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Selective enrichment and characterization of *Roseospirillum parvum*, gen. nov. and sp. nov., a new purple nonsulfur bacterium with unusual light absorption properties

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Abstract A new type of phototrophic purple bacterium, strain 930I, was isolated from a microbial mat covering intertidal sandy sediments of Great Sippewissett Salt Marsh (Woods Hole, Mass., USA). The bacterium could only be enriched at a wavelength of 932 (± 10) nm. Cells were vibrioid- to spirilloid-shaped and motile by means of bipolar monotrichous flagellation. The intracytoplasmic membranes were of the lamellar type. Photosynthetic pigments comprised bacteriochlorophyll *a* and the carotenoids spirilloxanthin and lycopenal. The isolated strain exhibited an unusual, long-wavelength absorption maximum at 911 nm. Sulfide or thiosulfate served as electron donor for anoxygenic phototrophic growth. During growth on sulfide, elemental sulfur globules formed outside the cells. Elemental sulfur could not be further oxidized to sulfate. In the presence of sulfide plus bicarbonate, fructose, acetate, propionate, butyrate, valerate, 2-oxoglutarate, pyruvate, lactate, malate, succinate, fumarate, malonate, casamino acids, yeast extract, L(+)-alanine, and L(+)-glutamate were assimilated. Sulfide, thiosulfate, or elemental sulfur served as a reduced sulfur source for photosynthetic growth. Maximum growth rates were obtained at pH 7.9, 30 °C, 50 μmol quanta m^{-2} s^{-1} of daylight fluorescent tubes, and a salinity of 1–2% NaCl. The strain grew microaerophilically in the dark at a partial pressure of 1 kPa O_2. The DNA base composition was 71.2 mol% G + C. Sequence comparison of 16S rRNA genes indicated that the isolate is a member of the α-Proteobacteria and is most closely related to *Rhodobium orientis* at a similarity level of 93.5%. Because of the large phylogenetic distance to known phototrophic species of the α-Proteobacteria and of its unique absorption spectrum, strain 930I is described as a new genus and species, *Roseospirillum parvum* gen. nov. and sp. nov.

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Abbreviations *BChl* Bacteriochlorophyll · *LH* Light-harvesting complex

Introduction

Anoxygenic phototrophic bacteria grow with light as energy source and with reduced sulfur species or organic compounds as electron-donating substrates. Because light and reduced substrates usually occur in opposing gradients, these bacteria form dense populations in the chemocline of lakes (Guerrero et al. 1985; Overmann et al. 1991) or sulfide-rich sediments (Stal et al. 1985; Nicholson et al. 1987; Van Gemerden et al. 1989). In sediments, the gradients of light, oxygen, and sulfide are much steeper than in the pelagic environment (Jørgensen 1982), and light attenuation is strongly influenced by the sediment matrix (Jørgensen and Des Marais 1986; Lassen et al. 1992; Kühl et al. 1994).

In sandy sediments, light attenuation occurs preferentially in the wavelength range of blue light due to the reflection by sand grains (Jørgensen and DesMarais 1986; Kühl et al. 1994). Colonization by cyanobacteria and diatoms causes a significant increase of the light attenuation coefficient in the wavelength range of 400–700 nm (Lassen et al. 1992). In contrast to pelagic systems, however, absorption of infrared radiation is very low within the euphotic zone of sandy sediments. As a consequence, mainly far-red and near-infrared light reaches the anoxic layers of intertidal sandy sediments (Jørgensen and DesMarais 1986; Pierson et al. 1990; Lassen et al. 1992; Garcia-Pichel et al. 1994; Kühl et al. 1994). The most complex microbial communities in intertidal sediments consist of a vertical sequence of five layers formed (from the top) by diatoms and cyanobacteria, cyanobacteria, purple sulfur bacteria with bacteriochlorophyll (BChl) *a*, purple

sulfur bacteria with BChl *b*, and green sulfur bacteria (Nicholson et al. 1987). The vertical structure has been explained by a successive absorption of different wavelength bands of red and infrared light by these different types of photosynthetic microorganisms (Pierson et al. 1990).

If the absorption spectra of all major types of photosynthetic microorganisms are compiled (Fig. 1A), a wavelength region between 900 and 950 nm that is not harvested by any of the known types of phototrophic bacteria becomes evident. The only known exception is the thermophilic (optimal growth temperature, 48–50 °C) *Chromatium tepidum*, which exhibits an absorption maximum at 920 nm (Garcia et al. 1986). Because of the strong competition for infrared light in sediment ecosystems, an effective absorption of the wavelengths between 900 and 950 nm should be of selective advantage in microbial mats. The recent isolation of a purple nonsulfur bacterium containing a new type of photosynthetic light-harvesting (LH) complex with an absorption maximum at 986 nm (Pfennig et al. 1997) indicates that the diversity of the pigment-protein complexes in anoxygenic phototrophic bacteria is greater than previously assumed.

In the present study, we selectively enriched and isolated a small spirilloid bacterium with a novel type of absorption spectrum. As indicated by 16S rRNA gene sequence comparison, the newly isolated strain 930I is distantly related to phototrophic members of the α-subgroup of the Proteobacteria. On the basis of these properties, the new isolate is described as the first representative of a new genus and species, *Roseospirillum parvum* gen. nov. sp. nov.

Materials and methods

Origin of samples and enrichment scheme

Samples for the enrichment of phototrophic bacteria were obtained from four different locations. Two types of laminated micobial mats were sampled in Great Sippewissett Salt Marsh (Cape Cod, Mass., USA) using plexiglass corers. One type represented the macroscopically visible assemblage of colored layers described by Nicholson et al. (1987). The mat was dissected, and the two layers of purple sulfur bacteria (pink and peach-colored) were used for the enrichment cultures. The second type of microbial mat consisted of a black, leathery surface layer of cyanobacteria and an underlying layer of purple sulfur bacteria. A third and fourth sample originated from a small pond (diameter, 1 m) within Great Sippewissett Salt Marsh and from brackish Oyster Pond near Woods Hole (Cape Cod, Mass., USA). The sandy sediments of both ponds had a pinkish surface layer of purple sulfur bacteria. All four samples were obtained during the Microbial Diversity Course at the MBL, Woods Hole, on July 1–2, 1994. The fifth sample was obtained on November 29, 1994 from a cyanobacterial mat in the littoral of Solar Lake (Sinai, Egypt) and was kindly provided by A. Teske (Max Planck Institute of Marine Microbiology, Bremen, Germany).

All samples were stored in gas-tight glass containers at 4 °C. Enrichments were carried out in 22-ml screw-capped tubes filled with CR or RS medium (see below) that were inoculated with 0.5 g (wet mass) of sediment. Since a previous analysis of the absorption spectra of different layers of the Sippewissett microbial mats (Pierson et al. 1990) had not yielded any indications of an absorption peak between 900 and 950 nm, we employed highly selective conditions for the enrichment of new types of anoxygenic phototrophic bacteria. The tubes were incubated behind an interference band pass filter with maximum light transmission at a center wavelength of 932 nm and a half-power band width of ± 10 nm (L.O.T. Oriel, Langenberg, Germany; Fig. 1A). For comparison, a second set of enrichment cultures with the same media and inocula was incubated behind an infrared barrier filter (no. 530, cutoff < 780 nm; Göttinger Farbfilter, Göttingen, Germany). Tungsten lamp bulbs (60 W) served as the light source in all enrichment experiments.

