomycin (15 μg), oleandomycin (15 μg), carbenicillin (100 μg) and novobiocin (50 μg). Anaerobic growth was assessed on MA under

anaerobic (with 4–10 % CO₂) conditions using the GasPak Plus system (BBL) at 30 °C for 15 days.

Strain Y26^T grew at temperatures between 15 and 35 °C (optimum 25–30 °C), at pH 6.0–8.0 (optimum 6.5–7.5) and in the presence of 1.5–6.0 % (w/v) NaCl (optimum 2.0–3.0 %). Bacterial cells were Gram-stain negative, strictly aerobic, motile cocci (0.8–1.2 μm in diameter) with a single flagellum at 30 °C on MA (Fig. 2). The strain was not able to grow in media with NaCl as a sole salt, but was able to grow in media containing Na⁺ and Mg⁺ ions. Detailed phenotypic features of strain Y26^T are presented in the description and compared with those of closely related taxa in Table 1.

Isoprenoid quinones were analysed using an HPLC (model LC-20A, Shimadzu) equipped with a diode array detector (SPD-M20A, Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil, Akzo Nobel) as described by Komagata & Suzuki (1987). For analysis of fatty acid methyl esters, cells of strain Y26^T were harvested after incubation at 30 °C for 3 days on MA. Analysis of fatty acid methyl esters was performed according to the

Table 1. Phenotypic characteristics that differentiate strain Y26^T from other related members of the family *Rhodobacteraceae*

Strains: 1, strain Y26^T (data from this study); 2, *Maribius pelagius* B5-6^T (data from Choi *et al.*, 2007); 3, *Maribius salinus* CL-SP27^T (Choi *et al.*, 2007); 4, *Palleronia marisminoris* LMG 22959^T (Martínez-Checa *et al.*, 2005; Cho & Giovannoni, 2006); 5, *Maritimibacter alkaliphilus* HTCC2654^T (Lee *et al.*, 2007). All strains are negative for nitrate reduction. +, Positive; –, negative; (+), weakly positive; R, resistant; S, sensitive.

Characteristic	1	2	3	4	5
Origin	Tidal flat, Yellow Sea	Seawater, Sargasso Sea	Hypersaline water	Hypersaline soil	Seawater, Sargasso Sea
Pigmentation	Pink	Beige	Beige	Pink	Beige
Morphology	Cocci	Rod	Rod	Short rod	Short rod
Flagellar	+	–	–	–	–
Temperature range (°C)	15–35	10–35	10–35	20–37	16–37
pH range	6–8	6–9	6–9	5–10	4–12
Oxidase	+	+	+	–	+
Growth at 6 % NaCl	(+)	+	+	+	+
API 20E test:*					
Indole production	+	–	–	–	–
Voges–Proskauer test	+	–	–	–	–
β-Galactosidase	–	+	–	+	–
Hydrolysis of:*					
Gelatin	+	(+)	–	–	–
Aesculin	+	+	+	–	–
Antibiotic susceptibility:*					
Penicillin G	S	S	S	S	R
Tetracycline	R	S	S	R	S
Streptomycin	R	S	R	S	S
Major cellular fatty acids	C _{18:1} ω7c, C _{16:0}	C _{18:1} ω7c, C _{16:0} 2-OH, C _{16:0}	C _{18:1} ω7c, C _{19:0} cyclo ω8c	C _{18:1} ω7c, C _{19:0} cyclo ω8c	C _{18:1} ω7c, C _{16:0} 2-OH
Major hydroxy acid	C _{10:0} 3-OH	C _{16:0} 2-OH, C _{18:1} 2-OH	C _{10:0} 3-OH	C _{10:0} 3-OH	C _{16:0} 2-OH, C _{18:1} 2-OH
DNA G + C content (mol%)	61.0	70.0	66.7	64.2	61.7

*Data from this study.

instructions of the Microbial Identification System (MIDI; Microbial ID). The DNA G+C content of strain Y26^T was determined by using an HPLC fitted with a reversed-phase column (250 × 4.6 mm, GROM-SIL 100 ODS-2FE, GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strain Y26^T was ubiquinone Q-10 (Q-10). The major cellular fatty acids (>2% of the total fatty acids) were C_{18:1}ω7c (66.2%), C_{16:0} (12.4%), C_{10:0} 3-OH (5.0%), C_{19:0} cyclo ω8c (3.7%), unknown 11.799 (3.4%), 11-methyl C_{18:1}ω7c (2.6%) and C_{18:0} (2.7%). Although the overall fatty acid profile of strain Y26^T was similar to those of phylogenetically related taxa (Table 2), significant

differences in the respective proportions of several components clearly distinguished the novel strain from related taxa (Table 2). The DNA G+C content of strain Y26^T was 61.0 mol%. Therefore, the physiological, biochemical and phylogenetic properties of strain Y26^T support its description as a novel genus within the family *Rhodobacteraceae* of the class *Alphaproteobacteria*, for which the name *Hwanghaeicola aestuarii* gen. nov., sp. nov. is proposed.

Description of *Hwanghaeicola* gen. nov.

