

Isolation and characterization of spirilloid purple phototrophic bacteria forming red layers in microbial mats of Mediterranean salterns: description of *Halorhodospira neutriphila* sp. nov. and emendation of the genus *Halorhodospira*

Agnès Hirschler-Réa,¹ Robert Matheron,¹ Christine Riffaud,¹ Sophie Mouné,^{2,4} Claire Eatock,³ Rodney A. Herbert,³ John C. Willison² and Pierre Caumette⁴

Correspondence
Pierre Caumette
pierre.caumette@univ-pau.fr

¹Laboratoire de Microbiologie, IMEP, Faculté des Sciences et Techniques de Saint Jérôme, 13397 Marseille cedex 20, France

²Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, DBMS/BBSI, CEA Grenoble, 38054 Grenoble, France

³Division of Environmental and Applied Biology, Biological Sciences Institute, University of Dundee, Dundee DD1 4HN, UK

⁴Laboratoire d'Ecologie Moléculaire-Microbiologie, IBEAS, BP 1155, Université de Pau, F 64013 Pau cedex, France

Microbial mats developing in the hypersaline lagoons of a commercial saltern in the Salin-de-Giraud (Rhône delta) were found to contain a red layer fully dominated by spirilloid phototrophic purple bacteria underlying a cyanobacterial layer. From this layer four strains of spirilloid purple bacteria were isolated, all of which were extremely halophilic. All strains were isolated by using the same medium under halophilic photolithoheterotrophic conditions. One of them, strain SG 3105 was a purple non-sulfur bacterial strain closely related to *Rhodovibrio sodomensis* with a 16S rDNA sequence similarity of 98.8%. The three other isolated strains, SG 3301^T, SG 3302 and SG 3304, were purple sulfur bacteria and were found to be very similar. The cells were motile by a polar tuft of flagella. Photosynthetic intracytoplasmic membranes of the lamellar stack type contained BChl *a* and spirilloxanthin as the major carotenoid. Phototrophic growth with sulfide as electron donor was poor; globules of elemental sulfur were present outside the cells. In the presence of sulfide and CO₂ good growth occurred with organic substrates. Optimum growth occurred in the presence of 9–12% (w/v) NaCl at neutral pH (optimal pH 6.8–7) and at 30–35 °C. The DNA base composition of strains SG 3301^T and SG 3304 were 74.5 and 74.1 mol% G + C, respectively. According to the 16S rDNA sequences, strains SG 3301^T and SG 3304 belonged to the genus *Halorhodospira*, but they were sufficiently separated morphologically, physiologically and genetically from other recognized *Halorhodospira* species to be described as a new species of the genus. They are, therefore, described as *Halorhodospira neutriphila* sp. nov. with strain SG 3301^T as the type strain (=DSM 15116^T).

INTRODUCTION

Spiral cell morphology is widely distributed among several genera of both sulfur and non-sulfur phototrophic purple bacteria. Among the purple non-sulfur bacteria, eight genera composed of spiral-shaped bacteria have been

described: *Rhodospirillum*, *Phaeospirillum*, *Rhodovibrio*, *Rhodothalassium*, *Roseospira* (Imhoff *et al.*, 1998), *Rhodocista* (Kawasaki *et al.*, 1992; Favinger *et al.*, 1989), *Rhodospira* (Pfennig *et al.*, 1997) and *Roseospirillum* (Glaeser & Overmann, 1999). Among the purple sulfur bacteria, spiral-shaped bacteria are present in the *Chromatiaceae* (*Thiospirillum*) and the *Ectothiorhodospiraceae* as exemplified by the genera *Ectothiorhodospira*, *Halorhodospira* and *Thiorhodospira* (Imhoff & Süling, 1996; Bryantseva *et al.*, 1999).

The EMBL accession numbers for the 16S rDNA sequences of *Halorhodospira neutriphila* strains SG 3301^T and SG 3304 are AJ318525 and AJ318526, respectively.

The majority of these bacteria require salt for optimal growth except for members of the genera *Rhodospirillum*, *Phaeospirillum*, *Rhodocista* and *Thiospirillum*. The salt-requiring bacteria have been isolated from coastal marine environments or inland salt lakes. Several have been isolated from hypersaline environments and described as moderately halophilic bacteria belonging to the genera *Rhodovibrio*, *Rhodothalassium*, *Roseospira* and *Ectothiorhodospira*. Only a few strains have been described as extreme halophiles and have been included in a single genus, *Halorhodospira*. Among all these spiral-shaped phototrophic moderate or extreme halophilic bacteria, a number have been isolated from sediment layers or deposits in hypersaline ponds or salt flats in evaporitic coastal environments. Within the purple non-sulfur bacteria, *Rhodospirillum salexigens* (Drews, 1981), later renamed *Rhodothalassium salexigens* (Imhoff *et al.*, 1998), was isolated from hypersaline evaporated pools on the Oregon coast, USA, and *Rhodospirillum salinarum* (Nissen & Dundas, 1984), later renamed *Rhodovibrio salinarum* (Imhoff *et al.*, 1998), was isolated from a Portuguese saltern. Similarly, among the purple sulfur bacteria *Ectothiorhodospira mobilis* (Trüper, 1968) was isolated from evaporitic salt flats in the Galapagos Islands. Other moderately to extremely halophilic purple sulfur or non-sulfur bacteria have been isolated from inland salt lakes or the Dead Sea.

During our investigation of microbial mats that develop in some lagoons within commercial salterns in the Rhône Delta (France), we observed a purple layer composed mainly of spiral-shaped bacteria below a thin gypsum crust. A number of strains were isolated and characterized. According to phenotypic and phylogenetic features, strain SG 3105 was related to *Rhodovibrio sodomensis* which was originally isolated by Mack *et al.* (1993) from the Dead Sea. The other isolated strains, SG 3301^T, SG 3302 and SG 3304, were also characterized. According to physiological and phylogenetic features these strains are closely related to the genus *Halorhodospira* that contains extreme halophilic species isolated from inland salt lakes or the Dead Sea (Imhoff *et al.*, 1998). However, none of the strains could be related to any currently described species of this genus. They are described in this paper together with *Rhodovibrio sodomensis* strain SG 3105 which was isolated from the same layer using the same growth medium. Strains SG 3301^T, SG 3302 and SG 3304 represent a new species within the genus *Halorhodospira* for which we propose the name *Halorhodospira neutriphilila* sp. nov. Strain SG 3301^T (= DSM 15116^T) is the type strain of the species.

