

## *Maribius salinus* gen. nov., sp. nov., isolated from a solar saltern and *Maribius pelagius* sp. nov., cultured from the Sargasso Sea, belonging to the *Roseobacter* clade

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Two strictly aerobic, Gram-negative bacteria, designated strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup>, were isolated from the hypersaline water of a solar saltern in Korea and from the surface water of the Sargasso Sea, respectively. The two strains were rod-shaped, non-motile and grew on marine agar 2216 as beige colonies. Phylogenetic analyses of 16S rRNA gene sequences revealed a clear affiliation of the novel strains to the family *Rhodobacteraceae*. However, the novel strains were only distantly related to members of the *Roseobacter* clade, forming a distinct lineage. Although the 16S rRNA gene sequence similarity between strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> was very high (99.6%), DNA–DNA relatedness between the strains was 48.4%, suggesting that the strains be categorized as two genospecies. Additionally, the two novel strains could be differentiated by DNA G+C contents, fatty acid profiles, carbon source utilization patterns, antibiotic susceptibilities and biochemical characteristics. Based on taxonomic data obtained in this study, strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> represent separate species within a novel genus of the family *Rhodobacteraceae*, for which the names *Maribius salinus* gen. nov., sp. nov. (type species) and *Maribius pelagius* sp. nov. are proposed. The type strains of *Maribius salinus* and *Maribius pelagius* are CL-SP27<sup>T</sup> (=KCCM 42113<sup>T</sup>=JCM 13037<sup>T</sup>) and B5-6<sup>T</sup> (=KCCM 42336<sup>T</sup>=JCM 14009<sup>T</sup>), respectively.

Since the first description of a bacterium belonging to the *Roseobacter* clade within the  $\alpha$ -3 subgroup of the phylum *Proteobacteria* (Shiba, 1991), many novel species in the *Roseobacter* clade have been isolated from various marine environments (seawater, sediment, marine algae, invertebrates, vertebrates, hypersaline microbial mats and coastal

biofilms). Nowadays, the *Roseobacter* clade within the family *Rhodobacteraceae* is known to be one of the most abundant groups in marine environments (Giovannoni & Rappé, 2000; Selje *et al.*, 2004; Buchan *et al.*, 2005). Members of the *Roseobacter* clade show diverse physiological and morphological features (e.g. phototrophy, aerobic sulfite oxidation, organic sulfur compound degradation, methylotrophy, gas vacuoles, poly  $\beta$ -hydroxybutyrate granules, rosette formation) (Arahal *et al.*, 2005; Buchan *et al.*, 2005). In this study, two strains, designated CL-SP27<sup>T</sup> and B5-6<sup>T</sup>, were isolated from hypersaline water of a solar saltern in Korea and surface seawater from the Sargasso Sea, Atlantic Ocean. Although the two novel strains were isolated from different geographical origins, the 16S rRNA gene sequences of the strains were 99.6% similar. A polyphasic taxonomic approach was used to determine whether the two novel strains represent the same or separate species. Based on these data, it is proposed that the isolates represent two novel species in a new genus within the family *Rhodobacteraceae*.

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Abbreviation: PHB, poly  $\beta$ -hydroxybutyrate.

