

Fulvimarina pelagi gen. nov., sp. nov., a marine bacterium that forms a deep evolutionary lineage of descent in the order 'Rhizobiales'

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Three brownish-yellow bacterial strains were isolated from the western Sargasso Sea by high-throughput culturing methods and characterized by polyphasic approaches. All isolates were Gram-negative, strictly aerobic, chemoheterotrophic, non-motile short rods that contained carotenoid pigments. Phylogenetic analyses based on 16S rRNA gene sequences, DNA–DNA hybridization and DNA G+C content, along with phenotypic characteristics, revealed that they belonged to the same species. The strains utilized a wide range of substrates, including pentoses, hexoses, oligosaccharides, sugar alcohols, organic acids and amino acids, as sole carbon sources. The DNA G+C content of the isolates ranged from 57.6 to 59.9 mol%. The predominant cellular fatty acid constituent was $C_{18:1}\omega7c$, whilst $C_{16:0}$, $C_{18:0}$ and $C_{19:0}\omega8c$ cyclo were also abundant. The organism related most closely to these strains, as determined by 16S rDNA sequence comparison, was the recently described species *Aurantimonas coralicida* (93.3–93.8% similarity). Phylogenetic analyses indicated that the strains formed a distinct and deep evolutionary lineage of descent, together with *A. coralicida*, within the order 'Rhizobiales' of the α -Proteobacteria. This lineage could not be associated with any of the ten known families in the order 'Rhizobiales'. From polyphasic evidence, it is proposed that the strains be placed into a novel genus and species, *Fulvimarina pelagi* gen. nov., sp. nov. (type strain, HTCC2506^T = ATCC BAA-666^T = KCTC 12091^T = DSM 15513^T).

INTRODUCTION

Since Woese *et al.* (1984) suggested the taxonomic outline of the α -Proteobacteria, the name $\alpha 2$ -Proteobacteria has been used historically to describe *Rhizobium*-, *Agrobacterium*-, *Caulobacter*-, *Hyphomicrobium*- and *Rhodomicrobium*-like bacteria (Bhat *et al.*, 1991; Rainey *et al.*, 1998; Abraham *et al.*, 1999; Young *et al.*, 2001). The order 'Rhizobiales', the nomenclature of which was suggested in the most recent edition of *Bergey's Manual of Systematic Bacteriology* (Garrity & Holt, 2001), is the biggest group within the $\alpha 2$ -Proteobacteria. According to *Bergey's Manual*, the α -Proteobacteria are divided into six orders, and the order 'Rhizobiales' into ten families, on the basis of 16S rDNA phylogenetic analyses. Among the six orders of the α -Proteobacteria, marine bacteria are mainly distributed

in three orders: 'Rhodobacterales', 'Sphingomonadales' and 'Caulobacterales' (Jannasch & Jones, 1960; Shiba *et al.*, 1991; Giovannoni & Rappé, 2000). Only a few members of the order 'Rhizobiales', such as the genera *Rhodobium* (Hiraishi *et al.*, 1995), *Roseibium* (Suzuki *et al.*, 2000) and *Aurantimonas* (Denner *et al.*, 2003), were retrieved from marine environments; nearly all members of this order were isolated from terrestrial ecosystems, such as soil and freshwater.

In this study, three strains were isolated from the Sargasso Sea by using high-throughput culturing (HTC) methods (Connon & Giovannoni, 2002) and characterized by polyphasic approaches (Vandamme *et al.*, 1996). Polyphasic taxonomic analyses indicated that these marine bacteria represented a novel family-level taxon and formed a deep evolutionary lineage of descent within the order 'Rhizobiales'; therefore, it is proposed that they should be classified as *Fulvimarina pelagi* gen. nov., sp. nov.

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Abbreviation: HTC, high-throughput culturing.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the strains in this study are AY178860–AY178862.

