Isolation and Characterization of *Rhodovulum strictum* sp. nov. and Some Other Purple Nonsulfur Bacteria from Colored Blooms in Tidal and Seawater Pools

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Several strains of phototrophic purple nonsulfur bacteria were isolated from colored blooms occurring in tidal and seawater pools in Japan. All of these isolates had ovoid to rod-shaped cells that were motile by means of single polar flagella and contained vesicular intracytoplasmic membranes together with bacteriochlorophyll *a* and carotenoids of the spheroidene series. They produced ubiquinone 10 as the major quinone and contained straight-chain fatty acids, with C_{18:1} predominating. They were mesophilic, halophilic, and photoheterotrophic, utilized sulfide and thiosulfate as electron donors for phototrophic growth, and photoassimilated a wide variety of organic compounds as carbon sources. Our results suggested that all of these isolates are members of the recently described genus *Rhodovulum*. The isolates were classified into four groups (designated groups I through IV) on the basis of phenotypic and genotypic data. The group I isolates, which were the most abundant purple nonsulfur bacteria recovered from the blooms, grew in the presence of NaCl concentrations ranging from 0.5 to 3.0% (optimum NaCl concentration, 0.8%) and at pH values ranging from 7.5 to 9.0 (optimum pH, 8.0 to 8.5). On the basis of these unique physiological traits, together with genotypic and phylogenetic data, we propose that the group I isolates should be classified as members of a new species, *Rhodovulum strictum*. The group II isolates were identified definitely as *Rhodovulum sulfidophilum*, and the group III and IV isolates were phenotypically most similar to *R. sulfidophilum* and *Rhodovulum adriaticum*, respectively, but could be differentiated from these species by DNA-DNA pairing data.

Sunlight-exposed stagnant water in tidal and seawater pools in which sulfide is produced during rapid decomposition of organic matter provides growth conditions favorable for anoxygenic phototrophic bacteria. In such environments, these bacteria often occur in great numbers and are visible as pink, red, brown, or green blooms. In general, blooms of phototrophic bacteria contain sulfur bacteria as their major constituents (25, 29), whereas color development caused by purple nonsulfur bacteria has rarely been encountered (20). However, there have been scattered reports concerning the frequent occurrence of purple nonsulfur bacteria in tidal pool blooms (12, 28), apart from the question of whether these bacteria contribute to bloom formation. Because of these reports we became interested in identifying and determining the ecophysiological significance of the purple nonsulfur bacteria found in seawater pools and similar marine environments in which colored blooms occur.

We have isolated several strains of purple nonsulfur bacteria from phototrophic bacterial blooms occurring in coastal areas in Japan. In this paper, the taxonomic characteristics of these isolates are described, and we propose that some of them should be classified as members of a new species of the genus *Rhodovulum*, which is a recently described genus of phototrophic bacteria proposed (15).

MATERIALS AND METHODS

Bloom samples. Surface bloom samples were collected from tidal and seawater pools that were located at a sewage sludge disposal site and in mud flat and rocky

coastal areas in Japan (Table 1). The samples were collected in autoclave-sterilized polyethylene bottles, placed in an insulated cooler for transport to the laboratory, and examined within 6 h. The bloom samples were diluted decimally with a sterile 2.5% NaCl solution containing 2 mM sodium ascorbate (filter sterilized), and 1-ml portions of each of the dilutions were used for enrichment cultures.

Enrichment and isolation. A liquid medium (pH 7.0) consisting of mineral base RM2 (containing 7.6 mM ammonium sulfate as the sole nitrogen source) (12), 10 mM sodium acetate, 10 mM sodium succinate, 1 mM sodium thiosulfate, 1 mM cysteine (filter sterilized), 2 mM sodium ascorbate (filter sterilized), 2.5% NaCl, 0.01% yeast extract, and 1 ml of vitamin solution VA (20) per liter was used as the enrichment medium. Screw-cap test tubes (capacity, 20 ml), each of which contained 10 ml of the enrichment medium, were inoculated with 1-ml portions of appropriately diluted samples, completely filled with the same medium, and incubated at 28°C and 2,000 lx of incandescent illumination. The agar pour plating technique and a GasPak anaerobic system were used to isolate colonies from the enrichment cultures; to do this, we used 1.5% agar and the medium described above. Single colonies that exhibited colored growth were picked from the plates for standard purification by streaking.

