

# RHODOMICROBIUM VANNIELII, A NEW PHOTOHETEROTROPHIC BACTERIUM

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Studies of the photoheterotrophic, or nonsulfur purple, bacteria have shown that these microorganisms are closely related morphologically, and can be placed either in the genus *Rhodospirillum* or *Rhodopseudomonas* (van Niel, 1944). These two genera comprise the family *Athiorhodaceae*. In view of the morphological homogeneity of the nonsulfur purple bacteria, it was, therefore, of considerable interest when one of a number of enrichment cultures prepared for organisms of this family supported the development of a photoheterotrophic bacterium in which the morphology and mode of cell division differed markedly not only from members of the *Athiorhodaceae* but from other *Schizomycetes* as well. Subsequent studies of the morphology and physiology of several pure cultures have led us to create a new genus, *Rhodomicrobium*, for these organisms. The type species of the new genus is *R. vannielii*. We have chosen this species name in honor of Professor C. B. van Niel, whose studies of the photosynthetic bacteria have added so much to our knowledge of this group of microorganisms. Definitions of the new genus and species are given in a later section of this paper.

## METHODS

The original culture was obtained following the inoculation of mud into the medium suggested by van Niel (1944), which consists of  $\text{NaHCO}_3$ , 0.5 per cent;  $\text{NaCl}$ , 0.2 per cent;  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 per cent;  $\text{K}_2\text{HPO}_4$ , 0.05 per cent;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 per cent;  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , 0.01 per cent; organic hydrogen donor (ethanol in the case above), 0.2 per cent; pH, 7.0. Glass-stoppered bottles were completely filled with the inoculated medium and incubated under continuous illumination at 25 to 30 C. *R. vannielii* was the predominant organism in the culture after 7 days' incubation. Many similar enrichment cultures have subsequently been made employing a variety of organic donors and inocula from different sources. Although *Rhodomicrobium* has been seen a number of times in these cultures, it ordinarily has been so outnumbered by other nonsulfur purple bacteria that isolation was impossible. Nevertheless, one additional strain was obtained from such enrichment cultures. Two other strains have been isolated from enrichment cultures for *Thiorhodaceae* that contained 0.1 per cent  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  in place of an organic hydrogen donor. Growth of *Rhodomicrobium* in the sulfide-containing enrichment cultures was slow and very sparse, and occurred subsequent to the development of the sulfur purple bacteria.

The agar medium used for the shake cultures in isolation and purification was prepared according to the procedures suggested by van Niel (1944). The composition was the same as for the enrichment cultures except that 0.2 per cent by

volume of yeast autolyzate was added to stimulate growth. The medium, with the exception of the bicarbonate, sulfide, and ethanol, was autoclaved in Erlenmeyer flasks, and to it, after it had been cooled to 50 C, appropriate amounts of the foregoing three constituents were added aseptically from solutions that had been sterilized by pressure filtration. The pH was then adjusted to 6.8 to 7.2 with sterile 5 per cent phosphoric acid. The molten medium was dispensed into sterile soft-glass test tubes and immediately inoculated with dilutions of material from the enrichment cultures. Since these organisms are strict anaerobes, it was necessary to seal the shake tubes with a layer of sterile "vaspar" to obtain consistently successful cultures. The selection of colonies of *R. vannielii* in shake tubes seeded from a mixed population was facilitated by the deep red color and characteristic rough, convoluted surface of the colonies (figure 1). Isolated strains remained viable in stab cultures for at least six months.

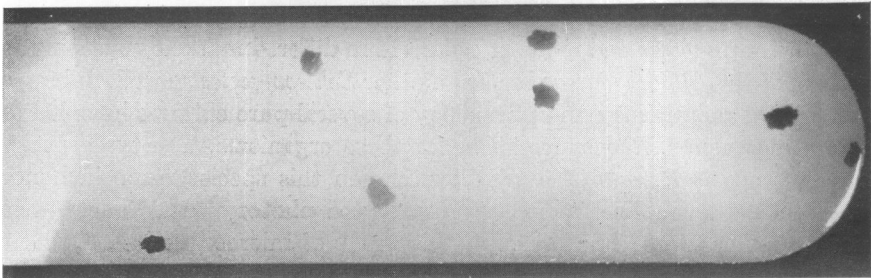


Figure 1. Colonies of *R. vannielii* in shake culture.  $\times 2$ .