Isolation and cultivation

Pure cultures were obtained by repeated deep-agar dilution series (Pfennig 1978). Purity was checked microscopically and by

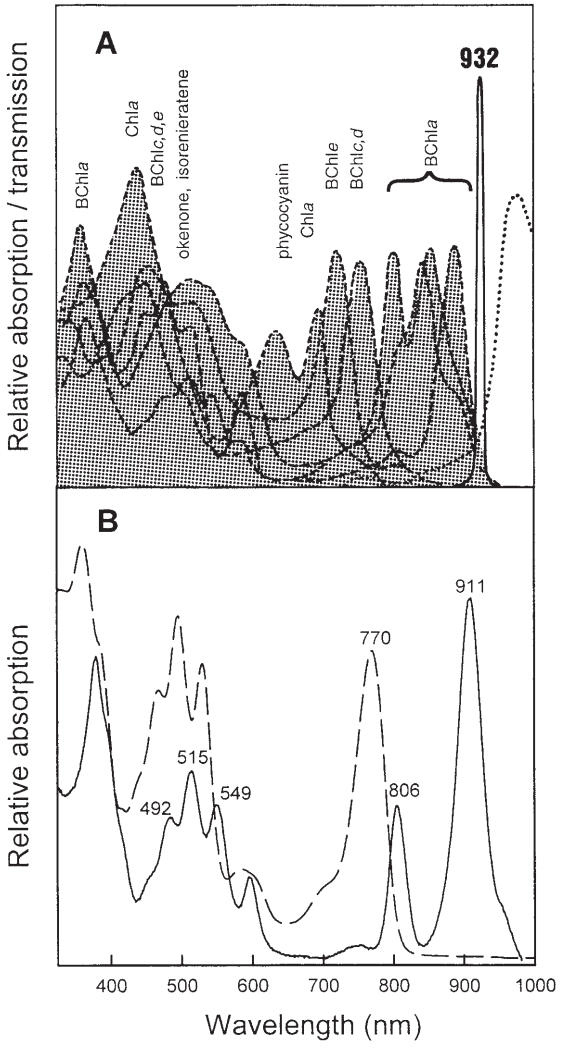

Fig. 1 **A** Cumulative representation of absorption spectra of pigment-protein complexes in the major groups of phototrophic microorganisms except strain 930I (*shaded area*), absorption spectrum of water (····), and wavelength range used for the enrichment of strain 930I. The latter is depicted as a transmission curve of the interference filter (—). The absorption spectrum of bacteriochlorophyll(BChl)-*b*-containing bacteria (maximum located at 1,025 nm) is not included in this graph. **B** Absorption spectra of whole cells (—) and of acetone/methanol extracts (7:2, v/v) (- - -) of strain 930I. The positions of absorption maxima of diagnostic value are given

growth tests in basal medium supplemented with casamino acids and yeast extract. The basal medium CR used for isolation and the initial cultivation of the organism contained per liter of distilled water: 0.25 g KH_2PO_4, 0.34 g NH_4Cl, 0.34 g KCl, 20 g NaCl, 2.8 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 1.5 g $NaHCO_3$, 0.60 g $Na_2S \cdot 9H_2O$, 1 ml solution of seven vitamins (Pfennig 1978), and 1 ml trace element solution SL12 (Overmann et al. 1992). The medium was sterilized, the pH was adjusted to 7.2–7.3, and the medium was aseptically distributed into culture vessels as described by Pfennig and Trüper (1989). After initial growth tests, substrate tests were performed in a modified version of the medium for purple nonsulfur bacteria (RS medium). This medium contained per liter of distilled water: 1.0 g KH_2PO_4, 0.50 g NH_4Cl, 20 g NaCl, 2.80 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.20 g yeast extract, 0.60 g $Na_2S \cdot 9H_2O$, and 1 ml trace element solution SL12. The pH was also set to 7.2–7.3.

Stock cultures were grown photosynthetically in 50-ml screw-capped bottles at 100 μmol quanta m^{-2} s^{-1} light intensity of a tungsten lamp bulb. Repeated addition of neutralized sulfide solution (Siefert and Pfennig 1984) was used to obtain higher cell yields. Stock cultures were stored at 4 °C in the dark.

Growth optima for light intensity, temperature, pH, and salinity were determined in CR medium. In order to assess the selective advantage of the new isolate, growth rates were also determined at different light intensities of a daylight fluorescent tube (Osram daylight 5000 de luxe; no infrared light is emitted by these tubes). The light intensities in front of (I_1) and behind (I_2) the cultures were monitored employing an LI-189 quantum meter plus an LI-200 pyranometer sensor (sensitivity range, 400–1,100 nm; Li Cor, Lincoln, Neb., USA). The mean values of light intensity within the bacterial cultures $\bar{I}$ were calculated as:

$$\bar{I} = (I_1 - I_2)/\ln(I_1/I_2) \quad (1)$$

(Van Liere and Walsby 1982). $\bar{I}$ was adjusted daily to account for the increase in self-shading during the growth experiments. The increase in cell numbers was followed by counting subsamples in a Thoma chamber after fixation with formaldehyde (4 vol.%).

Chemotrophic growth in the dark was tested in 200-ml batch cultures under an atmosphere of 1% O_2 and 0.5% CO_2 in N_2. Cultures were incubated in 1,000-ml Erlenmeyer flasks on a rotary shaker at 60 rpm. Different combinations and concentrations of electron donors and carbon sources were employed. One culture received thiosulfate (2 mM) and no additional carbon source besides hydrogen carbonate. A second culture contained 200 μM thiosulfate and 3 mM acetate. Acetate alone (3 mM final concentration) was used in a third culture, and the control consisted of only RS medium without further additions. The gas atmosphere was exchanged every second day, and growth was monitored by measuring optical density at 650 nm.

Analytical procedures

Absorption spectra of intact pigment-protein complexes were recorded in a Lambda 2 S UV/VIS spectrometer (Perkin Elmer, Überlingen, Germany) after passage of cell suspensions through a French pressure cell (at 6.9×10^6 Pa; Aminco, Silver Spring, Md., USA). After this treatment, the light scattering was significantly reduced. In order to obtain more distinct absorption peaks, especially in the short-wavelength range, the photosynthetic pigments in the sample were bleached by addition of 2.7% (w/v, final concentration) H_2O_2. Afterwards, a second scan was recorded that was then subtracted from the original scan. Comparison with absorption spectra of untreated cells confirmed that neither the disruption of cells by French pressure treatment nor the correction for the light scattering of bleached cells changed the position of the absorption peaks.

The type of bacteriochlorophyll was identified according to its long-wavelength absorption band (Q_y) after extraction of cell pellets with acetone/methanol (7:2, v/v). For the identification of carotenoids, bacterial cells were concentrated on Whatman GF/F glass-fiber filters and were extracted with acetone/methanol (7:2, v/v) under a nitrogen atmosphere. After concentration by rotary evaporation (40 °C), the extracts were loaded onto Merck 60 silica gel TLC plates. Chromatography proceeded in a petroleum ether/acetone mixture (80:20, v/v). Carotenoids were identified by comparison with reference compounds from strains with known carotenoid composition and by their absorption maxima in pure acetone (Züllig 1985). Carotenoid concentrations were calculated from the specific absorption coefficients listed by Züllig (1985).