Bacterial strains and cultivation. A total of 23 isolates recovered from the blooms were studied. The organisms used for comparison were *Rhodovulum adriaticum* DSM 2781^T (T = type strain), *Rhodovulum euryhalinum* DSM 4868^T, and *Rhodovulum sulfidophilum* DSM 1374^T; these strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. MMYS-I medium (pH 7.0), which contained mineral base RM2, 20 mM sodium DL-malate, 2 mM thiosulfate, 2.5% NaCl, and 0.1% yeast extract, was used for routine cultivation of the test organisms. For certain isolates, MMYS-I medium was modified by decreasing the concentration of NaCl to 0.8% and adjusting the pH to 7.8; the resulting medium was designated MMYS-II medium. MMYS-I medium supplemented with 0.5 mM sulfide was used for *R. euryhalinum*. All media contained 0.8, 1.4, and 1.8% agar when they were used for stab, slant, and plate cultures, respectively. Cells were grown anaerobically in the light in screw-cap test tubes or bottles filled with medium. Cultures on agar media were incubated either in GasPak anaerobic jars in the light or in an air incubator in the dark.

Analyses of quinones and fatty acids. Quinones were extracted with a chloroform-methanol mixture, purified by thin-layer chromatography, and analyzed by high-performance liquid chromatography (HPLC) as described previously (9, 10). Whole-cell fatty acids were analyzed by gas-liquid chromatography of their methyl ester derivatives as described previously (13).

Phenotypic characterization. Unless indicated otherwise, all test media were incubated at 30°C and 5,000 lx provided by incandescent lamps. Morphology and related properties were studied with an Olympus phase-contrast microscope and a JEOL transmission electron microscope. The photosynthetic membrane systems present in cells were determined by electron microscopy of ultrathin sec-

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TABLE 1. Bloom samples investigated and recovery of phototrophic purple nonsulfur bacteria

Sample no.	Date	Location ^a	Color of bloom	Recovery of phototrophic purple nonsulfur bacteria ^b
1	August 1985	Sludge disposal site, Tokyo	Pink	+
2	October 1985	Sludge disposal site, Tokyo	Pink	—
3	July 1986	Rocky coast, Chiba	Brown	—
4	July 1986	Rocky coast, Chiba	Red	—
5	August 1986	Rocky coast, Kanagawa	Brown	—
6	August 1986	Rocky coast, Kanagawa	Green	+
7	September 1986	Mud flat, Chiba	Pink	+
8	September 1986	Mud flat, Chiba	Red	+
9	August 1987	Mud flat, Chiba	Pink	—
10	August 1987	Rocky coast, Kanagawa	Green	—
11	August 1987	Rocky coast, Kanagawa	Yellow	—
12	August 1988	Rocky coast, Kanagawa	Red	—
13	August 1988	Rocky coast, Kanagawa	Yellow	—
14	September 1989	Rocky coast, Kanagawa	Red	—
15	September 1989	Rocky coast, Kanagawa	Green	—

^a All locations are in Japan.^b +, bacteria recovered; —, bacteria not recovered.

tions, using a standard method as described previously (11). Absorption spectra of intact cells and cell extracts were determined with a Hitachi model U-2000 spectrophotometer. Tests to determine NaCl ranges for growth were performed with MMYS-I or MMYS-II medium containing different concentrations of NaCl. Tests to determine pH ranges for growth were performed with the media described above; the pH was adjusted with 0.1 to 1 M HCl and NaOH and, for alkaline pH values, with 0.5 M sodium carbonate. Carbon source utilization tests were performed in screw-cap test tubes containing mineral base medium RM2 supplemented with 0.8 or 2.5% NaCl, a neutralized, filter-sterilized, organic compound as the carbon source, and 0.01% yeast extract as the growth factor. For isolates that required a reduced sulfur compound as the sulfur source, 2 mM thiosulfate or 2 mM cysteine (filter sterilized) was added. Sulfur source utilization tests were performed in a medium similar to that used for carbon nutrition tests; in these tests the nitrogen source was replaced by 5 mM ammonium chloride and malate was used as the carbon source. The sulfur source was either 2 mM sodium sulfate, 2 mM sodium thiosulfate, 2 mM cysteine (filter sterilized), or 0.5 mM sodium sulfide (neutralized). Photoautotrophic growth was determined by using the mineral medium supplemented with either 0.5 mM sodium sulfide (neutralized) or 2 mM sodium thiosulfate as the electron donor, 20 mM sodium bicarbonate (filter sterilized), and 0.01% yeast extract. The final oxidation products during photoautotrophic growth with the sulfur compounds were determined by measuring elemental sulfur (1) and sulfate (4) contents in the growth medium. In all growth response tests, growth was monitored turbidometrically at 660 nm with a model ANA-75 spectrophotometer (Tokyo Photoelectric Co., Tokyo, Japan), with the final reading performed after 2 weeks of incubation. Test tubes in which the optical density at 660 nm (OD_{660}) was greater than the OD_{660} of a negative control containing no test substrate were considered positive for growth. Hydrolysis of gelatin and hydrolysis of Tween 80 were determined by using cells grown either anaerobically in the light or aerobically in the dark on the agar media of Hoshino and Satoh (16) and Sierra (27), respectively; both test media were modified by adding 0.8 or 2.5% NaCl and 0.05% yeast extract.

