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Rhodopseudomonas globiformis, sp. n., a New Species of the Rhodospirillaceae

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Abstract. Enrichments of pH: 5.1, inoculated from a warm, acidic sulfur spring of the Yellowstone Park, yielded purple red cultures of a mobile, spherical organism belonging to the Rhodospirillaceae, described herein as a new species *Rhodopseudomonas globiformis*. Under favourable conditions, the cells are 1.6–1.8 μm in diameter, smaller and larger cells may occur. The photopigments consist of bacteriochlorophyll a_P and new aliphatic methoxylated ketocarotenoids. The organism grows either under anaerobic conditions in the light or under microaerophilic conditions in the dark. Biotin, p-aminobenzoic acid and a source of reduced sulfur are required as growth factors. Gluconate, mannitol, fructose and ethanol are the best carbon sources at a pH of 4.9. Growth is inhibited by low concentrations of sulfide.

Key words: Rhodospirillaceae – *Rhodopseudomonas globiformis* sp. n. – Taxonomic characteristics.

A succinate-mineral salts medium of pH 5.2–5.6 proved to be particularly suitable for the isolation and culture of *Rhodopseudomonas acidophila* and *Rhodomicrobium vannielii* (Pfennig, 1969). Both species differ from other purple nonsulfur bacteria in their low pH-optimum in the presence of all carbon sources except fatty acids; in addition, none of the strains studied requires growth factors (e.g. vitamins, amino acids).

The present study reports on the isolation and characterization of a new purple red, spherical purple nonsulfur bacterium by the use of the acidic culture medium. Pure cultures of the new organism grow only in the presence of growth factors and a reduced sulfur source. Thus, the organism is the first known purple nonsulfur bacterium which depends on reduced sulfur compounds for the synthesis of sulfur containing cell compounds due to a lack of an assimilatory sulfate reduction. The new organism differs from all known species of the Rhodospirillaceae and is therefore described as a new species of the genus *Rhodopseudomonas*: *R. globiformis* sp.n., strain 7950 (DSM 161).

Abbreviations: DSM = Deutsche Sammlung von Mikroorganismen.

Materials and Methods

1. Source of the Organism. Professor Thomas Brock, Madison, Wisconsin, kindly provided a water and sludge sample (L IX, 197) of a warm sulfur spring of pH 3.0 near the Gibbon River in the Yellowstone Park, Wyoming, U.S.A. At the natural habitat a weak red layer of nonmobile, spherical cells had been observed.

2. Media and Culture Conditions. Unless otherwise indicated, the following mineral base was used for all media: KH_2PO_4 0.5 g; NH_4Cl 0.4 g; $MgSO_4 \cdot 7H_2O$ 0.4 g; NaCl 0.4 g; $CaCl \cdot 2H_2O$ 0.05 g; $Na_2S_2O_3 \cdot 5H_2O$ 0.2 g; Fe-citrate 0.005 g; trace element solution (SL 6) 10 ml; water to 1000 ml. The pH was adjusted to 5.6. The trace element solution (SL 6) has the following composition: $ZnSO_4 \cdot 7H_2O$ 10 mg; $MnCl_2 \cdot 4H_2O$ 3 mg; H_3BO_3 30 mg; $CoCl_2 \cdot 6H_2O$ 20 mg; $CuCl_2 \cdot 2H_2O$ 1 mg; $NiCl_2 \cdot 6H_2O$ 2 mg; $Na_2MoO_4 \cdot 2H_2O$ 3 mg; distilled water 1000 ml. For enrichments, deep agar tube cultures and stock cultures, 1.0 g disodium fumarate and 0.5 g yeast extract per liter were added as carbon sources. Yeast extract is replaceable by biotin and p-aminobenzoic acid as growth factors. Pure cultures grow particularly well if fumarate is replaced by 1.5 g of mannitol plus 0.5 g gluconate per liter and the pH being adjusted to pH 4.9—5.0. Screw cap bottles (50 ml) were used for cultivation. A pure culture was obtained by repeated application of serial dilutions in deep agar tubes sealed with a sterile mixture of paraffin plus paraffin oil (1:3 w/w). Tests for the utilization of simple organic substrates (sodium salts were used in case of organic acids) were performed by adding the substrates from sterile stock solutions (final concentration 0.1% w/v in most cases) to the autoclaved mineral medium. Tests were carried out in triplicate at pH 5.6. Growth was estimated from measurements of the optical density at 650 nm in 1 cm cuvettes by using a Zeiss spectrophotometer PM QII, final readings being made after incubation for 2 weeks at the incubation temperature 28° C and 50 to 100 ft-c from a tungsten lamp. The capacity to grow in the dark under aerobic, microaerophilic and anaerobic conditions was tested in uniformely inoculated deep agar tube cultures (1% Difco-agar, 10 ml per tube); anaerobic conditions were prepared by sealing the agar surface with a sterile mixture of paraffin plus paraffin oil.