Concentrations of hydrogen sulfide were determined by the methylene blue method (Cline 1969). A modification (Overmann et al. 1994) of the cyanolysis method of Steinmetz and Fischer (1981) was used to determine particulate elemental sulfur recovered on cellulose nitrate membrane filters (pore size, 0.2 μm; Sartorius, Göttingen, Germany). Sulfate and thiosulfate were measured by ion chromatography using a Sykam ion chromatography system (Sykam, Gilching, Germany) equipped with an S 1000 solvent delivery system, an S 4110 column oven, an S 2210/S 6330 suppressor unit, an S3110 conductivity detector, and a C-R3 A integrator. An LCA-A09 polystyrene column was employed for separation of the anions.

Dry cell mass of cultures was determined after filtration of 40-ml subsamples on predried GF/F glass-fiber filters (nominal pore size, 0.7 μm; Whatman, Maidstone, England). A control of the filtrate confirmed that cells of strain 930I are quantitatively retained on GF/F filters. After filtration, the samples were washed with 10 ml 50 mM ammonium actetate solution, dried at 105 °C over night, and the weights determined. The dry weight values were corrected for the amount of sulfur determined also on filters and for the same culture.

Photosynthetic sulfide oxidation

Rates of sulfide oxidation were determined in a 4-ml reaction chamber equipped with a custom-made Ag/Ag_2S sulfide electrode and a commercial pH electrode (Cypionka 1989). The chamber was filled with dense cell suspensions at a final concentration of $4.7 \times 10^9 - 1.3 \times 10^{10}$ cells ml^{-1} of sulfide-free CR medium, and the electrodes were calibrated in the dark by repeated addition of small volumes of sulfide solution with a microliter syringe. Sulfide oxidation was initiated by illuminating the electrode chamber with infrared light that was generated by a 100-W tungsten reflector lamp bulb combined with the 932-nm interference band pass filter (see above). During these measurements, the temperature was kept constant at 30 °C (the optimal temperature of growth; see Results) by means of a thermostat.

Scotophobotaxis assay

The scotophobic response of cell suspensions was assessed in small microscopic chambers of 100-μm depth at a 60x magnification using a Leitz DMR microscope (Wetzlar, Germany). An interference filter (L.O.T. Oriel, Langenberg, Germany) was used to generate a continuous spectrum between 570 and 1,100 nm in the optical plane of the microscopic chamber. Image acquisition and processing were done after an incubation period of 15 min, as described previously (Fröstl and Overmann 1998).

Electron microscopy

Samples were negatively stained according to Cole and Popkin (1981) with a 5% solution of uranyl actetate (pH 5). For ultra-thin sectioning, cell pellets were fixed in glutaraldehyde [2% (v/v) in 0.1 M Na-K-phosphate buffer (pH 7.0)]. After four washing steps in phosphate buffer (15 min each), a post-fixation step in OsO_4 (1% in 0.1 M phosphate buffer) followed. Cells were embedded in 4% (w/v) agarose and were dehydrated in an ethanol series. The samples were finally embedded in Epon (Spurr 1969) and stained according to Reynolds (1963). Electron micrographs were taken with a Zeiss EM 109 electron microscope (Zeiss Oberkochen, Germany).

Analysis of the 16S rRNA gene sequence

Extraction and purification of genomic DNA, amplification, and cycle sequencing of the 16S rRNA gene were performed as described previously (Tuschak and Overmann 1997). The 1,310-base-pair sequence was compared to all presently available 16S rRNA gene sequences of anoxygenic phototrophic bacteria of the α-subgroup of Proteobacteria that were available in the RDP (Maidak et al. 1997) and GenBank (Benson et al. 1998) databases. For the phylogenetic comparison of the new isolate with nonphototrophic taxa of the α-Proteobacteria, the most closely related sequences were retrieved from RDP and GenBank employing the SIMILARITY RANK and BLASTN (Altschul et al. 1997) tools, respectively. Sequences were aligned with the aid of CLUSTAL W (Thompson et al. 1994). Gap open penalty was set to 10, and gap extension penalty to a value of 5. Distance matrices were calculated with the algorithm of Jukes and Cantor (1969) employing the DNADIST program of the PHYLIP 3.57c package (Felsenstein 1989) and after excluding those nucleotide positions that differed in more than 50% of all sequences. Approximately 1,300 base positions were used to generate similarities. Phylogenetic trees were inferred from evolutionary distances by means of the FITCH program of the package. The sequence of the new isolate was deposited with EMBL under accession no. AJ011919.

Mol% G+C

The G+C content of the genomic DNA was determined by high-performance liquid chromatography as outlined by Meshbah et al. (1989).

Results

Isolation

After incubation for 1–3 months at 20 °C, pink, red, or brown-red cultures developed in all the samples. A spectroscopic analysis of the ten cultures revealed that the anoxygenic phototrophic bacteria enriched behind the narrow-band pass filter at a wavelength of 932 nm differed from those enriched behind the barrier filter at > 780 nm. The five cultures illuminated through the > 780 nm barrier filter had absorption maxima that are characteristic of the light-harvesting complex I (LH I; 890–895 nm) and LH II (800–806, 860–865 nm) of many purple sulfur and purple nonsulfur bacteria (Table 1). The absorption maxima at 1,020–1,025 nm could be attributed to phototrophic bacteria containing BChl *b*. In contrast, the five enrichment cultures incubated in infrared light at 932 (± 10) nm exhibited unusual absorption maxima positioned above 900 nm. This maximum was most pronounced in the sample from the black-colored microbial mat and centered around 913 nm (Table 1). The position of this long-wavelength absorption maximum indicated a selective enrichment of photosynthetic bacteria harboring a new type of light-harvesting complex.

In deep-agar dilution series inoculated with subsamples from the latter enrichment, small pink-colored colonies developed. Individual colonies were picked and used as inoculum for subsequent second and third deep-agar dilutions. Finally, pure cultures were isolated, and one strain (930I) was maintained as a stock culture.

Photosynthetic pigments

Pure cultures of strain 930I had a rose-red color. The absorption spectrum of whole cells (Fig. 1B) confirmed that the same type of phototrophic bacteria had been isolated as had been observed in the enrichment culture. Absorption maxima of whole cells were found at 380, 492, 515, 549, 595, 806, and 911 nm. In acetone/methanol extracts, absorption maxima were centered at 360, 466, 496, 530, 584, and 770 nm. Based on the long-wavelength absorption maximum at 770 nm, BChl *a* constitutes the antenna of the new type of light-harvesting complex in strain 930I. High-performance liquid chromatography of the pigment extracts confirmed that BChl *a* was the only bacteriochlorophyll present in strain 930I (H. Permentier and J. Amesz, University of Leiden, The Netherlands, personal communication).

The in vivo absorption peaks at 492, 515, and 549 nm are characteristic of the carotenoid spirilloxanthin. Based on our analysis of carotenoids by thin-layer chromatography, spirilloxanthin represents 59% of the total carotenoid content of the cells. A second, purple-red-colored band was observed on the TLC plates and was identified as lycopenal.

