Roseivirga marina sp. nov., isolated from seawater

Jie Pan,[1] Cong Sun,[1] Rui-Jun Wang[2] and Min Wu[1]

[1]College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

[2]Ocean College, Zhejiang University, Hangzhou 310058, PR China

Correspondence
Min Wu
wumin@zju.edu.cn

Strain PSR^T was isolated from seawater of the Pacific Ocean. Cells of the strain were Gram-stain-negative, strictly aerobic, rod-shaped and motile by gliding. Growth was observed at 4–40 °C (optimum 25–30 °C), at pH 6.0–9.5 (optimum pH 7.0–7.5) and with 0.5–8 % (w/v) NaCl (optimum 2–3 %). The major fatty acids were iso-$C_{15:1}$ G (18.9 %), iso-$C_{15:0}$ (26.3 %) and iso-$C_{17:0}$ 3-OH (17.9 %). The predominant isoprenoid quinone was MK-7, and the DNA G+C content was 49.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain PSR^T was most closely related to *Roseivirga spongicola* UST030701-084^T (96.9 % 16S rRNA gene sequence similarity), and they formed a distinct clade in neighbour-joining, maximum-likelihood and maximum-parsimony phylogenetic trees with significant bootstrap supports. Based on phenotypic, chemotaxonomic and phylogenetic characteristics, strain PSR^T represents a novel species of the genus *Roseivirga*, for which the name *Roseivirga marina* sp. nov. is proposed. The type strain is PSR^T (=MCCC 1K00459^T=KCTC 42444^T).

The genus *Roseivirga* was phylogenetically classified into the class *Cytophagia*, phylum *Bacteroidetes*. It was proposed by Nedashkovskaya *et al.* (2005a) with the type species *Roseivirga ehrenbergii*. Soon after that, another novel genus *Marinicola* was proposed by Yoon *et al.* (2005) with the type species *Marinicola seohaensis*. However, the 16S rRNA gene sequences of *Marinicola seohaensis* and the recognized species of the genus *Roseivirga* demonstrated similarities of 99.1–99.9 %. Combining phylogenetic, phenotypic and genotypic characteristics of *Marinicola seohaensis*, it was suggested to place the genus *Marinicola* and the species *Marinicola seohaensis* in the genus *Roseivirga* and to emend the description of the genus *Roseivirga* (Nedashkovskaya *et al.*, 2005b). Based on the results of the phylogenetic analysis, Lau *et al.* (2006) proposed to reclassify [*Marinicola*] *seohaensis* in the genus *Roseivirga* as *Roseivirga seohaensis*. In addition, Lau *et al.* (2006) reported a novel species *Roseivirga spongicola*. Finally, Nedashkovskaya *et al.* (2008) reported the study of the genotypic and phenotypic characteristics of all species of the genus *Roseivirga* and suggests that *R. seohaensis* is a later synonym of *R. ehrenbergii*. At the time of writing, there are three species of the genus *Roseivirga* with validly published names: *Roseivirga echinicomitans* (Nedashkovskaya *et al.*, 2005b), *R. ehrenbergii* (Nedashkovskaya *et al.*, 2005a) and *R. spongicola* (Lau *et al.*, 2006). Members of the genus *Roseivirga* were defined as Gram-stain-negative, strictly aerobic rods that possess oxidase, catalase and alkaline phosphatase activities. Major fatty acids of the genus were iso-$C_{15:1}$, iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH and iso-$C_{17:0}$ 3-OH, and the predominant respiratory quinone is MK-7. In this study, we report the characterization of a novel strain, designated PSR^T, which was isolated from seawater collected in the Pacific Ocean.

