Thalassomonas eurytherma sp. nov., a marine proteobacterium

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Two Gram-staining-negative, aerobic, rod-shaped bacterial strains, designated Za6a-12^T and Za6a-17, were isolated from seawater of the East China Sea. Cells of Za6a-12^T and Za6a-17 were approximately 1.5–2.0 μm×0.5–0.7 μm and motile by a single polar flagellum. Strains grew optimally at pH 7.5-8.0, 28 °C, and in the presence of 2.5–3.0 % (w/v) NaCl. Chemotaxonomic analysis showed that the predominant respiratory quinone of strains Za6a-12^T and Za6a-17 was ubiquinone-8 (>97 %), and the major fatty acids were $C_{14:0}$, $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{17:1}\omega8c$. Their DNA G+C contents were 42.7 mol% and 42.8 mol%, respectively. 16S rRNA gene sequence analysis revealed that the isolates belonged to the genus *Thalassomonas* and showed the highest sequence similarity to *Thalassomonas loyana* CBMAI 722^T (95.9 %). Strains Za6a-12^T and Za6a-17 could be differentiated from *T. loyana* CBMAI 722^T according to their phenotypic and chemotaxonomic features, DNA G+C contents and fatty acid composition. On the basis of these features, we propose strains Za6a-12^T and Za6a-17 to be representatives of a novel species of the genus *Thalassomonas* with the name *Thalassomonas eurytherma* sp. nov. suggested. Strain Za6a-12^T (=CGMCC 1.12115^T=JCM 18482^T) is the type strain of this novel species.

The genus *Thalassomonas* was first proposed by Macián *et al.* (2001), and the genus description was later emended by Jean *et al.* (2006). Members of this genus are Gram-staining-negative rods belonging to the class *Gammaproteobacteria*. They are catalase-positive and oxidase is usually present. Cells are non-motile or motile by means of a single polar or subpolar flagellum and most strains are halophilic growing in 2–4 % NaCl.. They are also mostly mesophilic, growing at 20–35 °C, but not at 45 °C; some can grow at 4–37 °C. Cells contain either $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH or $C_{16:0}$ as the most abundant fatty acid(s) and Q-8 as the major respiratory quinone. At the time of writing, the genus *Thalassomonas* comprised seven species with validly published names: *Thalassomona viridans* (Macián *et al.*, 2001), *Thalassomona ganghwensis* (Yi *et al.*, 2004), *Thalassomona loyana* (Thompson *et al.*, 2006), *Thalassomona agarivorans* (Jean *et al.*, 2006), *Thalassomona actiniarum* (Hosoya *et al.*, 2009), *Thalassomona haliotis* (Hosoya *et al.*, 2009) and *Thalassomona agariperforans* (Park *et al.*, 2011). In the present study, two marine strains, Za6a-12^T and Za6a-17, were isolated from a seawater sample collected from the Zhoushan Islands in the East China Sea. The aim was to determine the taxonomic position of these strains using a polyphasic approach, to include the identification of their phenotypic and chemotaxonomic features and a phylogenetic analysis.

The seawater sample was collected in July 2010 from the Zhoushan Islands (122° 59′ 37″ E 29° 25′ 27″ N) of the East China Sea at a depth of 58 m (temperature 19.5 °C, salinity 31.3 %). The sample was diluted, using a tenfold series dilution method, spread on modified ZoBell 2216E agar medium (Oppenheimer & ZoBell, 1952) and incubated at 25 °C. The modified ZoBell 2216E agar medium

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains Za6a-12^T and Za6a-17 are JQ288724 and JQ288725, respectively.

Two supplementary tables are available with the online version of this paper.

contained (per litre distilled water): yeast extract 0.5 g, peptone 0.1 g, ferric citrate 0.1 g, NaCl 19.45 g, $MgCl_2 . 6H_2O$ 8.8 g, $CaCl_2 . 2H_2O$ 1.8 g, KCl 0.55 g, $NaHCO_3$ 0.16 g, Na_2SO_4 3.24 g, KBr 0.08 g, $SrCl_2$ 34 mg, H_3BO_4 22 mg, $NaSiO_4$ 4 mg, NaF 2.4 mg, NH_4NO_3 1.6 mg, Na_2HPO_4 8 mg, agar 20 g, pH 7.4 adjusted with NaOH. After 48 h of incubation, two cream colonies were collected and designated Za6a-12^T and Za6a-17. After repeated purifying, the strains were routinely cultured on ZoBell 2216E agar medium (marine agar; MA; Oppenheimer & ZoBell, 1952). For normal cultivation, all strains used in this study (Za6a-12^T, Za6a-17, *T. loyana* CBMAI 722^T and *T. viridans* CECT 5083^T) were cultured on MA, at 28 °C.

