Huaishuia halophila gen. nov., sp. nov., isolated from coastal seawater

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A novel Gram-negative, non-motile bacterium, designated ZXM137^T, was isolated from seawater collected from a coastal region of Qingdao, China, during a massive green algae (*Enteromorpha prolifera*) bloom. Strain ZXM137^T was strictly aerobic and did not accumulate poly- β -hydroxybutyrate. Growth occurred with 0.5–11.0% (w/v) NaCl, at pH 6–9 (optimum of pH 7) and at 4–45 °C (optimum at 28 °C). It contained Q-10 as the predominant ubiquinone and the major polar lipids were phosphatidylglycerol, phospholipids, and an unidentified aminolipid and lipid. The major cellular fatty acids of strain ZXM137^T were C_{18:1} ω 7c, C_{18:1} ω 6c and 11-methyl C_{18:1} ω 7c. The DNA G + C content was 60.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain ZXM137^T formed a distinct evolutionary lineage within the *Roseobacter* group in the class *Alphaproteobacteria*. On the basis of phenotypic, chemotaxonomic and phylogenetic evidence, strain ZXM137^T represents a novel species in a new genus, for which the name *Huaishuia halophila* gen. nov., sp. nov. is proposed; the type strain is ZXM137^T (=CGMCC 1.8891^T=LMG 24854^T).

In recent years, many novel taxa within the class *Alphaproteobacteria* have been described. This class has been divided into nine orders and the *Roseobacter* group falls within the order *Rhodobacterales*. The *Roseobacter* group is one of the major groups of marine bacteria and is the only group in which cultivated bacteria are closely related to environmental clones (Buchan *et al.*, 2005). Organisms belonging to the *Roseobacter* group have been detected in various marine environments, especially in association with marine algae (González *et al.*, 2000). The phylogenetic diversity of the cultivable bacterial community in the coastal water of Qingdao during the *Enteromorpha prolifera* bloom was investigated and a novel strain (ZXM137^T) belonging to this group was isolated. The phylogenetically most closely related species to the isolate was *Jannaschia pohangensis* (Kim *et al.*, 2008). The aim of the present study was to determine the exact taxonomic position of strain ZXM137^T by using a polyphasic taxonomic approach.

A 10-fold-diluted surface seawater sample (diluted with sterile seawater) collected from the coastal region of

Qingdao, China (36.027° N 120.184° E) on June 28th 2008 during a massive green algae (*Enteromorpha prolifera*) bloom, was spread on marine agar 2216 (MA; Difco) and incubated at 28 °C for 5 days. A whitish colony, strain ZXM137^T, was isolated on the plate and subsequently purified three times on MA at 28 °C. Working cultures were maintained on MA at 28 °C, and stocks were kept as suspensions in sterile 0.9% (w/v) saline supplemented with 15% (v/v) glycerol at –70 °C.

Jannaschia helgolandensis DSM 14858^T, obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) GmbH, Braunschweig, Germany, was used as the reference strain. Standard protocols, including those for Gram staining, catalase and oxidase activities, degradation of casein, starch, gelatin, DNA, Tween 80 and urea, nitrate reduction, and H₂S production from thiosulphate were used (Tindall *et al.*, 2007). Cell morphology was studied using light microscopy (CX; Olympus) and transmission electron microscopy (JEOL; JEM-1200EX), with cells grown for 24 h at 28 °C on MA. The presence of flagella was determined using transmission electron microscopy after cells had been negatively stained with 1% (w/v) phosphotungstic acid. Poly- β -hydroxybutyrate (PHB) accumulation was tested by fluorescence microscopy (BH2; Olympus) using Nile Blue A as a fluorescent stain (Tindall *et al.*, 2007). Growth on trypticase soy agar (Difco) was tested. Growth under anaerobic conditions was

Abbreviations: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; PHB, poly- β -hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Huaishuia halophila* ZXM137^T is FJ436725.

