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

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A yellowish brown, phototrophic, purple non-sulfur bacterium, strain JA924T, 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 JA924T had complex growth requirements. Strain JA924T was mesophilic and moderately halophilic. The DNA G+C content was 64 mol% (HPLC). The major cellular fatty acids were C18:1ω7c/C18:1ω6c, C16:0 and C18:0. The major quinone was ubiquinone-10 (Q-10). Phosphatidylglycerol, phosphatidylethanolamine, sulfolipid and an aminolipid were the main polar lipids of strain JA924T. EzTaxon-eBLAST searches based on the 16S rRNA gene sequence of JA924T revealed highest similarity with Rhodovulum mangrovi AK41T (98.19 %) and other members of the genus Rhodovulum (95.71 %). Strain JA924T was further identified to be distantly related to Rhodovulum mangrovi AK41T (29 % based on DNA–DNA hybridization and ΔTm (5°C)). Phenotypic, chemotaxonomic and molecular differences indicate that strain JA924T represents a novel species of the genus Rhodovulum, for which the name Rhodovulum aestuarii sp. nov. is proposed. The type strain is JA924T (=LMG 29031T =KCTC 15485T).

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 C18:1ω7c, C18:0 and C16:0. 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 (JA924T) isolated from a brackish water body, located near Somnath in Gujarat on the western coast of India (20° 8ʾ88ʾ E 70° 41ʾ 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.
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 JA924ᵀ (1388 nt) shared highest similarity with that of Rhodovulum mangrovi AK41ᵀ (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 JA924ᵀ 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 11775ᵀ 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.](image-url)
tree ([NJ, ML, MP; Fig. 1]) of strain JA924<sup>T</sup> and other members of the genus *Rhodovulum* as examined based on nearly complete (1588 nt) 16S rRNA gene sequences revealed that the new isolate and *Rhodovulum mangrovi* AK41<sup>T</sup> 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<sup>T</sup> were studied in detail as per recommended minimal standards (Imhoff & Caumette, 2004) together with the closely related *Rhodovulum mangrovi* JCM 19220<sup>T</sup> (=AK41<sup>T</sup>). Genomic DNA of strain JA924<sup>T</sup> and *Rhodovulum mangrovi* JCM 19220<sup>T</sup> 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 using HPLC (Shimadzu SPD-10A) with a reversed-phase lose nitrate filters and the G-C content was determined using HPLC (Shimadzu SPD-10A) with a reversed-phase G<sub>18</sub> column [eluted with the solvent system used by Mesbah et al. (1989); at 37 °C; flow rate 1 ml ms<sup>−1</sup>; 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<sup>T</sup> and *Rhodovulum mangrovi* JCM 19220<sup>T</sup> 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<sup>T</sup> as compared with *Rhodovulum mangrovi* JCM 19220<sup>T</sup>.

The taxonomic relationship between strain JA924<sup>T</sup> and *Rhodovulum mangrovi* JCM 19220<sup>T</sup> was examined using DNA–DNA hybridization, which was performed using a membrane filter technique (Tourouva & 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<sup>T</sup> was radioactively labelled, the level of DNA–DNA reassociation with *Rhodovulum mangrovi* JCM 19220<sup>T</sup> 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<sup>T</sup> in the reciprocal reaction, the reassociation value was 28 ± 3 % (Fig. S2).

Although divergence between the thermal denaturation midpoint (ΔT<sub>m</sub>) 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 photo-trophs. 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<sup>T</sup> and the type strain of *Rhodovulum mangrovi* was additionally examined by the ΔT<sub>m</sub> approach. The ΔT<sub>m</sub> of homoduplex DNA (ssDNA of JA924<sup>T</sup> hybridized with ssDNA of itself) and heteroduplex DNA (ssDNA of JA924<sup>T</sup> hybridized with ssDNA of *Rhodovulum mangrovi* JCM 19220<sup>T</sup>) 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<sub>or</sub>), which was approximated according to the method of De Ley et al. (1970) using the equation T<sub>or</sub>=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<sub>or</sub> 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<sub>m</sub>). Using the associated software, realplex, T<sub>m</sub> 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<sub>m</sub> values (Gonzalez & Saiz-Jimenez, 2005). The comparison of melting curves generated between homologous and hybrid DNA indicated ΔT<sub>m</sub>&gt;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<sub>m</sub> values are within generally accepted limits for species delineation (Wayne et al., 1987; Stackebrandt & Goebel, 1994), indicating that strain JA924<sup>T</sup> and *Rhodovulum mangrovi* JCM 19220<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> formed chains/aggregates and were non-motile (Fig. S4C), while cells of *Rhodovulum mangrovi* JCM 19220<sup>T</sup> were motile and always occurred singly.
Transmission electron micrographs of ultrathin sections of strain JA924\textsuperscript{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, lactate, glyceraldehyde, methanol and ethanol as carbon sources/electron donors was tested at 0.1 % (v/v) along with NaHCO\textsubscript{3} (0.1 %, w/v), while other substrates were tested at 0.3 % (w/v) without NaHCO\textsubscript{3}. Growth was measured turbidometrically at 660 nm. Carbon sources that supported growth of strain JA924\textsuperscript{T} were acetate, Casamino acids, fructose, formate, methanol, ethanol, glutamate or glycerate. +, Good growth; −, no growth.