Strain PSR^T was isolated from seawater samples collected from the Pacific Ocean (17° 10′ 20.22″ N 147° 46′ 17.78″ E) at a depth of 200 m using a CTD rosette sampler. The seawater sample was spread on modified marine agar 2216 (MA; Difco) where the ingredients were the same as those of MA except that peptone and yeast extract were reduced to 0.1 g l^{-1} and 0.5 g l^{-1}, respectively. Samples were incubated at 28 °C until diverse colonies were formed. Eleven strains were isolated, among which strain PSR^T showed relatively low 16S rRNA gene sequence similarities with recognized species and thus it was selected for further research. Purified strains were all preserved at −80 °C with 25 % (v/v) glycerol. Reference strains used in this study were *R. ehrenbergii* JCM 13514^T and *R. spongicola* JCM 13337^T.

Cell morphology and motility were observed by optical microscopy (BX40; Olympus) and electron microscopy (JEM-1230; JEOL) after incubation on MA at 28 °C for 3 days. Gliding motility was determined as described by Bernardet *et al.* (2002). Optimal growth conditions were determined in marine broth 2216 (MB; Difco) in duplicate.

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PSR^T is KP261822.

Four supplementary figures are available with the online Supplementary Material.

The temperature range for growth was tested at 4 °C and 10–45 °C at intervals of 5 °C. The pH range for growth was measured at pH 4.5–9.5 (at intervals of 0.5), in the presence of 25 mM MES (for pH 4.5–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5) or CAPSO (pH 9.0–9.5). The effect of salt was tested with different concentrations of NaCl (0, 0.5, 1, 2, 3, 4, 6, 8 and 10 %, w/v). Anaerobic growth was determined in the modified MB, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite, 20 mM sodium nitrate, 5 g l^{-1} L-arginine and 0.5 g l^{-1} cysteine were respectively added as electron acceptors. Hungate tubes filled with N_2 were used for incubation.

Catalase and oxidase activities were individually determined by using 3 % (v/v) hydrogen peroxide solution and oxidase reagent (bioMérieux), respectively. Hydrolysis of starch and Tweens 20, 40, 60 and 80 were determined as previously described (Gerhardt *et al.*, 1994; MacFaddin, 2000). H_2S production, methyl red tests and Voges–Proskauer reaction were performed according to methods described by Zhu (2011). Hydrolysis of casein, tyrosine, chitin, CM-cellulose and filter paper were determined according to Dong & Cai (2001). Flexirubin production was tested as described by Bernardet *et al.* (2002). API 20NE, API 50CH (with API 50 CH B/E medium) and API ZYM strips (bioMérieux) were used to examine the acid production, activities of constitutive enzymes and some other physiological characteristics. In addition, Biolog GN2 microplates were used to detect the utilization of organic substrates according to the manufacturer's instructions, except that the inoculating fluid was replaced by the modified MB in which peptone and yeast extract were reduced to 0 g l^{-1} and 0.1 g l^{-1}, respectively, and 0.03 % pluronic F-68 (Sigma) and 0.02 % gellan gum were added. After incubation at 28 °C for 1 week, the data were read using the Biolog Microbial ID System.

Antibiotic sensitivity was tested on MA with antibiotic discs containing ampicillin (10 μg), bacitracin (0.04 U), carbenicillin (100 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), rifampicin (5 μg), streptomycin (10 μg) or tetracycline (30 μg). Plates were incubated at 28 °C for 3 days then inhibition zones were observed. The sensitivity to antibiotic was determined by the diameter of the inhibition zone (>13 mm as susceptible, 10–12 mm as partly susceptible, <10 mm as resistant) (Nokhal & Schlegel, 1983).

For determination of fatty acid composition, cell mass of strain PSR^T and the two reference strains in the exponential growth phase was used, obtained by culture in MB at 28 °C for 3 days. Fatty acids were analysed as described by Kuykendall *et al.* (1988). Isoprenoid quinones were analysed using reversed-phase HPLC as described previously (Komagata & Suzuki, 1987). The polar lipids were extracted and separated on silica gel plates (10 × 10 cm, Merck 5554) and further analysed as described by Minnikin *et al.* (1984) and Fang *et al.* (2012).