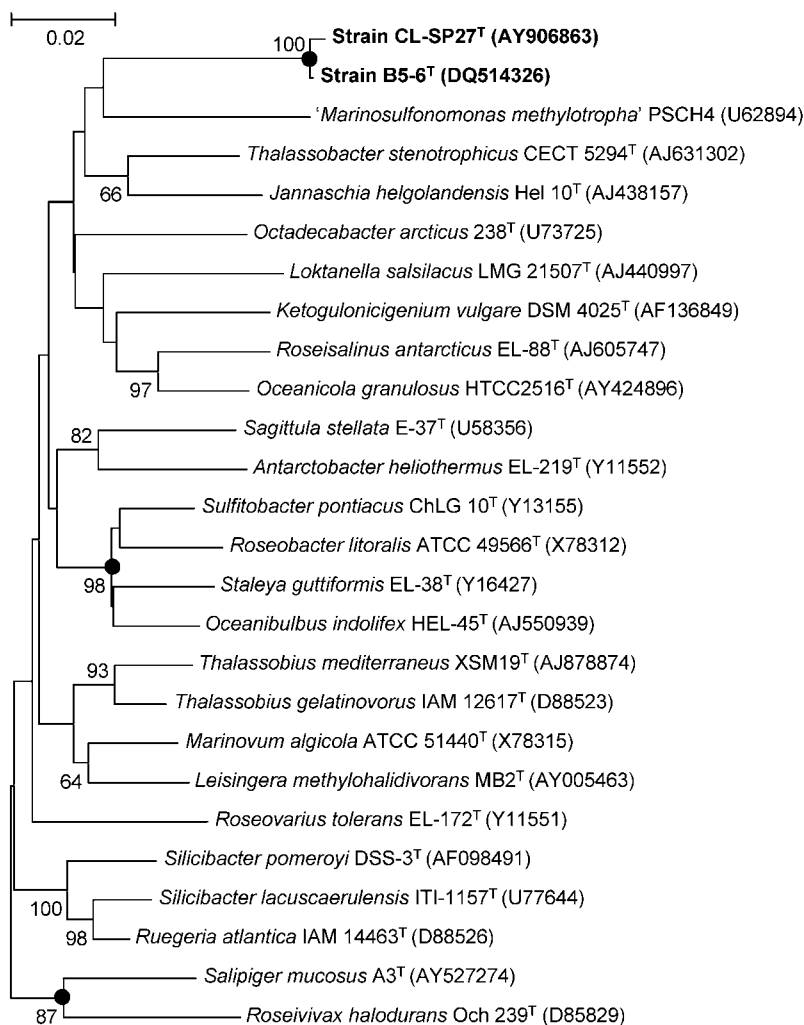
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain CL-SP27<sup>T</sup> and B5-6<sup>T</sup> are AY906863 and DQ514326, respectively.

A table giving the major characteristics that differentiate strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> from other related members of the family *Rhodobacteraceae* is available as supplementary material in IJSEM Online.

Strain CL-SP27<sup>T</sup> was isolated from a hypersaline water sample (31.8% salinity) from a solar saltern in Seosin, Korea, using a standard dilution plating method on marine agar 2216 (MA, Difco) plates. After incubation of the plates at 30 °C for 2 weeks, strain CL-SP27<sup>T</sup> was purified as single colonies. Strain B5-6<sup>T</sup> was isolated from a surface (5 m) seawater sample from the western Sargasso Sea on R2A seawater agar medium (Lanoil *et al.*, 2000) and routinely maintained on MA at 30 °C.

Nearly complete 16S rRNA gene sequences for strains CL-SP27<sup>T</sup> (1385 bp) and B5-6<sup>T</sup> (1405 bp) were obtained as described previously (Cho & Giovannoni, 2003; Choi *et al.*, 2006) and used for phylogenetic analyses. The 16S rRNA gene sequences were aligned with those of members of the family *Rhodobacteraceae* using the jPHYDIT program (Jeon *et al.*, 2005). Phylogenetic trees were generated by neighbour-joining (Saitou & Nei, 1987) with the Jukes & Cantor model (1969), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods, using MEGA3 (Kumar *et al.*, 2004) and PAUP 4.0 (Swofford, 1998). The robustness of the tree topologies

was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and maximum-parsimony trees and 100 replications for the maximum-likelihood tree. Likelihood parameters were estimated by the hierarchical ratio tests in MODELTEST, version 3.04 (Posada & Crandall, 1998). The 16S rRNA gene sequence similarity between strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> was 99.6%. Phylogenetic analyses based on the 16S rRNA gene sequences as well as BLASTN search (Altschul *et al.*, 1990) results showed that strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> belong to the *Roseobacter* clade within the family *Rhodobacteraceae*. The novel strains showed 93.0–93.2% gene sequence similarity to *Jannaschia helgolandensis* and *Oceanicola granulosis* and 90.4–92.9% to various type species belonging to the *Roseobacter* clade. In the phylogenetic trees generated by the three phylogenetic reconstruction methods, the novel strains formed a distinct clade within the family *Rhodobacteraceae*, but were not specifically associated with any species in the family (Fig. 1). Phylogenetic analyses and the low levels of 16S rRNA gene sequence similarities between the novel strains and other members of the family *Rhodobacteraceae* clade indicate that strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> represent a new genus in the family.