Growth at various NaCl concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 7.5 and 10 %, w/v) was determined in marine broth (MB). The pH range for growth was determined by adding 40 mM MES (pH 5.0–6.0; BBI), MOPS (pH 6.5–7.5; BBI), Tricine buffer (pH 8.0–8.5 BBI) and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic (CAPSO; pH 9.0–10.0; BBI) to marine broth (MB), respectively. The temperature range for growth was determined on marine broth (MB) at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 42 and 45 °C. Cell morphology was examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) using exponentially growing cells incubated in MA for 24 h.

Single carbon source assimilation tests were performed in basal medium (BM; Baumann *et al.*, 1984) supplemented with 0.01 % (w/v) yeast extract and the corresponding filter-sterilized sugar (0.2 % w/v), alcohol (0.2 % v/v), organic acid (0.1 % w/v) or amino acid (0.1 % w/v). The basal medium (BM) contained (per litre distilled water): NH_4Cl 1.0 g, $K_2HPO_4 . 3H_2O$ 0.075 g, $FeSO_4 . 7H_2O$ 0.028 g, Tris/HCl (1M, pH 7.5) 50 ml and half-strength artificial seawater (ASW). ASW contained (per litre distilled water): NaCl 50 g, $MgSO_4 . 7H_2O$ 24.6 g, KCl 1.5 g, $CaCl_2 . 2H_2O$ 2.9 g. Oxidation of 1 % (w/v) *p*-aminodimethylaniline oxalate was used to detect oxidase activity. Catalase activity was determined by observing bubble production in 3 % (v/v) H_2O_2 solution with optical microscopy (BX40; Olympus). MA containing 0.2 % (w/v) soluble starch was used to examine degradation of starch using the method of Smibert & Krieg (1994). Hydrolysis of Tweens 20, 40, 60 and 80 was tested on MA supplemented with 1 % (v/v) Tween 20, 40, 60 or 80. MA containing 1 % (w/v) skimmed milk (Difco) was used to determine the degradation of casein. MA supplemented with 0.2 % (w/v) DNA (salmon sperm; BBI) and 0.015 % (w/v) ammonium methylbenzene blue (BBI) was used to determine the hydrolysis of DNA. Degradation of L-tyrosine was tested on MA supplemented with 0.5 % (w/v) L-tyrosine. Nitrate reduction, urease activity and the ability to hydrolyse aesculin, casein and gelatin were determined according to the method of Dong & Cai (2001). Sensitivity to antibiotics was detected on MA with discs containing the following antibiotics (μg per disc unless stated otherwise): amoxicillin (10), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), ceftriaxone (30), cefoxitin (30), chloramphenicol (30), erythromycin (10), kanamycin (30), neomycin (30), nitrofurantoin (300), novobiocin (30), nystatin (100), penicillin (10 IU), polymyxin B (300 IU), rifampicin (5), streptomycin (10), tetracycline (30) and tobramycin (10). Acid production was tested using API 50CH (bioMérieux) strips. Leifson modified O/F medium (MOF; Leifson, 1963) was used to suspend cells for inoculation in the API 50CH test. API 50CH strips were read after 24 h and 48 h. Additional physiological characteristics and enzyme activities were tested by API 20NE and API ZYM (bioMérieux), and read after 24 h and 4 h, respectively. H_2S production, methyl red and Voges–Proskauer reactions were determined as described by Wu *et al.* (2010).

Isoprenoid quinones were analysed using reversed-phase HPLC (Komagata & Suzuki, 1987). The cells for fatty acid methyl ester (FAME) analysis were incubated on MA at 28 °C for 24 h and analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID) with standard MIS Library Generation Software version 4.5. Genomic DNA was collected using the method described by Marmur & Doty (1962) and hydrolysed with P1 nuclease. The nucleotides were dephosphorylated with calf intestine alkaline phosphatase. The G+C content of these deoxyribonucleosides was determined by reverse-phase HPLC and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Mesbah & Whitman, 1989). DNA–DNA hybridizations were performed by the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huss *et al.* (1983), using a Beckman DU 800 Spectrophotometer.