The genomic DNA G+C content was determined by reversed-phase HPLC (Mesbah *et al.*, 1989) with salmon sperm DNA as the calibration standard. The 16S rRNA gene was amplified and cloned into pMD 19-T vector (TaKaRa) for sequencing. The resulting sequence was subjected to pairwise sequence alignment by the EzTaxon-e server (Kim *et al.*, 2012). Multiple sequences were aligned and neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) phylogenetic trees were reconstructed using the MEGA 5 software package (Tamura *et al.*, 2011). Evolutionary distances for NJ and ML trees were calculated following the algorithm of Kimura's two-parameter model (Kimura, 1980). Bootstrap analyses for the trees were based on 1000 replications.

Cells of strain PSR^T were Gram-stain-negative rods, 0.2–0.5 μm wide and 1.5–2.5 μm long (Fig. S1, available in the online Supplementary Material), and could move by gliding. Colonies were orange-pigmented on MA. Growth occurred at 4–40 °C, at pH 6.0–9.5 and with 0.5–8 % (w/v) NaCl. Optimal growth occurred at 25–30 °C, at pH 7.0–7.5 and with 2–3 % (w/v) NaCl. Flexirubin-type pigments were not produced. Casein, starch and gelatin could be hydrolysed. Detailed phenotypic properties of strain PSR^T are given in Table 1 and the species description.

The major fatty acids of the strain PSR^T were iso-$C_{15:1}$ G (18.9 %), iso-$C_{15:0}$ (26.3 %) and iso-$C_{17:0}$ 3-OH (17.9 %) (Table 2). The predominant isoprenoid quinone was MK-7 (Table 1). The polar lipids of strain PSR^T consisted of phosphatidylethanolamine, an unknown aminolipid, an unknown phospholipid, two unknown glycolipids (GL1–2) and five unknown lipids (L1–5) (Fig. S2).

Results of the sequence alignment showed that strain PSR^T shared the highest sequence similarity with three type strains: *R. spongicola* UST030701-084^T (96.9 %), *R. ehrenbergii* KMM 6017^T (95.3 %) and *R. echinicomitans* KMM 6058^T (95.0 %). According to the NJ (Fig. 1), ML (Fig. S3) and MP (Fig. S4) phylogenetic trees based on 16S rRNA gene sequences, strain PSR^T and the type strains of species of the genus *Roseivirga* formed a distinct clade, in which strain PSR^T and *R. spongicola* UST030701-084^T formed a subclade with significant bootstrap support. The genomic DNA G+C content of strain PSR^T was determined to be 49.3 mol%.

Based on the results presented here, strain PSR^T was similar to members of the genus *Roseivirga*. All were Gram-stain-negative, strictly aerobic rods, and were catalase-, oxidase- and alkaline phosphatase-positive. Colonies were pink to orange. The predominant respiratory quinone in strain PSR^T and the closely related members of the genus *Roseivirga* was MK-7, and iso-$C_{15:1}$ G and iso-$C_{15:0}$ were the main cellular fatty acids. The polar lipid profile of strain PSR^T was very similar to those of *R. ehrenbergii* JCM 13514^T and *R. spongicola* JCM 13337^T. Moreover, according to the results of the 16S rRNA gene sequence analysis, strain PSR^T and species of the genus

Table 1. Differential phenotypic characteristics of strain PSR^T and type strains of closely related species of the genus *Roseivirga*

Strains: 1, PSR^T; 2, *R. spongicola* JCM 13337^T; 3, *R. ehrenbergii* JCM 13514^T. Unless otherwise indicated, all data are from this study. Data in parentheses were different from Lau *et al.* (2006) (column 2) and Nedashkovskaya *et al.* (2005a) (column 3). +, Positive; −, negative; W, weak.