#### MORPHOLOGY AND MODE OF CELL DIVISION

The distinguishing morphological features of *R. vannielii* are the attachment of the cells by means of a slender, branched filament and the mode of reproduction, which we believe to be by budding rather than fission. Figure 2 illustrates typical cell groups taken from a 7-day-old culture and photographed while suspended in dilute gentian violet. The individual cells at maturity are ovoid in shape with dimensions of approximately 1.2 by 2.8 microns. The connecting filaments vary greatly in length but are uniformly about 0.3 microns in diameter. The electron micrograph shown in figure 3 reveals that a short portion of the filament connecting two mature cells is generally constricted and considerably more opaque to the electron beam than the remainder of the filament.

It can be observed that many of the terminal cells of cell groups possess filaments that vary in length from very short protuberances to structures several microns long. The tips of the filaments may be undifferentiated (figure 6, left) or swollen (figure 6, right) to various degrees. We believe that these globose structures at the tips of the filaments are new cells in various stages of development. The size range of the terminal cells is well illustrated in figure 2. Cell multiplication appears to be initiated by the outgrowth of a new filament from the pole of a mature or immature cell, or from some point along a filament connect-

ing two cells (figures 7, 5). Following a period of elongation of the new filament, its tip enlarges to form a daughter cell. It appears that branching of the filaments is due almost exclusively to lateral outgrowths from the filaments con-

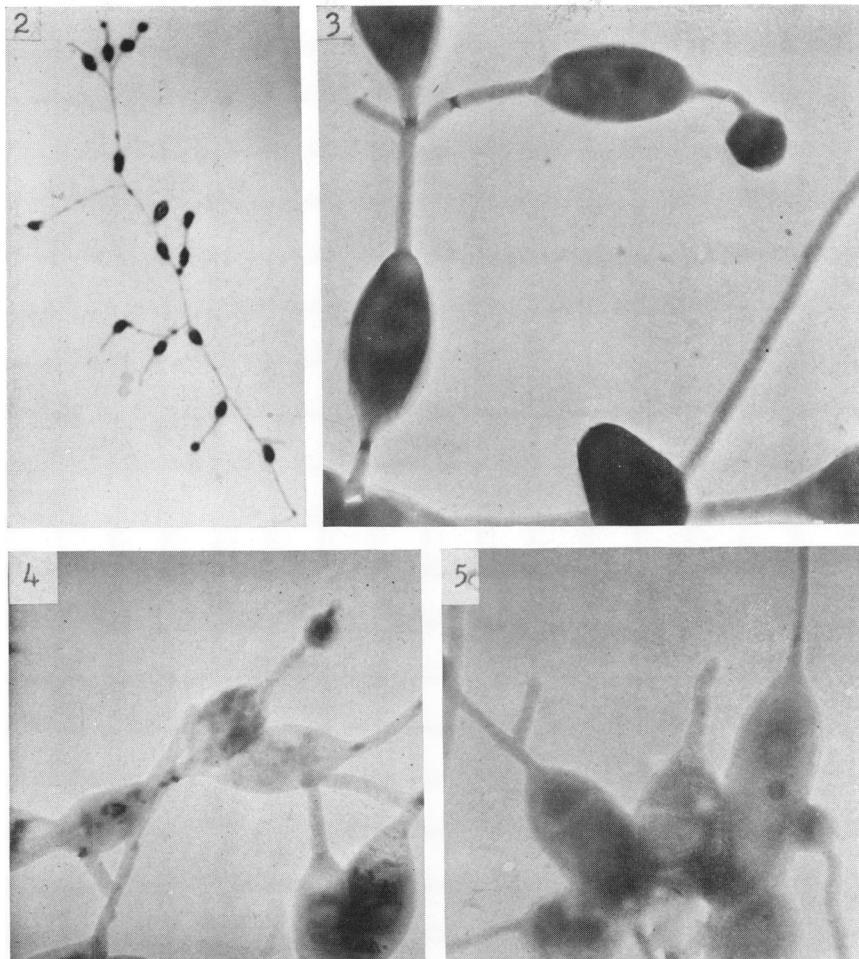


Figure 2. *R. vannielii*. Seven-day-old culture photographed in dilute gentian violet  $\times 1,800$ .

Figure 3. *R. vannielii*. Seven-day-old culture showing constriction of the filament. Electron micrograph,  $\times 10,000$ .