The absorption spectrum of living cells was measured in a 60% saccharose solution using a Zeiss spectrophotometer with an integrating sphere to compensate for light scattering in the suspension (Göbel, 1970). The DNA-base ratio has been determined by Dr. Manley Mandel, Houston, Texas, by $CsCl_2$-density gradient centrifugation.

Results

Isolation and Culture. Various modifications of a culture medium for purple and green sulfur bacteria (pH 6.7 and 7.2) as well as a succinate mineral salts medium of pH 5.2 (Pfennig, 1969) had been inoculated with the acidic water and mud sample from the warm sulfur spring of the Yellowstone Park. After 6—8 weeks incubation at room temperature and 20—50 foot candles light intensity a purple red bottom layer of nonmobile spherical cells developed only in the acidic succinate medium. Subsequent transfers grew only when succinate was replaced by fumarate and 0.05% yeast extract were added; 0.1—0.2% yeast extract inhibited growth. The fact that growth was slow and the cell yield never corresponded to the amount of added substrate pointed to an unknown growth-

Table 1. Utilization of reduced sulfur compounds by *Rhodopseudomonas globiformis*

Source of reduced sulfur	Concentration of sulfur compound in %	Optical density at 650 nm against control
Control	0	0
Thiosulfate	0.02	0.12
Cysteine	0.0035	0.11
Thioglycolate	0.02	0
Sulfide	0.01—0.015	Inhibition

limiting factor. Test of a large number of compounds revealed that the growth limitation could be overcome and normal growth rates obtained when small amounts of thiosulfate were added to the medium.

Requirement for Reduced Sulfur Compounds. In the presence of 0.1% fumarate and 0.05% yeast extract, the following reduced sulfur compounds were tested: thiosulfate, sulfide, thioglycolate and cysteine. It can be seen from Table 1 that sulfide completely inhibited growth and that thioglycolate is not used as a source of reduced sulfur; normal growth occurred in the presence of both thiosulfate and cysteine. No decrease in growth rate and cell yield was observed even after five consecutive transfers in the presence of these compounds. The fact that thiosulfate as a source of reduced sulfur is replacable by very small amounts of cysteine, shows that the added sulfur source is not utilized as an electron donor in photosynthesis but rather as a source of reduced sulfur for the synthesis of sulfur-containing cell substances. It can be concluded, therefore that the organism lacks an assimilatory sulfate reduction, a situation which has been demonstrated to occur in a number of purple sulfur bacteria and the green sulfur bacteria (Thiele, 1968; Lippert and Pfennig, 1969).