METHODS

Source of strains. All the spirilloid strains (SG 3301^T, SG 3302, SG 3304 and SG 3105) were isolated from the same red layer occurring immediately below a green layer of cyanobacteria in a laminated microbial mat in the hypersaline lagoons (Etang de Faraman) of the Salin-de-Giraud, a commercial saltern in the Camargue (Rhône Delta, France). This laminated mat was found below a thin gypsum

crust periodically covered by halite deposits. The salinity of the pond water ranges from 240 to 320 ‰ of total salinity. From October to June lower salinities (240–280 ‰) occur. During the summer months (June to September) the salinity increases up to 320 ‰, resulting in the deposition of halite; during this period, the microbial mats disappeared. The strains were isolated from samples collected when the microbial mats were fully developed in May to June.

Media, isolation and culture conditions. The strains were isolated from enrichment cultures of red-layer samples. The enrichment medium was prepared according to Pfennig & Trüper (1992). It contained (l⁻¹ distilled water): KH₂PO₄, 1 g; CaCl₂·2H₂O, 0.05 g; NH₄Cl, 0.5 g; NaCl, 100 g; MgCl₂·6H₂O, 2 g; MgSO₄·7H₂O, 1 g; NaHCO₃, 2 g; Na₂S·9H₂O, 0.75 g; vitamin solution V7, 1 ml; trace element solution SL12, 1 ml. The pH was adjusted to pH 7.2–7.4. For enrichments and for better growth of pure cultures, the growth medium was supplemented with acetate (2 mM), succinate (1 mM) and yeast extract (0.5 g l⁻¹).

Pure cultures were obtained after isolation from a deep-agar shake dilution series prepared according to the method of Pfennig & Trüper (1992) and incubated at 30 °C and at a light intensity of 500 lux from a tungsten lamp (16 h light, 8 h dark). Purity of cultures was checked by both microscopic observation and growth tests in deep agar AC medium (Difco) supplemented with NaCl and thiosulfate (final concentrations 10 and 0.05 %, w/v, respectively) and incubated in the dark. Pure cultures in liquid medium were stored in 60 ml screw cap bottles at 4 °C in the dark.

Microscopy. Microscopic observations and photomicrographs were made with a Zeiss photomicroscope according to the method of Pfennig & Wagener (1986). Flagella were observed by transmission electron microscopy (TEM) after negative staining with 1 % (w/v) tungstic acid neutralized to pH 7.2 (JEOL 1200 EX electron microscope). The fine structure of the cells was studied by TEM after fixation of a cell pellet with osmic acid and ultrathin sectioning of the cells according to Reynolds (1963).

Pigment analysis and DNA base composition. Absorption spectra of living cells were measured with a Kontron spectrophotometer (UVIKON 860) after suspension of a cell pellet in sucrose solution (Pfennig & Trüper, 1992). Carotenoids were extracted and separated by TLC according to the method described by Eichler & Pfennig (1986). They were determined by comparison with several standards.

The G + C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989), using bacteriophage λ DNA as the standard.

16S rDNA sequence. Isolation of genomic DNA, amplification of 16S rDNA by PCR, sequencing and phylogenetic analysis were all carried out as described previously (Mouné *et al.*, 1999, 2000). The accession numbers of all 16S rDNA sequences used in this study are given in Fig. 4.

Physiological tests. Utilization of carbon sources and electron donors was tested in the basal liquid medium supplemented with 1 mM Na₂S·9H₂O and using substrate concentrations as given in Table 1.

Growth tests of utilizable substrates, the determination of optimal concentrations of NaCl and MgCl₂ and the determination of optimal pH, light intensity and sulfide tolerance were performed in completely filled 25 ml screw cap tubes. Growth was measured by determining the OD₆₅₀ (Spectronic 20; Bausch and Lomb) over a 15 day period.

Micro-aerophilic growth was tested in uniformly inoculated deep agar tubes open to air and incubated in the dark at 25 °C (Kämpf &

Table 1. Utilization of substrates as electron donors or carbon sources by strains SG 3301^T, SG 3302, SG 3304 and SG 3105

+, Utilized; (+), poorly utilized; –, not utilized. The following substrates were not utilized by the four strains: thiosulfate (5 mM), sulfite (1 mM), formate (5mM), citrate (5 mM), caprylate (2 mM), pelargonate (2 mM), palmitate (2 mM), benzoate (2 mM), tartrate (5 mM), nicotinate (2 mM), glucose (5 mM), fructose (5 mM), sucrose (5 mM), trehalose (5 mM), methanol (5 mM), ethanol (5 mM), propanol (5 mM), butanol (5 mM), mannitol (5 mM), glycerol (5 mM), peptone (0.05 %), cysteine (2 mM), methionine (2 mM), thioglycolate (2 mM) and thioacetamide (2 mM).

Substrate*	SG 3301 ^T	SG 3302	SG 3304	SG 3105
Sulfide (3 mM)	(+)	(+)	(+)	–
Elemental sulfur (0.05 %)	(+)	(+)	(+)	–
Acetate	+	+	+	+
Propionate	–	+	+	+
Butyrate	–	–	–	+
Valerate	–	–	–	+
Crotonate	–	–	–	+
Lactate	–	–	–	+
Pyruvate	+	+	+	+
Malate	–	–	–	+
Fumarate	+	+	+	–
Succinate	+	+	+	+
2-Oxoglutarate	–	–	–	+
Glycolate	–	–	–	+
Betaine	–	–	–	+
Gluconate	–	–	–	+
Aspartate	–	–	–	+
Glutamate	–	–	–	+
Casamino acids (0.05 %)	–	–	–	+
Yeast extract (0.05 %)	+	–	–	+

*The concentration was 5 mM unless otherwise indicated.

Pfennig, 1980). Vitamin requirement and capacity for assimilatory sulfate reduction were tested in 60 ml screw cap bottles by growth tests through four consecutive transfers in synthetic liquid media free of vitamins and yeast extract or sulfide, respectively.

Nitrogen sources were tested in 60 ml aluminium cap sealed bottles with gas-impermeable butyl rubber septum stoppers filled one-third with liquid medium without nitrogen sources and supplemented with 10 mM acetate, and two-thirds with argon as gas phase. The tested nitrogen sources (ammonia, nitrate, organic nitrogen in cysteine, serine and glutamate) were added prior to inoculation. For nitrogen fixation, the gas phase was replaced by N₂ gas (100 kPa). Growth was tested through four consecutive transfers under the same conditions.