Four supplementary figures and a supplementary table are available with the online version of this paper.

determined after incubation in a dry-seal glass vacuum desiccator on MA for 3 days at 28 °C, with 10 g gallic acid/100 ml 10 % NaOH to consume oxygen. The temperature range for growth was determined on MA plates incubated at 4–55 °C for 10 days (Tindall *et al.*, 2007). Growth in 0–15 % (w/v) NaCl was investigated using synthetic marine ZoBell broth (5 g Bacto peptone, 1 g yeast extract and 0.1 g FePO₄ in 1 l modified artificial seawater) supplied with various concentrations of NaCl (0–15.0 %, w/v), and this artificial seawater was modified from Lyman & Fleming (1940), in which all of the Na⁺ was replaced by appropriate K⁺. The pH range for growth was determined at pH 4–11 (at 1 pH unit intervals) in marine broth 2216E (MB; Difco) prepared with citrate/phosphate buffer (0.2 mol l⁻¹), Tris/HCl buffer (0.05 mol l⁻¹) or Na₂CO₃/NaOH buffer (0.025 mol l⁻¹) (Breznak & Costilow, 1994) by measuring changes in OD₆₀₀ over time. Susceptibility to antibiotics was investigated on MA plates by using discs containing different antibiotics. Acid production and oxidation of substrates was determined by using the API 50CH system (bioMérieux) and Gram-negative MicroPlates (Biolog); other phenotypic and enzymic properties of strain ZXM137^T were tested using the API 20E, API 20NE and API ZYM systems with incubation at 28 °C, according to the manufacturer's instructions except that sterile 1.5 % (w/v) NaCl was used to prepare the inocula. Morphological, cultural, physiological and biochemical characteristics of strain ZXM137^T are given in the genus and species descriptions (see below) or are shown in Table 1 (transmission electron micrographs of strain ZXM137^T are available as Supplementary Fig. S1, available in IJSEM Online).

For fatty acid methyl ester, respiratory quinone and polar lipid analyses, 250 mg cell mass of strain ZXM137^T or reference strain *J. helgolandensis* DSM 14858^T was harvested from MB after incubation at 28 °C for 24 h, when both of the communities reached the logarithmic stage. Analyses of respiratory quinones and polar lipids were carried out by the Identification Service and B. Tindall, DSMZ, Braunschweig, Germany. Fatty acid methyl esters were prepared and analysed according to the standard protocol described in the MIDI (Microbial Identification) system (TSBA version 6.0; Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed using nuclease P1 (Boehringer Mannheim) and the resultant nucleotides were analysed by reversed-phase HPLC.

For 16S rRNA gene sequencing, DNA was extracted from strain ZXM137^T and purified by standard methods (Ausubel *et al.*, 1995). The 16S rRNA gene was amplified by PCR using two universal primers: B8F (5'-AGAGTTTGATCCTGG-CTCAG-3') and B1510 (5'-GGTTACCTTGTTACGACTT-3'). The PCR product was purified using an agarose gel DNA purification kit (TaKaRa Biotechnology) and sequenced in both directions with primers B8F, B1510, interF1 (5'-TCG-ATACTGACGCTGAGGTG-3') and interR1 (5'-AGCGTCA-GTATCGAGCCAGT-3') by Shanghai Biosune (China) with an Applied Biosystems automatic sequencer (ABI3730). The

near-complete 16S rRNA gene sequence (1370 nt) of strain ZXM137^T was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequences were aligned using CLUSTAL x 1.8 (Thompson *et al.*, 1997) and edited manually using BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999). Phylogenetic trees were constructed using the neighbour-joining method and maximum-parsimony methods with Kimura 2-state parameter model analyses implemented in the program MEGA version 4 (Tamura *et al.*, 2007). Relationships among taxa were also established with the maximum-likelihood method in the PHYLIP 3.69 package (Felsenstein, 2009). In each case, bootstrap values were calculated based on 1000 replicates.