A table showing habitats and isolation environments of the families in the order 'Rhizobiales' is available as supplementary material in IJSEM Online.

METHODS

Bacterial cultures and isolation procedure. A sea-water sample was collected from a depth of 10 m at the Bermuda Atlantic Time Series (BATS) station in the western Sargasso Sea, Atlantic Ocean, in August 2001. Initial liquid cultures of three strains, designated

HTCC2506^T, HTCC2615 and HTCC2619, were isolated by using the recently developed HTC approaches described by Connon & Giovannoni (2002) and Rappé *et al.* (2002). Liquid cultures of the strains were spread and purified as single colonies on marine agar 2216 (Difco) after incubation for 10 days at 30 °C. Thus, the strains described here are from the subset of HTCC strains that can be grown on agar (HTCC stands for the high-throughput culture collection that is being maintained by our laboratory at Oregon State University, USA). Most HTCC strains cannot be grown on agar. The strains were maintained as viable cultures on marine agar 2216 slants at 4 °C and also stored as 10% (v/v) glycerol suspensions in liquid nitrogen.

Phenotypic characterization. Unless indicated otherwise, standard methods for phenotypic characterization of the strains were employed as described by Smibert & Krieg (1994). Biochemical tests were carried out by using API 20NE strips (bioMérieux) following the manufacturer's instructions. Cellular pigments were extracted by using a methanol/acetone mixture (1:1, v/v) from cultures grown on marine agar 2216; their absorption spectra were determined by using a scanning UV/visible spectrophotometer (Biospec-1601; Shimadzu). Motility was examined from wet mounts of exponential-phase cells under dark field microscopy (DMRB; Leica). For electron microscopy, exponential-phase cells were concentrated by centrifugation, washed twice with PBS (pH 8.0), fixed with 1.5% (v/v) glutaraldehyde and negatively stained with 2% (v/v) aqueous ammonium molybdate (pH 6.3) on copper grids overlaid with a film of Formvar. Transmission electron microscopy was carried out on a Phillips CM12 transmission electron microscope, operated at 60 kV in transmission mode. Temperature range and optimum for growth were tested at 4–44 °C on marine agar 2216. pH range and optimum for growth were examined at pH 4.0–12.0 at 30 °C. NaCl concentration range and optimum for growth were determined at NaCl concentrations of 0–20% (w/v). Anaerobic growth was tested by using the Oxoid Anaerobic system.

Custom-made 48-well microplates that contained 47 different carbon compounds at a final concentration of 0.2% (w/v or v/v) were used for sole carbon source utilization tests. Strains were grown on marine agar plates and cell densities were adjusted to approximately 1.0×10^3 cells ml⁻¹ in artificial sea-water medium (25.0 g NaCl, 1.0 g MgCl₂·6H₂O, 5.0 g MgSO₄·7H₂O, 0.7 g KCl, 0.15 g CaCl₂·2H₂O, 0.5 g NH₄Cl, 0.1 g KBr, 0.27 g KH₂PO₄, 0.04 g SrCl₂·6H₂O, 0.025 g H₃BO₃ l⁻¹). After incubating microtitre plates in triplicate at 30 °C for 5 days, cellular growth and purity were examined by DAPI-stained epifluorescence microscopy. In addition to the sole carbon source test, Biolog GN2 microplates were used to test the oxidation of 95 carbon sources (Rüger & Krambeck, 1994). Susceptibility to antibiotics was determined by the disc-diffusion plate method. The following antibiotics were tested (concentration per disc in parentheses): chloramphenicol (25 µg), nalidixic acid (25 µg), kanamycin (30 µg), carbenicillin (25 µg), tetracycline (30 µg), streptomycin (50 µg), ampicillin (10 µg), puromycin (25 µg), erythromycin (15 µg), vancomycin (30 µg), rifampicin (50 µg), benzylpenicillin (100 U), gentamicin (10 µg) and cycloheximide (50 µg).

