Rhodoplanes piscinae sp. nov. isolated from pond water

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Two strains (JA266 T and JA333) of Gram-negative, rod-shaped, phototrophic, purple non-sulfur bacteria were isolated from a freshwater fish pond and an industrial effluent. Both strains were capable of phototrophic and chemotrophic growth. Bacteriochlorophyll a and carotenoids of the spirilloxanthin series were present as photosynthetic pigments. The major fatty acid for both strains was C18:1ω7c (65%), with minor amounts of 11-methyl C18:1ω7c, C16:0, C16:1ω7c and C18:0 also present. Both strains have the lamellar type of intracellular photosynthetic membranes. Ubiquinone-10 (Q10) and rhodoquinone-10 (RQ10) were present as primary quinone components. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine were the major polar lipids, while minor amounts of amino lipids (L1, L2) and an unidentified lipid (L1) were common to both strains. The DNA G+C contents of strains JA266 T and JA333 were 71.3 and 69.9 mol%, respectively. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that both strains clustered with members of the genus Rhodoplanes in the class Alphaproteobacteria. Strains JA266 T and JA333 had gene sequence similarity of 98.7 and 98.9% with Rhodoplanes serenus TUT3530 T, 96.4 and 96.5% with Rhodoplanes elegans AS130 T, respectively, and less than 96% with other members of the genus Rhodoplanes. 16S rRNA gene sequence similarity between the two strains was 99.3% and they exhibited high (84.7%) relatedness based on DNA–DNA hybridization. Furthermore, both strains had less than 65% DNA–DNA relatedness with the type strain R. serenus TUT3530 T. On the basis of phenotypic and genotypic data, it is proposed that strain JA266 T be classified as a novel species of the genus Rhodoplanes, with the species name Rhodoplanes piscinae sp. nov. The type strain of the proposed novel species is JA266 T (=JCM 14934 T =KCTC 5627 T), while strain JA333 (=NBRC 107574 =KCTC 5962) is an additional strain.

At the time of writing, the genus Rhodoplanes comprises four species with validly published names: Rhodoplanes roseus, Rhodoplanes elegans (Hiraishi & Ueda, 1994), Rhodoplanes serenus (Okamura et al., 2009) and Rhodoplanes pokkaliisoli (Lakshmi et al., 2009). Though ‘Rhodoplanes cryptolactis’ was effectively published (Okamura et al., 2007), this species name has not been validly published. Phylogenetically, the genus Rhodoplanes (Hiraishi & Ueda, 1994) is classified under the family Hyphomicrobiaceae of the order Rhizobiales in the class Alphaproteobacteria. In this communication, we propose a novel species of the genus Rhodoplanes.

Strain JA266 T was isolated from a surface water sample from a freshwater fish pond (pH ~7, 30°C) that was collected on 28 January 2007 at Mangalore, India (12.5°N 74.5°E). Enrichment was made with a medium containing (g l−1; pH 7.0): sodium succinate (0.5), sodium acetate (0.5), sodium glutamate (0.5), sodium glutamate (0.5), sodium ascorbate (0.5), yeast extract (0.3), Casamino acids (0.3), (NH4)2SO4 (0.5), NaCl (0.4), KH2PO4 (0.38), K2HPO4 (0.39); 1 ml l−1 vitamin solution and 5 ml l−1 basal salt solution. Two millimolar Na2S2O3·5H2O (final concentration) was added, after autoclaving, to the medium. The vitamin solution contained (mg 100 ml−1): nicotinic acid (100); thiamine-HCl (100); biotin (5); p-aminobenzoic acid (50);
vitamin-B_{12} (1); Ca-pantothenate (50); pyridoxyl-HCl (50); and folic acid (50). The basal salt solution contained (l{sup −1}): tri-sodium EDTA (4.53 g); FeSO_{4}.7H_{2}O (1.11 g); MgSO_{4}.7H_{2}O (24.6 g); CaCl_{2}.2H_{2}O (2.94 g); KCl (23.4 g); MnSO_{4}.4H_{2}O (111 mg); ZnSO_{4}.7H_{2}O (28.8 mg); Co(NO_{3}).6H_{2}O (29.2 mg); CuSO_{4}.5H_{2}O (25.2 mg); Na_{2}MoO_{4.2H_{2}O} (24.2 mg); H_{3}BO_{3} (60 mg). The strain was incubated in 50 ml, fully filled, screw-cap bottles at a light intensity of 2400 lx, 28–30 °C, for 7 days. Strain JA333 was isolated from a sample collected on 5 May 2006 from an industrial effluent treatment pond (JETL) at Jeedimetla, Hyderabad, India (17.3°N 78.3°E). The same medium, without glutamate, was used for the enrichment of strain JA333.