Hydrogen utilization was tested by the same method in liquid medium without electron donors with only 0.5 mM Na₂S₉H₂O as sulfur

source and with H₂/CO₂ (80:20, v/v; 100 kPa) as the gas phase. The presence of hydrogenase was tested according to the method of Trüper (1968).

Growth on sulfide, sulfide+CO₂ and on sulfide+acetate was tested in 1 l cultures grown in synthetic liquid medium for 15 days. Growth was measured periodically by OD₆₅₀ (Spectronic 20; Bausch and Lomb). Sulfide, sulfur and sulfate concentrations were determined according to the methods of Cline (1969), Stal *et al.* (1984) and Tabatabaï (1974), respectively.

The presence of catalase was determined by adding a few drops of 3 % (v/v) H₂O₂ to 2 ml of a dense cell suspension.

Determination of compatible solutes by ¹³C-NMR spectroscopy. Isolate SG 3301^T was grown as an 8 l batch culture in synthetic growth medium supplemented with 2 mM acetate and 1 mM succinate plus 15 % (w/v) NaCl but without yeast extract. Cultures were incubated at room temperature (25 °C) in the light and continuously sparged with oxygen-free nitrogen to maintain anaerobic conditions. Mid-exponential phase (OD₆₅₀=0.4) cultures were harvested, extracted and analysed for compatible solutes by natural abundance NMR according to the methods of Welsh & Herbert (1994).

RESULTS

Description of microbial mats and isolation of pure strains

Thin greenish and reddish laminated mats have been observed in particular hypersaline lagoons in the commercial salterns in the Salin-de-Giraud (Camargue, France) (Cornée, 1983; Caumette *et al.*, 1988, 1991, 1994). These mats are present for most of the year and are fully developed in spring and summer, from May to September. The thickest mats (10–20 mm thick) were observed at the sediment surface of a series of lagoons below a 2–3 cm thick gypsum crust. In these lagoons the salinity of the water column (20–40 cm depth) ranged from 130 to 200 ‰. These microbial mats consisted principally of a green layer of cyanobacteria overlying a purple layer of phototrophic sulfur bacteria belonging to the family *Chromatiaceae* (Caumette *et al.*, 1988, 1991, 1994).

In contrast, in other lagoons with higher water salinities ranging from 200 to 320 ‰, the gypsum crust at the sediment surface was thinner (2–5 mm thick) and it was periodically covered by halite crystals when the salinity increased above 300 ‰. In these lagoons, from May to June, a laminated mat developed at the sediment surface, below the gypsum crust. The sediment was composed of a closed anoxic system containing high amounts of AVS (total acid-volatile sulfide) up to 20–40 mmol kg⁻¹ (Caumette *et al.*, 1991, 1994). When the salinity increased up to 300 ‰ in July, the mat decreased gradually and then disappeared by the end of the summer. This mat consisted mainly of a green layer of cyanobacteria (*Phormidium*-like). Below this layer, a red layer occurred. Microscopic observations revealed the sole presence of actively motile spirilloid cells that formed the red layers (Fig. 1). Free sulfur globules were also present and distributed in the entire layer. From red layer samples, enrichment cultures of spirilloid purple sulfur bacteria

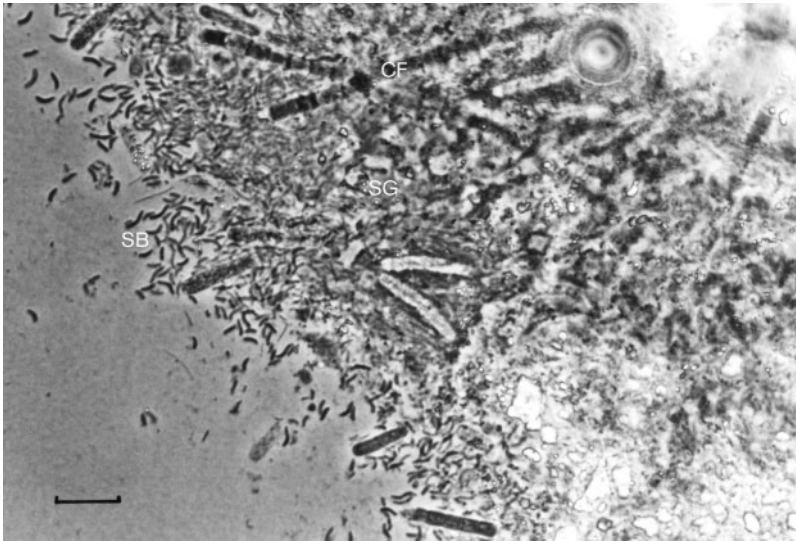


Fig. 1. Photomicrograph of the purple layer of the microbial mat below the gypsum crust at the sediment surface of the sampling site in the Salin-de-Giraud salterns. Note the presence of filamentous cyanobacteria (CF), many vibrioid to spirilloid bacterial cells (SB) and sulfur globules (SG) in the mat. Bar, 10 μm .

