Rhodovulum aestuarii sp. nov., isolated from a brackish water body

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A yellowish brown, phototrophic, purple non-sulfur bacterium, strain JA924^T, was isolated in pure culture from a brackish water sample collected from an estuary. Single cells were oval to rod-shaped, non-motile and Gram-stain-negative and had a vesicular architecture of intracellular photosynthetic membranes. Bacteriochlorophyll-*a* and carotenoids of the spheroidene series were present as photosynthetic pigments. Photolithoautotrophy, chemo-organoheterotrophy and photo-organoheterotrophy were the growth modes observed. Strain JA924^T had complex growth requirements. Strain JA924^T was mesophilic and moderately halophilic. The DNA G+C content was 64 mol% (HPLC). The major cellular fatty acids were $C_{18:1}\omega7c$/$C_{18:1}\omega6c$, $C_{16:0}$ and $C_{18:0}$. The major quinone was ubiquinone-10 (Q-10). Phosphatidylglycerol, phosphatidylethanolamine, sulfolipid and an aminolipid were the main polar lipids of strain JA924^T. EzTaxon-e BLAST searches based on the 16S rRNA gene sequence of JA924^T revealed highest similarity with *Rhodovulum mangrovi* AK41^T (98.19 %) and other members of the genus *Rhodovulum* (<95.71 %). Strain JA924^T was further identified to be distantly related to *Rhodovulum mangrovi* AK41^T (<29 % based on DNA–DNA hybridization and ΔT_m (>5 °C). Phenotypic, chemotaxonomic and molecular differences indicate that strain JA924^T represents a novel species of the genus *Rhodovulum*, for which the name *Rhodovulum aestuarii* sp. nov. is proposed. The type strain is JA924^T (=LMG 29031^T=KCTC 15485^T).

With the advent of 16S rRNA gene sequencing, marine and halophilic species that depend on NaCl were transferred from the genus *Rhodobacter* to the new genus *Rhodovulum* (Hiraishi & Ueda, 1995). In several phenotypic properties, species of the genus *Rhodovulum* resemble members of *Rhodobacter* and *Rhodobaca* and differentiation between these genera is possible based on 16S rRNA gene sequences and by hybridization (Imhoff, 2005). Members of the genus *Rhodovulum* are ovoid to rod-shaped, motile or non-motile and multiply by binary fission. Phototrophically grown cells form vesicular internal photosynthetic membranes. Pigments are bacteriochlorophyll-*a* and carotenoids of the spheroidene series. Ubiquinone-10 (Q-10) is the major quinone and the major fatty acids are $C_{18:1}$, $C_{18:0}$ and $C_{16:0}$. At the time of writing, the genus accommodates 18 recognized species which were isolated from different habitats. In this communication, we propose a novel species of the genus *Rhodovulum* to accommodate a bacterial strain (JA924^T) isolated from a brackish water body, located near Somnath in Gujrat on the western coast of India (20° 88′ E 70° 41′ N).

The culture was originally enriched in a medium described previously (Shivali *et al.*, 2011; but without supplementing with sodium bicarbonate), and incubated at 2400 lx and 30 °C for 7 days in 45 ml fully filled screw-capped bottles. Purification was by repeated streaking on agar slants (Lakshmi *et al.*, 2011). Purified cultures were grown in completely filled screw-capped test tubes (10 × 100 mm) under phototrophic conditions as described above. Cultures were maintained on agar slants or as lyophilized cultures preserved at 4 °C.

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA924^T is LN866627.

One supplementary table and seven supplementary figures are available with the online Supplementary Material.

Well-isolated colonies were used for 16S rRNA gene amplification by using PCR master mix (GeNei) as described previously (Subhash *et al.*, 2013a). 16S rRNA gene sequencing was performed on a 3130xl Applied Biosystems ABI prism automated DNA sequencer as described previously (Subhash *et al.*, 2013b). Levels of 16S rRNA gene sequence similarity were determined by BLAST search analysis on the EzTaxon-e server (Kim *et al.*, 2012). The EzTaxon BLAST search analysis indicated that the 16S rRNA gene sequence of strain JA924^T (1388 nt) shared highest similarity with that of *Rhodovulum mangrovi* AK41^T (98.19 %) and other members of the genus *Rhodovulum* (<95.71 %).

