Rhodocyclus purpureus gen. nov. and sp. nov., a Ring-Shaped, Vitamin B₁₂-Requiring Member of the Family *Rhodospirillaceae*

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The dominant phototrophic bacterium in a water sample from a purplish red waste-water lagoon was isolated in pure culture. The individual cells of this organism were half-ring-shaped and ring-shaped before cell division; the half-rings were 0.6 to 0.7 μm wide and 2.5 to 3.0 μm long. Open or compact coils of variable length were also formed. Acetate, pyruvate, and cyclohexane carboxylate were the carbon sources best utilized by this organism; vitamin B₁₂, *p*-aminobenzoic acid, and biotin were required as growth factors. The photosynthetic pigments produced by the organism are bacteriochlorophyll *a*_P and carotenoids of the rhodopinal series. The new bacterium was facultatively aerobic and was unable to photooxidize sulfide or thiosulfate to sulfur or sulfate. It belongs to the family *Rhodospirillaceae* and is described herein as a member of a new genus, *Rhodocyclus*. The name proposed for this new species is *Rhodocyclus purpureus*. The type strain of *R. purpureus* is "Ames" 6770 (= DSM 168).

Most species of the phototrophic family *Rhodospirillaceae* have a requirement for one or more growth factors. Biotin, *p*-aminobenzoic acid, nicotinic acid, and thiamine are the most commonly required substances (3, 5, 12, 16). The growth factor requirements are usually satisfied by the addition of yeast extract to the culture media (3, 16). Yeast extract does not contain vitamin B₁₂. Therefore, enrichments and isolations of *Rhodospirillaceae* were mostly carried out with culture media devoid of vitamin B₁₂, and none of the strains at present in pure culture has a requirement for this growth factor. On the other hand, vitamin B₁₂ was recognized as a necessary growth factor for several species of the phototrophic sulfur bacteria (families *Chlorobiaceae* and *Chromatiaceae*) (9, 11, 12). Vitamin B₁₂ was, therefore, regularly added to culture media for these bacteria. Using the culture medium for *Chromatiaceae* with the addition of 0.05% acetate, a new phototrophic bacterium was isolated from a sewage-lagoon sample in which this bacterium was the dominant phototrophic species. The new bacterium showed a requirement for vitamin B₁₂ and could be grown in media for *Rhodospirillaceae* when vitamin B₁₂ was added. The new bacterium is morphologically different from all known species and genera of the phototrophic bacteria (12), and it is recognized herein as a member of a new genus, for which the name *Rhodocyclus* (*Rho.do.cy'clus*. Gr. n. *rhodum* the rose; Gr. n. *cyclus* a circle; M. L. mas. n. *Rhodocyclus* a red

circle) is proposed. The type species of this genus, which is placed in the family *Rhodospirillaceae*, is *R. purpureus* sp. nov. (*pur.pu're.us*. L. adj. *purpureus* purple, or red-violet colored).

MATERIALS AND METHODS

Bacterial strain. In October 1969, J. Holt, Ames, Iowa, kindly sent me a water sample from a dirty-red-colored swine-waste lagoon. This sample was directly used as an inoculum for agar-shake cultures. Most of the colored colonies which developed contained the new bacterium, which was designated strain "Ames" 6770.

Media and culture conditions. The sulfide-bicarbonate medium of Pfennig and Lippert (11) for the cultivation of vitamin B₁₂-requiring *Chromatiaceae* was used with the addition of 0.05% acetate and 0.05% yeast extract. Agar-shake cultures were prepared by mixing 3 ml of liquid 3% agar (Difco) (60°C) with 6 ml of the culture medium (40°C); the tubes were kept at 40°C, and serial dilutions were carried out, at first of the original water sample and later of cell suspensions of single, isolated colonies. The tubes were sealed against air with a sterile mixture of paraffin (1 part) plus paraffin oil (3 parts). Incubation occurred at 20 to 22°C and 200 to 500 lx from a tungsten lamp for 3 to 4 weeks. Repeated application of the agar-shake-culture method yielded a pure culture, which we designated strain 6770.

The pure culture grew very well in the simpler culture medium 2, which contained (per liter of distilled water): KH₂PO₄, 0.5 g; ammonium acetate, 0.5 g; MgSO₄·7H₂O, 9.4 g; NaCl, 0.4 g; CaCl₂·2H₂O, 0.1 g; yeast extract 1 g; ferrous citrate, 5 mg; and trace element solution SL6 (10), 10 ml. The pH was adjusted

to 6.8 before autoclaving. Twenty micrograms of vitamin B₁₂ per liter and 1.0 g of NaHCO₃ per liter were added to the cold, sterile medium from sterile-filtered stock solutions; the pH before inoculation was 6.8 to 7.0.