DNA base composition and DNA-DNA hybridization. Genomic DNA was extracted and purified by the method of Marmur (23). The guanine-plus-cytosine (G+C) content of genomic DNA was determined by HPLC of nuclease P1 hydrolysates of the DNA, using external nucleotide standards as described previously (11). DNA-DNA reassociation studies were performed by the quantitative dot blot hybridization method with photobiotin labeling and colorimetric detection (5, 11), and the levels of hybridization were determined by measuring the color intensities of spots with a Shimadzu model CS-9000 two-dimensional computing densitometer (11).

16S rRNA gene sequencing and phylogenetic analysis. 16S ribosomal DNA (rDNA) fragments that corresponded to positions 8 to 1510 of *Escherichia coli* 16S rRNA (2) were amplified by PCR from cell lysates and were sequenced directly by the cycle sequencing method modified for automated fluorescence detection with a Pharmacia DNA sequencer. Detailed information concerning the PCR and sequencing procedures used has been given previously (8, 14). Sequence data were compiled and levels of binary sequence similarity were calculated with the GENETYX computer program (Software Development Co., Tokyo, Japan). Set sequences were aligned and nucleotide substitution rates (K_{muc} values) (21) were calculated with the CLUSTAL V program (7). A phylogenetic tree was reconstructed by the neighbor-joining method (26).

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in this study have been deposited in the DDBJ, EMBL, GSDB, and NCBI nucleotide sequence databases under accession numbers D32245 and D32246.

The accession numbers for the sequences used to construct the distance matrix tree are as follows: *R. sulfidophilum*, D16423 and D-16430; *R. euryhalinum*, D16426; *R. adriaticum*, D16418; strain MB260, D16420; strain MB-G2^T, D16419; *Rhodobacter capsulatus*, D-16427 and D16428; *Rhodobacter sphaeroides*, D-16424 and D16425; *Rhodobacter veldkampii*, D16421; *Rhodobacter blasticus*, D16429; *Paracoccus denitrificans*, X69159; *Roseobacter denitrificans*, M59070; and *Rhodospirillum rubrum*, D30778.

RESULTS

Microscopic studies of bloom samples and isolation. A total of 15 bloom samples were obtained from different coastal areas in Japan (Table 1). Microscopic observations (data not shown) revealed that large cells (1.5 to 3.5 by 2.0 to 7.0 μm) of *Thiopedia*- and *Chromatium*-like purple bacteria with elemental sulfur globules inside and of *Lamprobacter*-like bacteria with gas vacuoles occurred in high numbers in the red blooms, whereas much smaller bacteria (0.5 to 0.8 by 0.9 to 1.2 μm) morphologically similar to members of the genus *Prostecochloris* predominated in the green blooms. Of particular importance was the finding that small ovoid to rod-shaped cells (0.5 to 0.8 by 1.0 to 1.5 μm), possibly cells of purple nonsulfur bacteria, were present in significant numbers in some of the red blooms.

We first attempted to enrich and isolate purple nonsulfur bacteria from all bloom samples. In most cases, the sulfur bacteria overgrew the other organisms in the enrichment tubes during the initial stage of incubation (within 7 days), as determined with a phase-contrast microscope. In some cases, however, prolonged incubation resulted in sedimentation of the overgrowing sulfur bacteria and enrichment of the purple nonsulfur bacteria in the upper layer of the tubes. This was the case in the red samples, in which we detected small ovoid to rod-shaped cells microscopically, and in a green sample (Table 1). Agar plate cultures prepared from these enrichment samples yielded a number of single colonies of purple nonsulfur bacteria at high levels of dilution. As a result of these investigations, we successfully isolated 23 strains of purple nonsulfur bacteria from four bloom samples.

As suggested by their habitats and the cultural conditions used for enrichment and isolation, all of the isolates which we recovered were halophilic.