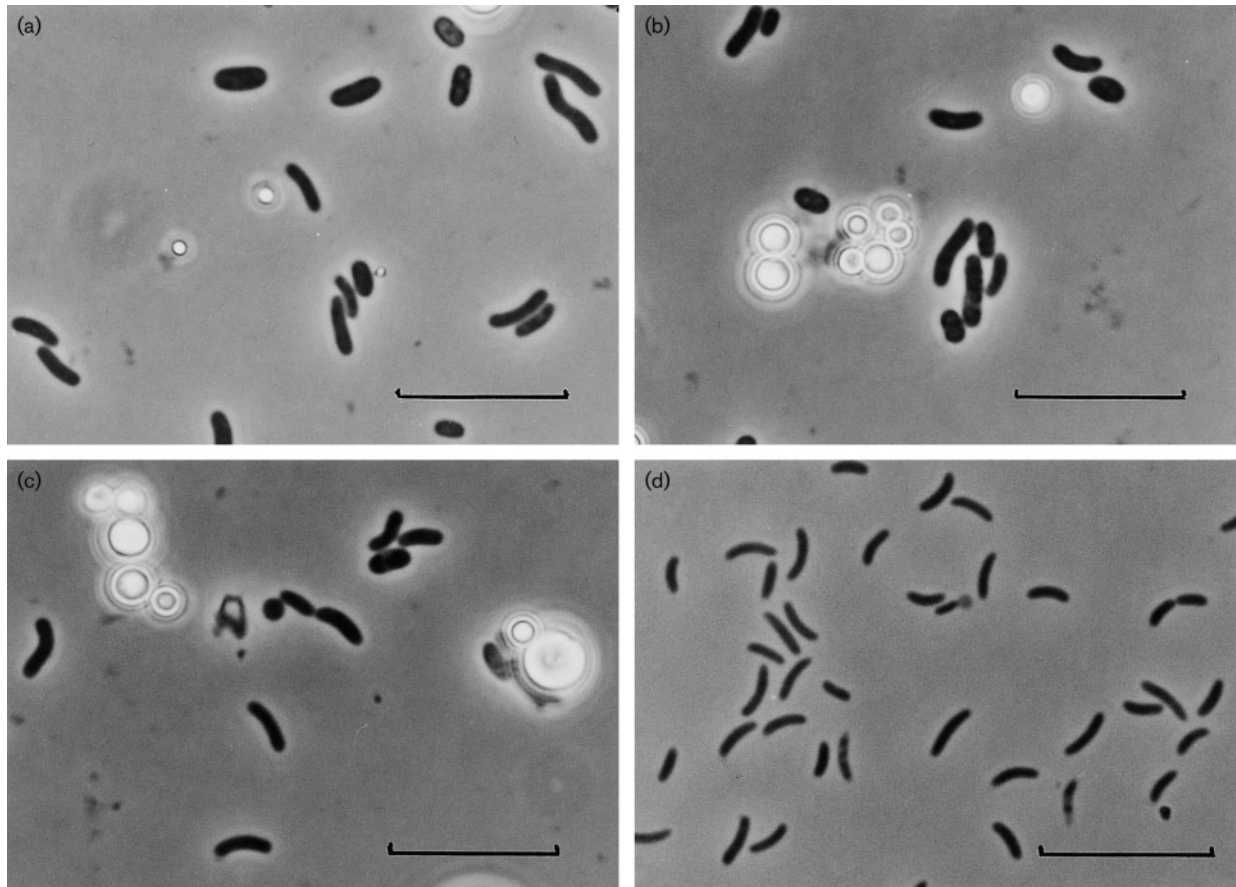


Fig. 2. Phase-contrast micrographs of strains SG 3301^T (a), SG 3302 (b), SG 3304 (c) and SG 3105 (d). Bars, 10 μm .

were obtained. Several strains were then isolated in the same synthetic growth medium with 10 % (w/v) NaCl. Four strains (SG 3301^T, SG 3302, SG 3304 and SG 3105) were isolated, maintained in pure culture and characterized.

Description of the isolated strains

Morphology. When examined by phase-contrast microscopy, three strains (SG 3301^T, SG 3302 and SG 3304) exhibited a similar morphology consisting of actively

motile curved rods, 2–5 μm long and 1.0–1.2 μm wide. When grown with sulfide, sulfur globules were present in the growth medium, outside the cells (Fig. 2a, b and c). In the presence of acetate or succinate, some larger non-curved cells containing reserve material (PHB) occurred with a width of 1.4–1.6 μm . The morphology of strain SG 3105 consisted of motile spirilloid cells of 2–4 μm length and 0.5–0.6 μm width. In contrast to the other strains, sulfur globules were never observed in the growth medium of strain SG 3105 (Fig. 2d). The cells of all strains divided by binary fission.

Pigments. For strains SG 3301^T, SG 3302 and SG 3304, the colour of the cell suspensions were identical, pink to pinkish-red. As shown in the absorption spectra of the living cells (Fig. 3a, b), cells of strains SG3301^T and SG 3304, and also SG 3302 (not shown), contained similar pigments: BChl *a* with absorption maxima at 376, 589, 786–787 and 883–884 nm; carotenoids of the normal spirilloxanthin series with absorption maxima at 486–488, 519–520 and 556 nm. Spirilloxanthin was the dominant

carotenoid present as determined by TLC. The colour of the cell suspension of strain SG 3105 was red to brown-red. Absorption spectra of whole cells (Fig. 3c) showed maxima at 379, 591, 804 and 857 nm typical for BChl *a* containing cells, and at 486, 516 and a shoulder at 545 nm, indicative of the presence of carotenoids of the normal spirilloxanthin series. The absorption maximum at 486 nm indicated the presence of rhodopin as a major carotenoid pigment and this was confirmed by TLC.

Substrate utilization by the four strains. Substrate utilization tests were performed for strains SG 3301^T, SG 3302, SG 3304 and SG 3105 (Table 1). Three strains, SG 3301^T, SG 3302 and SG 3304, were able to weakly use sulfide and sulfur. However, with these compounds as electron donors and CO₂ as sole carbon source, the growth of the three isolates was very poor. In contrast all three strains showed good growth with a limited range of organic substrates as electron donors and carbon sources: pyruvate, fumarate, succinate, acetate and for certain strains, propionate and yeast extract. Strain SG 3105 is an obligate photo-organotroph and able to use a large variety of organic substrates (Table 1).

Effect of salt on the growth of the four strains. The four strains SG 3301^T, SG 3302, SG 3304 and SG 3105 grew at NaCl concentrations ranging from 6–8 % to 25–30 % (w/v) NaCl. The optima for all strains ranged from 9 to 12 % (w/v) NaCl. These results demonstrate a good adaptation of the strains to their environment where the total salinity of 240–320 ‰ corresponds approximately to 17–25 % (w/v) NaCl in the overlying water.

The requirement for Mg²⁺ was tested for the four strains, between 0 and 200 mM Mg²⁺. Strains SG 3301^T, SG 3302 and SG 3304 required a minimum of 0.5 mM with optimal growth recorded at 50, 100 and 20 mM respectively. Strain SG 3105 required at least 7.5 mM for growth with an optimum at 100 mM. None of the isolates showed improved growth at Mg²⁺ concentrations above the optimum concentrations for growth.

Genetic properties and 16S rDNA sequences of the isolated strains. The G+C of the DNA of strains SG 3301^T, SG 3304 and SG 3105 was 74.5, 74.1 and 69.2 mol%, respectively.