Cellular fatty acid analysis. Cells were grown on marine agar 2216 at 30 °C for 5 days. Cellular fatty acid methyl esters were prepared and analysed by using GC according to the instructions of the Microbial Identification system (MIDI). Fatty acid profiles were analysed by Microbial ID, Newark, DE, USA.

Determination of DNA base composition. Genomic DNA was extracted and purified by using a Qiagen DNeasy Tissue kit. DNA G+C content was analysed by HPLC according to Mesbah *et al.* (1989) by using a Platinum EPS reverse-phase C18 column (150 mm, 4.6 mm, 5 µm pore size; Alltech).

DNA–DNA hybridization. Levels of genomic DNA relatedness among the strains were determined by DNA–DNA dot-blot hybridization. Probe DNA of strain HTCC2506^T was prepared by using DIG-High Prime DNA Labeling and Detection Starter kit I (Roche Molecular Biochemicals). Genomic DNA from strains HTCC2506^T, HTCC2615 and HTCC2619 was denatured by boiling for 10 min in 6 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) and transferred onto positively charged nylon membranes. Prehybridization, hybridization, stringency washing and detection were performed according to the manufacturer's instructions. Hybridization temperature was 50 °C and stringency washing was carried out in 0.1 × SSC and 0.1% SDS at 65 °C in a hybridization chamber.

16S rRNA gene sequence analyses. 16S rRNA genes of the strains were amplified by PCR with the slightly modified universal

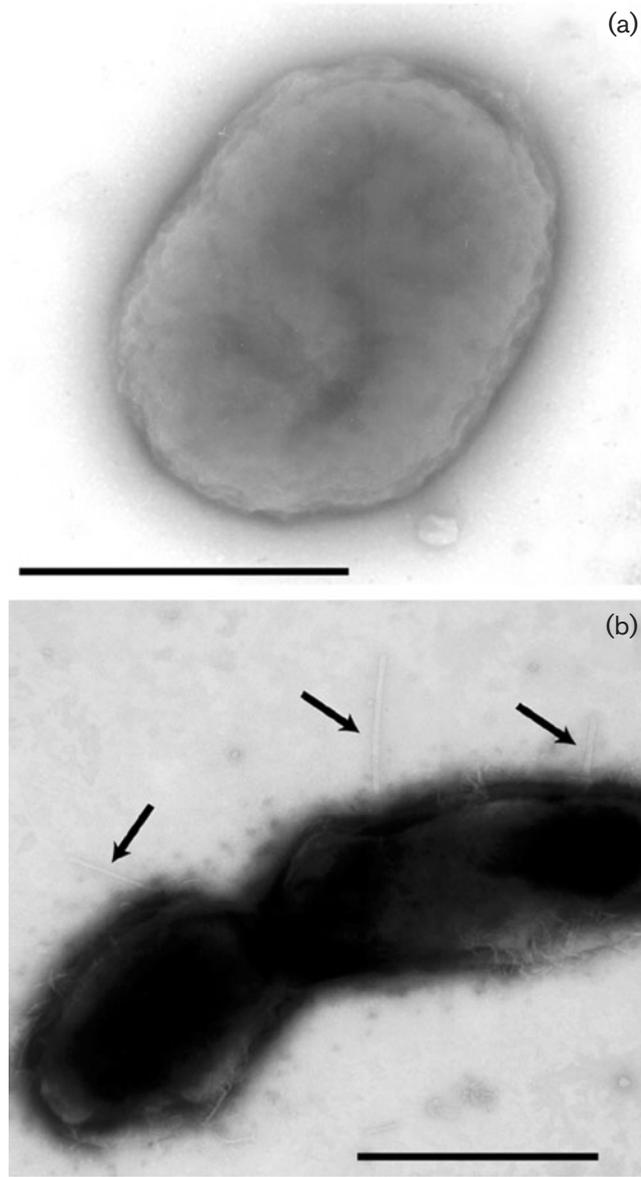


Fig. 1. Electron micrographs of negatively stained cells of strains (a) HTCC2506^T and (b) HTCC2516. Arrows indicate fimbriae around cells. Bars, 1 µm.