Morphology

Cells of strain 930I were gram-negative. Individual cells measured 0.5 (± 0.1) μm in width and 2.2 (± 0.4) μm in length, and had a spirilloid shape with one turn (Fig. 2). In addition, vibrio-shaped cells occurred. In the stationary growth phase, some of the cells reached a length of 10 μm. The morphology of cells grown with thiosulfate as electron donor either photolithoautotrophically or chemolitho-

Table 1 Absorption maxima of enrichment cultures from various natural samples incubated behind a 930-nm interference filter and behind a > 780-nm barrier filter after incubation for 3 months. *Values in parentheses* indicate absorption shoulders

Sample	Infrared absorption maxima (nm) after incubation at	
	932 (± 10) nm	> 780 nm
Sippewissett black mat	806, (874), 913	800, 870, 1,025
Sippewissett mat	806, 881, 930	805, 860, (890), 1,020
Sippewissett pond	803, 880–905 (broad peak)	800, 870
Oyster Pond	800, (875), 905	805, 865, (895), 1,020
Solar Lake mat	806, 885–905 (broad peak)	805, 865, (895)

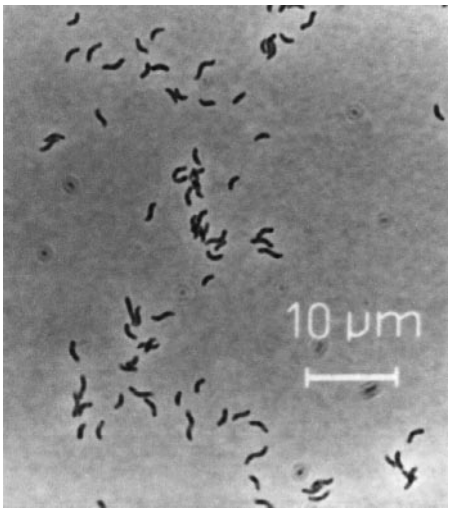

Fig. 2 Phase-contrast microphotograph of strain 930I

trophically in the dark was different. In these latter cultures, cells had a diameter of 0.7–0.9 µm and contained highly refractile inclusions.

Cells were highly motile by means of a single polar flagellum at each cell pole. Ultra-thin sectioning revealed the presence of lamellar stacks of intracellular membranes (Fig. 3A,B), which are rarely found among the phototrophic members of the α-subgroup of Proteobacteria (compare Fig. 7).

Physiology

Strain 930I was capable of photolithoautotrophic growth using sulfide or thiosulfate as electron donor (Table 2).

Fig. 3 A, B Electron microphotographs of strain 930I. **A** Ultra-thin longitudinal section showing the lamellar stacks of the intracellular membrane system. **B** Ultra-thin transverse section (*bars* = 0.3 µm)

Microscopic examination of cultures growing with sulfide as electron donor and at a pH of 7.2–7.7 revealed that cells of strain 930I formed extracellular sulfur globules. At pH values above 7.7, the culture fluid turned yellow during the exponential growth phase. This latter observation indicated that polysulfides were formed abiotically from extracellular sulfur and the sulfide still present in the medium. Upon addition of neutralized sulfide solution (final concentration, 1 mM) to sulfide-depleted cultures, an initial inhibition of growth was observed.

In medium containing 2 mM sulfide and 2 mM acetate, strain 930I formed 1.99 mM of sulfur and 93.5 mg dry cell mass l^{-1} (corrected for the amount of elemental sulfur present on the filter). No formation of sulfate was observed. If acetate could only be used by reductive carboxylation according to:

$$19H_2S + 8CH_3COOH + 8CO_2 + 6NH_3 \rightarrow$$
$$19\ S + 6 < C_4H_8O_2N > + 20\ H_2O,$$

a significantly lower cell yield of 64.5 mg dry cell mass l^{-1} would have been expected. Consequently, utilization of acetate is not limited to its assimilation by reductive carboxylation in strain 930I, and it has to be assumed that part of the acetate can serve as an additional electron-donating substrate.

Besides acetate, cells of strain 930I used 15 different substrates in the presence of sulfide and hydrogen carbonate. Twenty-five other substrates were tested but not used (Table 2). The addition of yeast extract enhanced the growth of strain 930I significantly. Elemental sulfur (supplied as sulfur flower) was used as sulfur source for biomass formation, whereas sulfite or sulfate were not assimilated.

Growth of strain 930I became light-saturated at intensities from 50 µmol quanta m^{-2} s^{-1} of daylight fluorescent tubes (Fig. 4). No growth was obtained at ≤ 20 µmol quanta m^{-2} s^{-1}. The optimal growth temperature was 30 °C. Maximum growth rates were observed at pH 7.9 and at NaCl concentrations of 1–2% (w/v; equivalent to 155–310 mM). High growth rates (88% of the maximum growth rates) were measured even at 6.2% NaCl. The

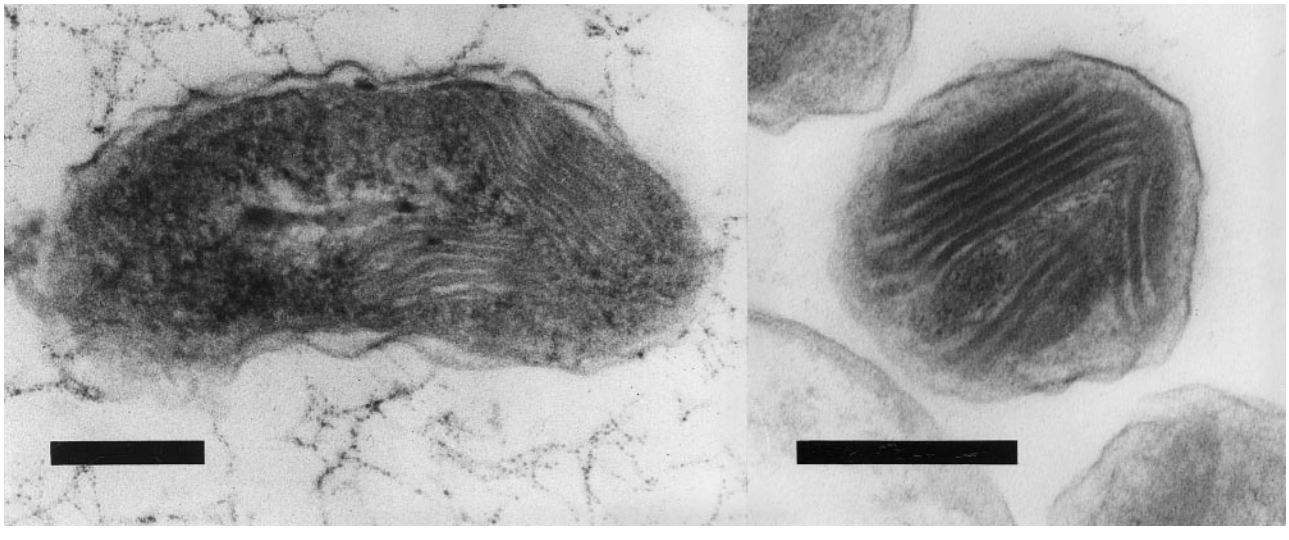

Table 2 Physiological characteristics of strain 930I as compared to all other known spirilloid mesophilic phototrophic bacteria of the α-Proteobacteria subgroup. Substrate concentrations are in mM (as used for growth tests with strain 930I); complex substrates are in % (w/v). Substrates tested but not utilized by strain 930I (concentrations in mM): glucose (2), formate (1), methanol (1), ethanol (1), propanol (1), butanol(1), pentanol (1), 1,2-ethanediol (1), 1,2-propanediol (1), 1,2-butanediol (1), 2,3-butanediol (1), glycerol (2), mannitol (2), caproate (2), caprylate (2), tartrate (2), glycolate (2), citrate (2), gluconate (2), acetone (2), benzoate (1), trimethoxybenzoate (1), ascorbate (2), L(+)-arginine (2), and peptone (0.025%). (+) Slight increase in cell yield (OD_{650} > 150% of that of the negative control), + high increase in cell yield (OD_{650} > 300% of that of the negative control), +/– utilization by only some of the strains, and – no utilization (*nd* not determined, *Psp. Phaeospirillum*, *Rdv. Rhodovibrio*, *Rha. Rhodospira*, *Rsa. Roseospira*, *Rsp. Rhodospirillum*, and *Rtl. Rhodothalassium*)