The phylogenetic trees obtained from the comparison of 16S rDNA sequences are presented in Fig. 4(a, b). The sequence of Fig. 4(a) shows the phylogenetic tree obtained for strain SG 3105 and the closest sequences. According to the homologies, this strain is closely related to the species *Rhodovibrio sodomensis* (98.8 % similarity). Fig. 4(b) shows the phylogenetic tree obtained for the sequences of strains SG 3301^T and SG 3304 among the closest sequences. These two strains are identical (100 % homology) among the genus *Halorhodospira* and close to *Halorhodospira halophila* DSM 244^T (94.6 % similarity) which is the type species

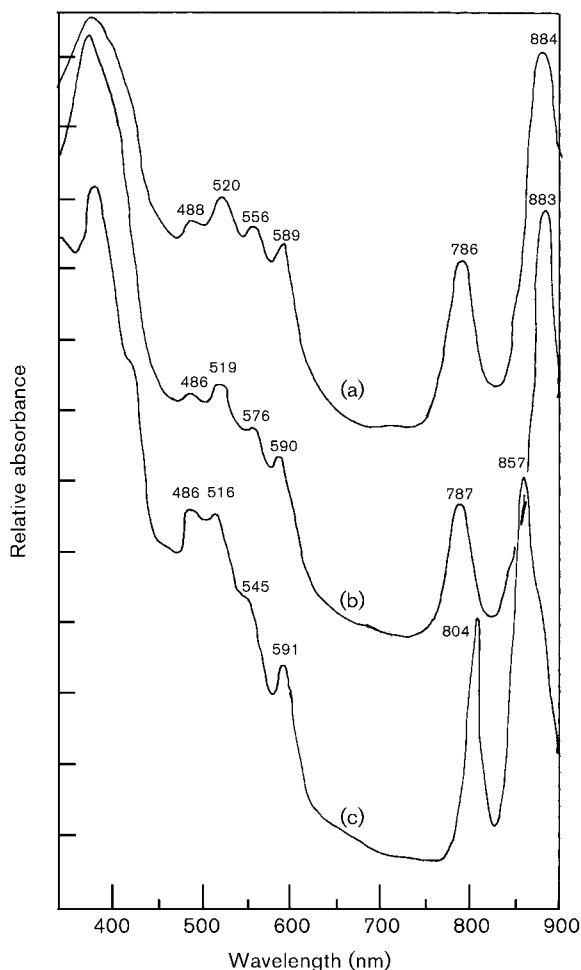


Fig. 3. Absorption spectra of living cells of strain SG 3301^T (a), SG 3304 (b) and SG 3105 (c).

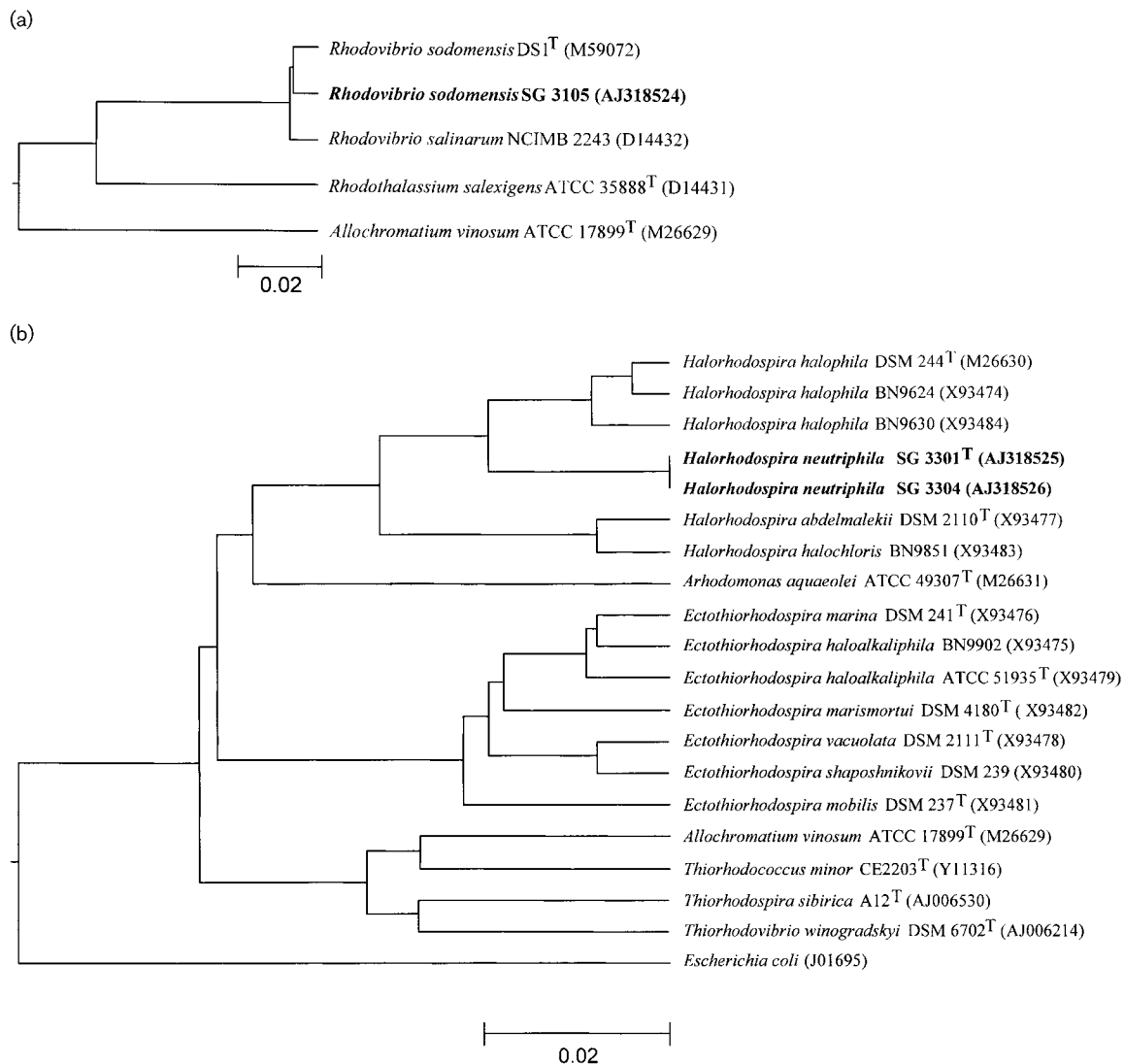


Fig. 4. (a) Phylogenetic position of *Rhodovibrio sodomensis* strain SG 3105 in relation to other *Rhodovibrio* and selected other organisms. Scale bar represents 2 nt substitutions per 100 nt. (b) Phylogenetic position of strains SG 3301^T and SG 3304 in relation to *Halorhodospira* and selected other organisms. Scale bar represents 2 nt substitutions per 100 nt. 16S DNA accession numbers are given for each strain in parentheses.

of the genus. The other two strains of the species (BN 9624 and BN 9630) are less closely related to our strain, with percentage similarities of 91.4 and 90.7 %, respectively. These percentage similarities indicate that strains SG 3301^T and SG 3304 are sufficiently distant from *H. halophila* to be considered as representatives of a new species.