The following six vitamins were tested in six combinations of five vitamins, each combination lacking a different one of the six vitamins (the final vitamin concentrations are given in brackets): biotin (10 ng/ml), calcium pantothenate (25 ng/ml), thiamine (50 ng/ml), *p*-aminobenzoic acid (50 ng/ml), nicotinic acid (100 ng/ml), and pyridoxamine (250 ng/ml). The inoculum was pregrown twice in the absence of vitamins and yeast extract and proved to be vitamin limited as no growth was obtained in the vitamin-free controls.

The utilization of simple organic substrates was tested under anaerobic conditions in the light by adding the substrates from sterile stock solutions (final concentrations, 0.05% [wt/vol]) to the autoclaved mineral medium 2 containing 0.5 g of NH₄Cl instead of NH₄ acetate and only 0.5 g of yeast extract per liter. Tests were carried out in triplicate at a pH of 7.2 and a light intensity of 500 to 1,000 lx. The utilization of the same substrates under aerobic conditions in the dark was tested using 25 ml of medium 2 in 50-ml screw-cap bottles; 3 g of yeast extract per liter was uniformly present in addition to the organic substrates to be tested. Growth was estimated from measurements of the optical density at 650 nm in 1-cm cuvettes, using a Zeiss PMQ2 spectrophotometer; final readings were made after 1 week of incubation at 30°C.

The dry-weight yield on vitamin B₁₂ was determined in medium 2 to which a growth-limiting concentration of 0.2 ng of vitamin B₁₂ per ml was added. The inoculum consisted of cell material pregrown under vitamin B₁₂ limitation; no growth was obtained with vitamin B₁₂-free controls.

The ability to grow in the dark under aerobic or microaerophilic conditions was tested in uniformly inoculated agar-shake cultures with 10 ml of 1% agar medium per tube; control tubes were sealed with paraffin to exclude air.

The absorption spectrum of living cells was measured as described by Pfennig (10). The deoxyribonucleic acid (DNA) base ratio was determined by Manley Mandel, Houston, Tex., by CsCl₂ density gradient centrifugation.

Electron microscopy. The study of the fine structure was carried out by G. Cohen-Bazire, Berkeley, Calif. The cells were fixed, stained, and embedded by the procedure of Ryter and Kellenberger (13). The period of main fixation was reduced to 2 h. Epon was used as embedding material. Sections were cut with a diamond knife in a Porter-Blum microtome MT2 and mounted on uncoated 300-mesh copper grids. After staining with lead hydroxide (8), the sections were examined in a Siemens Elmiskop I, operating at 80 kV.

RESULTS

Isolation and culture. Based on the number of typical purple-violet colonies present in the first series of agar-shake cultures as well as the microscopic examination of the waste lagoon sample, it is reasonable to assume that the new

bacterium described here was quantitatively the dominant phototrophic bacterium of the lagoon. The pure culture grew in the sulfide-bicarbonate medium only when supplemented with acetate and yeast extract (0.05% each); hydrogen sulfide was not utilized and did not inhibit growth. In the acetate- and yeast extract-containing medium 2 for purple nonsulfur bacteria, growth was obtained only when vitamin B₁₂ (20 ng/ml) was added.

Requirement for vitamin B₁₂. The requirement for vitamin B₁₂ was quantitatively measured by the determination of the dry-weight yield at a vitamin B₁₂ concentration in the range of the linear relationship between vitamin B₁₂ concentration and growth yield. In three different experiments with three parallels each, the following dry-weight yields were obtained: 180, 220, and 236 µg of dry cell material per 0.2 ng of vitamin B₁₂, that is, about 106 µg per 0.1 ng of vitamin B₁₂. The formation of 1 g of cell material requires 0.94 µg of vitamin B₁₂. In the case of the green sulfur bacterium *Chlorobium limicola*, Pfennig and Lippert (11) found a similar requirement (1.4 µg of vitamin B₁₂) for 1 g of cell material.

Requirement for other growth factors. *R. purpureus* "Ames" 6770 required the addition of both vitamin B₁₂ and yeast extract for growth. The yeast extract was fully replaceable by a vitamin solution. Tests for individual vitamins of the vitamin solution revealed that the new bacterium had an absolute requirement for *p*-aminobenzoic acid and biotin in addition to vitamin B₁₂.