The CLUSTAL W algorithm within the MEGA 6 (Tamura *et al.*, 2013) package was used for phylogenetic analyses. Distances were calculated by using the Kimura two-parameter (Kimura, 1980) method in a pairwise deletion procedure. The neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) methods in the MEGA 6 software were used to reconstruct phylogenetic trees. Phylogenetic analysis [combined phylogenetic

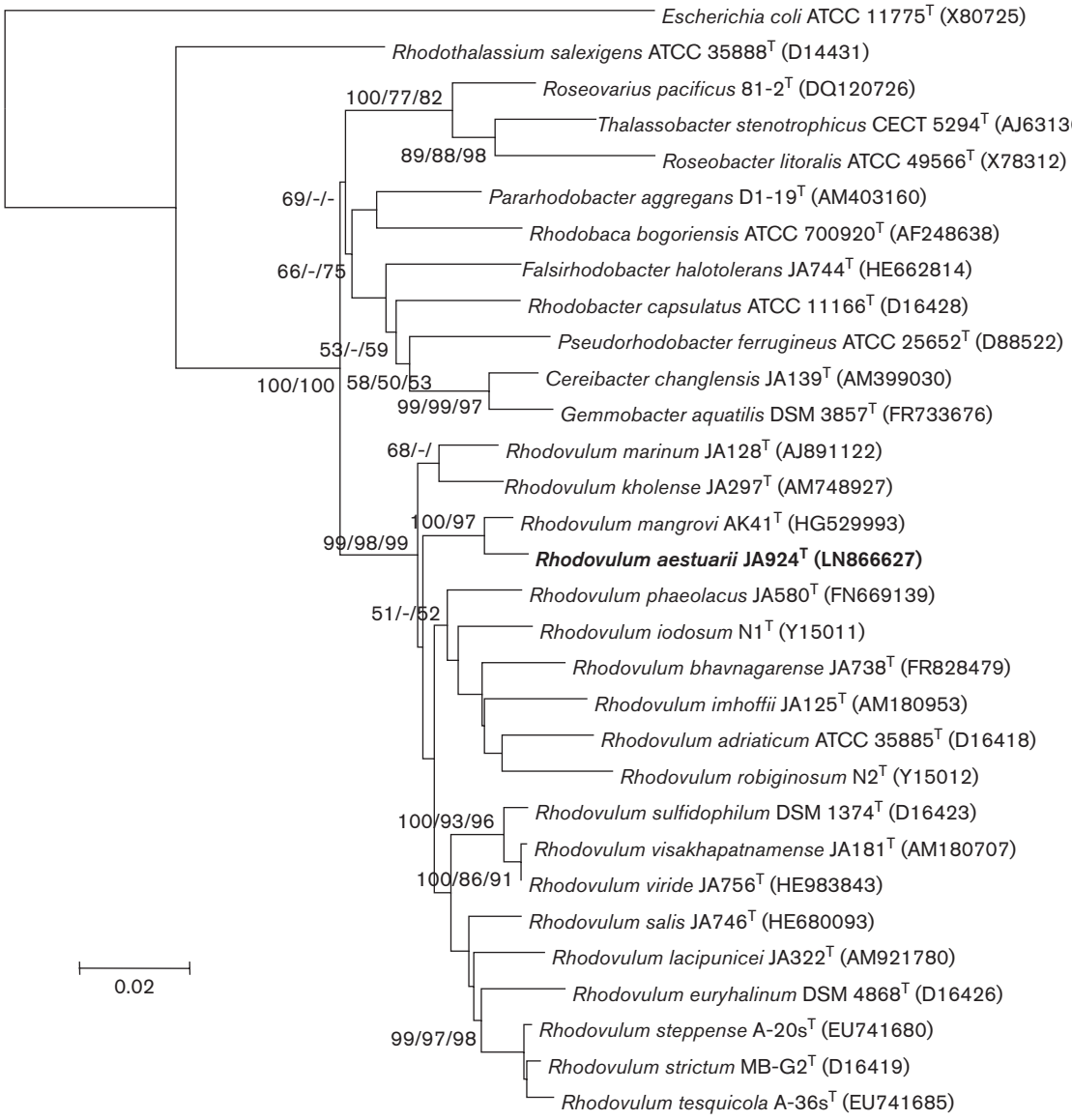

Fig. 1. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship of strain JA924^T with the phototrophic and chemotrophic members of the family *Rhodobacteraceae*. The tree was reconstructed by the NJ method using the MEGA 6 software and rooted by using *Escherichia coli* ATCC 11775^T as the outgroup. Numbers at nodes represent bootstrap values (based on 1000 resamplings). Bootstrap percentages refer to NJ/ML/MP analysis. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 2 nt substitutions per 100 nt.

tree (NJ, ML, MP; Fig. 1)] of strain JA924^T and other members of the genus *Rhodovulum* as examined based on nearly complete (1388 nt) 16S rRNA gene sequences revealed that the new isolate and *Rhodovulum mangrovi* AK41^T comprised a separate subclade that is positioned distinctly outside the clades formed by the next most closely related species of the genus *Rhodovulum*, and the levels of sequence similarity with the nearest phylogenetic members were in agreement with the EzTaxon-e server result.

