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Thalassobius aestuarii sp. nov., Isolated from Tidal Flat Sediment

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A strictly aerobic, non-motile, ovoid-shaped *Alphaproteobacteria*, designated strain JC2049^T, was isolated from a tidal flat sediment sample. The results of 16S rRNA gene sequence analysis indicated that this isolate belonged to the genus *Thalassobius*, with a sequence similarity of 96.9-97.3% to other valid *Thalassobius* spp. The cells required 1-7% NaCl for growth (optimum 2%) and accumulated poly-β-hydroxybutyrate. Nitrite was reduced to nitrogen, but nitrate was not reduced to nitrite. No genetic potential for aerobic anoxygenic photosynthesis was detected. The primary isoprenoid quinone (Ubiquinone-10), predominant cellular fatty acids ($C_{18:1}\omega7c$, 11 methyl $C_{18:1}\omega7c$ and $C_{16:0}$) and DNA G+C content (61 mol%) were all consistent with the assignment of this isolate to the genus *Thalassobius*. Several phenotypic characteristics clearly distinguished our isolate from other *Thalassobius* species. The degree of genomic relatedness between strain JC2049^T and other *Thalassobius* species was in a range of 20-43%. The polyphasic data presented in this study indicates that our isolate should be classified as a novel species within the genus *Thalassobius*. The name *Thalassobius aestuarii* sp. nov. is therefore proposed for this isolate; the type strain is JC2049^T (= IMSNU 14011^T = KCTC 12049^T = DSM 15283^T).

Keywords: *Thalassobius aestuarii*, *Roseobacter* clade, *Rhodobacteraceae*, tidal flat, taxonomy

Bacteria belonging to the *Roseobacter* clade in the *Rhodobacteraceae* family, thus far, comprise 24 genera and 45 species. However, many species belonging to this group have been misclassified in unrelated genera, due largely to problematic 16S rRNA gene tree topologies, which can vary significantly according to minor changes in sequence alignment or tree-inferring methods (Yi and Chun, 2004). In the course of our study regarding Korean marine microbial diversity, a number of strains from the *Roseobacter* clade were isolated and analyzed on the basis of their 16S rRNA gene sequences. However, the majority of these remained unidentified, due to the complexity inherent to genus delimitation in this group. One of the isolates, designated JC2049^T, which had evidenced the most profound, albeit still uncertain, relationship with *'Ruegeria gelatinovorans'*, was recently identified via the establishment of the genus *Thalassobius* Arahal *et al.* 2005, and is the subject of the taxonomic investigation described in this work.

The genus *Thalassobius* has been proposed to accommodate a novel *Roseobacter* clade isolated from the Mediterranean Sea, namely *Thalassobius mediterraneus* (Arahal *et al.*, 2005), as well as a misclassified *Ruegeria* species, namely *'R. gelatinovorans'* (Rüger and Höfle, 1992; Uchino *et al.* 1998), which was subsequently transferred to the genus *Thalassobius*, as *Thalassobius gelatinovorus* (Arahal *et al.*, 2005). Based on the polyphasic evidence presented in this study, our isolate appears to represent a distinct novel species in the *Thalassobius* genus, for which the name *Thalassobius aestuarii* sp. nov. has been proposed.

Methods

Bacterial strains

A sediment sample was collected from Korean tidal flat of Ganghwa island, Korea (N 37°35' 31.9", E 126°27'24.5"). This sample was diluted with sterilized artificial seawater (ASW; Lyman and Fleming, 1940), spread onto marine agar 2216 (MA; Difco) and incubated for 3 weeks. The isolate was then routinely cultured on MA at 30°C and maintained as a glycerol suspension (20%, w/v) at –80°C.