Characteristic	1	2	3
Range for growth			
pH	6.0–9.5	5.0–10.0*[a]	5.5–9.5[b]
Temperature (°C)	4–40	12–44[a]	4–40[b]
Salinity (%, w/v)	0.5–8	0–16[a]	1–8[b]
Gliding motility	+	+	−
Hydrolysis of:			
Casein	+	−	−
Gelatin	+	−	− (+)
Starch	+	− (W)	−
Tween 40	+	+	−
Urea	−	−	+
N-Acetyl-β-glucosaminidase	−	+	−
Arginine dihydrolase	−	− (+)	+
Utilization of:			
α-Cyclodextrin	−	+	+
α-D-Glucose 1-phosphate	+	−	−
Citric acid	+	−	+ (−)
DL-α-Glycerol phosphate	−	+	−
D-Arabitol	+	−	−
Dextrin	−	+	+
D-Mannitol	−	+	−
Glycogen	−	+	+
Sucrose	+	− (+)	−
Voges–Proskauer test	+	+	−
DNA G+C content (mol%)	49.3	42.5[a]	40.2[b]

*Data from: *a*, Lau *et al.* (2006); *b*, Nedashkovskaya *et al.* (2005a).

Table 2. Cellular fatty acid profiles of strain PSR^T and type strains of closely related species of the genus *Roseivirga*

Strains: 1, PSR^T; 2, *R. spongicola* JCM 13337^T; 3, *R. ehrenbergii* JCM 13514^T. All data are from this study. Values are percentages of the total fatty acids. Fatty acids that amounted to <1.0 % of the total fatty acids in all strains are not shown. −, Not detected; TR, traces.

Fatty acid	1	2	3
iso-$C_{13:0}$	1.9	2.8	1.4
iso-$C_{14:0}$	1.2	1.4	−
iso-$C_{15:1}$ G	18.9	18.0	29.5
anteiso-$C_{15:0}$	5.0	5.6	2.1
$C_{15:0}$	TR	3.5	1.4
iso-$C_{15:0}$	26.3	25.9	45.2
iso-$C_{16:1}$ G	1.0	−	TR
iso-$C_{16:0}$	TR	2.5	TR
$C_{16:0}$	TR	3.2	TR
iso-$C_{15:0}$ 3-OH	7.8	4.8	4.6
$C_{15:0}$ 3-OH	TR	1.1	TR
iso-$C_{17:0}$	1.2	−	TR
iso-$C_{16:0}$ 3-OH	6.6	8.2	TR
$C_{16:0}$ 3-OH	TR	4.6	TR
$C_{18:0}$	−	1.1	−
iso-$C_{17:0}$ 3-OH	17.9	7.9	4.2
$C_{17:0}$ 2-OH	2.4	1.3	0.2
Summed feature 3*	2.0	−	−
Unknown 13.565	2.0	5.0	6.9
Unknown 16.582	1.2	−	TR

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC using the Microbial Identification System. Summed Feature 3 consisted of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

Roseivirga shared high sequence similarity (95.0–96.9 %), and were clustered in a distinct clade based on the NJ, ML and MP phylogenetic trees. The above characteristics supported the point that strain PSR^T was a member of the genus *Roseivirga*. However, some properties of strain PSR^T showed that the novel strain could be distinguished from the closest phylogenetic neighbour, *R. spongicola* JCM 13337^T. For example, as shown in Table 2, the percentage of iso-$C_{17:0}$ 3-OH from total fatty acids was significantly higher in strain PSR^T in contrast with *R. spongicola* JCM 13337^T and *R. ehrenbergii* JCM 13514^T. Fig. S2 showed that an additional glycolipid (GL3) was detected in the reference strains but not in strain PSR^T, and one of the unknown lipids (L2) was only detected in strain PSR^T. Strain PSR^T possessed caseinase, amylase and gelatinase, which were absent in the two reference strains. In addition, strain PSR^T, in contrast to *R. spongicola* JCM 13337^T, could use α-D-glucose 1-phosphate, citric acid, D-arabitol and sucrose as sole carbon and energy sources, but not α-cyclodextrin, DL-α-glycerol phosphate, dextrin, D-mannitol or glycogen. Based on the findings obtained in this study, we suggest that strain PSR^T represents a novel species of the genus *Roseivirga*, for which we propose the name *Roseivirga marina* sp. nov.