Substrate (mM)	930I	*Rtl. salexigens*	*Psp. molischianum*	*Psp. fulvum*	*Rsp. rubrum*	*Rsp. photometricum*	*Rha. trueperi*	*Rdv. sodomense*	*Rdv. salinarium*	*Rsa. mediosalina*
Fructose (2)	+	–	–	–	+/–	+	nd	nd	–	–
Acetate (2)	+	+	+	+	+	+	+	+	–	+
Propionate (1)	+	nd	+	+	+	+/–	+	–	–	+
Butyrate (2)	+	nd	+	+	+	+	+	–	nd	+
Valerate (2)	+	nd	+	+	+	+	+	nd	nd	–
2-Oxoglutarate (2)	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pyruvate (2)	+	+	+	+	+	+	+	+	–	+
Lactate (2)	(+)	–	+/–	–	+	+	+	nd	+	+
Malate (2)	+	nd	+	+	+	+	+	+	–	+
Succinate (2)	+	+	+	+	+	+	+	+	–	+
Fumarate (2)	+	nd	+	+	+	+	+	nd	–	+
Malonate (2)	(+)	nd	–	nd	nd	nd	nd	nd	nd	–
L(+)-Alanine (2)	(+)	nd	nd	nd	+	+	nd	nd	nd	+
L(+)-Glutamate (2)	+	+	nd	nd	+	+	nd	nd	–	+
Casamino acids (0.1%)	(+)	+	+	nd	nd	nd	nd	nd	+	+
Yeast extract (0.1%)	+	nd	+	nd	+	nd	nd	+	+	+
Electron donors (mM)										
H_2S (1)	+	–	–	–	+	–	+	–	–	+
$S_2O_3^{2-}$ (2)	+	–	nd	nd	nd	nd	–	nd	nd	–
Assimilatory sulfate reduction	–	+	nd	+	+	+	–	nd	nd	+

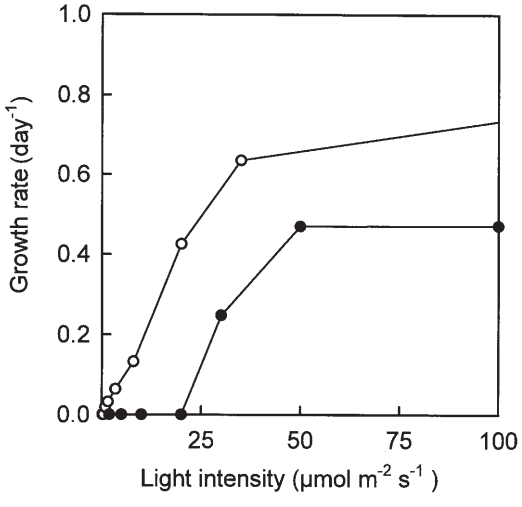

Fig. 4 Light-dependence of growth of strain 930I (●) and of the purple sulfur bacterium *Amoebobacter purpureus* strain ML1 (○). Daylight fluorescent tubes were used for illumination

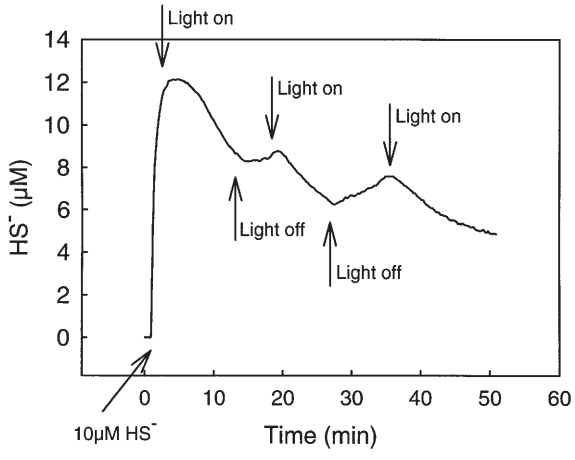

Fig. 5 Light-dependence of photosynthetic sulfide oxidation in dense cell suspensions of strain 930I illuminated with infrared light at a wavelength of 932 nm (see transmission curve in Fig. 1A). Increases in sulfide during dark phases are caused by reduction of sulfur to sulfide in the course of the fermentation of intracellular storage compounds

maximum growth rate of strain 930I determined during the present work was 2.38 day^{-1}, corresponding to a doubling time of 7 h. Microaerophilic growth was observed at a partial pressure of 1 kPa O_2 in the presence of thiosulfate, or with thiosulfate plus acetate.

The capacity of strain 930I to utilize infrared light at 932 nm for sulfide oxidation was demonstrated in short-term experiments in electrode chambers (Fig. 5). The oxidation of sulfide clearly correlated with the availability of

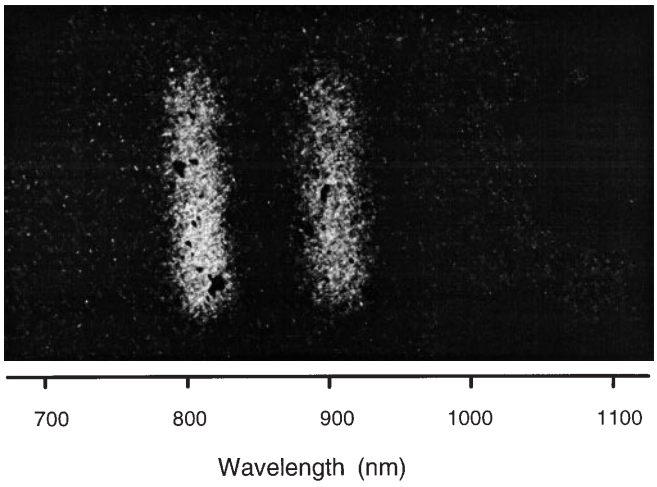

Fig. 6 Scotophobic accumulation of cells of strain 930I in the long-wavelength range between 650 and 1,150 nm. A low-resolution dark-field photomicrograph is shown; each *white dot* represents a single bacterial cell

infrared light. During the dark periods of the experiment, sulfide formation was observed that has to be attributed to the fermentation of intracellular glycogen with a concomitant reduction of elemental sulfur to sulfide (Van Gemerden 1968).

Scotophobic response to infrared light

Cells of strain 930I were capable of scotophobic accumulation at wavelengths above 900 nm. When light spectra between 650 and 1,100 nm were projected onto microcuvettes on a microscope stage, the cells accumulated in two distinct bands at wavelengths of 800–810 and 900–910 nm (Fig. 6). These wavelengths correspond to the absorption maxima of whole cells (compare Fig. 1B), indicating that the light-harvesting pigment-protein complexes also act as the photosensor for scotophobic response.

Phylogenetic affiliation

The DNA base composition was 71.2 mol% G+C. Sequence comparison of 16S rRNA genes indicated that strain 930I is most closely related to *Rhodobium orientis* (Table 3; Figs. 7, 8), albeit at a low similarity level of 93.5%.

Discussion

Ecophysiology of the new isolate

In the present study, a selective enrichment of strain 930I could only be achieved by incubation behind a narrow-band pass filter instead of an IR barrier filter. Our results (Table 1) indicate that bacteria containing unusual light-harvesting complexes with absorption maxima above 900 nm are widely distributed among the different types of microbial mats, but that they have a competitive advantage only in light of a narrow wavelength range.