Requirement for Other Growth Factors. In addition to small amounts of a reduced sulfur source the new organism required the addition of at least 0.02% yeast extract for growth. While vitamin-free casamino acids instead of yeast extract did not support growth, the yeast extract was fully replacable by a vitamin solution. Using the mineral medium with 0.1% fumarate and 0.01% thiosulfate, the following seven vitamins were tested in 7 combinations of six vitamins, each combination lacking a different one of the seven vitamins: p-aminobenzoic acid, thiamine, biotine, cyanocobalamine, nicotinic acid, Ca-pantothenate and pyridoxamine. Cultures without added vitamins and others with 0.05% yeast extract instead of the vitamin mixtures served as controls. The inoculum

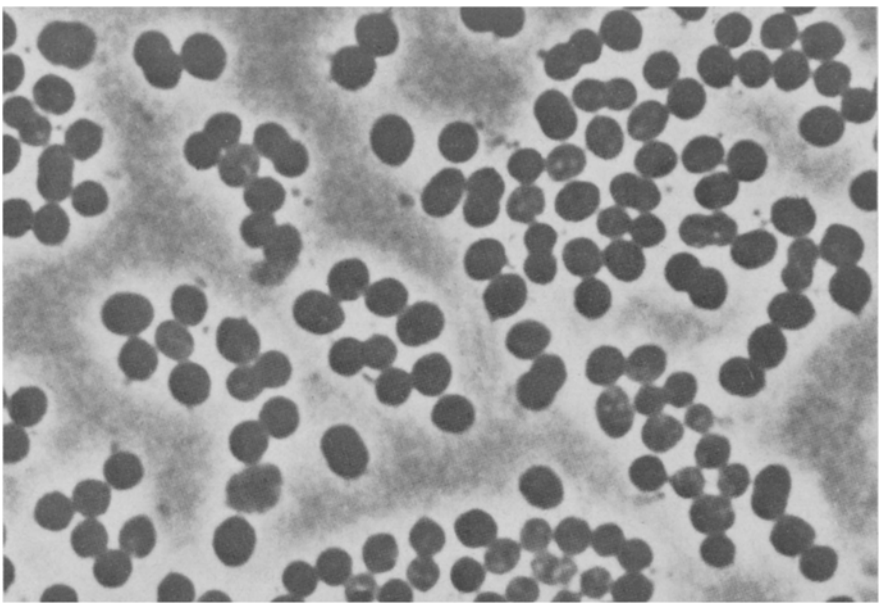

Fig. 1. Cell form of *Rhodopseudomonas globiformis* strain 7950. Phase contrast, × 3000

for these tests was grown in a medium without added yeast extract or vitamins. No growth occurred in the cultures without added vitamins and in the cultures containing vitamin combinations lacking biotin and p-aminobenzoic acid. All cultures with the other vitamin combinations showed growth as good and as fast as the controls with yeast extract. A series of consecutive growth tests using the growth factors biotin and p-aminobenzoic acid confirmed that these two vitamins fully replace yeast extract as the growth factor. The fact that it was possible to obtain growth limitation already after one subculture in vitamin-free medium indicates that the organism must have a higher requirement for biotin than other biotin-requiring bacteria. Quantitative measurements are in progress.

Morphology. *Rhodopseudomonas globiformis* has spherical to ovoid cells which appear diplococcus shaped before cell division (Fig. 1). Depending on the culture conditions the diameter of the cells varies between 1 and 2.5 μm; the most common diameter is from 1.6 to 1.8 μm. In addition to single cells, more or less long chains of cells occur in which the individual cells are connected by a very short and thin filament. Under unfavourable conditions particularly small cells (~ 1 μm) or

Table 2. Utilization of single organic substrates and electron donors by *Rhodopseudomonas globiformis*, strain 7950; for comparison results with *R. sphaeroides* and *R. capsulata* of van Niel (1944) are added