Morphology and ultrastructure. All of the new isolates were quite similar to each other in general morphology. The cells were ovoid to rod shaped (0.5 to 1.0 by 1.0 to 2.5 μm) when the

organisms were grown phototrophically in mineral media supplemented with malate as the carbon source and 0.1% yeast extract or a vitamin mixture as the growth factor. The cells were elongated or swollen when the organisms were grown in media containing either peptone or yeast extract at a concentration of 0.2% or more. The isolates were gram negative and reproduced by binary fission. Motile cells were observed at the exponential phase of growth in all cases. Electron microscopy of negatively stained cells revealed that all of the isolates had single polar flagellum. Phototrophically grown cells of the isolates contained vesicular intracytoplasmic membranes. The cell morphology and ultrastructure of strain MB-G2^T, a representative isolate, are shown in Fig. 1.

Photopigments, quinones, and fatty acids. The photopigments, isoprenoid quinones, and cellular fatty acids of the isolates were also similar. When the organisms were grown anaerobically in the light, they produced yellow-brown to yellow-green cultures, and these cultures had similar absorption spectra as determined with a spectrophotometer. For example, the absorption spectrum of a strain MB-G2^T cell extract is shown in Fig. 2; this spectrum had absorption maxima at 377, 460, 481, 512, 590, 803, and 860 nm, indicating that bacteriochlorophyll *a* and carotenoids of the spheroidene series were present. Thin-layer chromatography of lipid extracts of the isolates confirmed that spheroidene was the major carotenoid component. Phototrophic cultures of the isolates turned red gradually when they were exposed to the air because of oxidation of spheroidene to spheroidenone. This is characteristic of members of the genera *Rhodobacter* and *Rhodovulum* (18).

Thin-layer chromatography and HPLC experiments revealed that the new isolates, as well as all of the reference strains of the *Rhodovulum* species studied, produced ubiquinones as their sole quinone components. The major homolog was ubiquinone 10, which accounted for more than 93% of the total ubiquinone content in all cases.

The fatty acid profiles of our isolates and the *Rhodovulum* test strains were also similar. These organisms contained straight-chain unsaturated and monosaturated fatty acids; C_{18:1} was the predominant fatty acid (65 to 82% of the total fatty acid content), and C_{16:0} (4 to 12%) and C_{18:0} (11 to 21%) were minor components. Similar results have been obtained previously for *Rhodovulum* (formerly *Rhodobacter*) species (17, 19).

Growth and physiological characteristics. All of the isolates were halophilic, facultatively photoheterotrophic organisms and had similar morphological, cultural, and chemotaxonomic characteristics, as described above. Experiments to determine basic physiological and biochemical characteristics also revealed that all of the isolates had an optimum temperature for growth of 30 to 35°C, utilized sulfide and thiosulfate as electron donors for photoautotrophic and photoheterotrophic growth (with production of sulfate as the end oxidation product), and grew phototrophically in the presence of 2 mM sulfide and 0.05% yeast extract. Most of the isolates did not clearly produce extracellular elemental sulfur during sulfide oxidation; the exceptions were the isolates designated the group IV isolates below. Our isolates did not hydrolyze gelatin or Tween 80 or exhibit growth during gelatin and Tween 80 hydrolysis tests. These results suggested that all of the new isolates could be placed in the genus *Rhodovulum*. However, the isolates fell into four groups, designated groups I through IV, on the basis of NaCl concentration and pH ranges for growth and other primary physiological properties (Table 2).

The group I isolates, which were the most abundant purple nonsulfur bacteria recovered from the red blooms, were characterized by their ability to grow in relatively narrow NaCl

concentration and pH ranges. The effects of different NaCl concentrations and pH values on the phototrophic growth of strain MB-G2^T, a representative of group I, are shown in Fig. 3. This bacterium grew in the presence of 0.25 to 3.0% NaCl (optimum NaCl concentration, 0.8%) and at pH 7.5 to 9.0 (optimum pH, 8.0 to 8.5). Some strains belonging to this group were able to grow slowly at pH 7.0 but required a lag period of 5 days or more to do this. These findings showed that the group I isolates are slightly halophilic and slightly alkaliphilic. Aerobic chemotrophic growth in the dark occurred on agar media and in liquid media with shaking; under these conditions the colonies and cell suspensions were red. The group I isolates also differed from the other isolates in requiring biotin, *p*-aminobenzoic acid, and thiamine as growth factors.