bacterial primers, 27F-B and 1492R (Lane, 1991) and sequenced directly by the chain-termination method on an ABI 377 automated sequencer. Nearly full-length 16S rRNA gene sequences were aligned by using the ARB software package (Ludwig *et al.*, 1998) and 1140 unambiguously aligned nucleotide positions were used for phylogenetic analyses in PAUP* 4.0 beta 10 (Swofford, 2002). Distance matrices were calculated from sequence similarities according to Jukes & Cantor (1969). Phylogenetic trees were inferred by three different algorithms: neighbour-joining with the Kimura two-parameter model; maximum-parsimony with a heuristic search; and maximum-likelihood with a heuristic search, tree bisection-reconnection (TBR)-branching and a T_i/T_v ratio of 1.368336. Tree topologies from neighbour-joining and maximum-parsimony were evaluated by bootstrap analyses based on 1000 resamplings.

RESULTS AND DISCUSSION

Phenotypic characteristics of sea-water isolates

All strains were Gram-negative (by Gram-staining and KOH test), non-motile short rods, 0.8–1.7 μm long and 0.5–1.1 μm wide, which divided by binary fission (Fig. 1). No flagella were observed on negatively stained cells. The strains contained neither endospores nor poly- β -hydroxybutyrate granules. Strain HTCC2615 had several fimbriae that were scarcely visible around cells (Fig. 1b), whereas strain HTCC2506^T did not (Fig. 1a). Some major differentiating characteristics for the strains are represented in Table 1. Colonies were brownish-yellow, 0.8–1.8 mm in diameter, uniformly circular, convex, dry and opaque after growth on marine agar 2216 at 30 °C for 5 days. No isolates grew under anaerobic conditions, even with prolonged incubation for 30 days. Temperature range for growth was 4–40 °C (optimum, 30 °C), pH range for growth was 5.5–10.0 (optimum, 7.5–8.0) and NaCl concentration range for

growth was 0–10% (w/v) (optimum 2.0–2.5%, w/v). Although the strains were isolated from sea water, they could grow in the absence of NaCl. However, growth rates in the absence of NaCl were much lower than those at optimum concentrations of NaCl.

All HTCC strains were catalase-, oxidase- and urease-positive. No denitrification activity was detected. Tests for indole production, arginine deaminase activity, gelatin and aesculin hydrolysis and acid production from glucose were negative. Tests for sole carbon source utilization and antibiotic susceptibility differentiated the strains from each other (Table 1). The strains utilized a wide range of substrates, including pentoses, hexoses, oligosaccharides, sugar alcohols, organic acids and amino acids, as sole carbon sources (custom-made 48-well plate tests). The HTCC isolates also oxidized various substrates, including polysaccharides, oligosaccharides, pentoses, hexoses, sugar alcohols, organic acids and amino acids (Biolog tests). All strains were susceptible to chloramphenicol, tetracycline, streptomycin, puromycin, vancomycin, rifampicin and benzylpenicillin, and resistant to nalidixic acid, kanamycin, carbenicillin, ampicillin and cycloheximide.