Pairwise analysis revealed that strain ZXM137^T exhibited highest 16S rRNA gene sequence similarity with the type strain of *Celeribacter neptunius* (96.1 %), a recently described species, and showed 95.3 % similarity with the type strain of *J. pohangensis*, which belongs to the family *Rhodobacteraceae*. Similarities between ZXM137^T and other members of the *Alphaproteobacteria* were below 94.7 %. The sequence similarities between ZXM137^T and the type strains of *Roseovarius aestuarii*, *Thalassobacter stenotrophicus*, *Loktanelle fryxellensis*, *Loktanelle vestfoldensis* and *Loktanelle koreensis* were 94.3, 94.3, 92.2, 92.0 and 92.9 %, respectively. In the phylogenetic tree based on the neighbour-joining algorithm, strain ZXM137^T formed a clade with *C. neptunius* KMM 6012^T that was related to members of the genus *Roseovarius* (Fig. 1), but formed a separate branch. According to the maximum-parsimony tree (Supplementary Fig. S2, available in IJSEM Online) and maximum-likelihood tree (Supplementary Fig. S3, available in IJSEM Online), ZXM137^T also formed a clade with *C. neptunius* KMM 6012^T. Strain ZXM137^T formed a phylogenetic distinct lineage within the family *Rhodobacteraceae* in these three phylogenetic trees and related to members of the genera *Celeribacter*, *Roseovarius*, *Jannaschia* and *Thalassobacter*. Thus, ZXM137^T represents a novel species in a new genus that is separate from members of related genera based on 16S rRNA gene sequence analysis.

Strain ZXM137^T was Gram-negative, strictly aerobic, rod-shaped (0.8 × 0.3 µm in size) bacterium that lacked flagella. Growth occurred in 0.5–11.0 % (w/v) NaCl, at pH 6–9 (optimum growth at pH 7) and at 4–45 °C. Activities for lipase, amylase, gelatinase and casease were all negative. The distinctiveness of strain ZXM137^T was strongly supported by its physiological and biochemical characteristics compared with members of related genera (Table 1); growth at 45 °C enabled strain ZXM137^T to be differentiated from all other related genera.

The most abundant fatty acid in strain ZXM137^T was C_{18:1}ω7c (56.7 %) followed by C_{18:1}ω6c (13.0 %), 11-methyl C_{18:1}ω7c (11.7 %), C_{18:0} (6.9 %), C_{16:0} (3.2 %) and

Table 1. Phenotypic characteristics that differentiate *Huaishuia* gen. nov. from related genera

Genera: 1, *Huaishuia* gen. nov. (data from this study); 2, *Jannaschia* (Wagner-Döbler *et al.*, 2003; Adachi *et al.*, 2004; Macián *et al.*, 2005b; Choi *et al.*, 2006; Yoon *et al.*, 2007a; Kim *et al.*, 2008); 3, *Celeribacter* (Ivanova *et al.*, 2010); 4, *Loktanelia* (Lau *et al.*, 2004; Van Trappen *et al.*, 2004; Ivanova *et al.*, 2005; Weon *et al.*, 2006; Hosoya & Yokota, 2007; Yoon *et al.*, 2007b); 5, *Oceanicola* (Cho & Giovannoni, 2004; Gu *et al.*, 2007; Lin *et al.*, 2007; Yuan *et al.*, 2009); 6, *Roseisalinus* (Labrenz *et al.*, 2005); 7, *Thalassobacter* (Macián *et al.*, 2005a; Pujalte *et al.*, 2005; Kim *et al.*, 2009); 8, *Roseovarius* (Labrenz *et al.*, 1999; González *et al.*, 2003; Biebl *et al.*, 2005; Boettcher *et al.*, 2005; Wang *et al.*, 2009). +, Positive; –, negative; v, varies between species and/or strains; ND, not determined.