Infrared light > 780 nm has been used previously to selectively enrich a new type of purple sulfur bacterium from a littoral lake sediment (Overmann et al. 1992). The reason for the low competitive success of strain 930I in broad-band-width infrared light may be the inhibition of growth by high concentrations of sulfide in the growth medium (Hansen and Van Gemerden 1972). According to our results, strain 930I is inhibited by sulfide and, therefore, may be outcompeted by other anoxygenic phototrophic bacteria in the sulfide-reduced enrichment medium. This conclusion is also supported by the fact that purple sulfur bacteria (identified by their intracellular sulfur globules) were present in the sample from the black-colored microbial mat that was incubated at wavelengths > 780 nm. Another reason for the low competitive success in broad-band-width infrared light may be the requirement for growth factors, which were present at only low concentrations in the enrichment cultures. Indeed, growth of strain 930I was significantly stimulated by yeast extract, which was missing in the enrichment cultures.

The minimum light requirement of strain 930I was > 20 μmol quanta m^{-2} s^{-1} in fluorescent light and, thus,

Table 3 Partial evolutionary distance matrix of 16S rRNA sequences of strain 930I and the most closely related reference strains. Values *upper right* are uncorrected percentages of similarity, those *lower left* are K_{nuc} values corrected for multiple base change by the method of Jukes and Cantor (1969) (*Bcl. Blastochloris*, *Rdb. Rhodobium*, *Rmi. Rhodomicrobium*, *Rpl. Rhodoplanes*, *Rps. Rhodopseudomonas*, *Rtl. Rhodothalassium*)

		1	2	3	4	5	6	7	8	9	10
1	Strain 930I	–	89.8	89.5	90.8	91.8	91.5	91.2	92.1	**93.5**	90.5
2	*Rps. palustris* DSM123^T	0.1099	–	93.1	92.0	92.7	92.8	91.1	91.0	90.6	87.6
3	*Rps. acidophila* DSM137^T	0.1131	0.0728	–	92.7	93.5	93.3	90.9	90.8	91.6	88.3
4	*Bcl. viridis* DSM133^T	0.0985	0.0842	0.0765	–	95.8	95.6	91.6	93.1	93.5	90.3
5	*Rpl. elegans* AS130	0.0866	0.0765	0.0683	0.0429	–	98.6	93.0	92.7	93.3	89.4
6	*Rpl. roseus* DSM5909^T	0.0901	0.0759	0.0699	0.0454	0.0137	–	92.7	92.3	92.8	89.3
7	*Rmi. vannielii* ATCC51194^T	0.0933	0.0949	0.0970	0.0891	0.0732	0.0767	–	91.6	92.2	90.4
8	*Rdb. marinum* DSM2698^T	0.0832	0.0959	0.0984	0.0728	0.0766	0.0811	0.0891	–	95.1	91.4
9	*Rdb. orientis* JCM9337	**0.0683**	0.1007	0.0886	0.0679	0.0707	0.0752	0.0820	0.0510	–	91.6
10	*Rtl. salexigens* DSM2132^T	0.1012	0.1352	0.1272	0.1043	0.1141	0.1152	0.1032	0.0912	0.0887	–

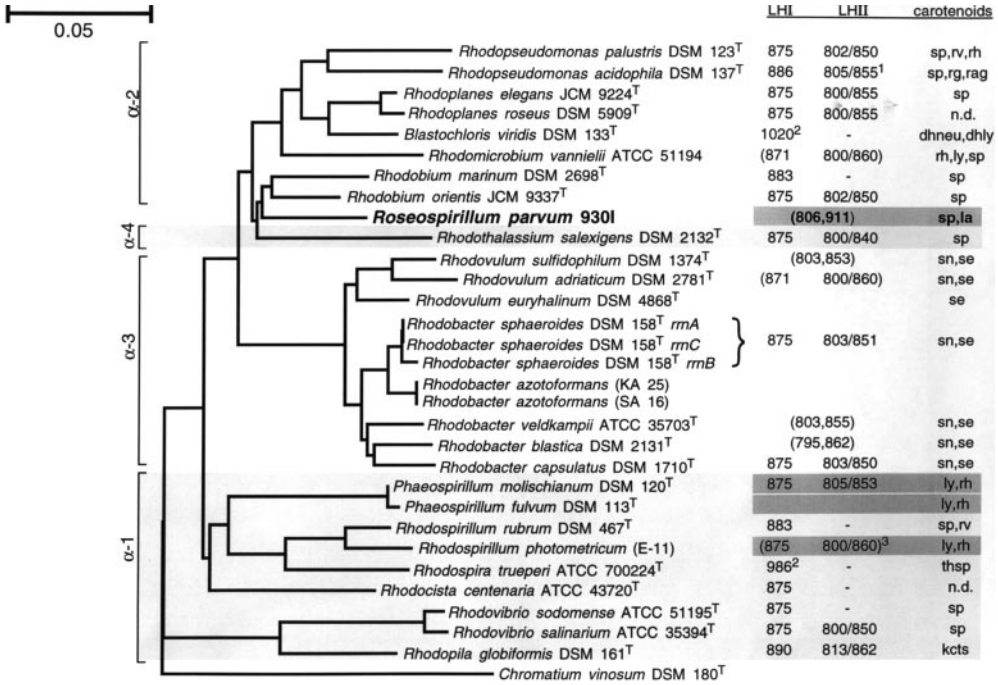

Fig. 7 Phylogenetic tree, photosynthetic light-harvesting (LH) complexes (Drews 1985; Angerhofer et al. 1986; Zuber and Cogdell 1995), and carotenoids (Imhoff 1995) of purple nonsulfur bacteria. [1] Depending on light intensity, an additional light-harvesting complex (B800-820, absorption maxima at 804 and 829 nm) is present in *Rhodopseudomonas acidophila*; [2] contains BChl *b* instead of BChl *a*; [3] low-temperature absorption spectra indicate the presence of a second light-harvesting complex (LHII) in *Rhodospirillum photometricum* DSM 2341 (Francke 1996). *Numbers in brackets* are the absorption maxima of species in which the type of antenna complexes are not known [taken from Pfennig and Trüper (1989); two numbers separated by a comma], or give antenna complexes found in other strains of the same species (Francke 1996). The new combinations of species names of the genus *Rhodospirillum* as introduced by Imhoff et al. (1998) were used. *Dark-shaded boxes* mark species with lamellar stacks of photosynthetic membranes. *Light-shaded boxes* mark α-1 and α-4 subgroups for easier comparison (*dhneu* 1,2-dihydroneurosporene, *dhly* 1,2-dihydrolycopene, *kcts* ketocarotenoids, *la* lycopenal, *ly* lycopene, *rag* rhodpinal glucoside, *rg* rhodopin glucoside, *rh* rhodopin, *rv* rhodovibrin, *se* spheroidene, *sn* spheroidenone, *sp* spirilloxanthin, *thsp* tetrahydrosprilloxanthin, *ATCC* American Type Culture Collection, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen, *JCM* Japan Collection of Microorganisms, *nd* not determined, and – not present; *bar* 5% estimated sequence divergence)