The HTCC isolates produced carotenoid pigments with absorption spectral peaks at 477, 453 and 419 nm. There were no differences between the spectral peaks of light- and dark-grown cultures and no bacteriochlorophyll peaks were detected. Therefore, energy metabolism of the isolates appears to be exclusively non-photosynthetic chemoheterotrophy. Generally, carotenoid pigments in non-photosynthetic micro-organisms, such as *Deinococcus radiodurans*, have a function for protecting cells from damage by UV radiation, whereas those in photosynthetic

Table 1. Differentiating characteristics for strains of *Fulvimarina pelagi*

Characteristic	<i>Fulvimarina pelagi</i> strain		
	HTCC2506 ^T	HTCC2615	HTCC2619
Fimbriae	–	+	–
Susceptibility to:			
Gentamicin	–	+	–
Erythromycin	+	–	+
Sole carbon source utilization of:			
L-Rhamnose	–	+	+
D-Trehalose	+	+	–
N-Acetyl-D-glucosamine	+	–	–
Adonitol	–	+	+
Methanol	+	+	–
Ethanol	+	+	–
Succinic acid	+	–	–
L-Ornithine	+	–	+
DNA G + C content (mol%)	57.6	58.1	59.9
DNA–DNA hybridization (with HTCC2506 ^T)	100	90.2	98.7

micro-organisms have auxiliary functions as accessory pigments during photosynthesis (Battista, 1997; Wynn-Williams *et al.*, 2002). As the HTCC strains were isolated from the oceanic surface, an environment of high solar radiation, carotenoids in these isolates may play an important role for their heterotrophic survival by means of UV protection and antioxidant activity.

DNA base composition and DNA relatedness

The DNA G + C content of the HTCC isolates ranged from 57.6 to 59.9 mol%, as determined by the HPLC method (Table 1). When the DNA of strain HTCC2506^T was used as a probe for DNA–DNA hybridization, strains HTCC2615 and HTCC2619 showed 90–99 % DNA relatedness to strain HTCC2506^T (Table 1). From the DNA G + C contents and the results of DNA–DNA hybridization, the HTCC isolates were considered to belong to the same genus.

Phylogenetic analyses based on 16S rRNA gene sequences

Nearly complete 16S rRNA gene sequences were determined for the three strains and used for phylogenetic analyses by employing three tree-generating algorithms. Results of preliminary BLAST network searches and ARB tree analyses indicated that all strains belonged to the order ‘*Rhizobiales*’ in the α -*Proteobacteria*. Sequence comparisons to bacteria with validly published names indicated that the strains were related most closely to the recently described species *Aurantimonas corallicida* (93.3–93.8 % similarity), the genera *Rhizobium* (90.1–90.7 %) and *Allorhizobium* (89.2–89.8 %) in the family *Rhizobiaceae* and the genera *Mesorhizobium* (90.9–91.7 %), *Aminobacter* (88.9–91.3 %) and *Pseudaminobacter* (88.6–90.1 %) in the family ‘*Phyllobacteriaceae*’. In all three phylogenetic trees, the HTCC isolates formed a distinct monophyletic clade with 100 % bootstrap support (neighbour-joining and maximum-parsimony) for a position within the order ‘*Rhizobiales*’; this clade was associated closely with the cluster that included *A. corallicida* and the coastal isolate *Aurantimonas* sp. HTCC2156 (Fig. 2).

Fatty acid composition

In total, 11 different kinds of fatty acid, containing 14–20 carbon atoms, were observed in the HTCC isolates (Table 2). The most abundant fatty acid in the isolates was *cis*-7-octadecenoic acid (C_{18:1}ω7c). The fatty acid components were similar between the HTCC isolates and their closest neighbour, *A. corallicida*; however, they were differentiated clearly by the proportions of several fatty acids, including C_{16:0}, C_{18:0}, C_{18:1}ω7c and C_{19:0}ω8c cyclo.

Taxonomic conclusions

Polyphasic approaches, including phenotypic data (Table 1), fatty acid profiles (Table 2), DNA G + C content (Table 1), DNA–DNA hybridization (Table 1) and 16S

rDNA phylogenetic analyses (Fig. 2) demonstrated that the sea-water isolates belonged to a novel genus within the α -*Proteobacteria*. The strains HTCC2506^T, HTCC2615 and HTCC2619 shared very similar phenotypic and genotypic characteristics, such as >99 % 16S rDNA sequence similarity and >90 % DNA–DNA hybridization, so they were regarded as members of the same species (Wayne *et al.*, 1987).