Figure 4. *R. vannielii*. Thirteen-day-old culture showing bud arising from the tip of an immature cell. Electron micrograph,  $\times 8,400$ .

Figure 5. *R. vannielii*. Seven-day-old culture showing terminal and lateral filaments. Electron micrograph,  $\times 9,000$ .

necting the cells rather than to longitudinal fission of terminal cells, although what seemed to be bifurcation of an undifferentiated tip has been observed on one occasion (figure 10).

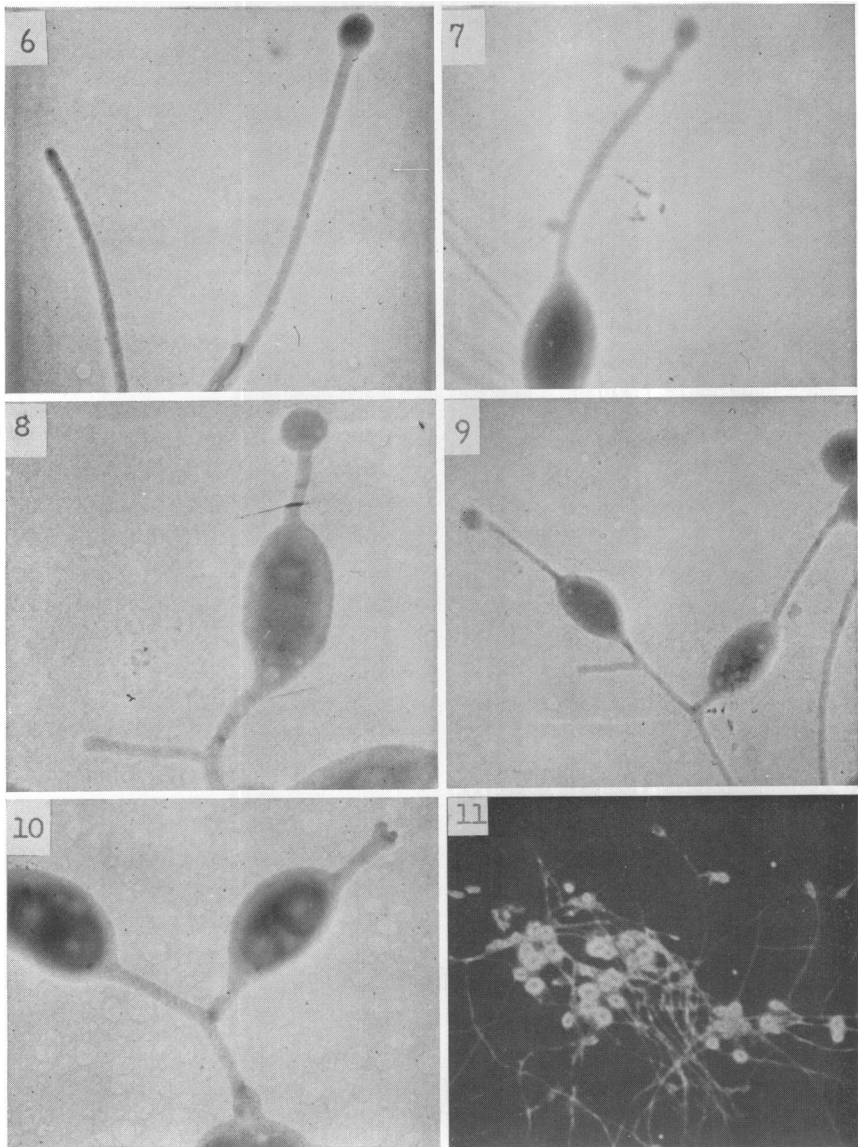


Figure 6. *R. vannielii*. Seven-day-old culture. On the left is a filament, the tip of which is undifferentiated. The tip of the filament on the right has swollen to form an immature cell. A broken filament can be seen lying alongside the filament on the right. Electron micrograph,  $\times 8,400$ .

Figure 7. *R. vannielii*. Fourteen-day-old culture showing buds arising from the filament connecting a mature and an immature cell. Electron micrograph,  $\times 8,400$ .

Figure 8. *R. vannielii*. Three-day-old culture. Electron micrograph,  $\times 9,000$ .

Figure 9. *R. vannielii*. Three-day-old culture. Electron micrograph,  $\times 5,600$ .

Figure 10. *R. vannielii*. Thirteen-day-old culture showing bifurcation of the tip of a filament. Electron micrograph,  $\times 9,000$ .