Purification was carried out by repeated streaking on agar slants (25 × 150 mm test tubes sealed with Suba Seals and the gas phase was replaced with argon) and the purified cultures were grown in the medium (g l{sup −1}; pH 7.0): KH_{2}PO_{4} (0.38), K_{2}HPO_{4} (0.39), MgSO_{4}.7H_{2}O (0.2), NaCl (0.4), NH_{4}Cl (0.6), CaCl_{2}.2H_{2}O (0.05), sodium succinate (1.5), sodium acetate (1.5), sodium ascorbate (0.5), yeast extract (0.2), ferric citrate (5 ml l{sup −1}); and folic acid (0.5). The basal salt solution contained AS130T (200), CuCl_{2}.H_{2}O (20), NiCl_{2}.6H_{2}O (20) and NaMoO_{4}.2H_{2}O (29.2 mg); H_{3}BO_{3} (60 mg). The strain was incubated in 50 ml, fully filled, screw-cap test tubes completely filled with the above growth (dark) by nitrate respiration was determined in India (17.3°N 78.3°E). The same medium, without glutamate, was used for the enrichment of strain JA333.

A taxonomic study was conducted based on a polyphasic approach as described by Srinivas et al. (2007). Anaerobic growth (dark) by nitrate respiration was determined in screw-cap test tubes completely filled with the above growth medium supplemented with 20 mM potassium nitrate (final concentration). N_{2} gas production by denitrification was observed in these test tubes, with Durham tubes compared with a negative control without nitrate. Spectral analysis was carried out as described by Anil Kumar et al. (2008). Carotenoids were extracted with an acetone–methanol mixture (7:2, v/v) and the carotenoid composition was analysed by using HPLC (solvent system used was 50:40:10, by vol., acetonitrile/methanol/ethylacetate, flow rate 1 ml min{sup −1}, column luna 5 μm, C_{18}, 250 × 4.6 mm; Phenomenex; Photo Diode Array detector (400–800 nm). Wavelengths monitored at 450, 500 and 550 nm).

For cellular fatty acids, photoheterotrophically grown cultures were harvested when growth was around 70% of its maximal optical density. Bacterial cells (40 mg) were then subjected to a series of four different reagents, followed by saponification and methylation of fatty acids, thus enabling their cleavage from lipids. The fatty acid methyl esters (FAME) thus obtained were analysed by GC equipped with Sherlock MIS software (Microbial ID; MIDI; Agilent 6850; Sasser, 1990). The peaks obtained were labelled and the equivalent chain-length values were computed by the Sherlock software (FAME analysis was outsourced to Royal Research Lab, Secunderabad, India). Quinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC and analysed by HPLC (Imhoff, 1984; Hiraishi & Hoshino, 1984).

Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2:1:0.8, by vol.) as described by Kates (1986). Lipids were separated using silica gel TLC (Kieselgel 60 F254; Merck) by two-dimensional chromatography using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) in the second dimension (Tindall, 1990a, b; Oren et al., 1996). Total polar lipid profiles were detected by spraying with 5% ethanolic molybdophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff (quaternary nitrogen) or α-naphthol (specific for sugars) (Kates, 1972; Oren et al., 1996).