The group II isolates, which were the second most common isolates obtained from the red blooms, were able to grow in the presence of NaCl concentrations ranging from 0 to 10% and at pH 5 to 9. They were able to grow in the absence of added NaCl, but their growth rates and cell yields were much higher in the presence of NaCl (optimum NaCl concentration, 2 to 5%). The growth factor requirement of the isolates belonging to this group was satisfied by four vitamins, biotin, niacin, *p*-aminobenzoic acid, and thiamine, a characteristic that distinguished them from the isolates belonging to other three groups. These phenotypic properties suggested that the group II isolates and *R. sulfidophilum* are closely related (6).

The group III isolates, which were also obtained from the red blooms, were quite similar to the group II isolates, but differed from the latter in two characteristics, growth at pH 5 and vitamin requirements. Experiments to determine growth factor requirements clearly showed that all of the group III isolates required niacin but not biotin as a growth factor. Curiously, the growth responses when one of the other vitamins added to the growth media was removed differed from experiment to experiment. Three serial transfers of cultures to the same medium lacking either thiamine or *p*-aminobenzoic acid resulted in a decrease in the growth yield, but this decrease was sometimes not so clear. Thus, we could not determine whether the group III isolates require either thiamine or *p*-aminobenzoic acid or both in addition to niacin.

The group IV isolates, which were obtained only from a green bloom, were able to grow in the presence of NaCl concentrations ranging from 1 to 10% and at pH 6 to 9. The characteristic features of the group IV isolates were their inability to grow chemotrophically in the presence of full atmospheric oxygen tension and their requirement for reduced sulfur compounds as sulfur sources. A requirement for biotin and thiamine as growth factors also distinguished the group IV isolates from the other isolates. In these respects, the group IV isolates most closely resembled *R. adriaticum* (24); a slight difference in motility between the two taxa was observed.

Photoassimilation of organic compounds. The isolates belonging to the four groups utilized a wide variety of organic substrates as electron donors and carbon sources for phototrophic growth (Table 3). Good substrates for all isolates were acetate, lactate, pyruvate, fumarate, malate, succinate, glycolate, and yeast extract. Some differences in photoassimilation of citrate, fructose, glucose, xylose, aspartate, glutamate, and leucine among the four groups were observed.

Genetic relationships. The results of the tests to determine DNA base composition and genomic DNA relatedness among the test strains are shown in Table 4. We observed high levels of hybridization (more than 67%) between isolates belonging to the same group, suggesting that each of the four groups is genetically coherent and corresponds to a single species. The levels of genomic DNA relatedness between the group I iso-

FIG. 1. Morphology and ultrastructure of cells of strain MB-G2^T, a representative of the isolates. (A) Phase-contrast micrograph showing cell morphology. Bar = 10 μ m. (B) Electron micrograph of an ultrathin section of cells showing vesicular intracytoplasmic membranes. Bar = 0.5 μ m.

FIG. 2. Absorption spectrum of a cell extract of strain MB-G2^T, a representative of the isolates.

lates and the members of the other groups or previously described *Rhodovulum* species were low enough to distinguish the group I isolates from the other organisms at the species level. The group II isolates were assigned to *R. sulfidophilum* on the basis of the high levels of hybridization between the DNAs of these isolates and *R. sulfidophilum* DSM 1374^T and labeled DNA from strain MB257, a representative of the group. The group III and IV isolates exhibited relatively high levels of reassociation with *R. sulfidophilum* (57%) and *R. adriaticum* (60%), respectively; however, these levels of reassociation suggest that these organisms belong to different spe-

TABLE 2. Differential characteristics of four groups of purple nonsulfur bacteria isolated from tidal pool blooms^a

Characteristic	Group I (n = 13) ^a	Group II (n = 4)	Group III (n = 3)	Group IV (n = 3)
Cell width (μm)	0.6–1.0	0.6–1.0	0.6–1.0	0.5–0.9
Aerobic chemotrophic growth in:				
Agar slabs	+	+	+	+
Liquid medium with shaking	+	+	+	–
Phototrophic growth in the presence of:				
0% NaCl	–	(+)	(+)	–
1% NaCl	+	+	+	(+)
3% NaCl	(+)	+	+	+
5% NaCl	–	+	+	+
10% NaCl	–	+	+	+
Phototrophic growth at:				
pH 5	–	+	–	–
pH 6	–	+	+	+
pH 7	–	+	+	+
pH 8	+	+	+	+
pH 9	+	+	+	+
Vitamin requirements				
Biotin	+	+	–	+
Niacin	–	+	+	–
p-Aminobenzoate	+	+	?	–
Thiamine	+	+	?	+
Utilization of sulfur sources				
Sulfate	+	+	+	–
Thiosulfate	+	+	+	+
Cysteine	+	+	+	+
Source (sample no.) ^c	1, 7, 8	7, 8	7	6

^a n is the number of strains isolated and tested.

