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Rhodovulum marinum sp. nov., a novel phototrophic purple non-sulfur alphaproteobacterium from marine tides of Visakhapatnam, India

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A yellowish-brown bacterium was isolated from enrichment cultures inoculated with seawater samples from the eastern coast of India (Visakhapatnam) under photoheterotrophic conditions. Enrichment and isolation in a medium containing 2 % NaCl (w/v) yielded strain JA128^T, which has ovoid to rod-shaped cells, also forms chains and is non-motile. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA128^T clusters with the *Alphaproteobacteria* and the sequence similarity with its closest relatives, *Rhodovulum iodosum* and *Rhodovulum sulfidophilum*, was 95 %. Strain JA128^T contained vesicular intracytoplasmic membranes, bacteriochlorophyll *a* and carotenoids of the spheroidene series. Strain JA128^T was mesophilic, slightly acidophilic, slightly halophilic and grew photoheterotrophically with a number of organic compounds as carbon source and electron donor. It was unable to grow photoautotrophically, chemoautotrophically or by fermentative modes. It did not utilize sulfide, thiosulfate or hydrogen as electron donors. Thiamine was required as a growth factor. Based on the 16S rRNA gene sequence analysis, morphological and physiological characteristics, strain JA128^T was significantly different from other species of the genus *Rhodovulum* and was recognized as a novel species for which the name *Rhodovulum marinum* sp. nov. is proposed. The type strain is JA128^T (=ATCC BAA 1215^T=CCUG 52183^T=JCM 13300^T).

Marine habitats represent excellent niches for anoxygenic phototrophic bacteria, which are widely distributed in numerous coastal marine habitats. Most common anoxygenic phototrophic bacteria have been isolated from estuarine salt pans, salt marshes (Imhoff *et al.*, 1998a, b; Imhoff & Pfennig, 2001), coastal lagoons with elevated salt concentrations (Imhoff *et al.*, 1998a, b), tidal waters, brackish waters (Pfennig & Trüper, 1989; Imhoff, 1988) and marine coastal sediments (Imhoff, 1983). They have even been found in the extreme marine habitats of Antarctica (Madigan *et al.*, 2000; Karr *et al.*, 2003).

Previously, the taxonomy of photosynthetic bacteria was based exclusively on phenotypical characteristics (Pfennig & Trüper, 1974, 1989). A 16S rRNA gene sequence comparison of these bacteria revealed phylogenetic differences among freshwater, true marine and halophilic bacteria (Imhoff *et al.*, 1998a). On the basis of these results, a number of taxa have been reclassified. The marine representatives of the genus *Chromatium* were transferred to the genera *Allochromatium*, *Marichromatium*, *Isochromatium* and *Halochromatium* (Imhoff *et al.*, 1998a), while the marine *Rhodobacter* species were reclassified as species of the genus *Rhodovulum* (Hiraishi & Ueda, 1994) and those of *Rhodospirillum* to *Rhodothalassium* and *Roseospira* (Imhoff *et al.*, 1998b).

The marine anoxygenic phototrophic *Gammaproteobacteria* are distributed among the families *Chromatiaceae* and *Ectothiorhodospiriaceae*. In the *Chromatiaceae*, the genera *Halochromatium*, *Thiohalocapsa*, *Thiococcus*, *Rhabdochromatium*, *Isochromatium*, *Thiorhodococcus*, *Marichromatium*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA128^T is AJ891122.

and *Thiorhodovibrio* (Imhoff *et al.*, 1998a) grow in NaCl concentrations from 1 to 11 %. The *Ectothiorhodospiraceae* genera include *Halorhodospira*, *Thiorhodospira* and *Ectothiorhodospira*, growing in NaCl concentrations from 1 to 32 % (Imhoff, 2001). Marine representatives of phototrophic *Alphaproteobacteria* that can grow in 1–12 % NaCl are found in the genera *Rhodospira*, *Roseospirillum*, *Roseospira*, *Rhodobium*, *Rhodovulum*, *Rhodobaca*, *Rhodothalassium* and *Rhodovibrio* (Imhoff, 2001). No marine representatives of anoxygenic phototrophic *Betaproteobacteria* have been reported so far. Apart from phylogenetic differences based on 16S rRNA gene sequences of marine and freshwater species of anoxygenic phototrophic bacteria, good correlations were also observed in the major quinone and fatty acid compositions, which were in accordance with the requirement of NaCl or sea salt for growth of some of these bacteria (Imhoff *et al.*, 1998b).