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Phylogenetic analysis

Bacterial DNA preparation, PCR amplification, and 16S rRNA gene sequencing were conducted as previously described (Chun and Goodfellow, 1995). The resultant sequence of the JC2049^T strain was manually aligned with the representative sequences of the *Rhodobacteraceae* family, which were obtained from the GenBank database on the basis of bacterial 16S rRNA secondary structure, using the jPHYDIT program (Jeon *et al.*, 2005; Kim *et al.*, 2005). The regions available for all sequences (positions 61-1448; *Escherichia coli* numbering system), excluding any positions likely to manifest ambiguous alignments (positions 76-94 and 1004-1037), were utilized in the preparation of phylogenetic trees. Fitch-Margoliash (Fitch and Margoliash, 1967) and neighbor-joining (Saitou and Nei, 1987) trees were inferred with jPHYDIT software. The evolutionary distance matrices for the distance methods were generated in accordance with the model established by Jukes and Cantor (1969). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees were prepared using the PAUP (version 4; Swofford, 1998) program with the heuristic search option. Resultant tree topologies were evaluated via bootstrap analyses (Felsenstein, 1985) on the basis of 1000 resamplings for the neighbor-joining tree.

Morphological and physiological characteristics

Cellular morphology and motility were evaluated via phase-contrast microscopy, SEM, and TEM, using cells grown on MA. Standard physiological and biochemical tests were conducted as was previously described (Smibert and Krieg, 1994) at 30°C. The hydrolysis of high molecular weight compounds was assessed, using MA as the basal medium. H_2S generation was detected in triple sugar iron agar (TSI; Difco) supplemented with 2% (w/v) NaCl. The accumulation of poly-β-hydroxybutyrate (PHB) was evaluated via Nile blue A staining. Other enzymatic activities were assessed using an API 20NE kit (bioMérieux) and an API ZYM kit (bioMérieux). Strips were inoculated with a heavy bacterial suspension in half-strength ASW and AUX (bioMérieux) medium, supplemented with 2% (w/v) NaCl. Carbon source utilization was evaluated in 96-well tissue culture microplates (Falcon), at a concentration of 0.1% (w/v). Basal medium (BM; Baumann *et al.*, 1972), supplemented with 1% (v/v) vitamin solution (Staley, 1968) and 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957), and modified by reducing the sea salt concentration to half-strength, was employed in the carbon source test. Growth was scored using a microtiter plate reader (PowerWave X, Biotech) at 600 nm.

The growth range for temperature (between 5°C and 50°C with an interval of 5°C), pH (between pH 4 and pH 11 with an interval of 1) and NaCl concentration (between 0% and 10% [w/v] with an interval of 1%) was determined using synthetic ZoBell medium (Zobell, 1941); growth was monitored after up to 4 weeks. Growth under anaerobic conditions was evaluated in an anaerobic chamber (10% CO_2, 10% H_2, 80% N_2; Sheldon Manufacturing).

Chemotaxonomy

Fatty acid methyl esters were prepared from a biomass scraped from the MA medium after 3 days of incubation at 30°C, then analyzed via gas chromatography in accordance with the instructions provided for the Microbial Identification System (MIDI). DNA G+C contents were determined via the HPLC of deoxyribonucleosides, as previously described by Mesbah *et al.* (1989), using a reverse-phase column. Isoprenoid quinones were isolated from freeze-dried biomass, in accordance with the protocols described by Minnikin *et al.* (1984), then analyzed using an HPLC apparatus equipped with a reverse-phase column, as described by Collins (1985).

DNA-DNA hybridization

DNA-DNA hybridization was conducted as described by De Ley *et al.* (1970), with modifications as described by Huss *et al.* (1983), using a Cary 300 Bio model UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller (Varian). Genomic DNA was extracted and purified with phenol/chloroform, via the procedure established by Wolff and Gemmill (1997), then sheared to a mean size of 1kb using a nebulizer (Invitrogen) under nitrogen gas. After the ethanol precipitation of the sheared genomic DNA samples, the pellets were resuspended in 2 × SSC and adjusted to an OD of 1.0 at 260 nm. After the 15 minutes of denaturation of the DNA samples at 99°C, hybridization was conducted at 69°C. The optimal temperature for renaturation (T_{OR}) was calculated via the formula $T_{OR} = 0.51 \times \% (G+C) + 47.0$, and lowered by the addition of 13% formamide.