Further characteristics of strain JA924^T were studied in detail as per recommended minimal standards (Imhoff & Caumette, 2004) together with the closely related *Rhodovulum mangrovi* JCM 19220^T (=AK41^T). Genomic DNA of strain JA924^T and *Rhodovulum mangrovi* JCM 19220^T was extracted and purified according to the method of Marmur (1961) and stored at −20 °C until use. The isolated DNA (5 mg) was subjected to acid hydrolysis [0.1 ml perchloric acid (70 %); Wyatt (1953)] in glass sealed bottles at 100 °C in a water bath for 1 h to release bases (Norris & Ribbons, 1972). The DNA lysate was centrifuged, the supernatant was filtered with 0.22 μm cellulose nitrate filters and the G+C content was determined using HPLC (Shimadzu SPD-10A) with a reversed-phase C_{18} column [eluted with the solvent system used by Mesbah *et al.* (1989); at 37 °C; flow rate 1 ml ms^{-1}; absorption at 260 nm] using a UV detector. Nucleobases (A, T, G and C; obtained from HiMedia) dissolved in 0.1 M HCl to a concentration of 1 mM were used as standards and the G+C content was calculated (all experiments were performed in triplicate). Strain JA924^T and *Rhodovulum mangrovi* JCM 19220^T had DNA G+C contents of 64±0.08 and 68±0.06 mol%, respectively (Fig. S1, available in the online Supplementary Material, shows the nucleobases of strain JA924^T as compared with *Rhodovulum mangrovi* JCM 19220^T).

The taxonomic relationship between strain JA924^T and *Rhodovulum mangrovi* JCM 19220^T was examined using DNA–DNA hybridization, which was performed using a membrane filter technique (Tourova & Antonov, 1988), with a nick translation kit supplied by BRIT. Hybridization was performed with three replications for each sample (control: reversal of strains was used for binding and labelling) and the mean values are quoted as DNA–DNA relatedness. When strain JA924^T was radioactively labelled, the level of DNA–DNA reassociation with *Rhodovulum mangrovi* JCM 19220^T was 29±5 % (mean±SD SD of three hybridizations). However, when the type strain of *Rhodovulum mangrovi* was labelled and used for DNA–DNA hybridization with JA924^T in the reciprocal reaction, the reassociation value was 28±3 % (Fig. S2).

Although divergence between the thermal denaturation midpoint (ΔT_m) determinations have proven to be valuable in the taxonomy of a variety of bacterial groups (Rosselló-Mora & Amann, 2001), this parameter has not been frequently used in species delineation of anoxygenic phototrophs. However, the thermal denaturation temperature approach represents an alternative method to the binding–labelling strategy of determining the degree of DNA–DNA relatedness. Moreover, the results of the two analyses are known to be in agreement with each other (Rosselló-Mora & Amann, 2001). In the present study, the degree of genomic divergence/relatedness between strain JA924^T and the type strain of *Rhodovulum mangrovi* was additionally examined by the ΔT_m approach. The ΔT_m of homoduplex DNA (ssDNA of JA924^T hybridized with ssDNA of itself) and heteroduplex DNA (ssDNA of JA924^T hybridized with ssDNA of *Rhodovulum mangrovi* JCM 19220^T) was determined using a real-time PCR thermocycler based on fluorescence determinations (Gonzalez & Saiz-Jimenez, 2005). Prior to analysis, isolated DNA was renatured at the optimum temperature for renaturation (T_{or}), which was approximated according to the method of De Ley *et al.* (1970) using the equation $T_{or}=0.51$ (% G+C)+47.0. Renaturation conditions consisted of a denaturation step of 99 °C for 10 min followed by an annealing period of 8 h at T_{or} and by progressive 60 min steps, each at 10 °C below the previous one, until room temperature was reached. To the renatured DNA, SYBR Green I dye was added at a final concentration of 1 : 100 000. The thermal denaturation of the labelled mixture and measurement of fluorescence during denaturation was carried out using a real-time PCR machine (Eppendorf Mastercycler ep *realplex* system). The thermal profile consisted of a 15 min hold at 25 °C followed by a 25–99 °C ramp in 0.2 °C steps with a 12 s hold. The mean of three replicates was used to determine the melting temperature (T_m). Using the associated software, *realplex*, T_m values of homologous and hybrid DNA were calculated as the temperatures corresponding to a 50 % decrease in fluorescence. Percentage similarity was estimated based on the ΔT_m values (Gonzalez & Saiz-Jimenez, 2005). The comparison of melting curves generated between homologous and hybrid DNA indicated $\Delta T_m>5$ °C (=7.8 °C; Fig. S3), which correlated well with the DNA–DNA reassociation values obtained using the binding–radioactive labelling strategy (28±3 to 29±5 %). In conclusion, both the DNA–DNA hybridization and ΔT_m values are within generally accepted limits for species delineation (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), indicating that strain JA924^T and *Rhodovulum mangrovi* JCM 19220^T are members of different species.