We have isolated several strains of marine purple bacteria from coastal areas of Andhra Pradesh, India. Strain JA128^T, isolated from photoheterotrophic enrichments of seawater from a tidal beach, had 95 % 16S rRNA gene sequence homology with the type strains of *Rhodovulum iodosum* and *Rhodovulum sulfidophilum*, and 93 % with *Rhodovulum robiginosum*. This isolate, based on phenotypic characteristics and significant differences in the 16S rRNA gene sequence, is described here as a novel species, *Rhodovulum marinum* sp. nov.

Tidal water samples from a beach at Visakhapatnam on the east coast of India (Bay of Bengal) were collected in polypropylene bottles during March 2004. The medium of Pfennig & Trüper (1992) supplemented with NaCl (2 % w/v), pyruvate (0·3 % w/v) as carbon source and ammonium chloride (0·12 %) as nitrogen source was used for photoheterotrophic growth under light (2400 lux) at 30 ± 2 °C. Purification was done by using the repeated agar shake dilution method (Pfennig & Trüper, 1992; Imhoff, 1988). Purified cultures were grown in completely filled screw cap test tubes (10×100 mm) for photoheterotrophic growth.

Morphological properties (cell shape, cell division, cell size, flagella) were observed by phase-contrast light microscopy (Olympus BH-2), ultrastructure of flagella was studied after staining with 1 % phosphotungstic acid and ultrathin sections for intracytoplasmic structures, such as the internal membrane system, were viewed through a transmission electron microscope (H-7500; Hitachi). *In vivo* absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer in sucrose solution (Trüper & Pfennig, 1981). Absorption spectra were also recorded from pigments extracted with acetone after eluting the cell suspension with acetone through a 10×200 mm column packed with aluminium oxide. Utilization of other inorganic and organic compounds as electron donors for phototropic growth was tested without any additional electron donor in the presence of yeast extract (0·03 %, w/v). Formic acid, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol were used at a concentration of 0·1 % (v/v); other compounds tested were used at 0·3 % (w/v), with benzoate (1 mM) and $NaHCO_3$ (0·1 %). For testing sulfur sources, $MgSO_4.7H_2O$ was replaced by $MgCl_2.5H_2O$ (0·01 %); sulfur sources ($Na_2S.9H_2O$, $Na_2S_2O_3$, sodium thioglycolate, cysteine, $MgSO_4.7H_2O$, all at 0·5 mM, and $FeSO_4$, 10 mM) were added to the medium in addition to $NaHCO_3$ (0·1 %). Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources at 0·12 % (w/v). Vitamin requirements were tested by replacing yeast extract with single and also combinations of vitamins as growth factors. Chemotrophy was determined by growing the cultures in Erlenmeyer flasks placed in an orbital shaker in the dark at 30 °C. Diazotrophy of the cultures was determined by growth under an N_2 atmosphere and was confirmed by repeated subculturing (four times). Growth was measured turbidometrically at 660 nm.