Morphology. *R. purpureus* "Ames" 6770 is morphologically similar to the organotrophic bacteria of the genera *Microcycilus* and *Spirosoma* (2). Individual cells are half-ring shaped and ring shaped (Fig. 1); they are 0.6 to 0.7 µm wide, and half-ring-shaped cells are about 2.7 µm long. The diameters of the ring-like cells are 2 to 3 µm. Under certain conditions, open or compact coils of variable length are formed. In sulfide-containing media, closely wound coils of cells are united in compact cell aggregates in which the shapes of the single cells cannot be discerned. All cell types are nonmotile under all conditions. The photosynthetic membrane system consists of the cytoplasmic membrane and very few single invaginations thereof (Fig. 2).

Physiological and biochemical characteristics. *R. purpureus* "Ames" 6770 is photoorganotrophic under anaerobic conditions and facultatively chemoorganotrophic under semi-aerobic to aerobic conditions. The pH range for growth on acetate was between 6.5 and 7.5, the pH for optimum growth being 7.2. In the presence of a utilizable carbon source, yeast extract

FIG. 1. Cell forms of *Rhodocyclus purpureus* 6770 in medium 2 with acetate and yeast extract as carbon source. Phase contrast. Magnification, $\times 3,000$.

stimulated growth at concentrations between 0.05 and 0.3%. When grown under anaerobic conditions in the light, the color of the cultures was purple-violet to violet, resembling the violet color of the strains of *Rhodospirillum tenue* (12). Aerobically grown cultures were colorless to pale violet. The results of growth tests with single organic carbon sources in the presence of yeast extract are presented in Table 1. Hydrogen served as an excellent electron donor; reduced sulfur compounds were not used. The spectrum of usable carbon sources is rather narrow; the most rapid and the heaviest growth occurred with acetate, butyrate, pyruvate, and cyclohexane carboxylate under both anaerobic conditions in the light and aerobic conditions in the dark.

Photosynthetic pigments. The characteristic absorption maxima of bacteriochlorophyll *a* are recognized in the absorption spectrum of living cells of *R. purpureus* "Ames" 6770 (Fig. 3). Künzler and Pfennig (6) showed that the bacteriochlorophyll *a* is esterified with phytol (bacteriochlorophyll *a_P*). The carotenoids of strain "Ames" 6770 were identified by Schmidt (14): the bacterium contains only the components of the rhodopinal series, with rhodopinal as the major component; in addition, rhodopin and small amounts of lycopinal and rhodopinol occur.

A condensed description of both *Rhodocyclus* and *R. purpureus* follows.

***Rhodocyclus* gen. nov. and *Rhodocyclus purpureus* sp. nov.** Cells are half-ring shaped

to ring shaped before cell division; they are 0.6 to 0.7 μm wide, and the length of half-circle-shaped cells is about 2.7 μm ; the diameter of the circles is 2.0 to 3.0 μm . Under certain conditions, open or compact spirals or coils of variable length are formed. In sulfide-containing media, closely wound spirals are united in compact cell aggregates. Cells are gram negative and non-motile. Multiplication is by binary fission. The cells possess a photosynthetic apparatus consisting of the cytoplasmic membrane and only a few invaginations into the cells as is characteristic of *Rhodopseudomonas gelatinosa* and *R. tenue*.

Photoorganotrophic, growing either anaerobically in the light or aerobically in the dark. The pH range for growth with acetate is 6.5 to 7.5; the pH for optimal growth is 7.2. The temperature for optimum growth is 30°C. The color of anaerobic cultures is purple-violet to violet. Aerobically grown cells are colorless to pale violet. Vitamin B₁₂, *p*-aminobenzoic acid, and biotin are required as growth factors; the growth rate is increased in the presence of yeast extract.

Phototrophic or chemotrophic growth occurs with acetate, butyrate, caproate, pyruvate, malate, fumarate, benzoate, or cyclohexane-carboxylate. Good growth occurs with either hydrogen or bicarbonate in the presence of 0.05% yeast extract or vitamins. No growth occurs in media with sulfide, thiosulfate, most fatty and organic acids, sugars, sugar alcohols, or ethanol.

Bacteriochlorophyll *a* is produced with phytol as the esterifying alcohol; carotenoids of the

FIG. 2. Thin section of *Rhodocyclus purpureus* 6770. The electron micrograph was prepared and kindly provided by G. Cohen-Bazire, Berkeley, Calif. Magnification, $\times 40,000$.

rhodopinal series are produced, with rhodopinal as the major component.

Hydrogenase and catalase activities are present.

Storage materials: Poly- β -hydroxybutyrate and polysaccharide.