Amplification of PufLM genes

The potential for aerobic anoxygenic photosynthesis was determined on the genetic level via the PCR amplification of the *pufLM* genes of the bacterial photosynthesis reaction center. The primer pair, pufLF (5'-CTK TTC GAC TTC TGG GTS GG-3') and pufMR (5'-CCC ATG GTC CAG CGC CAG AA-3'), was employed in the amplification procedure, in accordance with the protocols established by Allgaier *et al.* (2003). *Jannaschia cystaugens* LMG 22015^T,

Roseobacter denitrificans NBRC 15277^T and *Staleya guttiformis* LMG 19755^T were used as positive controls, and *Octadecabacter antarcticus* CIP 106731^T, *Ruegeria algicola* NBRC 16653^T and *T. gelatinovorus* NBRC 15761^T were used as negative controls, in accordance with the methods of Yi and Chun (2004).

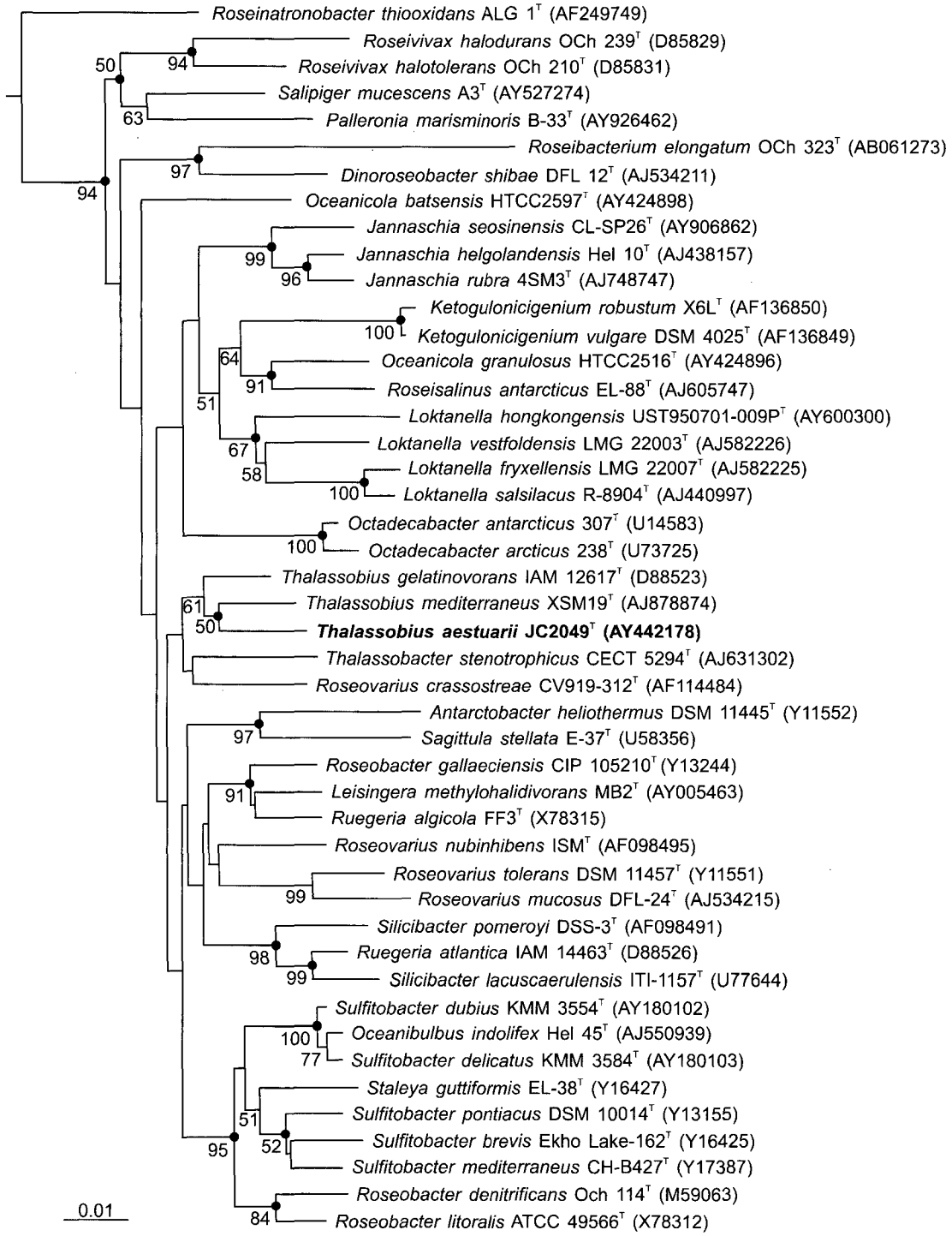

Fig. 1. Phylogenetic position of strain JC2049^T within the *Roseobacter* clade bacteria, based on 16S rRNA gene sequences. The tree was created using the neighbor-joining method; percentage numbers at nodes are levels of bootstrap support (> 50%) from 1000 resampled datasets. Solid circles indicate that the corresponding nodes (groupings) were also recovered in Fitch-Margoliash, maximum likelihood and maximum parsimony trees. *Rickettsia prowazekii* ATCC VR-142^T (M21789) was used as an outgroup (not shown). Bar, 0.01 nt substitution per position.

Results and Discussion

Phylogenetic analysis

The nearly complete 16S rRNA gene sequence of strain JC2049^T (1,369 bp) was obtained (Genbank accession number AY442178) and employed in an initial BLAST search against the GenBank database. The result indicated clearly that our isolate was a member of the *Roseobacter* clade, within the *Rhodobacteraceae* family. After manual alignment against representatives of the *Roseobacter* clade on the basis of the secondary structure of the bacterial 16S rRNA, strain JC2049^T evidenced the most profound 16S rRNA gene sequence similarity to *T. mediterraneus* XSM19^T (97.3%), followed by *T. gelatinovorus* IAM 12617^T (96.9%), *R. algicola* ATCC 51440^T (96.1%), *O. antarcticus* 307^T (95.5%), *Oceanicola batsensis* HTCC 2597^T (95.3%), *Octadecabacter arcticus* 238^T (95.2%) and *Silicibacter lacuscaerulensis* ITI-1157^T (95.1%). No other validly published species evidenced higher than a 95.0% sequence similarity to this test strain. The sequence similarity between *T. mediterraneus* and *T. gelatinovorus* was 97.7%. This close relationship between our isolate and the other *Thalassobius* species was also noted in the phylogenetic trees. Strain JC2049^T and *T. mediterraneus* formed a monophyletic clade in all of the inferred trees referenced in this study (Fig. 1), and also formed a monophyletic clade with *T. gelatinovorus,* in all trees except for the maximum-likelihood tree. However, despite the high degree of sequence similarity (97.3%) between the test strain and *T. mediterraneus*, the bootstrap value was as low as 50%.

Genotypic and phenotypic characteristics

DNA-DNA hybridization was conducted in order to ascertain the degree of genomic relatedness between strain JC2049^T and *T. mediterraneus* and *T. gelatinovorus*, the species with the highest (96.9-97.3%) levels of 16S rRNA gene sequence similarity to the strain. The JC2049^T strain evidenced reassociation values of 20 and 43%, respectively, with regard to the aforementioned species, thereby indicating that it was unrelated to them at the species level (Wayne *et al.*,

Table 1. Characteristics that differentiate strain JC2049^T from other *Thalassobius* species

Characteristics	strain JC2049^T	*T. gelatinovorus*	*T. mediterraneus*
Motility	–	+	–
Nitrate reduction to nitrite	–	+	–
Nitrite reduction to nitrogen	+	–	–
Growth at 40°C	–	+	–
Gelatinase	+	+	–
Utilization of:			
D-Fructose	–	+	+
D-Mannitol	–	+	+
D-Sorbitol	–	+	+
D-Xylose	+	ND	–
Inositol	–	+	+
API ZYM:			
Alkaline phosphatase	+	+	–
Valine arylamidase	+	+	–
Acid phosphatase	+	+	–
Naphtol-AS-BI-phosphohydrolase	+	–	–
β-galactosidase	–	+	–
α-glucosidase	–	+	–
DNA G+C content (mol%)	61	59	57

+, Positive; -, negative ; ND, not determined. Data from this and earlier studies (Arahal *et al.*, 2005; Rüger and Höfle, 1992)

1987). The *PufLM* genes were not amplified in strain JC2049^T, which implies the absence of aerobic anoxygenic photosynthesis. Other genotypic and phenotypic properties are provided in the species description and in Table 1.