Figure 11. *Hypomicrobium vulgare*. Dark-field photomicrograph,  $\times 1,400$ . After Kingma-Boltjes.

Because of difficulties encountered in growing *Rhodomicrobium* in slide cultures, we have not yet made direct observations of the mode of growth. Nevertheless, our failure to find any evidence of cells dividing by either transverse or longitudinal fission in cultures of various ages, together with the general morphological features of the organism, makes it reasonable to believe that this bacterium multiplies in the manner described above. Since the process of budding is understood to involve the formation of a new cell from a protuberance of part of another cell, we believe that the use of the term budding to describe the mode of cell multiplication in *Rhodomicrobium* is justified, even though the process differs considerably from that in yeasts in which the bud develops directly into a daughter cell without a preceding elongation to form a filament.

The morphology of *Rhodomicrobium* is quite constant regardless of the type of organic donor present in the culture medium. This is in contrast to the pronounced effect of different hydrogen donors on the morphology of *Rhodopseudomonas* and *Rhodospirillum* (van Niel, 1944).

Neither resting stages nor motile forms have been observed, and the gram reaction is negative. Mature cells contain refractive globules which by staining with Sudan black B have been shown to be fat.

#### PHYSIOLOGY AND BIOCHEMICAL ACTIVITY

An organic hydrogen donor, carbon dioxide furnished as bicarbonate, and light are required for growth. The organisms are obligately photosynthetic and obligately anaerobic. No growth has ever been obtained except in strictly anaerobic illuminated cultures. Preliminary investigations of the pigment system of one strain have demonstrated the presence of bacteriochlorophyll and a number of carotenoids (Volk and Pennington, 1949).

Growth factors are not required. About half-maximal growth is obtained in media containing ethanol as the only organic compound, and serial transfers in this medium have shown no diminution of growth. Growth in ethanol medium is stimulated by small amounts of yeast autolyzate, although the amount of growth obtained at the expense of the yeast autolyzate in the absence of ethanol is negligible (table 1). Yeast autolyzate as a growth stimulant could not be replaced by mixtures of B vitamins or amino acids. When the vitamin and amino acid supplements used by Henderson and Snell (1948) for the cultivation of lactic acid bacteria were added either separately or together to otherwise un-supplemented medium, no stimulation of growth was effected. In fact, an amino acid concentration of 0.01 per cent inhibited growth markedly, and at 0.05 per cent amino acid concentration inhibition was complete. The ability of *R. vannielii* to grow without an exogenous supply of growth factors is an important difference between the nutritional requirements of this organism and members of the *Athiorhodaceae*, for none of the latter organisms will grow in un-supplemented medium (van Niel, 1944; Hutner, 1946).

Hydrogen donors that give equally good cultures at 0.2 per cent concentration are ethanol, propanol, butanol, acetate, propionate, butyrate, valerate, caproate, and lactate. Malate is utilized slowly, but glucose, mannose, fructose,

sorbitol, mannitol, citrate, tartrate, formate, thiosulfate, and sulfide are not utilized. Since two of our four strains were isolated from enrichment cultures containing 0.1 per cent sulfide in place of an organic donor, the ability of pure cultures to use this donor has been tested a number of times at several different sulfide concentrations. However, we have been unable to obtain any evidence that sulfide is utilized at a significant rate (table 2). It must be concluded, therefore, that growth of *Rhodomicrobium* in the sulfide-containing enrichment cultures occurred at the expense of small amounts of organic matter present in

TABLE 1

*Effect of yeast autolyzate on growth of R. vannielii in media with and without ethanol*

(The medium contained inorganic salts plus the additions indicated in the table. Incubation period, 7 days at 28 to 30 C)

% BY VOLUME YEAST AUTOLYZATE	OPTICAL DENSITY	
	0.2% Ethanol	No ethanol
0	0.315	0
0.01	0.325	0.006
0.05	0.440	0.035
0.1	0.380	0.075
0.2	0.620	0.017
0.5	0.555	0.025

TABLE 2

*Effect of sulfide on growth of R. vannielii in media with and without ethanol*

(The basal medium contained inorganic salts plus 0.2 per cent by volume yeast autolyzate. Final pH 7.3; incubation, 7 days at 29 to 30 C)

Na <sub>2</sub> S·9H <sub>2</sub> O, %	OPTICAL DENSITY	
	0.2% Ethanol	No ethanol
0.01	0.72	0.038
0.02	0.75	0.041
0.04	0.67	0.044
0.06	0.76	0.047
0.08	0.49	0.035
0.10	0.58	0.032

the inoculum or elaborated by the sulfur purple bacteria that developed first in such cultures.