DNA base composition (buoyant density): 65.3 mol% guanine plus cytosine.

Habitat: Swine waste lagoon, Ames, Iowa.

Type strain: "Ames" 6770; a culture of this strain has been deposited in the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, under the number 168.

Because the genus *Rhodocyclus* is, at present, based on a single species, which itself is based on a single strain, the description given above serves as the description of the genus, the type species, and the type strain.

DISCUSSION

R. purpureus must be considered a member of the family *Rhodospirillaceae* because it does not oxidize sulfide or thiosulfate in the light. It is facultatively aerobic and is capable of growing in the dark with the same organic substrates that are photoassimilated under strictly anaerobic conditions. The DNA base composition of strain "Ames" 6770 is 65.3 mol% guanine plus cytosine, well in the range characteristic for several different genera and species of the *Rhodospirillaceae* (7).

Morphologically, the new bacterium, with its nonmotile, half-ring-shaped cells, which under many conditions remain attached to form rings and closed or open spirals of variable length, is unique among the *Rhodospirillaceae*.

The spectrum of carbon sources utilizable by

R. purpureus appears extremely limited when compared with that of other purple nonsulfur bacteria (12). Heavy growth is obtained only with acetate, butyrate, pyruvate, and cyclohexane carboxylate. The use of the latter compound

TABLE 1. Utilization of single organic substrates and electron donors by *Rhodocyclus purpureus* 6770^a

Carbon source and electron donor	Utilization under:	
	Anaerobic conditions, light (0.05% yeast extract present)	Aerobic conditions, dark (0.3% yeast extract present)
None	—	—
Acetate	+++	+++
Butyrate	++	+
Caproate	++	+
Pyruvate	+++	+++
Malate	++	++
Fumarate	++	++
Succinate	—	+
Benzoate	++	++
Cyclohexane carboxylate	+++	+++
95% H ₂ plus 5% CO ₂	+++	NT ^b

^a Not utilized either in the light or dark were: formate, propionate, valerate, caprylate, pelargonate, glycolate, lactate, citrate, tartrate, malonate, methanol, ethanol, glycerol, glucose, fructose, mannitol, asparagine, glutamate, arginine, Casamino Acids, yeast extract, thiosulfate, and sulfide. Symbols: —, growth not better than in control without added carbon source; +, light growth; ++, moderate growth; +++, heavy growth.

^b NT, Not tested.

as well as of benzoate indicates that *R. purpureus* may have the same pathway of anaerobic benzoate degradation as *Rhodopseudomonas palustris* (4). In the presence of vitamin B₁₂ and yeast extract as a source of growth factors, autotrophic growth with molecular hydrogen and bicarbonate is fast and profuse. *R. purpureus* resembles other species and genera of *Rhodospirillaceae* as follows: (i) the fine structure of *R. purpureus* is very similar to that of *R. tenue* and *Rhodopseudomonas gelatinosa* (1, 17); the photosynthetic apparatus consists of the cytoplasmic membrane and one or a few invaginations into the cells; (ii) the carotenoid composition of *R. purpureus* is nearly identical with that of the purple-violet strains of *R. tenue* (14).

Considering these similarities, the question arises whether *R. purpureus* should be considered to be a nonmotile, strongly coiled, morphological mutant of *R. tenue*. In this case, one would expect that the width of the cells of *R. tenue* and of *R. purpureus* should be the same. Actually, the cells of *R. purpureus* are nearly twice as wide as those of *R. tenue*, that is, 0.6 to 0.7 μ m as compared to 0.3 to 0.4 μ m. Also, the cells of *R. tenue* have conical to pointed ends while those of *Rhodocyclus* have flat to rounded ends. There are, in addition, differences in the utilization of single organic substances and in growth-factor requirements: *R. tenue* uses a wider range of fatty acids, organic acids, and alcohols and does not need vitamins for growth; on the other hand, it does not grow with benzoate or cyclohexane carboxylate.

R. purpureus "Ames" 6770 has now been maintained in pure culture in liquid medium for

FIG. 3. Absorption spectrum of living cells of *Rhodocyclus purpureus* 6770.

7 years, and it still shows its typical morphology and growth habits. Only further studies, including DNA hybridization and comparisons of ribosomal protein patterns with very similar species, can reveal the relationships of *R. purpureus* to other species and genera of the family *Rhodospirillaceae*. It is hoped that the use of vitamin B₁₂ for the isolation of members of the family *Rhodospirillaceae* will yield more strains of the new species.

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REPRINT REQUESTS

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