^b +, reaction positive; (+), reaction positive but weak or slow; –, reaction negative; ?, reaction ambiguous.

^c See Table 1.

FIG. 3. Effects of external NaCl concentrations and pH values on phototrophic growth of strain MB-G2^T, a representative of the isolates.

cies in view of the genetic criterion recommended for delineating bacterial species (30).

The 16S rDNA sequences of strains MB-G2^T and MB260, representatives of groups I and III, respectively, have been reported previously (15). A phylogenetic analysis showed that strain MB-G2^T occurs as an independent taxon within the cluster of the genus *Rhodovulum* and that strain MB260 is closely related to *R. sulfidophilum*. In this study, we also determined the 16S rDNA sequences of strains MB263 (group III) and MB253 (group IV). The sequences of strains MB263 and MB253 exhibited the highest levels of similarity to the sequences of strain MB260 and *R. adriaticum* DSM 2781^T, respectively; in both cases the level of similarity was 99.9%. The sequences which we determined were aligned with sequences published previously for members of the genera *Rhodovulum* (15) and *Rhodobacter* (18) and some other related species, and evolutionary distance (K_{nuc}) values were calculated by using the alignable 1,338 positions of all of the sequences studied. A phylogenetic tree was reconstructed by the neighbor-joining method on the basis of the distance matrix data (Fig. 4).

DISCUSSION

As reported above, all of the new isolates which we recovered from seawater blooms are mesophilic, halophilic, facultatively aerobic photoheterotrophs that have ovoid to rod-shaped cells and contain vesicular intracytoplasmic membranes together with bacteriochlorophyll *a* and carotenoids of the spheroidene group. Also, ubiquinone 10 is the sole quinone, C_{18:1} is the primary component of the cellular fatty acids, and sulfide and thiosulfate are utilized as electron donors for phototrophic growth, with sulfate as the end oxidation product. In view of these phenotypic data, we concluded that all of the isolates should be placed in the genus *Rhodovulum*. The 16S rDNA sequence-based phylogenetic information obtained in this study and elsewhere (15) supports this conclusion.

However, the majority of the isolates cannot be placed in any previously described *Rhodovulum* species. We were particularly interested in the group I isolates, since these organisms not only have unique physiological characteristics but also were the most frequently encountered purple nonsulfur bacteria recovered from colored blooms. The group I isolates grow in the presence of a relatively narrow range of NaCl concentrations (0.25 to 3%) and at a narrow pH range (pH 7.5 to 9.0), suggesting that they are slightly halophilic and slightly alkaliphilic phototrophs. To our knowledge, such a physiological type of purple nonsulfur bacteria has not been described previously among *Rhodovulum* species. The following three

TABLE 3. Photoassimilation of organic compounds as electron donors and carbon sources by phototrophic bacteria isolates

Substrate ^a	Utilization by:			
	Group I isolates (n = 7) ^b	Group II isolates (n = 3)	Group III isolates (n = 3)	Group IV isolates (n = 3)
Organic acids				
Acetate	+++ ^c	+++	++	++
Benzoate (0.05%)	—	—	—	—
Butyrate (0.05%)	+ / +++ ^d	+	+	+
Caprylate (0.05%)	—	—	—	—
Caproate (0.05%)	+	+	+	+
Citrate	+ / (+)	—	—	—
Formate	++	++	+	+
Fumarate	+++	+++	+++	++
Glycolate	+++	+++	+++	++
Lactate	+++	+++	+++	+++
Malate	+++	+++	+++	+++
Malonate	—	—	—	+
Peragonate (0.02%)	—	NT	NT	NT
Propionate (0.05%)	++	+	+	(+)
Pyruvate	+++	+++	+++	++
Succinate	+++	+++	+++	++
Tartrate	(+)	—	—	—
Valerate (0.05%)	+	NT	NT	NT
Sugars and alcohols				
Adonitol	—	—	—	NT
L-Arabinose	(+)	+	—	NT
Ethanol	(+) / —	+	(+) / —	— / (+)
Fructose	+++	++	— / (+)	— / (+)
Galactose	—	—	—	—
Glucose	+	++	—	+
Glycerol	(+) / —	—	—	—
Mannose	—	—	—	—
Mannitol	—	—	—	—
Methanol	—	—	—	—
Propanol	—	—	—	—
Sucrose	—	—	—	NT
Sorbitol	—	—	—	—
Xylose	(+) / —	++	—	NT
Amino acids and other compounds				
Alanine	+ / ++	NT	(+) / —	++
Asparagine	—	NT	+	NT
Aspartate	— / (+)	NT	+++	+
Glutamate	—	++	+++	NT
Leucine	+++	NT	— / (+)	—
Casamino Acids	+++	++	+++	NT
Peptone	+++ / ++	++	++	NT
Yeast extract	+++	+++	+++	+++