Phylogenetically, this novel taxon formed a new eleventh family, together with the recently described species *A. corallicida*, within the order ‘*Rhizobiales*’ of the α -*Proteobacteria*. The HTCC isolates, together with *A. corallicida*, could not be associated with any of the ten known families in the order ‘*Rhizobiales*’ of the α -*Proteobacteria*. The clade that contained the three strains and *A. corallicida* was placed at the deepest position from the outgroup species (*Rhodobacter capsulatus* ATCC 11166^T) in the maximum-likelihood and parsimony trees, indicating that the strains may be one of the evolutionary ancestral organisms of descent in the order ‘*Rhizobiales*’.

The strains were also different phenotypically and ecologically from members of the other families and some related genera in the order ‘*Rhizobiales*’. Nearly all members of the order ‘*Rhizobiales*’ have been isolated from soil, rhizosphere, freshwater, groundwater, wastewater, sewage and warm-blooded animals, but not from sea water (see supplementary table in IJSEM Online). A few exceptions are the genus *Rhodobium*, which contains marine budding phototrophic bacteria, the genus *Roseibium*, which are aerobic, bacteriochlorophyll-containing bacteria (Hiraishi *et al.*, 1995; Suzuki *et al.*, 2000) and *A. corallicida*, which are coral pathogens (Denner *et al.*, 2003). Therefore, genera that are related closely to our strains (*Rhizobium*, *Allorhizobium*, *Mesorhizobium*, *Aminobacter* and *Pseudaminobacter*) exhibit low tolerance to salt, with a maximum growth concentration of 3.0 % (Urakami *et al.*, 1992; Jarvis *et al.*, 1997; Kämpfer *et al.*, 1999; Young *et al.*, 2001). Our novel isolates grew in salt concentrations of up to 10 %. The HTCC isolates were also differentiated from these genera by flagellation, division type, growth temperature, pigmentation and fatty acid profiles. Members of the genera *Rhizobium*, *Allorhizobium* and *Mesorhizobium* form nitrogen-fixing nodules on the roots of leguminous plants (Jarvis *et al.*, 1997; Young *et al.*, 2001). The genus *Aminobacter* has flagella, divides by budding, contains poly- β -hydroxybutyric acid granules and does not contain pigments (Urakami *et al.*, 1992). Additionally, the strains were differentiated from the most closely related species, *A. corallicida*, by DNA G + C content (6.4–8.7 mol% difference), fatty acid profiles (Table 2), flagellation, NaCl requirement, acid production from glucose, carbon source utilization and antibiotic susceptibility (Denner *et al.*, 2003). Therefore, the novel strains cannot be characterized as a member of any known genus within the order ‘*Rhizobiales*’. Consequently, on the basis of both phylogenetic and phenotypic distinction, we propose the description of a novel genus and species, *Fulvimarina pelagi* gen. nov., sp. nov.