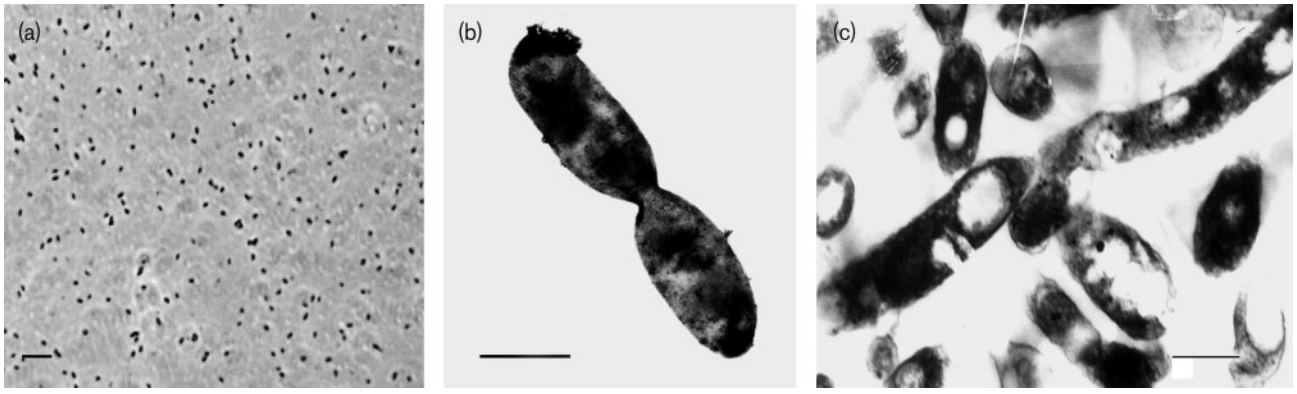

Fig. 1. (a) Phase-contrast micrograph of strain JA128^T. Bar, 5 μm. (b) Electron micrograph of negatively stained cells of strain JA128^T showing binary fission division. Bar, 1 μm. (c) Electron micrograph of ultrathin section of strain JA128^T showing the vesicular nature of the photosynthetic membranes extending throughout the cell. Bar, 0·5 μm.

Table 1. Differentiating characteristics of species of the genus *Rhodovulum*

Symbols: +, substrate utilized or present; (+), some strains utilizing the substrate and some not; −, substrate not utilized or absent; NT, not tested. References: a, Kompantseva (1985), Imhoff & Trüper (1992); b, Hiraishi & Ueda, (1995); c, Straub *et al.* (1999); d, Hansen & Veldkamp, (1973), Imhoff & Trüper (1992), Heising *et al.* (1996); e, Neutzling *et al.* (1984).

Characteristics	JA128^{T}*	*Rhodovulum euryhalinum*[a]	*Rhodovulum strictum*[b]	*Rhodovulum iodosum*[c]	*Rhodovulum robiginosum*[c]	*Rhodovulum sulfidophilum*[d]	*Rhodovulum adriaticum*[e]
Cell diameter (μm)	0·6–0·8	0·7–1·0	0·6–1·0	0·5–0·8	0·5–0·8	0·6–1·0	0·5–0·8
Shape	Ovoid to rods, chains	Ovoid to rods	Ovoid to rods	Ovoid to rods	Ovoid to rods	Ovoid to rods	Rods, chains
Motility	Non-motile	Motile, polar flagella	Motile, polar flagella	Non-motile	Non-motile	Motile, polar flagella	Non-motile
NaCl range (%)	0·05–8	0·5–10	0·25–3	2·5–5	2·5–5	0–10	1–10
pH range	5·5–7·5	6·0–8·5	7·5–9·0			5·0–9·0	6·0–8·5
Optima	6·0–6·8			6·5	6·5		
Chemo-organotrophy	+	−	+	+	+	+	−
Temperature range (optimum) (°C)	25–35 (30)	−	30–35	20–25	25–28	(25)	25–30
Colour of cell suspension	Yellow–brown	Yellow–brown	Yellow–brown	Yellow–brown	Yellow–brown	Brown	Brown
Vitamin requirement†	t	b, n, paba, t	b, paba, t	b, n	b, B12, n	b, n, paba, t	b, t
G+C (mol%)	62	62·1–68·6	67·3–67·7	66	69	66·3–66·6	64·9–66·7
Carbon/e^{-} donors							
Inorganic							
Hydrogen	−	NT	NT	+	+	+	−
Sulfide	−	+	+	+	+	+	+
Thiosulfate	−	+	+	+	+	+	+
Sulfur	−	NT	NT	+	+	+	NT
Ferrous iron	−	−	NT	+	+	+	−
Organic							
Formate	−	+	+	−	−	+	+
Acetate	+	+	+	+	+	+	+
Propionate	−	+	+	+	+	+	+
Butyrate	−	+	+	+	−	+	−
Valerate	−	+	+	+	−	+	−
Caproate	−	NT	+	−	−	+	+
Methanol	−	−	−	−	−	−	−
Ethanol	+	+	−	−	−	−	+
Pyruvate	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+
Succinate	+	+	+	+	+	+	+
Fumarate	+	+	+	+	−	+	+
Malate	−	+	+	+	+	+	+
Citrate	−	−	(+)	−	−	−	−
Benzoate	−	−	−	−	−	−	−
Aspartate	−	+	−	−	−	−	NT
Cysteine	−	NT	NT	+	−	NT	NT
Glutamate	−	+	−	+	+	+	NT
Glucose	+	+	+	−	−	+	+
Fructose	+	+	+	−	−	−	−
Glycerol	+	+	−	−	+	+	+
Mannitol	+	+	−	+	+	−	−

*Organic substrate utilization was tested during photoheterotrophic growth.

