Roseivivax pacificus sp. nov., isolated from deep-sea sediment

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A Gram-stain-negative, short-rod-shaped bacterium, designated 22DY03^T, was isolated from a sediment sample collected from the East Pacific Rise. The isolate required NaCl and grew best with 3–7 % (w/v) sea salts at temperature of between 30 and 35 °C at pH 7.0. It formed non-pigmented colonies and produced exopolysaccharide, but did not produce bacteriochlorophyll *a*. Strain 22DY03^T was positive for hydrolysis of aesculin and Tween 20 and negative for hydrolysis of casein, DNA, gelatin, starch and Tween 40, 60 and 80. The major respiratory quinone was ubiquinone-10. The polar lipid profile consisted of a mixture of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, diphosphatidylglycerol, two unidentified phospholipids and four unidentified polar lipids. The major fatty acids were $C_{19:0}$ cyclo ω8*c*, $C_{18:1}\omega$7*c* and 11-methyl $C_{18:1}\omega$7*c*. The genomic DNA G+C content was 64.6 mol%. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain 22DY03^T should be assigned to the genus *Roseivivax*. The 16S rRNA gene sequence similarities between the isolate and the type strains of species of the genus *Roseivivax* were in the range of 94.1–95.8 %. On the basis of phenotypic and genotypic data, it is concluded that strain 22DY03^T represents a novel species of the genus *Roseivivax,* for which the name *Roseivivax pacificus* sp. nov. (type strain 22DY03^T=CGMCC 1.12410^T=JCM 18866^T) is proposed.

The genus *Roseivivax*, a member of the family *Rhodobacteraceae* in the phylum *Proteobacteria*, was established by Suzuki *et al.* (1999) and emended by Park *et al.* (2010) and Chen *et al.* (2012). At the time of writing, the genus *Roseivivax* is comprised of five species with validly published names. All of them were isolated from saline environments such as a salt lake (Suzuki *et al.*, 1999), a salt mine (Xiao *et al.*, 2012), a tidal flat (Park *et al.*, 2010) and coral (Chen *et al.*, 2012). The members of the genus *Roseivivax* are Gram stain-negative, oxidase- and catalase-positive and rod-shaped bacteria. The major fatty acid is $C_{18:1}\omega$7*c*. The predominant respiratory quinone is ubiquinone-10. This study focuses on the description of a non-pigmented strain 22DY03^T that was isolated from deep-sea sediment collected from the East Pacific Rise.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 22DY03^T is KC018453.

A supplementary figure and two supplementary tables are available with the online version of this paper.

The deep-sea sediment samples were collected from the East Pacific Rise (102° 33′ 14″ W 3° 06′ 15″ S) at a depth of 3414 m by a television grab bucket operated during the DY22 cruise (R/V *Dayang Yi Hao* in 2011). Aboard the ship, the sediment samples were subsampled aseptically and used immediately. An approximately 100 mg subsample was suspended in 3 ml sterile seawater and vortexed for 15 min. The dispersed sediment suspension was diluted and spread on modified marine agar (prepared according to the Difco formula for marine 2216 agar, but with the amount of peptone and yeast extract reduced to 0.5 g and 0.1 g, respectively; final pH 7.2) by using the standard ten-fold dilution plating technique. After 3 days of aerobic incubation at 28 °C, one colony, designated 22DY03^T, was picked. The isolate was purified by repeated restreaking. Purity was confirmed by the uniformity of cell morphology. Unless otherwise stated, strain 22DY03^T was routinely cultured on marine 2216 broth (MB; BD) at 28 °C and preserved as a glycerol suspension (30 % v/v) at −80 °C.

The temperature range for growth was determined by incubation at 4, 10, 15, 20, 25, 30, 35, 37, 40, 42 and 45 °C. The pH range for growth was determined in MB that was adjusted to pH 5–10.5 (at 0.5 pH unit intervals) using appropriate biological buffers (MES for pH 5.0–6.0, PIPES for pH 6.5–7.0, Tricine for pH 7.5–8.5 and CAPSO for pH 9.0–10.5) at a concentration of 50 mM. Evaluation of the pH values after autoclaving revealed only minor changes. The optimal conditions for growth were tested by using NaCl-free MB with different NaCl concentrations (0, 0.5, 1.0, 3.0, 5.0, 7.5, 10.0, 12.0, 15.0 and 20.0 % as final concentration, w/v) (Chen *et al.*, 2012). The requirement of strain 22DY03^T for artificial sea salts was evaluated on trypticase/soy broth (BD) with the addition of 0, 1, 2, 3, 5, 7, 9, 10, 11, 12, 15 % (w/v) sea salts (Sigma). Cell motility and morphology were examined using optical microscopy (BX40, Olympus) and electron microscopy (S260, Cambridge; JEM-1230, JEOL).

Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 solution. Anaerobic growth was examined with an AnaeroPack (Mitsubishi) using potassium nitrate as a potential electron acceptor. The pigment absorption spectrum analysis was performed by the method described by Rainey *et al.* (2003) and Hildebrand *et al.* (1994), using a DU 800 Spectrophotometer (Beckman; absorption spectrum from 300 to 1000 nm). EPS production was investigated based on the methods of Poli *et al.* (2007) and Wang *et al.* (1998). Tests for hydrolysis of aesculin, casein, DNA, gelatin, starch and Tween 20, 40, 60 and 80 were performed according to the protocols of Dong & Cai (2001). Acid production was tested using marine oxidation–fermentation (MOF) medium supplemented with 0.5 % sugars (Leifson, 1963). Carbon utilization was examined on artificial seawater medium (Cho & Giovannoni, 2006) that had been supplemented with 0.01 % yeast extract. The corresponding filter-sterilized sugars (0.2 %), alcohols (0.2 %), organic acids (0.1 %) or amino acids (0.1 %) were added into liquid medium. Sensitivity to antimicrobial agents was determined on MA. Additional enzyme activities and biochemical characteristics were determined using API 20 NE, API 20E and API ZYM kits (bioMérieux) at 35 °C. Enzyme activities were tested using the API ZYM kit as recommended by the manufacturer. Strips were inoculated with a heavy bacterial suspension (MacFarland 5 standard) in AUX medium supplemented with 2 % (w/v) sea salts (Sigma) according to the method of Park *et al.* (2005). *Roseivivax isoporae* BCRC 17966^T was used as a control in the above tests.

Genomic DNA was obtained by using the method described by Marmur (1961). The G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the deoxyguanosine : thymidine ratio (Mesbah & Whitman, 1989). Cellular fatty acid methyl esters obtained from cells grown in MA for 3 days at 30 °C were analysed by using GC (Kuykendall *et al.*, 1988) according to the instructions of the Microbial Identification System (MIDI). Isoprenoid quinones were extracted from freeze-dried cells (200 mg) with chloroform/methanol (2 : 1) and analysed by reversed-phase HPLC. Total lipids were extracted by the modified method of Kamekura & Kates (1988) and identified by two-dimensional TLC.

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). PCR products were cloned into vector pMD 19-T (TaKaRa) and then sequenced to determine the almost-complete sequence of the 16S rRNA gene. The sequence was compared with sequences of closely-related reference organisms from the EzTaxon-e service (Kim *et al.*, 2012). Sequence data were imported into the ARB software package (Ludwig *et al.*, 2004) and aligned based on the secondary structure of 16S rRNA molecules. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981) with the MEGA 5 program package (Tamura *et al.*, 2011). Evolutionary

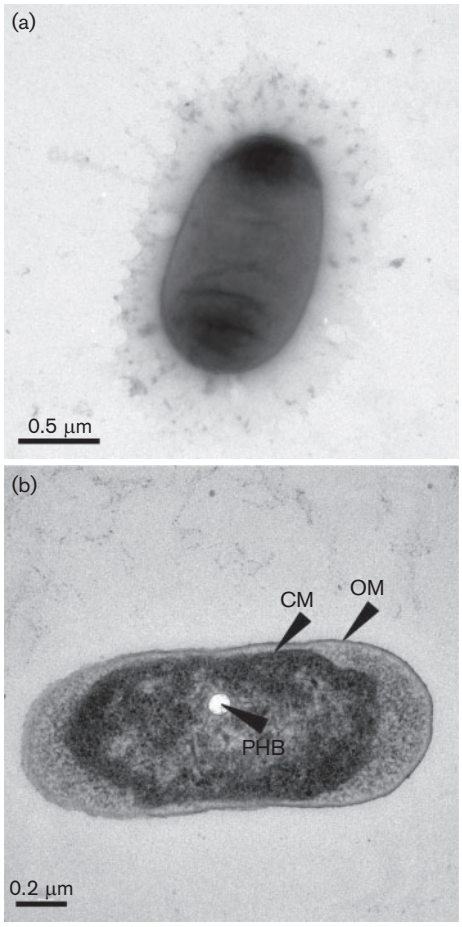

Fig. 1. Transmission electron micrographs showing the cell morphology and ultrastructure of strain 22DY03^T. Bars, 0.5 μm (a), 0.2 um (b). CM, Cytoplasmic membrane; OM, outer membrane; PHB, poly-β-hydroxybutyrate.

distances were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method.