#### TAXONOMIC POSITION AND POSSIBLE RELATIONSHIP TO HYPHOMICROBIUM VULGARE

Although *Rhodomicrobium* is closely related biochemically to the nonsulfur purple bacteria, its morphology and mode of cell division preclude its inclusion in the *Eubacteriales*, let alone the *Athiorhodaceae*. Since there is no other order of the *Schizomycetes* in which *Rhodomicrobium* may be placed, we believe it best to

include this organism in a provisional appendix to the *Schizomycetes* until more is known concerning the existence of other bacteria that may possess a similar mode of cell division. The inclusion of microorganisms of unknown relationships in an appendix to the *Schizomycetes* has been recommended by Stanier and van Niel (1941) on the ground that such a procedure stimulates further investigation of the organisms therein because of the tentative nature of such a treatment.

We believe there is some evidence to indicate a possible relationship between *Rhodomicrobium* and *Hyphomicrobium vulgare*, the chemoheterotrophic bacterium commonly found in enrichment cultures for nitrifying bacteria.

*Hyphomicrobium* has been described (Rullman, 1897, 1898; Stutzer and Hartleb, 1899) as a small, rod-shaped or egg-shaped bacterium that produces small threads which may be branched. Stutzer and Hartleb considered the threads as mycelial in nature and the bacterial cells as chlamydo spores from which the mycelium sprouts. Henrici and Johnson (1935), although they made no observation of *Hyphomicrobium* themselves, took an entirely different view of the nature of the filamentous structures of this bacterium since they considered them to be analogous to the lifeless stalks of the stalked bacteria. This interpretation was accepted by Stanier and van Niel (1941). The only recent studies of this organism have been made by Kingma-Boltjes (1934, 1936), who was unable to come to any conclusion concerning the significance of the filaments or the mechanism of cell division. He expressed the opinion, however, that cell division was probably different from that of other bacteria. Kingma-Boltjes' photomicrographs of *Hyphomicrobium*, one of which is reproduced in figure 11, clearly show the extensive branching of the filaments and the egg-shaped cells that occur at the tips of the filaments. These morphological features suggest to us that the filaments of *Hyphomicrobium* are not stalks in the sense that this term has been used by Henrici to describe the nonprotoplasmic attaching structures that are secreted by the true stalked bacteria, but are instead living structures analogous in function to the filaments found in *Rhodomicrobium*.

#### *Definition of the Genus and Species*

*Rhodomicrobium*, *nov. gen.* Oval to round bacteria, attached by means of a slender branched filament. Cell multiplication is initiated by the outgrowth of a new filament from the pole of a mature or immature cell, or from some point along a filament connecting two cells. The tip of the filament swells to form a round cell, which increases in size and eventually assumes an ovoid shape. Nonmotile, nonsporeforming, gram-negative. Contain bacteriochlorophyll, which enables them to have a photosynthetic metabolism dependent on extraneous oxidizable compounds and not accompanied by oxygen production. Contain carotenoid pigments, which give cultures a salmon-pink to a deep orange-red color, depending on the density of growth. The type species is *R. vannielii*.

*Rhodomicrobium vannielii*, *n. sp.* Morphology and mode of cell division as described above. Mature cells are ovoid, 1.2 by 2.8 microns. The filaments are approximately 0.3 microns in diameter. Growth occurs only in illuminated

anaerobic cultures and at the expense of organic hydrogen donors. Sulfide, thiosulfate, and sugars not utilized; organic growth factors not required. Gelatin not liquefied. Catalase-positive. Growth in fluid cultures flocculent, the color varying from a salmon pink to a deep orange red. Colonies are dark red and irregular in shape, and have a rough, convoluted surface.

#### ACKNOWLEDGMENTS

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#### SUMMARY

*Rhodomicrobium vannielii*, a new photoheterotrophic bacterium, has been described. The distinguishing morphological features of this microorganism are the attachment of the cells by means of a branched filament and the mode of reproduction, which we believe to be by budding.

The possibility of a relationship between *Rhodomicrobium* and *Hyphomicrobium vulgare* is discussed.

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