Genomic DNA was obtained from 1–2 ml well-grown liquid culture using the Genomic DNA Extraction kit (Qiagen). Recombinant Taq polymerase was used for PCR. The PCR was started with the primers 5’-GGTTGATCC-TGGCTCAG-3’ (F−27) and 5’-TACCTGTGACGACCTTGA-3’ (R’-1489) (Positions 11–27 and 1489–1506, respectively, according to the Escherichia coli 16S rRNA numbering system of the International Union of Biochemistry). PCR amplification was carried out as described previously by Imhoff et al. (1998) and 16S rRNA gene sequencing was outsourced to Eurofins Genomics India, Bangalore, India. The identification of phylogenetic neighbours and the calculations of pairwise 16S rRNA gene sequence similarities were achieved using the NCBI BLAST search (Altschul et al., 1990) and EzTaxon server (Chun et al., 2007). The CLUSTAL W algorithm of MEGA4 was used for sequence alignments and MEGA4 (Tamura et al., 2007) software was used for phylogenetic analyses. Distances were calculated by using the Kimura-2 parameter in a pairwise deletion procedure. Neighbour-joining, minimum evolution and maximum-parsimony methods in the MEGA4 software (http://www.megasoftware.net/mega4/mega.html) were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The taxonomic relationship between strains JA266T, JA333 and closely related type strains of members of the genus Rhodoplanes was examined using DNA–DNA hybridization, which was determined using a membrane filter technique (Tourova & Antonov, 1988) using a nick translation kit supplied by Jonaki, BRIT. Hybridization was performed with three replications for each sample (reciprocal experiments were conducted for binding and labelling) and the means are quoted as the DNA–DNA relatedness values. α-P^{32} dCTP was used for labelling the probe. The DNA immobilized on the blots (nylon membranes) was probed with labelled DNA and then exposed to a phosphor-imaging screen (Amersham Biosciences). The phosphor-imaging screen was scanned and quantified using a Typhoon (3480) variable mode Imager. The per cent hybridization was calculated according
### Table 1. Differentiating characteristics of species of the genus *Rhodoplanes*

Data pertains to comparative analysis in this study, unless stated otherwise. Strains: 1, JA266<sup>T</sup>; 2, JA333; 3, *R. serenus* TUT3530<sup>T</sup>; 4, *R. elegans* AS130<sup>T</sup>; 5, *R. roseus* 941<sup>T</sup>; 6, *R. pokkaliisoli* JA415<sup>T</sup>; 7, ‘*R. cryptolactis*’ DSM 9987. Organic substrate utilization was tested during photoheterotrophic growth for all taxa. Butyrate, lactate, malate, propionate, fumarate, succinate, acetate, citrate and valerate were utilized by most of the strains. Aspartate, benzoate, formate, glucose, glycolate, mannitol, methanol, propanol, sorbitol, sulfide, alanine and leucine were not utilized by any of the strains. Cells of all strains were motile. Internal membrane system was lamellar type in all strains. Phototrophically grown cultures were pink, while aerobically grown cultures were white for all strains. Bacteriochlorophyll <sub>a</sub> was present in all strains. There was no salt requirement for any of the strains. Denitrification was present in all strains. Aerobic dark growth was present in all the strains. Most strains could not ferment fructose. The major quinones for all strains were Q<sub>10</sub> and RQ<sub>10</sub>. +, Substrate utilized or present (OD values from 0.65 to 1.20 were considered as substrate utilized); 2, substrate not utilized or absent; (+), weak growth (OD values from 0.25 to 0.30 were considered as weak growth when compared with OD values from cultures incubated without the substrate); +/-, variable in different strains; tr, trace amounts (<0.5%); NA, not available.