^a The concentration of substrate used was 0.2%, unless indicated otherwise.^b n is the number of strains tested.^c +++, good growth (OD₆₆₀, >0.5); ++, moderate growth (OD₆₆₀, 0.2 to 0.5); +, poor growth (OD₆₆₀, 0.1 to 0.2); (+), negligible growth (OD₆₆₀, <0.1); —, no growth; NT, not tested.^d The first symbol indicates the more frequent results.

Rhodovulum species have been described previously: *R. adriaticum* (24), *R. euryhalinum* (22), and *R. sulfidophilum* (6). In addition to NaCl concentration and pH ranges for growth, the group I isolates differ from *R. adriaticum* in motility, aerobic growth in the presence of full atmospheric oxygen tension, the vitamins required, sulfate assimilation, and photoassimilation of butyrate, citrate, and fructose. They differ from *R. euryhalinum* in the vitamins required, sulfate assimilation, and photoassimilation of citrate and malonate. And they differ from *R. sulfidophilum* in the vitamins required and photoassimilation of citrate and glutamate. DNA-DNA hybridization studies re-

TABLE 4. DNA base compositions of and levels of DNA-DNA relatedness among strains of phototrophic bacteria

Strain	G+C content of DNA (mol%)	% Hybridization with labeled DNA from:			
		Strain MB-G2 ^T	Strain MB257	Strain MB263	Strain MB253
Group I					
MB-G2 ^T	67.5 ^a	100	28	23	23
MB-G10	67.7	94			
MB002	67.3	67	26	26	21
MB041	67.5	78			
Group II					
MB257	66.7	30	100	49	23
MB259	66.5	28	97		
Group III					
MB260	66.6 ^a	26	51	91	22
MB263	66.4	23	55	100	26
Group IV					
MB253	65.1	16	17	21	100
MB254	64.9	17	16	16	100
<i>R. adriaticum</i> DSM 2781 ^T	65.0 ^a	17	18	18	60
<i>R. euryhalinum</i> DSM 4686 ^T	65.5 ^a	22	20	18	30
<i>R. sulfidophilum</i> DSM 1374 ^T	66.3 ^a	25	89	57	24

^a Data from reference 15.

vealed that the genetic distance between the group I isolates and the three *Rhodovulum* species mentioned above is consistent with separation at the species level. Recently, a new marine species of purple nonsulfur bacteria, "*Rhodobacter marinus*," was described (3). Our isolates can be distinguished from this new species by a number of phenotypic characteristics, including oxygen sensitivity, NaCl concentration and pH ranges for growth, sulfide tolerance, and vitamin requirement. Therefore, we conclude that the group I isolates should be classified as a new species of the genus *Rhodovulum*, for which we propose the name *Rhodovulum strictum*. Differential char-

FIG. 4. Distance matrix tree showing phylogenetic relationships among the new isolates, previously described *Rhodovulum* species, and related organisms belonging to the alpha 3 subclass of the *Proteobacteria*. The sequence of *Rhodospirillum rubrum* was used as an outlier sequence to root the tree. For the species for which no strain designation is given we used the sequence data for the type strain.

TABLE 5. Differential characteristics of *Rhodovulum strictum* sp. nov. and related species of phototrophic bacteria^a

Characteristic	<i>Rhodovulum strictum</i>	<i>Rhodovulum sulfidophilum</i>	<i>Rhodovulum adriaticum</i>	<i>Rhodovulum euryhalinum</i>	" <i>Rhodobacter marinus</i> "
Cell diam (µm)	0.6–1.0	0.6–1.0	0.5–0.8	0.7–1.0	0.6–1.2
Motility	+	+	–	+	+
NaCl range for growth (%)	0.25–3	0–10	1–10	0.5–10	~4
pH range	7.5–9.0	5.0–9.0	6.0–8.5	6.0–8.5	6–8
Aerobic growth with shaking	+	+	–	–	–
Sulfide tolerance	+	+	+	+	–
Sulfate assimilated	+	+	–	–	+
Vitamins required ^c	b, p-ABA, t	b, n, p-ABA, t	b, t	b, n, p-ABA, t	–
Utilization of:					
Citrate	(+)	–	–	–	+
Tartrate	(+)	–	–	–	+
Glutamate	–	+	–	+	+
Sulfide	+	+	+	+	–
Thiosulfate	+	+	+	+	–
G+C content of DNA (mol%)	67.3–67.7	66.3–66.6	64.9–66.7	62.1–68.6	66.7

^a Data from references 3, 6, 15, 22, and 24 and this study.^b +, positive; (+), weakly positive, –, negative.^c b, biotin; n, niacin, p-ABA, *p*-aminobenzoic acid; t, thiamine.

acteristics of *R. strictum* and related species of phototrophic bacteria are shown in Table 5.