†b, Biotin; n, niacin; paba, *p*-aminobenzoate; t, thiamine.

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G+C content of the DNA was determined by thermal denaturation (Marmur & Doty, 1962). Cell material for 16S rRNA gene sequencing was taken from 1–2 ml of well grown liquid cultures. DNA was extracted and purified by using the Qiagen genomic DNA buffer set. PCR amplification and 16S rRNA gene sequencing was done as described previously (Imhoff *et al.*, 1998a; Imhoff & Pfennig, 2001). Sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) and the alignment was corrected manually. The distance matrix was calculated on the basis of the algorithm according to Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1989). The FITCH program in the PHYLIP package was used to fit a tree to the evolutionary distances.

Samples were collected during March 2004 from Ramakrishna beach, Visakhapatnam, Bay of Bengal, on the east coast of India (17·7° longitude, 83·32° latitude). The sample yielding strain JA128^T had a pH of 6·8 and a salinity of 2–3 % NaCl, and the temperature was 30 °C. The sample was inoculated into sterile screw cap tubes filled with medium for photoheterotrophic growth and incubated in the light for 1 week with intermittent shaking. Yellowish-brown enrichments were obtained and subcultured into the same medium. This culture was used for subsequent agar shake dilution series for purification. After 4 days incubation, small, convex, yellowish-brown colonies were observed. The colony and cell morphology were the same in all dilution series. The culture was maintained by repeated subculturing in the same medium and also in stabs. The stabs were preserved in a refrigerator at 4 °C. Individual cells of strain JA128^T were oval to rod-shaped (Fig. 1a), 0·6–0·8 μm wide and 1–2 μm long. The cells were non-motile and multiplied by binary fission (Fig. 1b). Electron microphotographs of ultrathin sections of the cells revealed vesicular internal membranes (Fig. 1c).

Strain JA128^T can grow photo-organoheterotrophically (optimum light intensity is 2000–4000 lux) and chemo-organoheterotrophically (aerobically in the dark). It tolerates the atmospheric level of oxygen. Photolithoautotrophic growth [anaerobic, light (2400 lux), H_2 (20 %, v/v)/Na_2S, $Na_2S_2O_3$ (0·5 mM), $NaHCO_3$ (0·1 %, w/v)], chemolithoautotrophic growth [aerobic, dark, thiosulfate (0·5 mM), $NaHCO_3$ (0·1 %, w/v)] and fermentative growth (anaerobic, dark with glucose/fructose (0·3 %, w/v)] could not be demonstrated. The substrates which were utilized (Table 1) as carbon/electron donor under photo-organoheterotrophic conditions include acetate, pyruvate, lactate, succinate, fumarate, oxaloacetate, tartrate, glucose, fructose, mannitol, sorbitol, glycerol, ethanol, yeast extract and Casamino acids. Those which could not be utilized include formate, propionate, butyrate, valerate, caproate, oleic acid, citrate, α-ketoglutarate, malate, sucrose, lactose, maltose, methanol, glutamate and benzoate. Thiosulfate, sodium sulfide and H_2 (with 0·1 % $NaHCO_3$) were not utilized as electron donors under photolithoautotrophic conditions. Ammonium chloride, molecular nitrogen, nitrate, glutamate and glutamine were utilized as nitrogen sources, while urea and nitrite did not support growth. Salt (NaCl) was obligatory for growth, and growth occurred from 0·05 to 8 % (w/v), the optimum NaCl concentration being 1–3 % (w/v). This is similar to the observed growth of another marine isolate from India, *Marichromatium indicum* JA100^T (Arunasri *et al.*, 2005), which also grows in a range of 0·05–8 % NaCl, reflecting adaptation to a natural habitat in which large variations in the salt concentration during various seasons occurs. The pH range for growth of strain JA128^T was 5·5–7·5 with the optimum at 6·0–6·8. The temperature range was from 25 to 35 °C and the optimum was at 30 °C. Thiamine was required as growth factor. The colour of photosynthetically grown cell suspensions was yellowish-brown to beige. The cell absorption spectrum (Fig. 2a) of strain JA128^T gave maxima at 378, 402, 488, 520, 590, 802, 884 nm, confirming the presence of bacteriochlorophyll *a* and most probably the carotenoids spheroidene and spheroidenone (Fig. 2b).