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7*</th>
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<td>Isolation source</td>
<td>Fish pond</td>
<td>Effluent pond</td>
<td>Pond water</td>
<td>Activated sludge</td>
<td>Lake sediment</td>
<td>Paddy soil</td>
<td>Hot spring</td>
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<td>Size (μm)</td>
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<td>0.9–1.0 × 1.5–13.5</td>
<td>0.8–1.0 × 2.0–13.0</td>
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<td>Tube</td>
<td>Tube</td>
<td>Tube</td>
<td>Sessile</td>
<td>Tube</td>
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<td>Rosette formation</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>In vivo absorption maxima (nm) in infrared region (shoulder at 875)</td>
<td>802, 821,</td>
<td>803, 824, 878</td>
<td>800, 850</td>
<td>799, 822, 878</td>
<td>801, 854</td>
<td>800, 866</td>
<td>802, 822, 857</td>
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<td>5.5–9.0</td>
<td>7.0</td>
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<td>38</td>
<td>35</td>
<td>45–46</td>
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<td>Growth factors</td>
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<td>Thiamine, p-aminobenzoic acid</td>
<td>Niacin</td>
<td>Niacin, p-aminobenzoic acid, pantothenate</td>
<td>Niacin, p-aminobenzoic acid, B&lt;sub&gt;12&lt;/sub&gt;</td>
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<td>Utilization of carbon/electron source for growth†</td>
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<td>Caproate (0.1)</td>
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<td>+</td>
<td>+</td>
<td>+/−</td>
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<td>−</td>
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<td>Glycerol (0.3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Fructose (0.3)</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Yeast extract (0.1)</td>
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<td>(+)</td>
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<td>+</td>
<td>+</td>
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<td>Citrate (0.3)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>Valerate (0.1)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>Pyruvate (0.3)</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Tartarate (0.1)</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>Electron donor</td>
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<td>Thiosulfate (2 mM)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Cellular fatty acids (%)</td>
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<td></td>
<td></td>
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<td>C&lt;sub&gt;12&lt;/sub&gt;:0</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>1.2</td>
<td>−</td>
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<tr>
<td>C&lt;sub&gt;16&lt;/sub&gt;:0</td>
<td>10.6</td>
<td>3.8</td>
<td>14.4</td>
<td>15.8</td>
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<td>C&lt;sub&gt;16&lt;/sub&gt;:0 3-OH</td>
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<td>1.5</td>
<td>−</td>
<td>1.5</td>
<td>1.3</td>
<td>−</td>
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<tr>
<td>C&lt;sub&gt;16,107c&lt;/sub&gt;</td>
<td>3.0</td>
<td>1.1</td>
<td>2.5</td>
<td>1.6</td>
<td>4.0</td>
<td>1.9</td>
<td>−</td>
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to the formula: \( \frac{\text{counts obtained from heterologous hybridization}}{\text{counts obtained from homologous hybridization}} \times 100. \)