The 16S rRNA gene was amplified by PCR. PCR products were cloned into pMD 19-T vectors (TaKaRa) for sequencing (Xu *et al.*, 2007). The complete 16S rRNA sequences of strains Za6a-12^T and Za6a-17 (1346 bp and 1370 bp, respectively) were identified on the EzTaxon-e service (Kim *et al.*, 2012) by using the EzTaxon-e tool. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with the MEGA5 program package (Tamura *et al.*, 2011). For the neighbour-joining method, evolutionary distances were calculated with the MEGA5 program package, according to the algorithm of the Kimura two-parameter model (Kimura, 1980).

16S rRNA gene sequence analysis indicated that strains Za6a-12^T and Za6a-17 belonged to the genus *Thalassomonas*. 16S rRNA gene sequence similarities between the isolates and species of the genus *Thalassomonas* were less than 97.0 %, ranging from 93.8 to 95.9 %, and exhibiting the highest sequence similarity to *T. loyana* CBMAI 722^T (95.9 %). The 16S rRNA gene sequence similarity of strains Za6a-12^T and Za6a-17 was 99.9 %. The phylogenetic trees reconstructed with all three treeing methods showed that strains Za6a-12^T and Za6a-17 fell into the clade comprising only species of the genus *Thalassomonas*, forming a cluster with *T. loyana* CBMAI 722^T, *T. agarivorans* TMA1^T, *T. agariperforans*

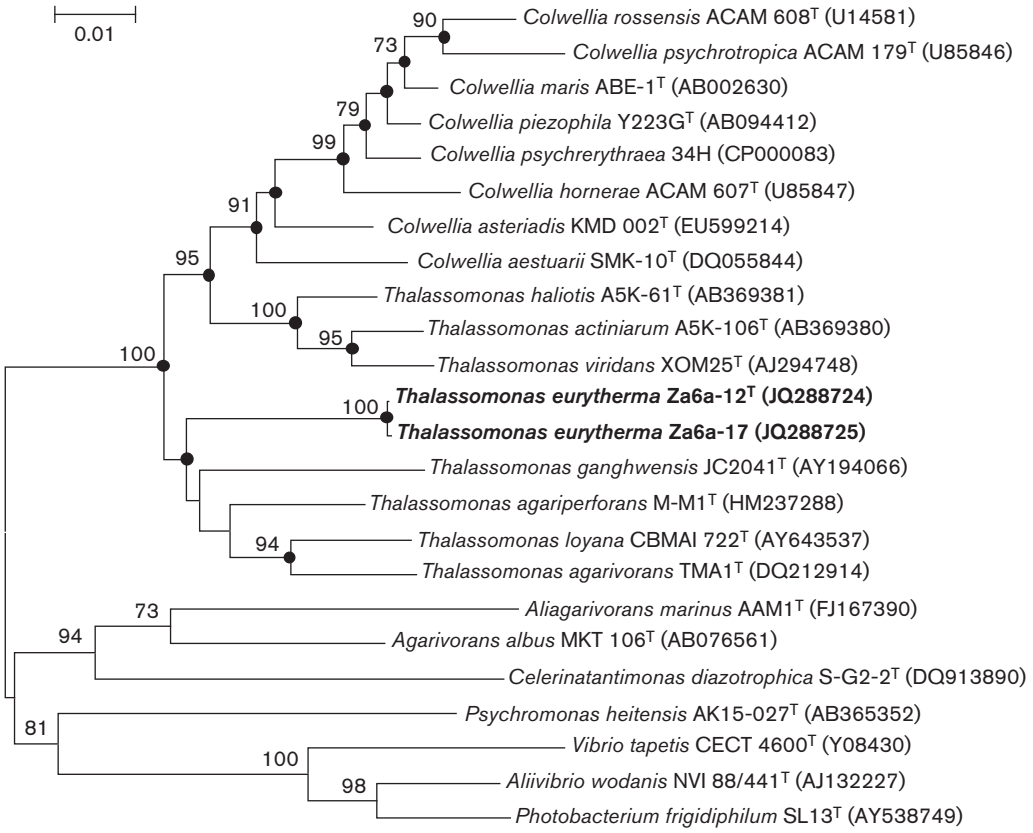

Fig. 1. Neighbour-joining tree using the Kimura two-parameter model based on 16S rRNA gene sequences, showing the phylogenetic relationships of the novel isolates and related members of the genus *Thalassomonas* and other relative genera. Bootstrap values are based on 1000 replicates; values >70 % are shown. Filled circles indicate nodes also obtained in both maximum-likelihood and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position.