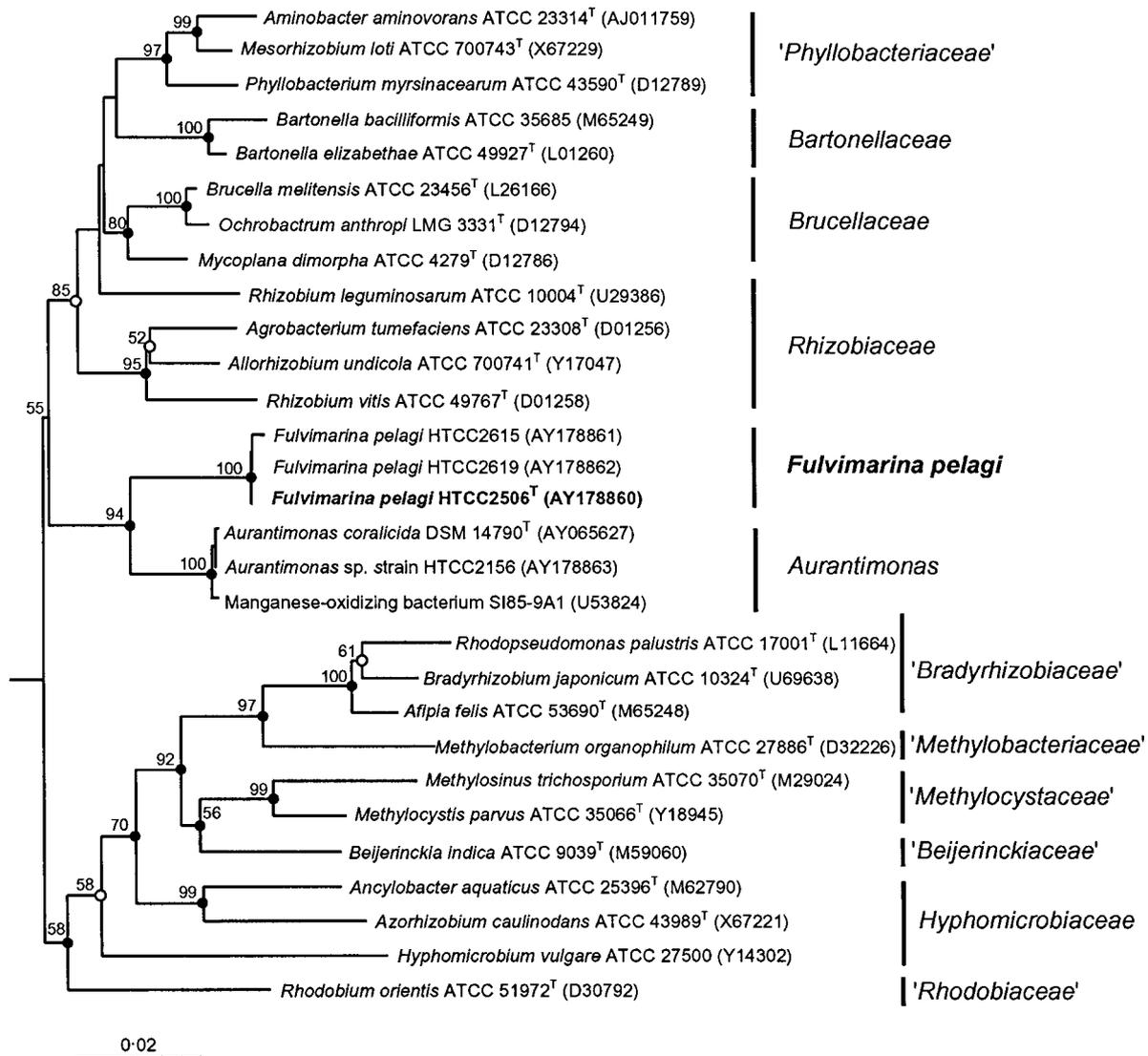


Fig. 2. Neighbour-joining 16S rRNA phylogenetic tree showing relationships between the strains studied and representatives of the order 'Rhizobiales'. Bootstrap proportions > 50% from neighbour-joining analysis are shown. Closed and open circles indicate nodes recovered by all treeing methods and by two treeing methods, respectively. *Rhodospirillum rubrum* ATCC 11166^T (D16428) was used as outgroup to define the root of the tree. Bar, 0.02 substitutions per nucleotide position.

Description of *Fulvimarina* gen. nov.

Fulvimarina (Ful.vi.ma.ri'na. L. adj. *fulvus* brownish-yellow; L. fem. adj. *marina* of the sea; N.L. fem. n. *Fulvimarina* brownish-yellow bacterium isolated from sea water).