For tests of the utilization of sulfur sources, MgSO\textsubscript{4} . 7H\textsubscript{2}O was replaced with MgCl\textsubscript{2} . 6H\textsubscript{2}O (0.2 %) and the respective sulfur sources [sodium sulfide, sodium thiosulfate, thiglucolate, 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\textsuperscript{T}, elemental sulfur, cysteine, thiglycolate 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{2}S . 9H\textsubscript{2}O/Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} . 5H\textsubscript{2}O (1 mM/5 mM) and NaHCO\textsubscript{3} (0.1 %, w/v)] and chemo-organoheterotrophically [anaerobically, dark, Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} . 5H\textsubscript{2}O (5 mM) and NaHCO\textsubscript{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\textsuperscript{T}. Although strain JA924\textsuperscript{T}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>JA924\textsuperscript{T}</th>
<th>Rhodovulum mangrovi JCM 19220\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter ((\mu\text{m}))</td>
<td>2–3</td>
<td>0.5–1.0</td>
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<td>Chain/aggregate formation</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Motility</td>
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<td>+</td>
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<td>NaCl range (optimum) (%, w/v)</td>
<td>0–8 (2–4)</td>
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<td>pH range (optimum)</td>
<td>6.0–10 (7.5)</td>
<td>5.0–9.0 (7.0–8.0)</td>
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<tr>
<td>Temperature range (optimum) (°C)</td>
<td>25–35 (30)</td>
<td>25–38 (28)</td>
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<tr>
<td>Sulfide tolerance (up to 4 mM)</td>
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<td>−</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>64 ± 0.08</td>
<td>68 ± 0.06</td>
</tr>
</tbody>
</table>

Table 1. Differential characteristics between strain JA924\textsuperscript{T} and Rhodovulum mangrovi JCM 19220\textsuperscript{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), sulfoquinovosyl diacylglycerol (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.
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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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-<i>a</i> and carotenoids. The carotenoid composition of strain JA924<sup>T</sup> as determined by C<sub>18</sub>-HPLC (eluted with methanol; flow rate 2 ml ms<sup>−1</sup>; 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<sup>T</sup>.

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<sub>660</sub> 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, RTSSA6) (Sasser, 1990; www.midi-inc.com) which was outsourced to Royal Research Laboratories, Secunderabad, India. The major fatty acids (>10 %) of strain JA924<sup>T</sup> were C<sub>16</sub> : 1ω7c/C<sub>18</sub> : 1ω6c, C<sub>16</sub> : 0 and C<sub>18</sub> : 1ω7c 11-methyl; minor amounts (<5 % but >1 %) of C<sub>20</sub> : 1ω7c, C<sub>10</sub> : 0 3-OH, C<sub>14</sub> : 0 3-OH/iso-C<sub>16</sub> : 1 L, C<sub>19</sub> : 0 cyclo ω8c, C<sub>18</sub> : 1ω5c and C<sub>16</sub> : 1ω7c/C<sub>16</sub> : 1ω6c (Table S1) were also found. Strain JA924<sup>T</sup> differed from the type strain of *Rhodovulum mangrovi* in the presence of ω5c and ω8c and absence of C<sub>20</sub> : 1ω6,9c, C<sub>16</sub> : 1ω9c and iso-C<sub>19</sub> : 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<sub>254</sub>; 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 % ethanol molybdophosphoric acid and was further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff reagent (quaternary nitrogen) or z-naphthol (specific for sugars) (Kates, 1972; Oren et al., 1996) and creosyl violet (for sulfolipids; Soto et al., 2000). Strain JA924<sup>T</sup> had major proportions of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sulfolipid (SL), an aminolipid (AL1) and two unidentified lipids (L1, L2; Fig. S7). Strain JA924<sup>T</sup> shared the presence of major and a few minor polar lipids with *Rhodovulum mangrovi* JCM 19220<sup>T</sup>. 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<sup>T</sup> and *Rhodovulum mangrovi* JCM 19220<sup>T</sup>.

It is interesting to note that strain JA924<sup>T</sup> 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<sub>m</sub> (>5 °C) between strain JA924<sup>T</sup> and its nearest phylogenetic neighbour, *Rhodovulum mangrovi* JCM 19220<sup>T</sup>, there were several other phenotypic (motility, chain/aggregate formation, photolithoauototrophy, 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<sup>T</sup> 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.ari.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 fimbriae and divide by binary fission. Colour of photosynthetically grown cultures is yellowish brown. Bacteriochlorophyll-<i>a</i> 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, mallow, thiglycolate, 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}\_\text{cis-9}, C_{16:0}, C_{18:0} and C_{18:1}\_\text{cis-11}-methyl. Minor amounts of C_{20:0}, C_{10:0}, 3-OH, C_{14:0} 3-OH/iso-C_{16:1} \_\text{I}, C_{19:0} cyclo C_{8:0}, C_{18:2}\_\text{cis-5c} and C_{16:0} 17\_\text{cis}-\text{cis-10c} 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 (\text{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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