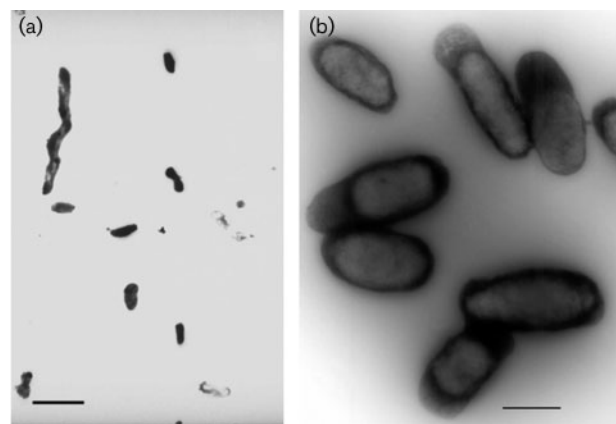
**Fig. 1.** Neighbour-joining tree showing relationships between strains CL-SP27<sup>T</sup>, B5-6<sup>T</sup> and other related species belonging to the *Roseobacter* clade of the family *Rhodobacteraceae*. Only bootstrap values above 60% are shown (1000 resamplings) at the branch nodes. Solid circles indicate that the corresponding nodes are also recovered in maximum-parsimony and maximum-likelihood trees. *Idiomarina seosinensis* (GenBank accession number AY635468) was used as the outgroup (not shown). Bar, 0.02 nucleotide substitutions per site.

Genomic DNA–DNA relatedness was determined by dot-blot hybridization. Pre-hybridization, hybridization and detection were performed using a DIG labelling and detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. When the genomic DNA of strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> was used as a probe, strains B5-6<sup>T</sup> and CL-SP27<sup>T</sup> showed  $51 \pm 2\%$  and  $46 \pm 3\%$  DNA–DNA relatedness, respectively, with the reciprocal strain. Although 16S rRNA gene sequence similarity between the two novel strains was quite high (99.6%), the DNA–DNA relatedness was lower than the level accepted as delineating species (Wayne *et al.*, 1987). In addition, there was a considerable difference in the DNA G + C contents between the two novel strains (70.0 and 66.7 mol% for strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup>, respectively) as measured by HPLC according to Mesbah *et al.* (1989). Based on these results, the novel strains represent two separate species in a new genus within the family *Rhodobacteraceae*.

Morphological and physiological analyses were also performed. Morphology and motility of cells grown on MA and marine broth 2216 (MB) were examined by phase-contrast microscopy. Accumulation of poly  $\beta$ -hydroxybutyrate (PHB) granules was determined by Nile blue A staining (Ostle & Holt, 1982). Transmission electron microscopic investigations were performed after negative staining of cells with 2% uranyl acetate. Catalase and oxidase activities were determined according to the protocols described by Smibert & Krieg (1994). Gelatinase, amylase, DNase, nitrate reductase activities and degradation of Tween 80 were examined as described by Hansen & Sørheim (1991). Anaerobic growth was assessed on MA using the GasPak anaerobic system (BBL). Bacteriochlorophyll *a* production was determined spectrophotometrically in 90% acetone extracts from cells cultured in the dark. In addition, nitrate reduction, the production of indole, arginine dihydrolase, urease, gelatinase and  $\beta$ -galactosidase activities, acid production from glucose and the hydrolysis of aesculin were tested using an API 20NE kit (bioMérieux). The kit was used according to the manufacturer's instructions, except that the cell suspension was prepared using artificial seawater [ASW (24 g NaCl, 5.1 g MgCl<sub>2</sub>, 4 g Na<sub>2</sub>SO<sub>4</sub>, 1.1 g CaCl<sub>2</sub>, 0.7 g KCl, 0.2 g NaHCO<sub>3</sub>, 0.1 g KBr, 0.027 g H<sub>3</sub>BO<sub>3</sub>, 0.024 g SrCl<sub>2</sub>, 0.003 g NaF, 1 l distilled water); Lyman & Fleming, 1940] as a suspension medium. Other enzyme activities were also assayed using the API ZYM kit (bioMérieux) with ASW as the suspension medium. The temperature range for growth was determined on the basis of colony formation on MA plates that were incubated at 5–45 °C with increments of 5 °C. The pH range (pH 4–12 at intervals of 1 pH unit) for growth was determined by changes in OD<sub>600</sub> with time in MB. Tolerance of sea salts was determined using synthetic ZoBell broth (distilled water 1<sup>-1</sup>, 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate) at various concentrations [0 to 10% at intervals of 1% and 15, 20 and 25% (w/v)] of sea salts (Sigma). Ionic requirements were determined using synthetic ZoBell agar with the following combinations of salts (all w/v): (i) 3% NaCl; (ii)