The DNA base composition of strain JA128^T was 62 mol% G+C (T_m). The phylogenetic relationship of strain JA128^T to other purple non-sulfur bacteria was examined by 16S rRNA gene sequencing. The data obtained revealed that the sequence of the new isolate formed a separate branch in the cluster of the genus *Rhodovulum* (Fig. 3), but was distinct from other genera of purple non-sulfur bacteria. The highest sequence similarities to strain JA128^T were

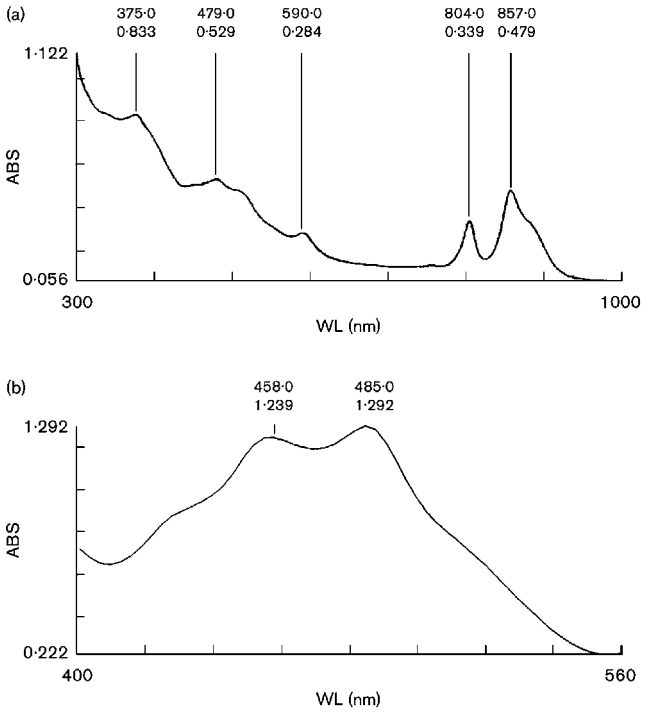

Fig. 2. Whole-cell absorption spectrum (a) of strain JA128^T and acetone spectrum (b) of extracted pigments.