Cells of strain 22DY03^T were Gram-stain-negative, aerobic and rod-shaped, 0.6–0.9 μm in width and 1.0–1.8 μm in length. Ultrathin sections revealed that strain 22DY03^T possesses a typical Gram-stain-negative cell periphery structure, including a cytoplasmic membrane and an outer membrane (Fig. 1). Flagella were not observed. Colonies were non-pigmented, circular, slightly convex and 1–2 mm in diameter after 2 days incubation at 30 °C on MA. Extracts of cell material in acetone/methanol (7 : 2, v/v) did not show absorption maxima in the visible and in the infrared range; extracts of the reference strain *R. isoporae* BCRC 17966^T showed typical maxima at 482 nm and at 775 attributed to carotenoids and to bacteriochlorophyll *a*. Also in the lipid extraction using chloroform/methanol/water (1 : 2 : 1), the layer of chloroform remained colourless. Strain 22DY03^T tested positive for urease, acid production from aesculin, D-fructose, *myo*-inositol, D-melezitose, sucrose and trehalose, growth on *myo*-inositol and α-D-lactose and susceptibility to ampicillin (10 μg) and penicillin (10 IU); whilst *R. isoporae* BCRC 17966^T was negative in these tests (Table 1). The detailed phenotypic characteristics of strain 22DY03^T are given in the species description. A comparison of the phenotypic properties between strain 22DY03^T and the type strains of recognized species in the genus *Roseivivax* are given in Table 1 as well as Table S2 (available in IJSEM Online).

The almost-complete 16S rRNA gene sequence (1424 nt) of strain 22DY03^T was obtained. Sequence similarity of strain 22DY03^T with the type strains of species of the genus

Table 1. Differential characteristics of the novel isolate 22DY03^T and its closest phylogenetic neighbours

Strains: 1, 22DY03^T; 2, *Roseivivax isoporae* BCRC 17966^T; 3, *Roseivivax halodurans* DSM 15395^T; 4, *Roseivivax sediminis* ACCC 10710^T. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4
Source	Deep-sea sediment	Reef-building coral	Saline lake	Salt mine
Colony colour	Non-pigmented	Greyish pink	Greyish pink	Cream-yellow
Bacteriochlorophyll *a* production	−	+	− (+)*	−
Presence of flagella	−	+[a]†	+[b]†	−[c]†
Enzyme activities				
Urease	+	−	−	+
α-Galactosidase	+	+	−	+
β-Galactosidase	+	+	−	−
Acid production from:				
Aesculin	+	−	−	−
D-Fructose	+	−	+‡	+(−)‖
myo-Inositol	+	−	−	−
D-Melezitose	+	−	−	−
Sucrose	+	−	−¶	+
Trehalose	+	−	−	−
Assimilation of:				
Adonitol	−	−	+	−
Ethanol	W	+	+ (−)*	+
myo-Inositol	W	−	−	−
L-Isoleucine	−	+	−	W
α-Lactose	+	−	+	+
Sucrose	W	+	+	+
Trehalose	W	+	+	+
L-Valine	−	+	−	−
Antibiotic susceptibility				
Ampicillin (10 μg)	+	−	+	−
Penicillin G (10 IU)	+	−	+#	−
DNA G+C content (mol%)	64.6	68.8[a]†	64.4[b]†	67.7[c]†

*Different result (shown in parentheses) reported by Suzuki *et al.*, 1999.
†Data from: *a*, Chen *et al.*, 2012; *b*, Suzuki *et al.*, 1999; *c*, Xiao *et al.*, 2012.
‡Identical result reported by Suzuki *et al.*, 1999, but different result reported by Park *et al.*, 2010.
‖Different result (shown in parentheses) reported by Xiao *et al.*, 2012.
¶Identical result reported by Xiao *et al.*, 2012, but different result reported by Suzuki *et al.*, 1999.
#Identical result reported by Xiao *et al.*, 2012, but different result reported by Suzuki *et al.*, 1999.