M-M1^T and *T. ganghwensis* JC2041^T (Fig. 1). In this cluster, strain Za6a-12^T was found to be closely related to strain Za6a-17 in a new branch; this was supported by a high bootstrap value (100 % with all three methods). The DNA–DNA relatedness value of 91 % between strain Za6a-12^T and Za6a-17 was significantly higher than the value of 70 % considered to be the threshold for the delineation of species (Wayne *et al.*, 1987).

Strain Za6a-12^T grew optimally at pH 7.5, at 28 °C, and in the presence of 2.5–3.0 % (w/v) NaCl. Strain Za6a-17 grew optimally at pH 7.5–8.0, at 28 °C, and in the presence of 2.5 % (w/v) NaCl. Other physiological and biochemical characteristics of strains Za6a-12^T and Za6a-17 are included in the species description. A comparison of the physiological and biochemical characteristics of strains Za6a-12^T, Za6a-17, *T. loyana* CBMAI 722^T and *T. viridans* CECT 5083^T are shown in Table 1 and Table S1 (available in the online Supplementary Material). Several characteristics were found to discriminate strains Za6a-12^T and Za6a-17 from *T. loyana* CBMAI 722^T and *T. viridans* CECT 5083^T.In particular, strains Za6a-12^T and Za6a-17 could grow at 4 and 42 °C, while other species of the genus could not. All strains were susceptible to (μg per disc unless stated otherwise) amoxicillin (10), ampicillin (10), carbenicillin (100), cefotaxime (30), ceftriaxone (30), cefoxitin (30), chloramphenicol (30), erythromycin (10), nitrofurantoin (300), novobiocin (30), penicillin (10 IU), polymyxin B (300 IU) and rifampicin (5), but not bacitracin (0.04 IU), neomycin (30), nystatin (100), streptomycin (10), tetracycline (30) or tobramycin (10). *T. loyana* CBMAI 722^T and *T. viridans* CECT 5083^T were susceptible to kanamycin (30), while Za6a-12^T and Za6a-17 were not.

Strains Za6a-12^T and Za6a-17 contained straight-chain fatty acids and unsaturated fatty acids such as $C_{14:0}$, $C_{16:0}$ and $C_{17:1}\omega8c$; these fatty acids were also considered to be major components of most species of the genus *Thalassomonas* (Macián *et al.*, 2001; Yi *et al.*, 2004; Thompson *et al.*, 2006; Jean *et al.*, 2006; Park *et al.*, 2011). The most abundant fatty acid of strains Za6a-12^T and Za6a-17 were summed features 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) as with most species of the genus *Thalassomonas* (Jean *et al.*, 2006). The fatty acid patterns of strains Za6a-12^T and Za6a-17 were similar to those of *T. loyana* CBMAI 722^T and *T. viridans* CECT 5083^T, but there were differences in the proportions of some fatty acids (Table S1). The predominant respiratory quinone of the isolates was ubiquinone-8 (>97 %) as with other species

Table 1. Differential characteristics of strains Za6a-12^T and Za6a-17, *Thalassomonas loyana* CBMAI 722^T and *Thalassomonas viridans* CECT 5083^T.

Strains: 1, Za6a-12^T; 2, Za6a-17; 3, *T. loyana* CBMAI 722^T; 4, *T. viridans* CECT 5083^T. +, Positive; –, negative; W, weakly positive. Unless stated otherwise, data were obtained from this study under identical growth conditions.