3% NaCl, 0.6% MgCl<sub>2</sub>·6H<sub>2</sub>O and 0.3% MgSO<sub>4</sub>·7H<sub>2</sub>O; (iii) 3% NaCl, 0.6% MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.3% MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.06% KCl, and (iv) 3% NaCl, 0.6% MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.3% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.06% KCl and 0.2% CaCl<sub>2</sub>·2H<sub>2</sub>O. Carbon utilization was tested on basal agar medium supplemented with yeast extract (23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 5.94 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.3 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 g NaNO<sub>3</sub>, 0.2 g NH<sub>4</sub>Cl, 15 g Bacto agar, 0.05 g yeast extract, 1 l distilled water; Choi *et al.*, 2006) containing 0.2% of the carbon source. Incubation was prolonged for 1 month and growth was scored as positive when visible colonies were observed. No visible colonies were observed on control plates with no carbon source. Susceptibility to antibiotics was determined by the diffusion plate method. The following antibiotics were tested ( $\mu$ g per disc): streptomycin (10), gentamicin (10), cephalixin (30), vancomycin (30), mitomycin (1), kanamycin (30), penicillin (10), erythromycin (15), tetracycline (30), nalidixic acid (30), chloramphenicol (30), ciprofloxacin (5) and ampicillin (10).

Cells of strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> were Gram-negative, non-motile rods approximately 0.7–1.4  $\times$  1.0–4.5  $\mu$ m and 0.4–0.8  $\times$  1.3–2.8  $\mu$ m, respectively. Irregular rod-forms were frequently found in both fresh and old cultures of strain CL-SP27<sup>T</sup>. Elongated cells of up to 30  $\mu$ m were observed in old cultures. PHB granules were identified in cells of both novel strains by transmission electron microscopy (Fig. 2) and Nile blue A staining. Detailed morphological, physiological and biochemical characteristics of the strains are listed in the genus and species descriptions. The novel strains were not able to grow in medium with NaCl as a sole salt, but were able to grow in medium containing Na<sup>+</sup> and Mg<sup>2+</sup>. Most biochemical characteristics tested were similar between the two novel strains except glucose fermentation and esterase activity. Carbon source utilization patterns, however, were quite different between the two novel species (Table 1). Biochemical characteristics, carbon source utilization



**Fig. 2.** Transmission electron micrographs of negatively stained cells of strains CL-SP27<sup>T</sup> (a) and B5-6<sup>T</sup> (b). Cells were grown at 30 °C on MA for 5 days. Bars, (a) 2  $\mu$ m; (b) 1  $\mu$ m.

**Table 1.** Selected characteristics that differentiate strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup>

+, Positive; –, negative; w, weakly positive; R, resistant; S, sensitive.