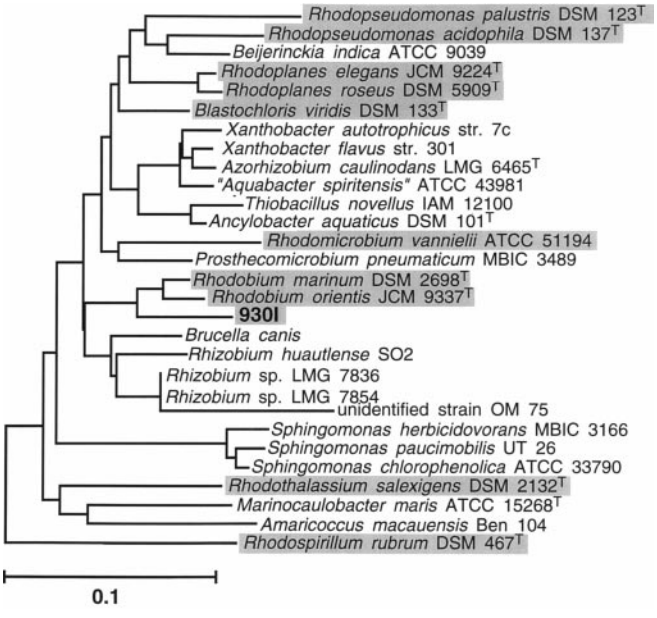

Fig. 8 Phylogenetic comparison between strain 930I and the most closely related phototrophic (*shaded boxes*) and nonphototrophic taxa of the α-Proteobacteria (*bar* 0.1 fixed changes per nucleotide base)

was significantly higher than for several species of the Chromatiaceae (5 μmol quanta m^{-2} s^{-1} for *Thiorhodovibrio winogradskyi* SSP1; 1 μmol quanta m^{-2} s^{-1} for *Amoebobacter purpureus* ML1 and *Thiocapsa roseopersicina* ML2) using the same light source (Overmann et al. 1991, 1992). At illumination with daylight fluorescent tubes, light is harvested largely by carotenoids. The higher light requirement of strain 930I compares well with the spectroscopical properties of its photosynthetic antenna complex. Fluorescence spectroscopy revealed that the efficiency of energy transfer from carotenoids to BChl *a* is only 21–24% in strain 930I, but > 80% in *Amoebobacter purpureus* (H. Permentier and J. Amesz, University of Leiden, The Netherlands, personal communication). The transfer efficiency of strain 930I is low compared to that of other purple nonsulfur bacteria (25–100%; Angerhofer et al. 1986; Cogdell and Frank 1987). Due to the absorption characteristics of lake water, the underwater light spectrum progressively narrows with depth. Therefore, mainly blue-green or green light reaches deeper water layers, and light energy can be harvested only by carotenoids (Van Gemerden and Mas 1995). Therefore, strain 930I will not be able to compete with many purple sulfur bacteria or other purple nonsulfur bacteria under these condi-

Table 4 Accession numbers of bacterial sequences used in the phylogenetic analysis of the α-subgroup of Proteobacteria (*ATCC* American Type Culture Collection, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen, and *JCM* Japan Collection of Microorganisms)

Species	Strain	EMBL/ GenBank accession no.
Rhodopseudomonas palustris	DSM 123^T	L11664
Rhodopseudomonas acidophila	DSM 137^T	M34128
Rhodoplanes elegans	JCM 9224^T	D25311
Rhodoplanes roseus	DSM 5909^T	D25313
Blastochloris viridis	DSM 133^T	D25314
Rhodomicrobium vanielii	ATCC 51194	M34127
Rhodobium marinum	DSM 2698^T	D30790
Rhodobium orientis	JCM 9337^T	D30792
Roseospirillum parvum	DSM 12498^T	AJ011919
Rhodothalassium salexigens	DSM 2132^T	M59070
Rhodovulum sulfidophilum	DSM 1374^T	D16423
Rhodovulum adriaticum	DSM 2781^T	D16418
Rhodovulum euryhalinum	DSM 4868^T	D16426
Rhodobacter sphaeroides	DSM 158^T (*rrnA*)	X83853
Rhodobacter sphaeroides	DSM 158^T (*rrnB*)	X83854
Rhodobacter sphaeroides	DSM 158^T (*rrnC*)	X83855
Rhodobacter azotoformans	KA 25	D70846
Rhodobacter azotoformans	SA 16	D70847
Rhodobacter veldkampii	ATCC 35703^T	D16421
Rhodobacter blastica	DSM 2131^T	D16429
Rhodobacter capsulatus	DSM 1710^T	D16428
Phaeospirillum molischianum	DSM 120^T	M59067
Phaeospirillum fulvum	DSM 113^T	M59065
Rhodospirillum rubrum	DSM 467^T	D30778
Rhodospirillum photometricum	E-11	D30777
Rhodospira trueperi	ATCC 700224^T	X99671
Rhodocista centenaria	ATCC 43720^T	D12701
Rhodovibrio sodomense	ATCC 51195^T	M59072
Rhodovibrio salinarium	ATCC 35394^T	M59069
Rhodopila globiformis	DSM 161^T	M59066
Chromatium vinosum	DSM 180^T	M26629

tions. In contrast, carotenoids are of minor importance in intertidal sandy sediments, in which mainly light of the far-red and near-infrared wavelength region reaches the anoxic layers (Jørgensen and DesMarais 1986; Pierson et al. 1990; Lassen et al. 1992; Garcia-Pichel et al. 1994; Kühl et al. 1994).

Bacteria with absorption maxima between 900 and 930 nm were predominantly enriched from the black-colored mat, but not from the other samples. Compared to other laminated microbial mats, the attenuation of visible light is even more pronounced within the black-colored cyanobacterial mats of Sippewissett Salt Marsh. The black coloration of these mats is caused by sunscreen pigments such as the yellow-brown sheath pigment scytonemin, which is synthesized by cyanobacteria under intense solar radiation (Garcia-Pichel and Castenholz 1991). The absorption spectrum of scytonemin has its maximum at 370 nm, but also a broad shoulder between 400 and 550 nm. Absorption extends into the far-red region to 750 nm (Garcia-Pichel and Castenholz 1991). This restricts the wavelength range of light available for photosynthesis. Therefore, it appears plausible that competition for infrared light will be more severe in the black mats as compared to other mats in sandy sediments. Obviously, species that are able to exploit infrared wavelengths not harvested by other phototrophic bacteria have a selective advantage, especially in the black-colored cyanobacterial mats.

In situ measurements of radiance or irradiance have mostly been limited to a wavelength range ≤ 900 nm (Lassen et al. 1992; Garcia-Pichel et al. 1994; Kühl et al. 1994). In those cases where the spectral light distribution has also been determined at longer wavelengths, no distinct absorption maxima have been detected between 900 and 950 nm (Jørgensen and Des Marais 1986; Pierson et al. 1990). It therefore remains unclear whether bacteria such as strain 930I can form dense accumulations under natural conditions or rather represent metabolically flexible ubiquists present in low cell numbers.

To date, the new type of light-harvesting complex with an absorption maximum at 911–920 nm has been found in two different phylogenetic lineages, namely the α-Proteobacteria (strain 930I) and the γ-Proteobacteria (*Chromatium tepidum*; Garcia et al. 1986). It is therefore likely that this type of light-harvesting complex is more widespread among the different phylogenetic groups of purple nonsulfur and purple sulfur bacteria.