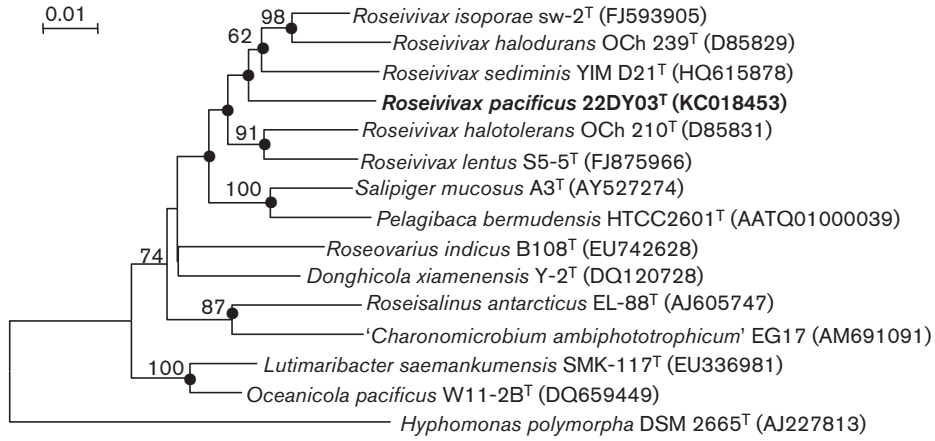

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of the novel isolate and related taxa. Bootstrap values (>60 %) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Hyphomonas polymorpha* DSM 2665^T (GenBank accession number AJ227813) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Roseivivax with validly published names was 94.1–95.8 %. The tree topologies showed that strain 22DY03^T fell within the cluster comprising species of the genus *Roseivivax* and formed a clade with *R. isoporae*, *Roseivivax sediminis* and *Roseivivax halodurans* (Fig. 2). Phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that strain 22DY03^T represented a member of the genus *Roseivivax*. The DNA G+C content of strain 22DY03^T was 64.6 mol%, a value in the range reported for members of the genus *Roseivivax*, i.e. 59.7–68.7 mol% (Suzuki *et al.*, 1999; Chen *et al.*, 2012).

The fatty acids profile of strain 22DY03^T was different from that of *R. isoporae* BCRC 17966^T, *R. halodurans* DSM 15395^T and *R. sediminis* ACCC 10710^T (Table S1). The content of $C_{19:0}$ cyclo $\omega 8c$ of strain 22DY03^T (39.3 %) was higher than that of *R. isoporae* BCRC 17966^T (17.9 %), *R. halodurans* DSM 15395^T (13.4 %) and *R. sediminis* ACCC 10710^T (37.3 %); whereas the content of $C_{18:1}\omega 7c$ of the former (27.5 %) was less than that of the latter (58.2 %, 44.6 % and 32.3 %, respectively). The predominant respiratory quinone detected in strain 22DY03^T was Q-10, consistent with that observed in all members of the genus *Roseivivax* (Suzuki *et al.*, 1999; Chen *et al.*, 2012). The polar lipid profiles comprise phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, diphosphatidylglycerol, two unidentified phospholipids and four unidentified polar lipids (Fig. S1). These chemotaxonomic data support the result of the phylogenetic analysis.

Strain 22DY03^T could be differentiated from the recognized species of the genus *Roseivivax* on the basis of some phenotypic characteristics, including production of bacteriochlorophyll *a*, presence of flagella, nitrate reduction, enzyme activities, utilization of substrates, acid production and antibiotic susceptibility (Table S1). Strain 22DY03^T also could be distinguished from its closest relatives (*R. isoporae* BCRC 17966^T, *R. halodurans* DSM 15395^T and *R. sediminis* ACCC 10710^T) by colony colour, acid production from aesculin, *myo*-inositol, D-melezitose and trehalose and assimilation of ethanol, *myo*-inositol, sucrose and trehalose (Table 1).

On the basis of the phenotypic data, chemotaxonomic and phylogenetic inferences obtained in this study, it is concluded that strain 22DY03^T represents a novel species within the genus *Roseivivax*, for which the name *Roseivivax pacificus* sp. nov. is proposed.