Carbon source and electron donor	Utilization[a] *R. globiformis* strain 7950, at pH 5.6	*R. sphaeroides* at neutral pH	*R. capsulata* at neutral pH
1. Formate	i	·	·
2. Acetate	i	+	+
3. Propionate	i	—	+
4. Butyrate	i	+	+
5. Valerate	i	+	+
6. Caproate	i	(+)	+
7. Caprylate	i	(+)	+
8. Pelargonate	i	—	+
9. Glycolate	i	·	·
10. Pyruvate	0—1	+	+
11. Lactate	i	·	+
12. Citrate	i	+	—
13. Malate	1	+	+
14. Fumarate	1	+	+
15. Succinate	0	+	+
16. Tartrate	1	+	—
17. Malonate	i	+	+
18. Benzoate	i	·	·
19. Cylohexane carboxylate	i	·	·
20. Methanol	0	·	·
21. Ethanol	2	+	—
22. Glycerol	0	+	—
23. Glucose	1	+	+
24. Fructose	3	+	+
25. Mannitol	3	+	—
25. Asparaginate	0	+	+
27. Glutaminate	0	+	—
28. Arginine	0	·	·
29. Casein amino acids	0	·	·
30. Yeast extract	2	+	+
31. Thiosulfate	0	—	—
32. Sulfide	i	—	—
33. Peptone	0	+	+
34. Gluconate	3	+	—
35. Mannose	0	+	—
36. Sorbitol	0	+	—

[a] Optical density (OD) at 650 nm: 0 = OD as in the control without added substrate; 1 = up to 0.1 OD; 2 = 0.2 to 0.5 OD; 3 = 0.6 to 0.9 OD and higher; i = growth completely inhibited; + = growth; — = no growth; · = not tested.

extremely large cells may occur (up to 5 μm); in the large cells a separation of the cytoplasm from the cell wall becomes visible. Only under favourable conditions—pH 4.8 to 5.0—the larger part of the cells of a

culture is mobile. Judging from flagella-stained preparations, the cells are mobile by means of a single flagellum.

Electron microscopic fine structure studies showed that the photosynthetic membrane system is of vesicular type comparable to that of *R. sphaeroides* and *R. capsulata* (Cohen-Bazire, personal communication).

Physiological and Biochemical Characteristics. The pH-optimum of *R. globiformis* proved to be dependent on the type of carbon source used. With mannitol, a pH-range for growth from 4.2 to 6.2 was observed, the pH-optimum being 4.8 to 5.0. When fumarate was the carbon source, a pH-range from 5.2 to 6.5 was observed, the pH-optimum being 5.6. During growth with glucose, fructose or mannitol under anaerobic conditions in the light, the pH of the culture dropped to 3.5. The results of growth tests in the presence of single organic carbon sources under anaerobic conditions in the light are presented in Table 2. The new organism is characterized by a rather narrow spectrum of usable carbon sources; best growth occurs in the presence of fructose, mannitol and gluconate.

Of the organic acids, only fumarate, malate and tartrate are used. Many organic acids, all fatty acids, benzoate, cyclohexanecarboxylate and sulfide completely inhibit growth. When grown under anaerobic conditions in the light, the color of the culture is intensively purple red, independent of the nature of the carbon source or the pH of the medium. In the presence of air, the color is less intensively purple red to pink; no characteristic change of color occurs. No growth was obtained under anaerobic conditions in the dark. Agar deep cultures open to air showed growth in the dark in a pinkish layer a few millimeters below the agar surface. With fumarate as the sole carbon and energy source, the growth-layer occurred about 6 mm below the surface; when mannitol plus gluconate (0.05% each) were the carbon- and energy sources, the growth layer was situated 2—4 mm below the surface. No growth occurred at the agar surface with full aerobic conditions. Therefore, *R. globiformis* is able to generate energy for growth by respiration under reduced oxygen tension, that is under microaerophilic conditions.

Photopigments. The absorption spectrum of living cells of *R. globiformis* exhibits the characteristic maxima of bacteriochlorophyll a (see Fig. 2); detailed analysis showed that it is bacteriochlorophyll a_P (phytylester, Künzler and Pfennig, 1973). The carotenoids of the organism were recently studied by Schmidt and Liaaen-Jensen (1973). The major carotenoids are new aliphatic, methoxylated ketocarotenoids. The major component has twelve conjugated double bounds, two methoxy-groups in 1,1′-position and one keto-group in 4-position; a second component—the end product of the biosynthesis series—has a second keto-group in 4′-position.