Cells of strains JA266\textsuperscript{T} and JA333 were rod-shaped. While cells of strain JA266\textsuperscript{T} were 0.7–1.0 µm wide and 2–3 µm long (Fig. S1, available in IJSEM Online), cells of strain JA333 were 0.9–1.0 µm wide and 1.5–3.5 µm long. Both strains multiplied by budding and asymmetrical cell division, with a slender prostheca occurring between the mother and daughter cells. On ageing, both strains formed rosette-like clusters or cell aggregates. Cells of both strains were motile by means of polar flagella (Fig. S2) and had lamellar-type internal membrane structures parallel to the cytoplasmic membrane (Fig. S3). Both strains grew photo-organoheterotrophically [anaerobically, in the light (2400 lx) with sodium acetate and sodium succinate (0.3 %, w/v) as carbon sources], and chemo-organoheterotrophically [aerobically, in the dark with sodium acetate and sodium succinate (0.05 %, w/v) as carbon sources]. Photolithoautotrophic [anaerobically, in the light (2400 lx)] and chemolithoautotrophic (aerobically, in the dark) growth [both growth modes with Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}·5H\textsubscript{2}O (0.5 mM) as an electron donor (electron donors not utilized were H\textsubscript{2}, 20 %, v/v; sulfide, 0.5 and 1 mM; sulfite or thioglycolate, 1 mM; or sulfur, 0.05 %, w/v) and NaHCO\textsubscript{3} (0.1 %, w/v) as a carbon source] were observed in both strains; however, the biomass yields were poor in strain JA266\textsuperscript{T}. Fermentative growth (anaerobic, dark with glucose/fructose/pyruvate, 0.3 %, w/v) could not be observed in either strain. Organic substrates utilized/not utilized by both strains are given in Table 1. Both strains could utilize ammonium chloride, urea, glutamate and glutamine as nitrogen sources, while nitrite and nitrate did not support phototrophic growth. Denitrification (anaerobically, in the dark) was positive for both strains. Niacin was required as a growth factor by both strains. Sulfate, sulfite, thiosulfate and thioglycolate were used as sulfur sources, while cysteine, elemental sulfur and sulfide did not support phototrophic growth of both strains. There was no requirement for salt (NaCl) for growth by either strain. The pH range for growth of strain JA266\textsuperscript{T} was 6.5–9.0, with an optimum of 6.5–7.0, while strain JA333 grew at pH 5.5–9.0, having an optimum of 7.0–8.0. Both strains were mesophilic with optimal growth at 30 °C (range 25–40 °C). Cell suspensions of both strains were pink when grown phototrophically and white when grown chemotrophically (aerobically, in the dark). Whole-cell absorption spectrum of both strains showed absorption maxima at 515–517, 550, 590, 802–824 nm, with a shoulder at 875 nm for strain JA266\textsuperscript{T}, and a peak at 878 nm for strain JA333 (Fig. S4). Carotenoid composition of both strains as detected by HPLC analysis was: rhodopin (45–46 %), anhydrorhodovibrin (20–22 %), rhodovibrin (15–18 %) and spirilloxanthin (16–19 %). We could not observe any difference in the whole-cell absorption spectra of both strains grown at high [5000 lx (incandescent)] and low [500 lx (incandescent)] light intensities, as observed previously in ‘R. cryptolactis’
(Okamura et al., 2007) and in R. serenus (Okamura et al., 2009). Whole-cell fatty acid analysis revealed that C_{18:1}ω7c was the major fatty acid in both strains, with 68.1% in JA266^T and 76.7% in JA333 (Table 1). Both strains contained both Q_{10} and RQ_{10} as primary quinone components (the molar ratio of Q_{10}/RQ_{10} in strains JA266^T and JA333 was 8.5:1.5 and 7.4:2.6, respectively). Menaquinones (MKs) were absent. The presence of Q_{10} and RQ_{10}, and the absence of MKs are specific for the genus *Rhodoplanes* (Hiraishi & Ueda, 1994). Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine are the major polar lipids, while minor amounts of unidentified amino lipids (AL1, AL2) and an unidentified lipid (L1) are common to both strains (Fig. S5). The novel isolates differed from *R. serenus* DSM 18633^T in the absence of the unidentified lipids L2–3 and unidentified phospholipids PL1–3.