Taxonomic conclusions

Strain JC2049^T clearly forms a monophyletic clade with the type species of the genus *Thalassobius* in the phylogenetic trees, with a 16S rRNA gene sequence similarity of 97.3%. This close relationship was corroborated by the determined chemotaxonomic characteristics, i.e. the predominance of $C_{18:1}\omega7$ fatty acids, the presence of ubiquinone-10, and the G+C content (61 mol%). However, the genomic relatedness between strain JC2049^T and other *Thalassobius* species was only in the range of 20-43%, and a number of phenotypic characteristics (Table 1) readily distinguished the test strain from other *Thalassobius* species. The polyphasic data acquired in this study clearly indicate that the test strain merits novel species status within the genus *Thalassobius*. The name *Thalassobius aestuarii* sp. nov. has therefore been proposed for strain JC2049^T.

Description of Thalassobius aestuarii sp. nov

Thalassobius aestuarii (aes.tu.a'ri.i. L. gen. n. *aestuarii*, of a tidal flat).

Gram-negative and oxidase- and catalase-positive. Cells are ovoid, approximately 0.4-0.6 × 1.1-5.3 μm, non-flagellated and non-motile. Colonies are convex, opaque, butyrous, circular with entire margins and cream-colored on MA after 2-3 days. Growth occurs aerobically at pH 6-11 (optimum pH 7), 1-7% NaCl (optimum 2%) and 15-35°C (optimum 35°C). Accumulates PHB. Decomposes gelatin, but not agar, alginate, casein, chitin, carboxymethyl cellulose, DNA, egg yolk, aesculin, starch, or Tween 80. Negative reaction for arginine dihydrolase, β-galactosidase, and urease. Does not produce indole or H_2S. Reduces nitrite to nitrogen, but not nitrate to nitrite. Does not produce acid from glucose. Alkaline phosphatase, leucine arylamidase, acid phosphatase, and naphtol-AS-BI-phosphohydrolase are positive; esterase (C4) and esterase lipase (C8) are weakly positive; lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galatosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative in API ZYM kits. Assimilates acetate, citrate, D-glucose, D-ribose (weakly), D-xylose, L-arginine, L-asparagine, L-lysine, L-ornithine and succinate as sole carbon sources, but not acetamide, D-cellobiose, D-fructose, D-galactose, D-mannitol, D-mannose, D-raffinose, D-salicin, D-sorbitol, D-trehalose, ethanol, inositol, inulin, isopropanol, lactose, L-arabinose, L-ascorbate, L-rhamnose, *N*-acetylglucosamine, polyethylene glycol, salicylate, sucrose, tartrate, or thiamine. Predominant cellular fatty acids are $C_{18:1}\omega7c$ (68.0%), 11 methyl $C_{18:1}\omega7c$ (12.0%) and $C_{16:0}$ (6.8%) and smaller amounts of $C_{16:0}$ 2-OH (3.8%), $C_{18:0}$ (3.0%), $C_{17:0}$ (1.4%) and $C_{10:0}$ 3-OH (1.3%) were also present. Major isoprenoid quinone is Ubiquinone-10. DNA G+C content is 61 mol%. No genetic potential for aerobic anoxygenic photosynthesis is detected.

The type and only strain is JC2049^T (= IMSNU 14011^T = KCTC 12049^T = DSM 15283^T). The Genbank accession number for the 16S rRNA gene sequence is AY442178. Isolated from a sediment sample of tidal flat, Ganghwa Island, Korea.

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