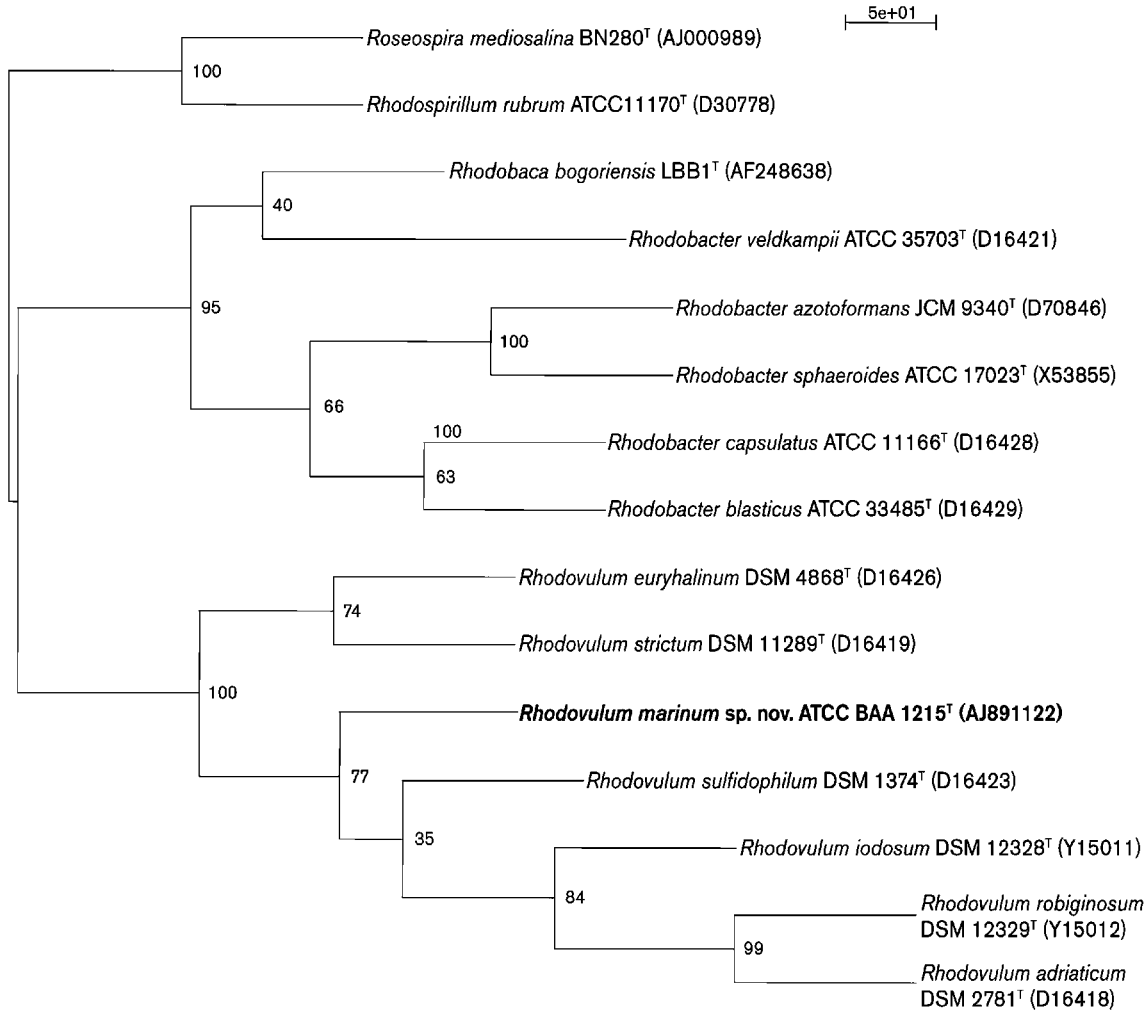

Fig. 3. Dendrogram depicting the phylogenetic relationships of strain JA128 within the family *Rhodobacteriaceae* determined using 16S rRNA gene sequence analysis. Bar, 1 nt substitution per 100 nt.

found with the type strains of *Rhodovulum iodosum*, *Rhodovulum sulfidophilum* (95 %) and *Rhodovulum robiginosum* (93 %). Apart from 16S rRNA gene sequence dissimilarity, strain JA128^T showed phenotypic differences to other *Rhodovulum* species (Table 1) that justify the description of this strain as a novel species.

Description of *Rhodovulum marinum* sp. nov.

Rhodovulum marinum (ma.ri′num. L. neut. adj. *marinum* of the sea, marine).

Cells are ovoid to rod-shaped, 0·6–0·8 μm wide, 1·0–2·0 μm long and form chains. Non-motile and division by binary fission. Gram-negative. Growth occurs under anaerobic conditions in the light (photo-organoheterotrophy) or under aerobic conditions in the dark (chemo-organoheterotrophy). Internal photosynthetic membranes are vesicular. The colour of phototrophic cultures is yellow–green to brown, while aerobic cultures are pink. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 375, 479, 590, 804 and 857 nm. Photosynthetic pigments are bacteriochlorophyll *a* and most probably carotenoids of the spheroidene series. The type strain is mesophilic (range 25–35 °C, optimum 30 °C), slightly acidophilic (pH range 5·5–7·5, optimum 6·0–6·8) and slightly halophilic with NaCl concentrations of 0·5 to 3·0 % required for optimal growth. Photo-organotrophy with various organic compounds is the preferred mode of growth. Good carbon sources are sorbitol, mannitol, pyruvate, lactate, intermediates of the citric acid cycle and some sugars. Growth on acetate, glycerol and ethanol also occurs. Photoautotrophic and chemoautotrophic growth is not possible in the presence of sulfide, thiosulfate or hydrogen as electron donor and $NaHCO_3$ as carbon source. Thiamine is required as growth factor. DNA G+C base composition: 62 mol% (T_m). Natural habitats are marine surface and tidal waters exposed to light. The EMBL accession number for the 16S rRNA gene sequence of strain JA128^T is

AJ891122. The type strain, JA128^T, has been deposited as ATCC BAA 1215^T = JCM 13300^T = CCUG 52183^T.

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