Cells are Gram-negative, non-motile short rods that occur singly or in pairs and multiply by binary fission. Neither endospores nor poly- β -hydroxybutyrate granules are formed. Brownish-yellow colonies are formed on marine agar. Metabolism is obligately aerobic and chemoheterotrophic. No denitrification activity is detected. Catalase, oxidase and urease are positive. Indole production, arginine deaminase activity, gelatin and aesculin hydrolysis and acid production are negative. Predominant fatty acids are

$C_{18:1\omega7c}$ (82.9%) and $C_{18:0}$ (8.2%). Other minor fatty acids are $C_{16:0}$, $C_{16:1\omega7c}$, $C_{18:1}$ 2-OH, $C_{18:0}$ 3-OH, $C_{19:0\omega8c}$ cyclo and $C_{20:1\omega7c}$. DNA G+C content is 57.6–59.9 mol% (by HPLC). Phylogenetically, the genus forms a novel eleventh family within the order 'Rhizobiales'. The type species of the genus is *Fulvimarina pelagi*.

Description of *Fulvimarina pelagi* sp. nov.

Fulvimarina pelagi (pe.la'gi. L. fem. adj. *pelagi* from the open sea).

The description of this species is the same as that given for the genus. Cells are 0.7–1.4 μ m wide and 0.4–1.0 μ m long. Colonies are 0.8–1.8 mm in diameter, uniformly circular,

Table 2. Cellular fatty acid composition of *Fulvimarina pelagi* and its closest neighbour, *Aurantimonas coralicida*

Values are percentages (mean \pm SD) of total fatty acids. Data for *Aurantimonas coralicida* are from Denner *et al.* (2003).

Fatty acid	<i>Fulvimarina pelagi</i> (<i>n</i> = 3)	<i>Aurantimonas coralicida</i>
C _{16:0}	2.6 \pm 0.6	6.7
C _{17:0}	–	0.6
C _{18:0}	8.2 \pm 0.9	1.5
C _{16:1} ω 7c	–	1.3
C _{18:1} ω 7c	82.9 \pm 3.1	76.9
C _{18:1} 2-OH	0.4 \pm 0.2	2.0
C _{18:0} 3-OH	0.4 \pm 0.2	–
C _{19:0} ω 8c cyclo	2.6 \pm 0.4	10.5
C _{20:1} ω 7c	1.7 \pm 0.1	0.5
C _{14:0} 3-OH + i-C _{16:1}	1.2 \pm 0.2	–

convex and opaque. Tolerates 10.0% NaCl and grows optimally at 2.0% NaCl. Absorption spectral peaks of the pigments are observed at 477, 453 and 419 nm. Utilizes D-ribose, D-arabinose, D-glucose, D-galactose, D-fructose, D-mannose, D-melibiose, D-mannitol, glycerol, pyruvic acid, formic acid, L-glutamic acid, L-lysine, L-proline, L-serine, L-isoleucine and L-arginine as sole carbon sources, but does not utilize DL-glyceraldehyde, β -lactose, D-melezitose, D-sorbitol, itaconic acid, citric acid, D-malic acid, malonic acid, myo-inositol, D-glucosamine, L-alanine or glycine (sole carbon source utilization tests by custom-made 48-well plate). According to Biolog tests, the following substrates were oxidized by all strains: α -cyclodextrin, dextrin, glycogen, Tween 40, adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β -D-glucoside, D-raffinose, D-sorbitol, sucrose, xylitol, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, formic acid, β -hydroxybutyric acid, α -ketobutyric acid, DL-lactic acid, propionic acid, D-saccharic acid, succinamic acid, glucuronamide, L-alaninamide, L-glutamic acid, glycol L-glutamic acid, L-leucine, L-proline, L-serine, uridine, thymidine and glycerol.

The type strain is HTCC2506^T (=ATCC BAA-666^T=KCTC 12091^T=DSM 15513^T). Isolated from the western Sargasso Sea, Atlantic Ocean.

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