Characteristic	CL-SP27 <sup>T</sup>	B5-6 <sup>T</sup>
Origin	Hypersaline water, Korea	Seawater, Sargasso Sea
Cell size (µm)	0.7–1.4 × 1.0–4.5	0.4–0.8 × 1.3–2.8
Glucose fermentation	–	+
Esterase (C4)	w	+
Temperature range (°C)	10–35	10–40
pH range	7–8	6–9
Sea salt range (%)	1–10	2–15
DNA G+C content (mol%)	70.0	66.7
Sole carbon source:		
D-Cellobiose	+	–
Formic acid	+	–
D-Glucose	–	+
Glycerol	–	+
<i>myo</i> -Inositol	+	–
L-Leucine	+	–
L-Lysine	+	–
D-Mannitol	+	–
L-Ornithine	+	–
D-Raffinose	+	–
D-Ribose	–	+
D-Sorbitol	+	–
Succinate	+	–
Antibiotic susceptibility:		
Streptomycin (10 µg)	R	S

patterns and antibiotic susceptibilities can be used to differentiate the two novel strains.

Isoprenoid quinones were isolated according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The major isoprenoid quinone in strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> is UQ-10. Fatty acid methyl esters (FAMES) in whole cells were analysed by GC according to the instructions of the Microbial Identification System (MIDI). Dominant fatty acids for the strains were 18:1 $\omega$ 7 $c$  (54.2–65.3%), followed by cyclo 19:0 $\omega$ 8 $c$  (8.2–21.0%) (Table 2). Strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> could be differentiated by the proportions of the major fatty acids (18:1 $\omega$ 7 $c$  and cyclo 19:0 $\omega$ 8 $c$ ) and by the presence or absence of several fatty acids, including 16:0, 17:0, 18:3 $\omega$ 6 $c$  and 11-methyl 18:1 $\omega$ 7 $c$  (Table 2).

In conclusion, comparative and phylogenetic analyses of 16S rRNA gene sequences of the two novel strains showed they should be assigned as members of a new genus. Furthermore, morphological, biochemical and genetic characteristics clearly differentiated the novel strains from phylogenetically related species (see Supplementary Table S1 in IJSEM Online). Polyphasic evidence, such as growth ranges and carbon source utilization patterns, fatty acid profiles and DNA–DNA relatedness, demonstrated that

strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup> represent two separate novel species in the same new genus. Consequently, we propose the genus name *Maribius* gen. nov., containing two novel species, *Maribius salinus* sp. nov. (the type species) and *Maribius pelagius* sp. nov.

**Table 2.** Cellular fatty acid contents (%) of strains CL-SP27<sup>T</sup> and B5-6<sup>T</sup>

–, Not detected. ECL, equivalent chain-length. Fatty acids that account for less than 1% of the total are not shown.

Fatty acid	CL-SP27 <sup>T</sup>	B5-6 <sup>T</sup>
16:0	4.6	–
17:0	1.3	–
18:0	5.4	2.7
18:3 $\omega$ 6 $c$ (6,9,12)	1.7	–
18:1 $\omega$ 7 $c$	65.3	54.2
Cyclo 19:0 $\omega$ 8 $c$	8.2	21.0
3-OH 10:0	3.5	3.8
ECL 11.799	2.3	2.8
11-methyl 18:1 $\omega$ 7 $c$	3.4	–
19:1 $\omega$ 6 $c$ /ECL 18.846/19:0 cyclo $\omega$ 10 $c$	1.7	2.4

### Description of *Maribius* gen. nov.

*Maribius* (Ma.ri.bi'us. L. neut. n. *mare* the sea; N.L. masc. n. *bios* from Gr. n. *bios* life; N.L. masc. n. *Maribius* sea life).

Cells are Gram-negative, non-motile rods. Growth is obligately heterotrophic and strictly aerobic. On MA medium, colonies are circular, entire, convex, opaque and beige in colour. Catalase- and oxidase-positive. Accumulate PHB granules. Require  $Mg^{2+}$  and  $Na^+$  for growth. The predominant isoprenoid quinone is UQ-10. Dominant fatty acids are 18:1 $\omega$ 7c and cyclo 19:0 $\omega$ 8c. Cells do not contain bacteriochlorophyll *a*. The G+C content of the genomic DNA is 66.7–70.0 mol%. Phylogenetically, the genus is a member of the family *Rhodobacteraceae*. The type species is *Maribius salinus*.