Hwanghaeicola (Hwang.hae.i'co.la. N.L. n. *Hwanghaeum* Hwanghae, the Korean name of the Yellow Sea in Korea; L. suff. -cola (from L. n. *incola*) a dweller, inhabitant; N.L. masc. n. *Hwanghaeicola* a dweller of the Yellow Sea).

Cells are Gram-negative, strictly aerobic, chemoheterotrophic and moderately halotolerant. Cells are motile cocci with a single flagellum. Oxidase- and catalase-positive. Neither nitrate nor nitrite is reduced. The only isoprenoid quinone detected is ubiquinone-10 (Q-10). The major fatty acids are C_{18:1}ω7c, C_{16:0} and C_{10:0} 3-OH. DNA G+C content is 61.0 mol% (HPLC). Phylogenetically, the genus *Hwanghaeicola* is a member of the family *Rhodobacteraceae* of the phylum *Alphaproteobacteria*. The type species is *Hwanghaeicola aestuarii*.

Description of *Hwanghaeicola aestuarii* sp. nov.

Hwanghaeicola aestuarii (aes.tu.a'ri.i L. gen. n. *aestuarii* of the tidal flat, from where the organism was first isolated).

In addition to the traits reported for the genus description, cells are 0.8–1.2 µm in diameter and colonies on MA are pale-pink, convex and round with entire margins. Grows at 15–35 °C (optimum 25–30 °C), pH 6.0–8.0 (optimum pH 6.5–7.5) and with 1.5–6.0% (w/v) NaCl (optimum 2.0–3.0%). Urea, aesculin and gelatin are hydrolysed. ONPG, indole, tryptophan deaminase and the Voges–Proskauer test are positive. H₂S is not produced and citrate is not utilized (API 20E kit). Negative for assimilation of L-arabinose, D-mannose, D-glucose, maltose, D-mannitol, malic acid, potassium gluconate, N-acetylglucosamine, capric acid, adipic acid, trisodium citrate and phenylacetic acid (API 20NE). Alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase activities are present, but trypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are not. Weak enzymic activities are observed for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucuronidase (API ZYM). Resistant to polymyxin B, streptomycin, gentamicin, kanamycin, ampicillin, oleanomycin, lincomycin, tetracycline and carbenicillin, but sensitive to penicillin G, chloramphenicol and novobiocin. Does not grow in media with NaCl as a sole salt, but grows in media containing Na⁺ and Mg²⁺ ions. The major

Table 2. Cellular fatty acid compositions (%) of strain Y26^T and related members of the family *Rhodobacteraceae*

Species: 1, strain Y26^T (this study); 2, *Maribius pelagius* B5-6^T (this study); 3, *Maribius salinus* CL-SP27^T (this study); 4, *Palleronia marisminoris* LMG 22959^T (Martínez-Checa *et al.*, 2005); 5, *Maritimibacter alkaliphilus* HTCC2654^T (this study). Data are expressed as percentages of total fatty acids. Fatty acids amounting to less than 0.5% in all species are not shown. tr, Trace amount (<0.5%); –, not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5
Straight					
C _{12:0}	1.3	–	0.7	–	–
C _{16:0}	12.4	12.6	3.5	4.2	11.2
C _{17:0}	–	tr	0.6	–	1.0
C _{18:0}	2.7	0.6	4.7	3.4	0.6
Unsaturated					
C _{17:1} ω8c	0.5	–	0.9	–	–
C _{18:1} ω7c	66.2	50.5	70.2	68.9	35.0
11-methyl C _{18:1} ω7c	2.6	9.5	3.0	–	12.4
C _{19:0} cyclo ω8c	3.7	tr	6.5	12.8	1.2
C _{20:1} ω7c	tr	–	1.4	–	–
Hydroxy					
C _{10:0} 3-OH	5.0	tr	3.7	5.0	0.6
C _{15:0} 2-OH	–	tr	–	–	1.0
C _{16:0} 2-OH	–	17.5	–	–	26.5
C _{17:0} 2-OH	–	tr	–	–	0.6
C _{16:1} 2-OH	–	0.5	–	–	0.7
C _{18:1} 2-OH	–	5.3	–	–	7.0
Summed features*					
2	1.7	–	–	–	–
3	0.5	1.2	–	–	1.3
7	–	–	2.0	–	tr
Unknown ECL 4.870	–	–	–	2.3	–
Unknown ECL 11.799	3.4	–	2.9	–	–

*Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 2 contained one or more of C_{14:0} 3-OH, iso-C_{16:1} I, an unidentified fatty acid with an equivalent chain-length of 10.928 and C_{12:0} ALDE. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 7 contained one or more of C_{18:1}ω7c, C_{18:1}ω9c and C_{18:1}ω12t.

cellular fatty acids are C_{18:1}ω7c, C_{16:0} and C_{10:0} 3-OH. The DNA G+C content of the type strain is 61.0 mol% (HPLC).

The type strain is Y26^T (=KACC 13705^T =DSM 22009^T), which was isolated from a tidal flat of the Taean coast in South Korea.

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