Morphological properties (cell shape, cell division, cell size, motility) were observed under a phase-contrast microscope (Olympus BH-2). The internal membrane structures were viewed with a transmission electron microscope (Hitachi H-7500), after the cells had been processed as described by Hanada *et al.* (2002). Cells of strain JA924^T were oval to rod-shaped, measured about 2–3 μm in diameter (Fig. S4A), possessed fimbriae (Fig. S4B) and multiplied by binary fission. Cells of strain JA924^T formed chains/aggregates and were non-motile (Fig. S4C), while cells of *Rhodovulum mangrovi* JCM 19220^T were motile and always occurred singly.

Table 1. Differential characteristics between strain JA924^T and *Rhodovulum mangrovi* JCM 19220^T

All data are from the present study. Both strains are oval to rod-shaped, divide by binary fission, have vesicles as intracytoplasmic membrane structures, have Q-10 as the quinone system, possess carotenoid pigments, and have phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sulfolipid (SL), aminolipid (AL1) and unidentified lipids (L1, L2) as polar lipids. Organic substrate utilization was tested during photoheterotrophic growth. Both strains utilize thiosulfate, acetate, glucose, pyruvate and succinate, but not citrate, malate, benzoate, methanol, ethanol, glutamate or glycolate. +, Good growth; −, no growth.

Characteristic	JA924^T	*Rhodovulum mangrovi* JCM 19220^T
Cell diameter (μm)	2–3	0.5–1.0
Chain/aggregate formation	+	−
Motility	−	+
NaCl range (optimum) (%, w/v)	0–8 (2–4)	0–12 (1–9)
pH range (optimum)	6.0–10 (7.5)	5.0–9.0 (7.0–8.0)
Temperature range (optimum) (°C)	25–35 (30)	25–38 (28)
Sulfide tolerance (up to 4 mM)	+	−
Photolithoautotrophy	+	−
Vitamin requirements	Complex	None
Carbon/electron donors		
Sulfide	+	−
Propionate	−	+
Butyrate	−	+
Valerate	−	+
Fumerate	−	+
Fructose	+	−
Glycerol	−	+
Crotonate	−	+
Casamino acids	+	−
Sucrose	+	−
DNA G+C content (mol%)	64 ± 0.08	68 ± 0.06

Transmission electron micrographs of ultrathin sections of strain JA924^T revealed the presence of a vesicular architecture of internal membrane structures (Fig. S4D).

Utilization of organic carbon compounds such as formate, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol as carbon sources/electron donors was tested at 0.1 % (v/v) along with $NaHCO_3$ (0.1 %, w/v), while other substrates were tested at 0.3 % (w/v) without $NaHCO_3$. Growth was measured turbidometrically at 660 nm. Carbon sources that supported growth of strain JA924^T were acetate, Casamino acids, fructose, formate, glucose, gluconate, methionine, peptone, pyruvate, sorbitol, succinate and sucrose, while those that could not be utilized included ascorbate, aspartate, butanol, butyrate, caproate, glucose, tartrate, glutamate, maltose, thioglycolate, mannitol, ethanol, methanol, glycerol, glycolate, 2-oxoglutarate, malate, fumarate, aspartate, butyrate, lactate, propionate, valerate, crotonate, citrate, benzoate and propanol (Table 1).