Additional characteristics of strains SG 3301^T and SG 3304

Electron microscopy. Fig. 5(a, b) shows electron micrographs of whole cells after negative staining (Fig. 5a) and a thin section of the cells of strain SG 3301^T (Fig. 5b). The cells are polarly flagellated with a tuft of a few flagella. Thin

sections of the cells revealed the presence of an intracellular membrane system comprising stacks of lamellar membranes.

Nitrogen and sulfur sources. Strains SG 3301^T and SG 3304 were only able to use NH₄⁺ and N₂ as nitrogen sources. With nitrate, serine or glutamate as sole nitrogen source, no growth was observed.

Cysteine served as sulfur source in the absence of sulfide. With sulfate as sole sulfur source, growth did not occur, indicating the absence of sulfate reduction.

Micro-aerophilic growth. Growth of strain SG 3301^T and SG 3304 was very poor or absent under micro-aerophilic conditions in the dark. Catalase was positive.

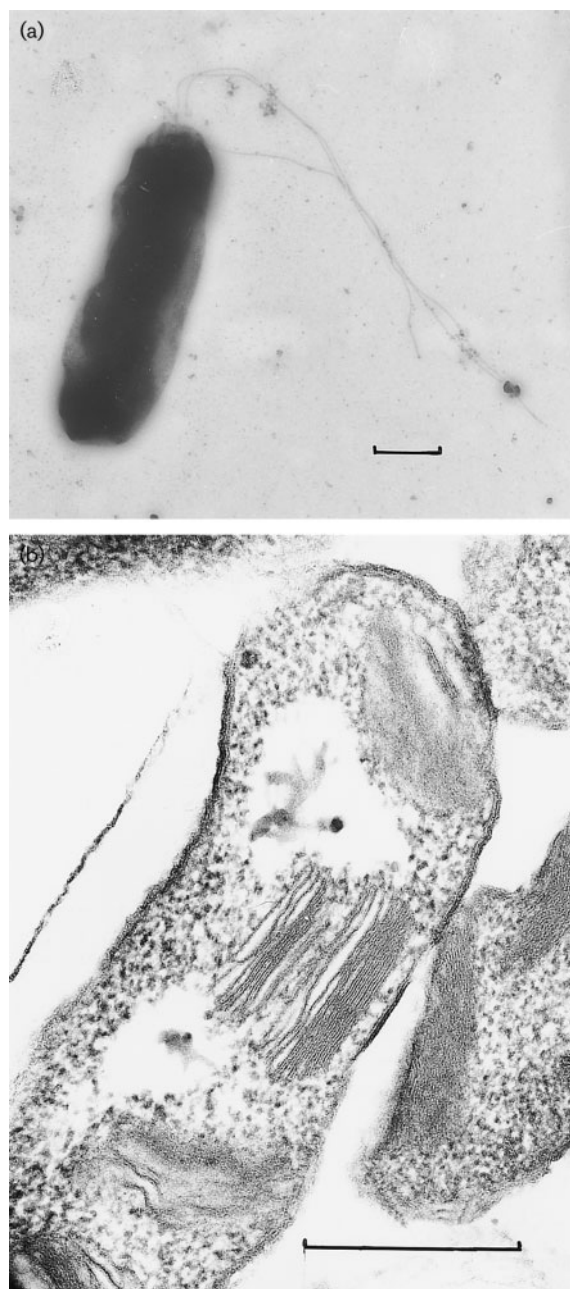


Fig. 5. Electron microscopy and fine structure of *Halo-rhodospira* strain SG 3301^T. (a) Negative staining showing polar flagella. Bar, 1 μm. (b) Ultrathin section of the cells showing the intracellular stacks of lamellar membranes and envelopes of the Gram-negative type. Bar, 0.5 μm.

pH, light. Strains SG 3301^T and SG 3304 grew in media with a pH between 6 and 8.5. The optimum pH was 6.8–7.0 for both strains. Growth at different light intensities was determined between 25 and 5000 lux using a tungsten lamp light source. For both isolates growth was very slow at 25 lux. Optimal light intensity was recorded at 5000 lux for both strains with growth rates of

0.034 h⁻¹ for strain SG3301 and 0.01 h⁻¹ for strain SG 3304 under optimal conditions.

H₂ and vitamin requirements. Both strains SG 3301^T and SG 3304 were unable to grow with H₂ as sole electron donor. In addition, hydrogenase activity was tested and the result was negative for both strains.

Both strains were able to grow in synthetic growth medium in the absence of vitamins or yeast extract after five consecutive transfers, indicating that neither isolate had a vitamin requirement.

Sulfide tolerance and utilization of sulfur compounds as electron donors. Strains SG 3301^T and SG 3304 were able to grow with up to 5.5–6 mM sulfide in the synthetic growth medium. The optimum was 1–3 mM for both strains. For strain SG 3301^T, with optimum sulfide, in the presence of CO₂ as sole carbon source, growth was always very poor, although sulfide and the produced sulfur were completely oxidized to sulfate (Fig. 6a). In contrast with acetate as electron donor and carbon source, growth was always very good and the equivalent amount of sulfide and produced sulfur were also completely oxidized to sulfate (Fig. 6b). Sulfide could therefore be considered as a good electron donor for this strain, with or without an organic substrate, even though growth with sulfide and CO₂ alone was very poor. This observation indicates that CO₂ alone only supported limited growth of strain SG 3301^T, as shown in Table 2. Maximal growth was obtained with sulfide, acetate and CO₂ as substrates in the growth medium.

Identification of compatible solutes. Strain SG 3301^T grew optimally in medium containing 12 % (w/v) NaCl. At supra-optimal salt concentrations growth progressively decreased due to an increased lag phase and decrease in growth rate (data not shown). Natural abundance ¹³C spectra of extracts prepared from strain SG 3301^T grown in medium containing 15 % (w/v) NaCl, but in the absence of yeast extract, showed strong signals at 53.70, 66.52 and 169.58 p.p.m. which correspond to authentic glycine betaine. In addition signals at 18.46, 21.70, 37.46, 53.70, 66.52 and 177.02 p.p.m. were also present which correspond to the osmolyte ectoine. Semi-quantitative estimation (based on peak height) of the identified osmolytes indicate that glycine betaine was the major osmolyticum present and ectoine the minor component.