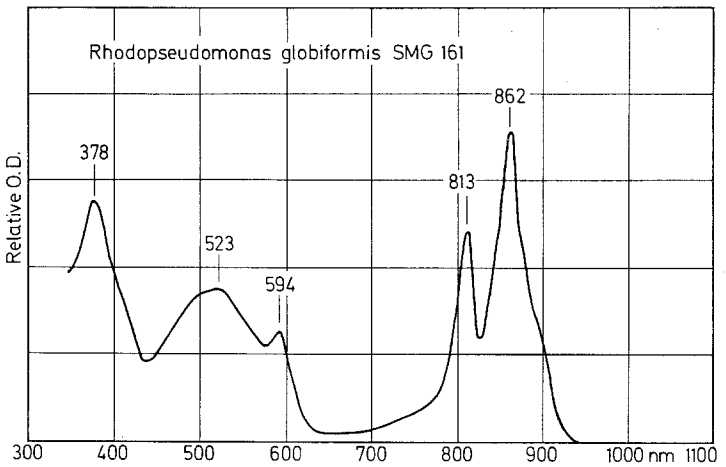

Fig. 2. Absorption spectrum of living cells of *Rhodopseudomonas globiformis* strain 7950

Discussion

The new organism *Rhodopseudomonas globiformis* strain 7950 is completely inhibited by low concentrations of H_2S and does not form and utilize elemental sulfur as an electron donor for photoautotrophic CO_2-assimilation; therefore, it has to be classified with the Rhodospirillaceae. On the basis of its morphological, cytological and biochemical characteristics, strain 7950 shows the closest resemblance to *Rhodopseudomonas sphaeroides*. There are, however, major differences in comparison to this species which do not permit to classify the new strain together with *R. sphaeroides*. These differences are (see also Table 3):

1. Physiological Characteristics. R. globiformis was isolated from an acid sulfur spring (pH: 3). The following two characteristics suggest, that the new organism is specially adapted to its natural environment: 1. The optimum pH for growth 4.8 or 5.6 (depending on the carbon source) is unusually low for phototrophic bacteria, and 2. the organism is the first purple nonsulfur bacterium so far isolated which requires reduced sulfur compounds for the synthesis of sulfur-containing cell constituents because it lacks an assimilatory sulfate reduction. Out of 34 carbon sources tested, *R. globiformis* utilizes in addition to six other compounds the following substrates particularly well: gluconate, mannitol, fructose and ethanol. These compounds are also very well utilized by *R. sphaeroides* which has, however, a much broader spectrum of usable carbon sources than the new organism. It is also typical for *R. sphaeroides* to grow best and fastest in the presence of higher concen-

Table 3. Characteristic features of *Rhodopseudomonas globiformis* compared with *R. sphaeroides* and *R. capsulata*

Organisms	Color of dim-light-grown anaerobic cultures	Major carotenoids under anaerobic growth conditions	Color of semi-aerobic cultures	Optimum pH-range	Size of cells in μm	Growth factors required	Growth conditions in the dark	Moles % guanine plus cytosine in DNA
R. globiformis	Purple-red	Aliphatic methoxylated keto-carotenoids	Pink	4.6—5.6	1.6—1.8	Biotin, p-aminobenzoic acid, cystein or thiosulfate	Micro-aerophilic	66.3
R. sphaeroides	Yellowish-brown with greenish tinge	Spheroidene and OH-spheroidene	Brownish red	6.5—7.5	2.0—2.5	Biotin, thiamin, nicotinic acid	Aerobic	68.4—69.9
R. capsulata	Yellowish-brown with greenish tinge	Spheroidene and OH-spheroidene	Brownish red	6.5—7.5	0.5—1.2 wide, 2—2.5 long	Biotin, thiamin, nicotinic acid	Aerobic	65.5—66.8