The G+C contents of the genomic DNA of strains JA266^T and JA333 were 70.3 and 69.9 mol% (by HPLC), respectively. The phylogenetic relationship (Fig. 1) of strains JA266^T and JA333 to other purple non-sulfur bacteria was examined by using 16S rRNA gene sequence analysis. The data obtained revealed that the novel isolates clustered with the type strains of species of the genus *Rhodoplanes*, but were distinct from other genera of purple non-sulfur bacteria. (Minimum evolution and maximum-parsimony trees also showed similar tree topology, Fig. S6a, b, respectively). Strains JA266^T and JA333 showed the highest gene sequence similarities to the type strains of *R. serenus* TUT3530^T (98.7 and 98.9%), *R. elegans* AS130^T (96.4 and 96.5%), 'R. cryptolactis' DSM 9987 (96.3 and 96.5%), *R. roseus* 941^T (95.8 and 96%) and *R. pokkaliioli* JA415^T (95.5 and 95.6%, respectively). At the whole genome level, as determined by DNA–DNA hybridization, when strains JA266^T and JA333 were radioactively labelled, the level of DNA–DNA relatedness with *R. serenus* TUT3530^T was 55±6.0% for each strain. However, when *R. serenus* TUT3530^T was labelled and used for DNA–DNA hybridization in the reciprocal reaction, the similarity was 52.6±4.8% for each strain. The DNA–DNA hybridization value between strains JA266^T and JA333 was 81±3.3%. Apart from the 16S rRNA gene sequence dissimilarity and DNA–DNA relatedness, strain JA266^T (and strain JA333) also showed clear phenotypic differences to *R. serenus* TUT3530^T in whole-cell absorption spectra (Fig. S4), whole-cell fatty acid profile (Table 1), lack of utilization of pyruvate and the requirement for niacin for growth and the differences in polar lipid composition. These phenotypic differences justify the description of JA266^T as a representative strain of a novel species, for which the name *Rhodoplanes piscinae* sp. nov. is proposed, and of JA333 as an additional strain of the same species.

**Description of *Rhodoplanes piscinae* sp. nov.**

*Rhodoplanes piscinae* (pi.sci.nae. L. gen. n. piscinae, of a fish pond, from where the type strain was isolated).
Cells are rod-shaped, 0.7–1.0 μm wide and 1.5–3.5 μm long. Cells are motile by means of polar flagella and multiply by budding and asymmetrical cell division, with slender prosthecae occurring between the mother and daughter cells. If cells age, they form rosette-like clusters or cell aggregates. Intracellular photosynthetic membranes are of the lamellar type, parallel to the cytoplasmic membrane. Phototrophically grown cultures are pink, while aerobic cultures are white. The in vivo absorption spectrum of intact cells in sucrose exhibits maxima at 515–517, 550, 590, 802–803 and 821–824 nm, with a shoulder at 875–878 nm. Bacteriochlorophyll $a$ and carotenoids of the spirilloxanthin series (rhodopin, anhydrorhodovibrin, rhodovibrin and spirilloxanthin) are present. A mesophilic bacterium with optimal growth at 30 °C (range 25–40 °C), 0–0.5 %, w/v, NaCl (range 0–0.5 %, w/v) and pH 6.5–8.0 (range 5.5–9.0). Growth occurs under anaerobic conditions in the light (photo-organoheterotrophy) or under aerobic conditions in the dark (chemo-organoheterotrophy). The preferred mode of growth is photo-organoheterotrophy with a few organic compounds. Acetate, propionate, butyrate, valerate, crotonate, lactate, malate, fumarate, succinate, citrate, 2-oxoglutarate, glutamate, ethanol, butanol, caproate and Casamino acids are good carbon sources. Growth is poor on fructose, peptone, propanol, tartrate and yeast extract. Formate, caprylate, glycolate, benzoate, pyruvate, glucose, glutamate, mannitol, aspartate, sucrose, methanol, sorbitol, leucine and alanine are not utilized. Some strains can use thiosulfate and thioglycolate as sulfur sources. Ammonium chloride, urea, glutamate and glutamine are used as nitrogen sources. Sulfate, sulfite, thiosulfate and thioglycolate are used as sulfur sources. Casamino acids are good carbon sources. Growth is poor in the presence of glucose, fructose or pyruvate as fermentable carbon sources. Niacin is required as a growth factor. The predominant Q10 and RQ10 are present as major quinones.

The type strain JA266$^T$ (=JCM 14934$^T$=KCTC 5627$^T$) was isolated from a water sample from a freshwater fish pond at Mangalore, facing the Arabian sea on the west coast of India. The DNA G+C content is 69.9–71.3 mol% (by HPLC). Strain JA333 (=NBRC 107574=KCTC 5962) was isolated from an industrial effluent treatment pond at Jeedimetla (JETL), Hyderabad, India, a second strain of the species.

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References