### Description of *Maribius salinus* sp. nov.

*Maribius salinus* (sa.li'nus. N.L. masc. adj. *salinus* salted, salty).

Displays the following properties in addition to those given in the genus description. Cells are 0.7–1.4  $\mu$ m wide and 1.0–4.5  $\mu$ m long. Growth occurs within the temperature range of 10–35 °C (optimum 30–35 °C), at pH values of between 7 and 8 and at sea salt concentrations of 1–10 % (w/v). Gives a positive result in tests for amylase, urease, esterase (C4), esterase lipase (C8), leucine arylamidase and  $\beta$ -galactosidase activities and Tween 80 and aesculin hydrolysis. Negative results in tests for DNase, gelatinase, nitrate reductase, alkaline and acid phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, *N*-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase,  $\alpha$ -fucosidase and arginine dihydrolase and  $\alpha$ -fucosidase activities, indole production and glucose acidification. Several fatty acids, including 16:0, 17:0, 18:0, 18:3 $\omega$ 6c, 3-OH 10:0, ECL 11.799, 11-methyl 18:1 $\omega$ 7c, are present at >1%. Growth occurs on citrate, D-cellobiose, D-fructose, D-mannitol, D-raffinose, D-sorbitol, D-xylose, ethanol, formic acid, L-arginine, L-leucine, L-lysine, L-ornithine, *myo*-inositol, *N*-acetylglucosamine, pyruvic acid and succinate. No growth occurs on D-galactose, D-glucose, D-mannose, D-ribose, D-trehalose, glycerol, glycine, L-glutamate, L-proline, L-rhamnose or sucrose. The type strain is susceptible to gentamicin, cephalixin, vancomycin, mitomycin, kanamycin, penicillin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin and ampicillin, but resistant to nalidixic acid and streptomycin. The DNA G+C content is 70.0 mol%.

The type strain, CL-SP27<sup>T</sup> (=KCCM 42113<sup>T</sup>=JCM 13037<sup>T</sup>), was isolated from hypersaline water from a solar saltern in Korea.

### Description of *Maribius pelagius* sp. nov.

*Maribius pelagius* (pe.la'gi.us. L. masc. adj. *pelagius* of or belonging to the sea).

Displays the following properties in addition to those given in the genus description. Cells are 0.4–0.8  $\mu$ m wide and 1.3–2.8  $\mu$ m long. Growth occurs within the temperature range of 10–40 °C (optimum 30–35 °C), at pH values of between 6 and 9 and at sea salt concentrations of 2–15 % (w/v). Gives a positive result in tests for amylase, urease, esterase (C4), esterase lipase (C8), leucine arylamidase,  $\beta$ -galactosidase activities, hydrolysis of Tween 80 and aesculin hydrolysis and glucose acidification. Negative results in tests for DNase, gelatinase, nitrate reductase, alkaline and acid phosphatases, lipase (C14), valine arylamidase, cystine arylamidase, trypsin,  $\alpha$ -chymotrypsin, naphthol-AS-BI-phosphohydrolase,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, *N*-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase,  $\alpha$ -fucosidase and arginine dihydrolase activities and for indole production. Several fatty acids, including 18:0, 3-OH 10:0 and ECL 11.799 are present at >1%. Growth occurs on citrate, D-fructose, D-glucose, D-ribose, D-xylose, ethanol, glycerol, L-arginine, *N*-acetylglucosamine and pyruvic acid. No growth occurs on D-cellobiose, D-galactose, D-mannitol, D-mannose, D-raffinose, D-sorbitol, D-trehalose, formic acid, glycine, L-glutamate, L-leucine, L-lysine, L-ornithine, L-proline, L-rhamnose, *myo*-inositol, sucrose or succinate. The type strain is susceptible to gentamicin, cephalixin, vancomycin, mitomycin, kanamycin, penicillin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin, ampicillin and streptomycin, but resistant to nalidixic acid. The DNA G+C content is 66.7 mol%.

The type strain, B5-6<sup>T</sup> (=KCCM 42336<sup>T</sup>=JCM 14009<sup>T</sup>), was isolated from surface water of the Sargasso Sea.

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