Description of *Roseivirga marina* sp. nov.

Roseivirga marina (ma.ri′na. L. fem. adj. *marina* of the sea, marine).

Cells are Gram-stain-negative, strictly aerobic rods (0.2–0.5 μm wide and 1.5–2.5 μm long) and can move by gliding. Colonies are orange, smooth and circular when grown on MA at 28 °C for 3 days. Growth occurs at 4–40 °C (optimum 25–30 °C), at pH 6.0–9.5 (optimum pH 7.0–7.5), with 0.5–8 % NaCl (optimum 2–3 %). Positive for catalase and oxidase activities. Casein, tyrosine, starch and Tweens 20, 40, 60 and 80 are hydrolysed, but agar, cellulose (CM-cellulose and filter paper) and chitin are not. Acetoin (Voges–Proskauer test) is produced, but H_2S and flexirubin-type pigments are not. Negative for

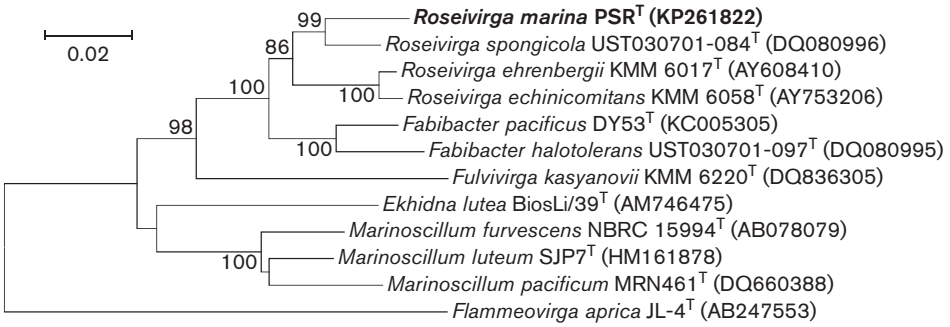

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain PSR^T and other related species. Numbers at branch points are bootstrap values (percentages based on 1000 replications); only values >70 % are shown. The 16S rRNA sequence of *Flammeovirga aprica* JL-4^T (GenBank accession no. AB247553) was used as the outgroup. Bar, 0.02 substitution per nucleotide position.

methyl red test. In the API ZYM strip, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase, chymotrypsin, acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphopydrase, trypsin, valine arylamidase, β-glucuronidase and lipase (C14) activities are absent. In API 20 NE strip, positive for fermentation of β-glucose and hydrolysis of aesculin and gelatin. Acid is produced from starch, amygdalin, arbutin, cellobiose, D-fructose, D-galactose, D-glucose, lactose (bovine origin), maltose, D-mannose, melezitose, melibiose, raffinose, sucrose, trehalose, turanose, D-xylose, aesculin ferric citrate, gentiobiose, glycogen, inulin, L-arabinose, L-sorbose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine and salicin. With Biolog GN2 MicroPlates the following substrates can be used as sole carbon and energy sources: α-D-glucose 1-phosphate, α-hydroxybutyric acid, citric acid, DL-lactic acid, D-arabitol, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, glucuronamide, inosine, itaconic acid, lactulose, L-alaninamide, L-alanine, L-alanyl glycine, L-arabinose, L-aspartic acid, L-fucose, L-histidine, L-pyroglutamic acid, sucrose, thymidine, Tween 40, Tween 80 and uridine.

The major fatty acids are iso-$C_{15:1}$ G, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The polar lipids consist of phosphatidylethanolamine, an unknown aminolipid, an unknown phospholipid, two unknown glycolipids and five unknown lipids. The predominant isoprenoid quinone is MK-7.

The type strain, PSR^T (=MCCC 1K00459^T=KCTC 42444^T), was isolated from a seawater sample of the Pacific Ocean. The DNA G+C content of the type strain is 49.3 mol%.

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