For tests of the utilization of sulfur sources, $MgSO_4 . 7H_2O$ was replaced with $MgCl_2 . 6H_2O$ (0.2 %) and the respective sulfur sources [sodium sulfide, sodium thiosulfate, thioglycolate, cysteine, magnesium sulfate, sodium sulfate and sodium sulfite (all at 0.5 mM concentration)] were added to the medium. While sulfate, sulfide and thiosulfate were used as sulfur sources by strain JA924^T, elemental sulfur, cysteine, thioglycolate and methionine did not support growth. Nitrogen source requirements for growth were tested by replacing ammonium chloride with different nitrogen sources at 7 mM. Nitrogen sources metabolized by strain JA924^T included ammonium chloride, aspartate, nitrate, glutamine and molecular nitrogen. Growth was not observed with nitrite or glutamate. Vitamin requirements were tested by replacing yeast extract with single and combinations of vitamins as growth factors, which revealed that strain JA924^T had complex growth requirements.

Chemotrophy was determined by growing the cultures in Erlenmeyer flasks placed in an orbital shaker (in the dark) at 30 °C. Strain JA924^T was able to grow photo-organoheterotrophically [anaerobically, light (2400 lx) with pyruvate (0.03 %, w/v) as a carbon source/electron donor], photolithoautotrophically [anaerobically, light (2400 lx), $Na_2S . 9H_2O/Na_2S_2O_3 . 5H_2O$ (1 mM/5 mM) and $NaHCO_3$ (0.1 %, w/v)] and chemo-organoheterotrophically [aerobically (respiration)/anaerobically (fermentation), dark with pyruvate (0.3 %, w/v)]. Chemolithoautotrophy [aerobically, dark, $Na_2S_2O_3 . 5H_2O$ (5 mM) and $NaHCO_3$ (0.1 %, w/v)] and fermentative growth [anaerobically, dark with pyruvate/glucose (0.3 %, w/v) as fermentable substrates] could not be demonstrated for strain JA924^T. Although strain JA924^T

tolerated sulfide concentrations up to 4 mM, the type strain of *Rhodovulum mangrovi* could not grow even in the presence of 0.5 mM sulfide. Strain JA924^T was able to grow without NaCl supplementation in the medium, and also with up to 8 % (w/v) NaCl (optimum 2–4 %). The temperature profile of strain JA924^T showed that it was mesophilic, growing at 25–35 °C (optimum growth occurred at 30 °C). Strain tolerated pH values in the range 6–10 with an optimum pH of 7.5 (Table 1).

The colour of photosynthetically grown cell suspensions of strain JA924^T was yellowish brown and the *in vivo* absorption spectrum as measured with a Spectronic Genesys 2 spectrophotometer in sucrose solution (Trüper & Pfennig, 1981) exhibited maxima at 377, 479, 593, 755, 800 and 854 nm (Fig. S5), indicating the presence of bacteriochlorophyll-*a* and carotenoids. The carotenoid composition of strain JA924^T as determined by C_{18}-HPLC (eluted with methanol; flow rate 2 ml ms^{-1}; absorption at 481 nm) using a photodiode array detector indicated the presence of spheroidene (55 %), demethylspheroidene (7 %), spheroidenone (15 %), demethylspheroidenone (6 %), neurosporene (6 %), dihydroxyneurosporene (4 %) and hydroxylspheroidene (2 %; Fig. S6). Pigments were identified by comparing their absorption spectra with those stated in the lipid bank database (http://lipidbank.jp/) and also by comparing the retention times with those of *Rhodovulum mangrovi* JCM 19220^T.

For fatty acid analysis, cells were harvested by centrifugation (10 000 ***g*** for 15 min at 4 °C) on reaching a cell density of 70 % of the maximum optical density (100 %=OD_{660} of 0.9) and the pellet was used for analysis. Cellular fatty acids were methylated, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI 6.0 version; method, RTSBA6) (Sasser, 1990; www.midi-inc.com) which was outsourced to Royal Research Laboratories, Secunderabad, India. The major fatty acids (>10 %) of strain JA924^T were $C_{18:1}\omega7c$/$C_{18:1}\omega6c$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}\omega7c$ 11-methyl; minor amounts (<5 % but >1 %) of $C_{20:1}\omega7c$, $C_{10:0}$ 3-OH, $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I, $C_{19:0}$ cyclo $\omega8c$, $C_{18:1}\omega5c$ and $C_{16:1}\omega7c$/$C_{16:1}\omega6c$ (Table S1) were also found. Strain JA924^T differed from the type strain of *Rhodovulum mangrovi* in the presence of $C_{18:1}\omega5c$ and $C_{19:0}$ cyclo $\omega8c$ and absence of $C_{20:2}\omega6,9c$, $C_{18:1}\omega9c$ and iso-$C_{19:0}$.