Characteristic	1	2	3	4
Pigment	Cream	Cream	Cream	Green*
Growth at 4 and 42 °C	+	+	–†	–*
NaCl range for growth (%)	1.5–3.5	1.5–3.0	0–10.0†	2–4*
Oxidase	+	+	–	+
Nitrate reduction	–	–	+	–
Hydrolysis of:				
Tween 60	–	–	–	+
Urea	+	–	+	–
Production of:				
Arginine dihydrolase	+	–	+	–
α-Chymotrypsin	–	–	W	–
β-Galactosidase	–	–	+	–
Acid production from:				
Amygdalin	+	+	–	–
Arbutin	–	–	–	+
Cellobiose	+	+	W	–
Gentiobiose	+	+	–	–
5-Ketogluconate	+	+	–	–
D-Glucose	+	+	–	W
Ribose	–	–	–	+
Starch	+	+	W	–
DNA G+C content (mol%)	42.7	42.8	39.3†	48.4*

*Data from Macián *et al.*, 2001.
†Data from Thompson *et al.*, 2006.

of the genus *Thalassomonas* (Yi *et al.*, 2004; Hosoya *et al.*, 2009; Park *et al.*, 2011). The G+C contents of strains Za6a-12^T and Za6a-17 were 42.7 mol% and 42.8 mol%, respectively (as determined by HPLC), which discriminates the isolates from *T. loyana* CBMAI 722^T (39.3 mol%, Thompson *et al.*, 2006) and *T. viridans* CECT 5083^T (48.4 mol%, Macián *et al.*, 2001).

On the basis of 16S rRNA gene sequence comparisons and their physiological and chemotaxonomic characteristics, it is proposed that strains Za6a-12^T and Za6a-17 represent a novel species of the genus *Thalassomonas* for which the name *Thalassomonas eurytherma* sp. nov. is proposed.

Description of *Thalassomonas eurytherma* sp. nov.

Thalassomonas eurytherma (eu.ry.ther′ma. Gr. adj. *eurys* wide; Gr. adj. *thermos* hot; N.L. fem. adj. *eurytherma* able to tolerate a wide range of temperatures.)

Cells are Gram-staining-negative, aerobic, rod-shaped, approximately 1.5–2.0 μm × 0.5–0.7 μm. Cells are motile by a single polar flagellum. After 2 days of incubation at 28 °C on MA colonies are 1–2 mm in diameter, circular, smooth, elevated and cream. The pH growth range is 6.5–9.0. The temperature range for growth is 4–42 °C. Growth occurs in the presence of 1.5–3.5 % (w/v) NaCl, optimally at pH 7.5–8.0, at 28 °C, and in the presence of 2.5–3.0 % (w/v) NaCl. Oxidase- and catalase- positive. Positive for the degradation of tyrosine, casein, starch, gelatin, aesculin, Tween 20, Tween 40 and Tween 80. DNA and Tween 60 are not hydrolysed. Nitrate is not reduced to nitrite. Negative for indole and H_2S production and in the methyl red test. Positive in the Voges–Proskauer test. In API 20NE tests, glucose fermentation is negative. β-Galactosidase and α-glucosidase are positive. In API ZYM tests, alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive. Esterase (C4), esterase lipase (C8), valine arylamidase, cystine arylamidase and α-glucosidase are weakly positive. Lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Acid is produced from D-glucose, *N*-acetylglucosamine, amygdalin, cellobiose, maltose, starch, gentiobiose, potassium 2-ketogluconate and potassium 5-ketogluconate, but not glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, arbutin, aslicin, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol or potassium gluconate (API 50CH). The following substrates are utilized for growth: L-arabinose, erythritol, mannitol, L-rhamnose, D-mannose, maltose, xylitol, D-sorbitol, dulcitol, melezitose, sucrose, D-glucose, pyruvate, butyrate, formate, propionate, L-glutamine, asparagine, L-ornithine, L-threonine, L-valine, L-leucine, L-alanine, L-proline, L-phenylalanine and L-isoleucine. The following compounds are not utilized as sole carbon sources: α-lactose, ethanol, tartrate, fumaric acid, succinate, bezonic acid, L-glutamic acid, L-cysteine, L-cystine, L-methionine, L-glycine and L-tryptophan. Assimilation of xylose, L-sorbose, D-ribose, glycerol and L-histidine are weakly positive. The predominant respiratory quinone is ubiquinone-8 (>97 %). The major fatty acids are $C_{14:0}$, $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{17:1}\omega8c$. The DNA G+C content is 42.7–42.8 mol% (type strain, 42.7 mol%).

The type strain is Za6a-12^T (=CGMCC 1.12115^T=JCM 18482^T), isolated from the Zhoushan Islands in the East China Sea; strain Za6a-17 (=CGMCC 1.12116=JCM 18483) was isolated from the same location.

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