Description of *Roseivivax pacificus* sp. nov.

Roseivivax pacificus (pa.ci′fi.cus. L. masc. adj. *pacificus* peaceful, pertaining to the Pacific Ocean).

Cells are Gram-stain-negative, aerobic, non-flagellated and short-rod-shaped, 0.6–0.9 μm in width and 1.0–1.8 in length. Colonies are non-pigmented, circular, slightly convex and 1–2 mm in diameter after 2 days incubation at 30 °C on MA. Requires natural seawater or artificial sea-salts for growth. Growth occurs at NaCl concentrations of 0.5–15.0 % (w/v), but not in trypticase soy broth without supplementation with sea salts or seawater. The pH and temperature ranges for growth are pH 5.5–8.5 and 10–40 °C (optimum at pH 7.0 and 30–35 °C). No growth is detected at 4 °C or above 42 °C. Does not produce bacteriochlorophyll *a*. Produces exopolysaccharide. No anaerobic growth occurs on MA supplemented with potassium nitrate. No growth on MacConkey agar and Cetrimide agar. Positive for oxidase, catalase, urease and Voges–Proskauer reaction. Negative for arginine dihydrolase, lysine and ornithine decarboxylases, tryptophan deaminase, nitrate reduction, indole formation and H_2S production. Aesculin and Tween 20 are hydrolysed. Casein, DNA, gelatin,

starch and Tween 40, 60 and 80 are not hydrolysed. According to the API ZYM tests, acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), α- and β-galactosidases, α- and β-glucosidases, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present; *N*-acetyl-β-glucosaminidase, α-chymotrypsin, β-fucosidase, β-glucuronidase, lipase (C14) and trypsin activities are absent; cystine arylamidase and α-mannosidase activities are weak. The following compounds are utilized as sole carbon and energy sources: acetate, *N*-acetylglucosamine, adipic acid, aesculin, L-alanine, L-arabinose, L-arginine, cellobiose, citrate, L-cysteine, ethanol, formate, D-fructose, D-galactose, D-gluconate, glucose, glycerol, *myo*-inositol, α-lactose, malate, malonate, maltose, mannitol, D-mannose, D-melezitose, L-ornithine, propionate, pyruvate, D-ribose, L-rhamnose, raffinose, D-salicin, L-serine, sorbitol, succinate, sucrose, trehalose and D-xylose. The following compounds are not utilized as sole carbon and energy sources: adonitol, capric acid, fumarate, glycine, L-isoleucine, L-lysine, phenylacetic acid and L-valine. Acid is produced from aesculin, L-arabinose, cellobiose, D-fructose, D-galactose, glucose, glycerol, *myo*-inositol, α-lactose, maltose, mannitol, D_melezitose, D-ribose, L-rhamnose, raffinose, D-salicin, sucrose, trehalose and xylose, but not from adonitol, ethanol, sorbitol, sorbose or starch. Susceptible to (μg per disc unless otherwise stated): ampicillin (10), cefalexin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), kanamycin (30), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (10 IU), rifampicin (5) and tetracycline (30) and weakly susceptible to amikacin (30), gentamicin (10), nalidixic acid (30), streptomycin (10) and tobramycin (10); but not to clindamycin (2), polymyxin B (300 IU) and vancomycin (30). The principal fatty acids (>10 %) are $C_{19:0}$ cyclo $\omega 8c$, $C_{18:1}\omega 7c$ and 11-methyl $C_{18:1}\omega 7c$. The predominant respiratory quinone is Q-10. The polar lipid profiles comprise phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, diphosphatidylglycerol, two unidentified phospholipids and four unidentified polar lipids.

The type strain, 22DY03^T (=CGMCC 1.12410^T=JCM 18866^T), was isolated from a deep-sea sediment sample collected from the East Pacific Rise. The DNA G+C content is 64.6 mol% (by HPLC).

Acknowledgements

This work was supported by grants from China Ocean Mineral Resources R & D Association (COMRA) Special Foundation (DY125-15-R-03 and DY125-14-E-02), the Scientific Research Fund of the Second Institute of Oceanography, State Oceanic Administration (JT0906 and JT1011), the National Natural Science Foundation of China (41276173) and the Open Research Program of the Key Laboratory of Marine Ecosystem and Biogeochemistry (LMEB), State Oceanic Administration (LMEB 201006).

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