Polar lipids were extracted from 1 g of 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 F_{254}; 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). The total lipid profile was visualized by spraying with 5 % ethanolic molybdophosphoric acid and was further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff reagent (quaternary nitrogen) or α-naphthol (specific for sugars) (Kates, 1972; Oren *et al.*, 1996) and cresyl violet (for sulfolipids; Soto *et al.*, 2000). Strain JA924^T had major proportions of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sulfolipid (SL), an aminolipid (AL1) and two unidentified lipids (L1, L2; Fig. S7). Strain JA924^T shared the presence of major and a few minor polar lipids with *Rhodovulum mangrovi* JCM 19220^T. Quinones were extracted with a chloroform/methanol (2 : 1, v/v) mixture, purified by TLC and analysed by HPLC (Imhoff, 1984; Hiraishi & Hoshino, 1984; Hiraishi *et al.*, 1984). Q-10 (>85 %) was observed as the major quinone in strain JA924^T and *Rhodovulum mangrovi* JCM 19220^T.

It is interesting to note that strain JA924^T was isolated from the north-west coast of India, while *Rhodovulum mangrovi* was from the north-east coast of India, two geographically separate locations. Apart from the genotypic distinctiveness observed in terms of DNA–DNA hybridization (well below 70 %) and ΔT_m (>5 °C) between strain JA924^T and its nearest phylogenetic neighbour, *Rhodovulum mangrovi* JCM 19220^T, there were several other phenotypic (motility, chain/aggregate formation, photolithoautotrophy, sulfide tolerance, NaCl tolerance, pH range for growth, organic substrate utilization, growth factor requirements) and chemotaxonomic (fatty acids; Table S1) differences that allow the clear placement of strain JA924^T to a novel species of the genus *Rhodovulum*, for which the name *Rhodovulum aestuarii* sp. nov. is proposed.

Description of *Rhodovulum aestuarii* sp. nov.

Rhodovulum aestuarii (aes.tu.a′ri.i. L. gen. n. *aestuarii* of an estuary, the environment from which the type strain was isolated).

Cells are oval to rod-shaped, 2–3 μm in diameter, capable of forming chains and aggregates, non-motile, possess fimbrae and divide by binary fission. Colour of photosynthetically grown cultures is yellowish brown. Bacteriochlorophyll-*a* and spheroidene are the photosynthetic pigments. Photo-organoheterotrophic, photolithoautotrophic and chemo-organoheterotrophic growth occurs and internal photosynthetic membranes are of the vesicular type. Chemolithoautotrophy and fermentative growth are absent. Tolerates sulfide concentrations up to 4 mM. Tolerates NaCl concentrations from 0 to 8 % (w/v) with optimum growth at 2–4 %. Temperature range for growth is 25–35 °C, with optimum growth at 30 °C; optimum pH is 7.5 (range pH 6–10). Assimilates sulfate, sulfide and thiosulfate. Ammonium chloride, aspartate, nitrate, glutamine and molecular nitrogen are used as nitrogen sources. Has complex growth requirements. Carbon source/electron donors for good growth are acetate, Casamino acids, fructose, formate, glucose, gluconate, methionine, peptone, pyruvate, sorbitol, succinate and sucrose. Does not utilize ascorbate, aspartate, butanol, butyrate, ethanol, methanol,

caproate, glucose, tartrate, glutamate, maltose, thioglycolate, mannitol, glycerol, glycolate, 2-oxoglutarate, malate, fumarate, aspartate, butyrate, lactate, propionate, benzoate, valerate, crotonate, citrate or propanol. Major cellular fatty acids are $C_{18:1}\omega7c/C_{18:1}\omega6c$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}\omega7c$ 11-methyl. Minor amounts of $C_{20:1}\omega7c$, $C_{10:0}$ 3-OH, $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I, $C_{19:0}$ cyclo $\omega8c$, $C_{18:1}\omega5c$ and $C_{16:1}\omega7c/C_{16:1}\omega6c$ are also present. Q-10 is the major quinone. The polar lipids consist of phosphatidylglycerol, phosphatidylethanolamine, sulfolipid, an aminolipid and two unidentified lipids (L1, L2).

The type strain, JA924^T (=LMG 29031^T=KCTC 15485^T), was isolated from brackish water, located near Somnath on the western coast of Gujarat, India. The DNA G+C content of the type strain is 64 ± 0.08 mol%.

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