DISCUSSION

Spirilloid purple bacteria are relatively common in the photic anoxic zones of marine and hypersaline environments. It is perhaps not surprising to observe these microorganisms in microbial mats that develop in marine salterns. In this study, we isolated both purple sulfur and purple non-sulfur bacteria from the same purple layer in a microbial mat occurring below a gypsum crust at total salinities of about 240 to 320 ‰. All the isolated strains grew between

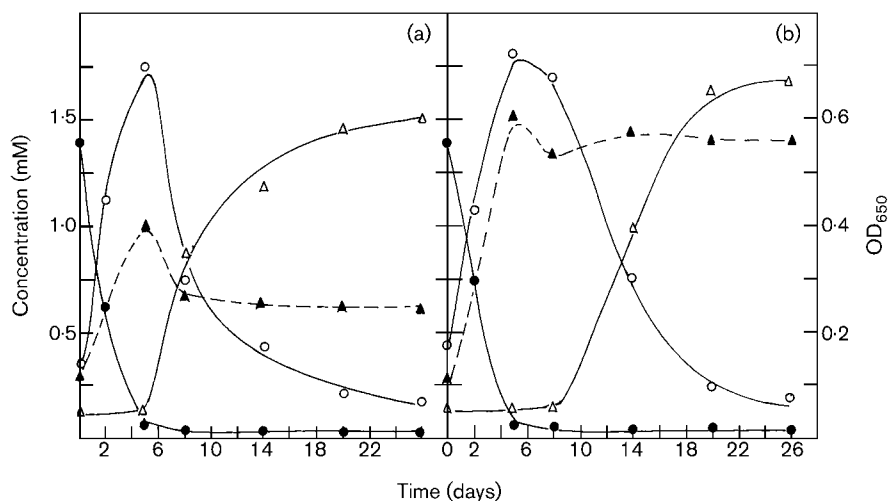


Fig. 6. Production of sulfide, sulfur and sulfate during growth of *Halorhodospira* strain SG 3301^T (a) with CO₂ as sole carbon source and sulfide as sole electron donor and (b) with acetate plus CO₂ as carbon sources, and acetate plus sulfide as electron donors. Filled circle, sulfide; open circle, sulfur; open triangle, sulfate; filled triangle, OD₆₅₀.

6–8 and 25–30 % (w/v) NaCl in the synthetic growth medium with optima at 9–12 % (w/v) NaCl. Thus all strains can be considered as strictly halophilic to extremely halophilic bacteria (Caumette *et al.*, 1999). Among these strains, the purple non-sulfur bacterium strain SG 3105 showed characteristics of the genus *Rhodovibrio* (Imhoff *et al.*, 1998) and was more closely related to *Rhodovibrio sodomensis* on a physiological and phylogenetical basis, rather than to *Rhodovibrio salinarum*. This latter organism has been isolated from marine salterns (Nissen & Dundas, 1984) like our strain. In contrast *Rhodovibrio sodomensis* was first isolated from a hypersaline pond close to the Dead Sea (Mack *et al.*, 1993). Thus our strain represents the first member of this species isolated from a marine saltern.

In this study, the three other isolated spirilloid strains (SG 3301^T, SG 3302 and SG 3304) deposited sulfur globules extracellularly when grown with sulfide as electron donor. Morphologically, physiologically and phylogenetically they are fully included in the family *Ectothiorhodospiraceae*. This family is composed of three genera: *Ectothiorhodospira*,

Halorhodospira and *Thiorhodospira* (Imhoff & Süling, 1996; Bryantseva *et al.*, 1999). Our three isolated strains were more closely related to the genus *Halorhodospira* according to the phylogenetic tree based on comparison of 16S rDNA sequences. The genus *Halorhodospira* comprises the most halophilic and alkaliphilic representatives of the *Ectothiorhodospiraceae*, isolated from athalassohaline environments like the representative of the genus *Thiorhodospira*. In contrast, members of the genus *Ectothiorhodospira* have been isolated principally from coastal marine environments at neutral pH and at lower salinity, although some representatives are alkaliphilic and have been isolated from soda lakes (Imhoff & Süling, 1996). Therefore, our three strains which are extremely halophilic are the first members of the genus *Halorhodospira* isolated from marine salterns and living at neutral pH, thus showing that a moderate to extreme salt requirement is an important factor in bacterial taxonomy at the genus level, rather than pH.

All previous isolations of *Halorhodospira* spp. and *Rhodovibrio sodomensis* have been from enclosed hypersaline lakes. This study is the first to demonstrate their presence in a modified marine environment, indicating that they have a wider distribution than previously considered. The presence of these populations in the same depth horizon within the mats suggests that both populations are living in same conditions and possible interactions may be occurring. Evidence to support this conclusion is the ability of the purple sulfur *Halorhodospira* isolates to preferentially use low-molecular-mass organic substrates for growth rather than reduced sulfur compounds. This characteristic is more typical for the photoheterotrophic growth of *Rhodovibrio sodomensis*.

The salinity range for growth of the strains indicates that all these isolates are well adapted to life in the hypersaline lagoons from which they were isolated. Data presented in this study show that strain SG 3301^T when grown at supra-optimal salt concentrations (15 %, w/v, NaCl) in the absence of yeast extract synthesized glycine betaine and

Table 2. Growth of *Halorhodospira* strain SG 3301^T in mineral medium supplemented with CO₂ or acetate

Growth was measured as OD₆₅₀ after 15 days (mean of duplicates).

Medium	OD ₆₅₀
Mineral medium without CO ₂	
No supplement	0.01
Sulfide (2 mM)	0.01
Acetate (10 mM)	0.58
Mineral medium with CO ₂	
No supplement	0
Sulfide (2 mM)	0.12
Acetate (10 mM)	0.66
Acetate (10 mM) + sulfide (2 mM)	0.82

Table 3. Major properties and differences between *Halorhodospira* species

+, Utilized; (+), poorly utilized; \pm , not utilized by this strain but can be utilized by other strains of this species; –, not utilized. All four species are vibrioid in shape, have a stacked internal membrane system and can use pyruvate and acetate.