Comparison with established species of anoxygenic phototrophic bacteria and with nonphototrophic members of the α-Proteobacteria

The most obvious difference of the newly isolated strain 930I to all known photosynthetic species of the α- and β-Proteobacteria is the long-wavelength absorption maximum of intact cells at 911 nm. The intracellular photosynthetic membranes are present as lamellar stacks. By comparison, only 3 out of 27 species of the phototrophic α-Proteobacteria, namely *Phaeospirillum molischianum*, *Phaeospirillum fulvum*, and *Rhodospirillum photometricum*, show this membrane arrangement (Imhoff 1995). Based on our analysis of all available 16S rDNA gene sequences, strain 930I is only distantly related to the other three species. Therefore, a lamellar arrangement of the intracellular photosynthetic membranes appears to have no phylogenetic implications (Fig. 7). In addition, the pigment composition of strain 930I differs from that of the other three species.

Cells of strain 930I utilize sulfide and thiosulfate as electron donor, and lack assimilatory sulfate reduction. Among the phototrophic members of the α-Proteobacteria with spirilloid cell morphology, *Rhodospira trueperi* is the only one known to date that also lacks assimilatory sulfate reduction (Pfennig et al. 1997) (Table 2).

The phylogenetic distance to the most closely related species, *Rhodobium orientis* JCM 9337, is comparable to distances between the different genera of other photo-

trophic members of the α-Proteobacteria (less than 93.5% similarity; Table 3; also compare *Rhodobacter*/*Rhodovulum* or *Rhodospirillum*/*Rhodospira* in Fig. 7). In addition, the phenotypic differences between strain 930I and the two known *Rhodobium* species are considerable and do not permit an affiliation of strain 930I with the genus *Rhodobium*. Similarly, *Rhodothalassium salexigens* is also only distantly related to strain 930I (similarity, 90.5%). Incidentally, our analysis revealed a closer phylogenetic relationship of *Rhodothalassium salexigens* to strain 930I, *Blastochloris viridis*, *Rhodomicrobium vannielii*, and the *Rhodobium* species (Fig. 7) than to *Rhodovibrio sodomense* and *Rhodovibrio salinarium*. This result is in contrast to a previous analysis that had been limited to the spiral-shaped phototrophic purple nonsulfur bacteria (Imhoff et al. 1998).

Based on our results, strain 930I contains a new type of light-harvesting pigment-protein complex that absorbs at wavelengths above 900 nm. Its distinct absorption characteristics imply that the pigment-protein complex of strain 930I differs in structure from those of all known species and genera of phototrophic α- and β-Proteobacteria. The sequence similarity to the phylogenetically most closely related strains of phototrophic α-Proteobacteria is at a level that is widely accepted for differentiating between genera. In addition, strain 930I exhibits a combination of features (lamellar stacks of intracellular membranes, lack of sulfate reduction, and spirilloid morphology) that are rare among the purple nonsulfur bacteria.

Furthermore, the phylogenetic distance of the 16S rRNA gene sequence of strain 930I to all other taxa of the α-Proteobacteria, including the nonphototrophic species, was investigated. The ten most closely related full 16S rRNA sequences were retrieved from the RDP and GenBank databases, respectively, as described in Materials and methods. In addition, all sequences of nonphototrophic relatives of the four most closely related phototrophic species were retrieved from the RDP database (2.14.1.7 *Rhodothalassium salexigens* group; 2.14.1.9.5 *Rhodopseudomonas viridis* subgroup; 2.14.1.9.6 *Rhodomicrobium vannielii* assemblage; and 2.14.1.9.16 *Rhodobium marinum* subgroup). A phylogenetic tree was constructed for the total of 29 sequences using *Rhodospirillum rubrum* as outgroup. This analysis (Fig. 8) demonstrated that the closest known relatives of strain 930I are the phototrophic *Rhodobium marinum* and *Rhodobium orientis* and, thus, fully confirmed the phylogenetic separatedness of the *Rhodobium*/*Roseospirillum* cluster.

Based on their phylogenetic position, pigment composition, and (in some cases) lipid composition, many phototrophic species of the α-subgroup of the Proteobacteria have been reclassified, or newly described, as members of new genera [*Blastochloris* (Hiraishi 1997); *Phaeospirillum*, *Rhodovibrio*, *Rhodothalassium*, and *Roseospira* (Imhoff et al. 1998); and *Rhodospira* (Pfennig et al. 1997)]. Applying the same criteria to the classification of strain 930I, the distinct differences between strain 930I and other phototrophic α-Proteobacteria require the description of a new genus, notwithstanding the fact that phylogenetic analyses based on 16S rRNA gene sequences have led to an enormous increase in the number of new genera and species. The unique absorption spectrum represents a valuable diagnostic feature that can be used to rapidly differentiate members of this genus from other purple nonsulfur bacteria.

Based on the cumulative physiological and phylogenetic evidence, the new genus *Roseospirillum* is proposed for our isolate, with the species name *Roseospirillum parvum* gen. nov. sp. nov.

Description of the genus *Roseospirillum* gen. nov.

Ro.se.o.spi.ril'lum. *roseus* L.adj., rosy; *spirillum* M.L.neut.n., a screw-shaped cell; *Roseospirillum* M.L.neut.n., the rosy *spirillum*.

Cells are vibroid- to spirilloid-shaped. Multiplication by binary fission; motile by means of flagella. Gram-negative. An intracytoplasmic membrane system is present in the form of lamellar stacks. Contains bacteriochlorophyll *a* and carotenoids as photosynthetic pigments. The absorption spectrum of the cells exhibit maxima at wavelengths of 806 and 911 nm, but no maxima at 850–870 nm (which are typical for light-harvesting complexes I or II of other anoxygenic phototrophic bacteria).

Growth occurs preferentially photomixotrophically, using sulfide or thiosulfate as electron donor and acetate or other organic acids as carbon substrates under anoxic conditions in the light. Lacks assimilatory sulfate reduction. A source of growth factors such as yeast extract is required.

Based on the 16S rRNA gene sequence of the type strain 930I (DSM 12498^T) of its type species, the genus belongs to the α-subgroup of the Proteobacteria.

Description of the type species *Roseospirillum parvum* sp. nov.

Ro.seo.spi.ril'lum. L.adj. *roseus* rosy; M.L. neut. n. *Spirillum* a screw-shaped cell. M.L. neut. n. *Roseospirillum* the rosy *Spirillum*.

Cells vibrioid to spirilloid, 0.4–0.6 μm × 1.8–2.6 μm. Motile by means of bipolar flagella. Anaerobically grown cultures are pink to pinkish-red. Living cell suspensions exhibit absorption maxima at 380, 492, 515, 549, 595, 806, and 911 nm. Photosynthetic pigments are bacteriochlorophyll *a* and the carotenoids spirilloxanthin and lycopenal.

Phototrophic growth under anoxic conditions. Sulfide and thiosulfate can serve as electron donors for photosynthesis. Elemental sulfur is used as sulfur source for biomass formation. Lacks assimilatory sulfate reduction. In the presence of sulfide and bicarbonate, fructose, acetate, propionate, butyrate, valerate, 2-oxoglutarate, pyruvate, lactate, malate, succinate, fumarate, malonate, L(+)-alanine, L(+)-glutamate, casamino acids, and yeast extract are used.

Optimal growth occurs at 30 °C, pH 7.9, 1–2% NaCl, and a light intensity above 50 µmol quanta m^{-2} s^{-1} (fluorescent light). Minimum light requirement is 25 µmol quanta m^{-2} s^{-1}.

The nucleotide sequence of the 16S rRNA gene of the type strain shows 93.5% similarity to the sequence of *Rhodobium orientis* JCM 9337. The G+C content of the DNA is 71.2 mol%. Habitat: anoxic layer of a black-colored cyanobacterial mat of Great Sippewissett Salt Marsh (Cape Cod, Mass., USA) Type strain: 930I, DSM 12498^T.

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