trations of complex organic carbon sources such as yeast extract, peptone, casamino acids. In contrast, 0.1 % yeast extract markedly inhibit growth of *R. globiformis*. *R. globiformis* is able to grow under microaerophilic—not aerobic—conditions in the dark while all strains of *R. sphaeroides* tested grow well under aerobic conditions. The DNA-base composition with 66.3 mole per cent guanine plus cytosine is not within the range of all *R. sphaeroides* strains studied so far with 68.4—69.9 moles per cent G + C.

2. The Photopigments. Characteristic for all strains of the species *R. sphaeroides* (as well as for *R. capsulata* and *R. gelatinosa*) are the aliphatic carotenoids spheroidene and hydroxyspheroidene which belong to the alternative spirilloxanthin-series and which in the presence of oxygen are being transformed into the corresponding keto-carotenoids. This transformation is accompanied by a change of the color of the cells from dirty yellow-greenish-brown to brown-red. Cultures of *R. globiformis* are under all conditions pinkish-red to purple-red colored. The carotenoids which are typical for *R. sphaeroides*, are entirely lacking; instead, *R. globiformis* contains as the major carotenoid components two new aliphatic methoxylated keto-carotenoids (Schmidt and Liaaen-Jensen, 1973). These carotenoids are a particular characteristic of the new organism. *R. globiformis* contains the classical bacteriochlorophyll a_P with phytol as the esterifying alcohol (Künzler and Pfennig, 1973).

The new organism differs in important physiological and biochemical characteristics from *R. sphaeroides*. Therefore, it appears proper to recognize the organism as a new species of the genus *Rhodospeudomonas*.

Rhodopseudomonas globiformis n.sp.

glo.bi.for.'mis. L. noun *globus* sphere; L. noun *forma* shape. M.L. noun *globiformis* sphere-shaped.

Morphology. Cells spherical to ovoid, diplococcus-shaped before cell division, 1.6 to 1.8 μm in diameter, larger cells may occur under unfavourable growth conditions; tendency to form chains of cells. Mobile by means of polar flagella. Gram-negative. Photosynthetic intracytoplasmic membrane system of vesicular type.

Culture. Photoorganotrophic, growing either anaerobically in the light or microaerobically in the dark. pH-range 4.2 to 6.5; optimum pH with mannitol 4.8—5.0, with fumarate 5.6. Optimum temperature 30—35° C, no growth at 40° C. Color of anaerobic light cultures intensively purple red; microaerophilically grown cells pink. Growth factors required: biotin, p-aminobenzoic acid and a source of reduced sulfur in the form of either cystein or thiosulfate. Growth rate somewhat increased in the presence of low concentrations (0.05 %) of yeast extract. Photoassimila-

tion of gluconate, mannitol, fructose and ethanol; growth possible also with glucose, tartrate, fumarate, malate, pyruvate and yeast extract. No growth with sulfide, fatty acids, lactate, citrate, glycerol, mannose, sorbitol, amino acids and benzoate.

Pigments. Absorption spectra of living cell suspensions show the maxima of bacteriochlorophyll-a-containing organisms (378, 594, 813, 862 and a shoulder at about 890 nm).

The bacteriochlorophyll a_P contains phytol as the esterifying alcohol.

The carotenoids are new aliphatic methoxylated ketocarotenoids; the major component has one ketogroup in 4-position; a second component has two keto-groups in 4,4'-position.

Storage Material. Polysaccharides.

DNA Base Composition (buoyant density). 66.3 mol-% guanine plus cytosine.

Habitat. Acidic sulfur spring at the Gibbon River, Yellowstone Park, Wyoming, U.S.A.

Type. Strain 7950 deposited with the German Collection of Microorganisms in Göttingen, number DSM 161.

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