Character	<i>Hlr. neutriphila</i> sp. nov. SG 3301 ^T	<i>Hlr. halophila</i> DSM 244 ^{T*}	<i>Hlr. abdelmalekii</i> DSM 2110 ^{T†}	<i>Hlr. halochloris</i> DSM 1059 [‡]
Cell size (width × length, μm)	1–1.2 × 2–5	0.6–0.9 × 2–8	0.9–1.2 × 4–6	0.5–0.6 × 2.5–8.0
Flagellation	Polar tuft	Bipolar	Bipolar	Bipolar
BChl	<i>a</i>	<i>a</i>	<i>b</i>	<i>b</i>
Carotenoids	Spirilloxanthin	Spirilloxanthin	Rhodopin	Rhodopin
Optimal NaCl (%)	12	11–22	14–16	14–25
Salinity range (%)	7–>25	3–30	5–30	10–35
Optimal pH	6.8–7.0	8.5–9.0	8.0–9.2	8.1–9.1
Sulfate assimilation	–	–	–	+
G + C (mol%)	74.5 (HPLC)	67.5–69.7 (T_m)	63.3–63.8 (T_m)	50.5–52.9 (T_m)
Sulfide used	(+)	+	+	+
Sulfur used	(+)	+	+	+
Thiosulfate used	–	+	–	–
Organic substrates:				
Lactate	–	\pm	–	–
Propionate	\pm	+	+	+
Malate	–	+	+	+

*Data from Raymond & Sistro (1969) and Pfennig & Trüper (1989).

†Data from Imhoff & Trüper (1981) and Pfennig & Trüper (1989).

‡Data from Imhoff & Trüper (1977) and Pfennig & Trüper (1989).

ectoine as compatible solutes. Semi-quantitative estimation of the relative amounts of the accumulated osmolytes indicate that glycine betaine was the major osmoticum synthesized by this isolate and ectoine was the minor component. These data are in agreement with those of Severin *et al.* (1992) who showed that *Halorhodospira halochloris*, *Halorhodospira abdelmalekii* and *Halorhodospira halophila* also synthesized glycine betaine and ectoine when subjected to salt stress. Strain SG 3301^T grows more rapidly when yeast extract is added to the growth medium. Since yeast extract contains significant amounts of glycine betaine (Dulaney *et al.*, 1968) this suggests that strain SG 3301^T may possess a functional glycine betaine uptake system. In hypersaline lagoons glycine betaine may account for >20 % of the total nitrogen present in the mats (King, 1988). Thus, from an ecological viewpoint the ability of an organism to accumulate a compatible solute such as glycine betaine would be advantageous since it is energetically more favourable than synthesis.

Among the *Halorhodospira* species, our strains (SG 3301^T and SG 3304) are related to *Halorhodospira halophila* according to the rDNA sequence. However, the degree of similarity (94.6 % similarity) is not sufficiently high to include these isolates within the species *Halorhodospira halophila*. Physiologically, strain SG 3301^T has several characteristics close to the *Halorhodospira halophila* type strain (DSM 244^T, Table 3). Both strains contain BChl *a*

and spirilloxanthin as the major carotenoid and have higher G + C contents in their DNA, whereas the two other *Halorhodospira* species (*Halorhodospira abdelmalekii* and *Halorhodospira halochloris*) are different with BChl *b* and rhodopin as the major photopigments, and a lower G + C content in their DNA. However, *Halorhodospira* strain SG 3301^T differs from *Halorhodospira halophila* by its flagella pattern with one polar tuft rather than bipolar flagellation for *Halorhodospira halophila* and also for other *Halorhodospira* (Table 3). Their DNA G + C content ratios are also different with 74.5 mol% for strain SG 3301^T and 67.5–69.7 mol% for *Halorhodospira halophila*. There are also additional physiological differences between both micro-organisms. *Halorhodospira halophila* is able to use thiosulfate and malate as electron donor whereas *Halorhodospira* strain SG 3301^T, is unable to use these substrates and grows poorly on all other sulfur compounds, which is not an usual characteristic of the purple sulfur bacteria. The most important difference between strain SG 3301^T and *Halorhodospira halophila* is the optimal pH for growth. All *Halorhodospira* species are alkaliphilic with pH optima between 8.0 and 9.0. In contrast, strain SG 3301^T grows optimally at neutral pH (6.8–7.0) like other isolated strains (SG 3302 and SG 3304). The three isolated strains are all very similar. According to their morphological, physiological and genetic differences with the described species in the genus *Halorhodospira*, it is justifiable to consider the three strains as members of a new

species for which the name *Halorhodospira neutriphila* sp. nov. is proposed with respect to their optimal growth at neutral pH. Strain SG 3301^T is proposed as the type strain of this new species.

Emended description of the genus

Halorhodospira

The description of the genus *Halorhodospira* is as given by Imhoff & Süling (1996), but with the following additional features: motile by polar tuft of flagella; growth is dependent on saline conditions and neutral or alkaline pH. *Halorhodospira* species are found in hypersaline and extremely saline environments with neutral to extremely alkaline pH that contain sulfide and that are exposed to light, such as salterns, salt flats, salt lakes and soda lakes. The G + C content of the DNA is 50.5–74.5 mol%.

Description of *Halorhodospira neutriphila* sp. nov.

Halorhodospira neutriphila (neu.tri.phi'la. L. adj. *neutralis* neutral; Gr. adj. *philos* liking, preferring; N.L. adj. *neutriphila* preferring neutral pH).

Cells are curved rods, 1–1.2 µm wide and 2–5 µm long. Multiplication is by binary fission. Gram-negative. Cells are motile by a polar tuft of flagella. Colour of cell suspension is pinkish-red. Photosynthetic membrane system, present as lamellar stacks, contains BChl *a* and spirilloxanthin as the major carotenoid. Electron donors for photoautotrophic growth are sulfide and sulfur. Globules of elemental sulfur are present outside the cells. Photoautotrophic growth is poor. In the presence of sulfide and CO₂, acetate, pyruvate, fumarate, succinate and yeast extract are photo-assimilated. Propionate can be used by some strains. Not capable of chemolithotrophic or chemo-organotrophic growth. Not capable of assimilatory sulfate reduction. N₂ and NH₄ serve as nitrogen sources. Cysteine is used as sulfur source. No vitamins are required. Optimal pH, 6.8–7.0; pH range for growth, 6.0–8.5; optimal temperature, 30–35 °C; NaCl range is between 6–8 and 25–30 % (w/v) NaCl; optimal concentration at 9–12 % (w/v) NaCl. G + C content of DNA is 74.1–74.5 mol% (HPLC). Habitat is anoxic mud, sediments and microbial mats exposed to light in hypersaline to extreme saline environments, such as coastal salterns with salt concentrations of approximately 20–30 % (w/v). Type strain is SG 3301^T (=DSM 15116^T) isolated from a benthic microbial mat in a commercial saltern, Salin-de-Giraud (Camargue, France). The 16S rDNA sequence has been deposited in the EMBL database under the accession number AJ318525. The second strain, SG3304, has also been deposited in the DSMZ as DSM 15115 and its 16S rDNA sequence accession number is AJ318526.

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- salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. *Int J Syst Bacteriol* **48**, 793–798.
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