Characteristic	1	2*	3	4	5	6	7	8
Colony colour†	W	O, W, B, R, Br (W)	W	P, O, W	Y, W	R	P, Br	Y, W, P, O, R
Motility	–	v (–)	+	v	–	+	+	v
PHB	–	v (+)	–	ND	+	+	+	ND
Bud formation	–	ND (–)	ND	ND	ND	+	+	+
NaCl range (%)	0.5–11.0	0.34–10.0	1–8	0–14	0–10	1–13	0.85–8.0	0.3–12.0
Growth at:								
4 °C	+	v (–)	–	v	v	+	–	v
45 °C	+	– (–)	–	–	–	–	–	–
ONPG	+	v (+)	ND	v	v	ND	v	–
Hydrolysis of (in API 20E):								
Urea	+	v (–)	+	v	v	ND	–	–
Aesculin	+	v (–)	ND	v	v	ND	+	–
Arginine dihydrolase	+	– (–)	–	–	–	ND	–	–
Lysine decarboxylase	+	– (–)	–	–	ND	ND	–	+
Ornithine decarboxylase	+	– (–)	–	–	ND	ND	–	–
Acid production from (in API 50CH):								
Glycerol	–	v (+)	ND	ND	–	–	+	v
Mannitol	+	v (+)	ND	v	v	–	v	v
Trehalose	–	– (–)	ND	+	v	–	–	v
Cellobiose	–	v (+)	ND	+	v	–	–	–
D-Fructose	+	v (+)	ND	+	v	–	+	–
D-Galactose	+	v (+)	ND	+	+	–	v	v
D-Glucose	+	v (+)	ND	v	v	–	+	v
D-Mannose	+	v (+)	ND	–	v	–	v	–
Arabinose	–	– (–)	ND	v	v	–	v	–
Sorbitol	–	v (+)	ND	ND	v	–	+	ND
Major fatty acids	C _{18:1} ω7c, C _{18:1} ω6c, 11-methyl C _{18:1} ω7c	C _{18:1} ω7c, C _{18:0} (C _{18:1} ω7c, C _{18:0})	C _{18:1} ω7c, C _{18:1} ω9c, C _{18:0}	C _{18:1} ω7c, C _{16:0} , C _{10:0} 3-OH	C _{18:1} ω7c, C _{16:0} , C _{19:0} cyclo, C _{18:1} methyl	C _{18:1} ω7c, C _{16:0} , C _{18:0}	C _{18:1} ω7c, 11-methyl C _{18:1} ω7c, C _{16:0}	C _{18:1} ω7c, C _{16:0} , C _{18:0}
DNA G + C content (mol%)	60.1	63.0–65.2	59	56.8–66.4	64.6–71.5	67	56.0 and 59.1	58.6–66.0

*Data in parentheses are for the type strain of *Jannaschia helgolandensis* (data from this study).

†B, Beige; Br, brown; O, orange; P, pink; R, red; W, white; Y, yellow.

Fig. 1. Phylogenetic dendrogram of *Huaishuia halophila* ZXM137^T and strains of related species based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method with the software package MEGA version 4.0 (Tamura *et al.*, 2007). Bootstrap values (%) based on 1000 replicates are given at branching points. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

10-methyl $C_{19:0}$ (2.9%). Consistent with neighbouring genera, ZXM137^T contained $C_{18:1\omega7c}$ as the major fatty acid. A large amount of $C_{18:1\omega6c}$ was detected in strain ZXM137^T but this fatty acid was not found in members of other related genera (Table 1 and Supplementary Table S1, available in IJSEM Online).

The predominant ubiquinone detected in strain ZXM137^T was Q-10 at a peak area ratio of 100%, which was consistent with some species in the family *Rhodobacteraceae*, such as *J. pohangensis*, *R. aestuarii* and *L. koreensis* (Kim *et al.*, 2008; Yoon *et al.*, 2008; Weon *et al.*, 2006). Major polar lipids in strain ZXM137^T were phosphatidylglycerol, phospholipids, and an unidentified aminolipid and lipid (Supplementary Fig. S4, available in IJSEM Online). Some species in the genera *Jannaschia*, *Thalassobacter* and *Roseovarius* were found to have phosphatidylglycerol but they also had diphosphatidylglycerol and phosphatidylcholine as major polar lipids (Wagner-Döbler *et al.*, 2003; Macián *et al.*, 2005a; Labrenz *et al.*, 1999), which were not found in ZXM137^T. The DNA G + C content of strain ZXM137^T was 60.1 mol%.

Therefore, on the basis of phenotypic, phylogenetic and genetic evidence, strain ZXM137^T should be classified as a representative of a novel species in a new genus, for which the name *Huaishuia halophila* gen. nov., sp. nov. is proposed.

Description of *Huaishuia* gen. nov.