Of the remaining isolates which we studied, the group II isolates can be assigned unequivocally to *R. sulfidophilum* on the basis of their phenotypic and genotypic properties. The group III isolates are also similar to *R. sulfidophilum*, although there are phenotypic differences between the two taxa in growth at pH 5 and the vitamins required. DNA-DNA pairing studies also showed that the group III isolates are genetically different from *R. sulfidophilum* and related species of phototrophic bacteria. Nevertheless, it may be inappropriate to propose a new species for these isolates because there are few diagnostic characteristics that can be used to differentiate them from *R. sulfidophilum*. This is also true for the group IV isolates, which are phenotypically and phylogenetically similar to *R. adriaticum* but can be distinguished on the basis of genomic DNA homology data.

The results of this study provide evidence that purple nonsulfur bacteria coexist with sulfur bacteria in colored blooms in tidal and seawater pools, which supports previous findings concerning the distribution of purple nonsulfur bacteria in similar environments (12, 28). On the basis of our results, we estimated that the purple nonsulfur bacteria constituted significant proportions of the microalgal flora of some of the blooms which we studied. These results suggest that in tidal and seawater blooms, the purple nonsulfur bacteria are neither predominant nor always present in detectable numbers but appear to occur more frequently or in higher numbers than once thought. The high levels of *R. strictum* and other members of the genus *Rhodovulum* in the seawater blooms indicate that these bacteria inhabit nutrient-rich tidal pools and similar marine environments.

Description of *Rhodovulum strictum* sp. nov. *Rhodovulum strictum* (stric'tum. L. part. adj. *strictus*, strict, accurate, referring to the fact that the cells require strict growth conditions). Cells are ovoid to rod shaped, 0.6 to 1.0 µm wide and 1.0 to 2.5 µm long. Motile by means of polar flagella. Gram negative. Facultative photoheterotrophs that can grow anaerobically in the light or aerobically in the dark in the presence of full atmospheric oxygen tension. Photosynthetic cultures are yellow-green to yellow-brown, while aerobic cultures grown in the dark are pink to red. Colonies that develop aerobically on agar media in the dark are circular, convex with entire margins, and

red; they are 2 to 3 mm in diameter after 7 days of incubation. Phototrophically grown cells contain intracytoplasmic membranes of the vesicular type together with bacteriochlorophyll *a* and carotenoids of the spheroidene series. Mesophilic, slightly halophilic, and slightly alkaliphilic. The optimum growth temperature is 30 to 35°C. Growth occurs in the presence of NaCl concentrations ranging from 0.25 to 3.0% (optimum NaCl concentration, 0.8%) and at pH 7.5 to 9.0 (optimum pH, 8.0 to 8.5). Biotin, *p*-aminobenzoic acid, and thiamine are required as growth factors. Good carbon sources for phototrophic growth are formate, acetate, propionate, butyrate, lactate, pyruvate, fumarate, malate, succinate, glycolate, fructose, alanine, leucine, Casamino Acids, peptone, and yeast extract. Weak or slow growth occurs with valerate, caproate, tartrate, citrate, arabinose, and glucose. No or little growth occurs with caprylate, pelargonate, malonate, sucrose, galactose, mannose, adonitol, mannitol, sorbitol, glycerol, methanol, ethanol, propanol, asparagine, aspartate, or glutamate. Sulfide and thiosulfate are used as electron donors for phototrophic growth, with sulfate as the final oxidation product. No reduced sulfur compounds are required as sulfur sources. The major quinone is ubiquinone 10. Straight-chain unsaturated and monosaturated fatty acids are present, with C_{18:1} predominating. The G+C content of the DNA ranges from 67.3 to 67.7 mol% (as determined by HPLC).

Source: tidal and seawater pools and similar marine environments.

Type strain: MB-G2, which has been deposited in the Japan Collection of Microorganisms, RIKEN, Wako, Japan, as *Rhodovulum strictum* JCM 9220.

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