Huaishuia (Hua.i.shu'i.a. N.L. fem. n. *Huaishuia* named after Professor Huai-Shu Xu, who discovered the special live form viable but non-culturable state of bacteria).

Gram-negative, strictly aerobic, slightly halophilic. Cells are non-motile rods. PHB accumulation is not observed. Positive for cytochrome oxidase and catalase. The major cellular fatty acids are $C_{18:1\omega7c}$, $C_{18:1\omega6c}$ and 11-methyl $C_{18:1\omega7c}$. The major polar lipids are phosphatidylglycerol, phospholipids, and an unidentified aminolipid and lipid. Phylogenetically, the genus *Huaishuia* is a member of *Roseobacter* group in the class *Alphaproteobacteria*. The type species is *Huaishuia halophila*.

Description of *Huaishuia halophila* sp. nov.

Huaishuia halophila [ha.lo.phi'la. Gr. n. *hals halos*, salt; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*) friend, loving; N.L. fem. adj. *halophila* salt-loving].

In addition to the characteristics that define the genus, the type species has the following characteristics. Cells are approximately 0.8 µm in length by 0.3 µm in width. Colonies on MA are light white, circular, smooth and convex with an entire edge. Growth occurs on trypticase soy agar. Grows in 0.5–11% (w/v) NaCl; Na⁺ is required

for growth and growth is not detected without ASW. Grows at pH 6–9 and 4–45 °C (optimum growth at pH 7 and 28 °C). No growth is detected at 0 °C or 50 °C. Hydrolyses aesculin and urea (in API 20E), but not starch, casein, gelatin, DNA or Tween 80. Negative for nitrate reduction, Voges–Proskauer test, and H₂S and indole production. Weakly positive for alginase. According to API 20E, positive for β -galactosidase, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, but negative for tryptophan deaminase. According to API ZYM, positive for alkaline phosphatase, esterase (C4), esterase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -glucosidase and α -glucosidase, but negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Citrate utilization is positive. According to API 50CH, acids are produced from the following substrates: D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol and D-mannitol. According to API 50CH, acids are not produced from the following substrates: glycerine, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, dulcitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, potassium 5-ketogluconate and potassium gluconate. According to API 20NE, capric acid, adipic acid and trisodium citrate are hydrolysed, but malic acid is not hydrolysed. According to Biolog GN, the following substrates are oxidized: dextrin, Tween 80, L-rhamnose, sucrose, monomethyl succinate, acetic acid, citric acid, α -, β - and γ -hydroxybutyric acid, α -ketoglutaric acid, succinic acid and L-glutamic acid. Resistant to 30 μ g vancomycin and 2 μ g clindamycin, but sensitive to 30 μ g midecamycin, 10 μ g norfloxacin, 5 μ g ofloxacin, 5 μ g ciprofloxacin, 100 U polymyxin B, 75 μ g sulfamethoxazole, 300 μ g furazolidone, 30 μ g tetracycline, 30 μ g ceftriaxone, 75 μ g cefoperazone, 30 μ g amikacin, 10 μ g gentamicin, 30 μ g kanamycin, 30 μ g neomycin, 30 μ g chloramphenicol, 30 μ g doxycycline, 30 μ g minocycline, 15 μ g erythromycin, 10 U penicillin, 1 μ g oxacillin sodium, 10 μ g ampicillin, 100 μ g carbenicillin, 100 μ g piperacillin, 30 μ g cephalothin IV, 30 μ g cephalothin V, 30 μ g cephalothin VI, 30 μ g cefuroxime and 30 μ g ceftazidime. The predominant ubiquinone is Q-10. Major polar lipids are phosphatidylglycerol, phospholipids, and an unidentified aminolipid and lipid.

The type strain is ZXM137^T (=CGMCC 1.8891^T=LMG 24854^T), isolated from seawater collected from a coastal region of Qingdao, China, during a massive green algae (*Enteromorpha prolifera*) bloom. The DNA G + C content is 60.1 mol% (determined by HPLC).

Acknowledgements

This work was supported by the National High Technology R&D Program of China (grant 2007AA09Z434) and the National Natural Science Foundation of China (grant 40876067).

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