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NOTES

Cultivation of *Spirillum volutans* in a Bacteria-Free Environment

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Despite the early description of *Spirillum volutans* Ehrenberg in 1832, attempts by various investigators to grow this organism in pure culture have been unsuccessful (Williams and Rittenberg, Intern. Bull. Bacteriol. Nomencl. Taxonom. **7**:49, 1957); however, by a most ingenious dialysis sac technique, Rittenberg and Rittenberg (Arch. Mikrobiol. **42**:138, 1962) were able to isolate the Pringsheim strain in pure culture. Growth did not occur independently, however, and the sac had to be suspended in a culture of other bacteria which were presumed to supply a nutritional factor required by *S. volutans*, although no chemical supplements added were able to replace this requirement.

Our strain of *S. volutans* was obtained from a pond water-hay infusion. After 36 serial transfers by the Rittenberg method, a pure sac culture was finally obtained. The spirillum so isolated appeared to fit the morphological description of the Pringsheim strain in all respects (Fig. 1). The cell diameter ranged from 1.5 to 1.8 μ, the wavelength from 16 to 24 μ, and the cell length from 18 to 40 μ. Volutin granules were abundant (Fig. 2). A most striking characteristic of the sac culture was the regular formation of a ring-shaped band of spirilla when covered wet mounts were prepared and allowed to stand for 10 min (Fig. 3); this suggested a possible microaerophilic nature. The Rittenbergs noted a similar ring formation in an uncovered culture drop, but apparently the ring did not contain the enormous numbers of organisms congregated in the sharply limited zone which we have observed in covered mounts.

Successful independent cultivation of the organism from our sac culture was finally accom-

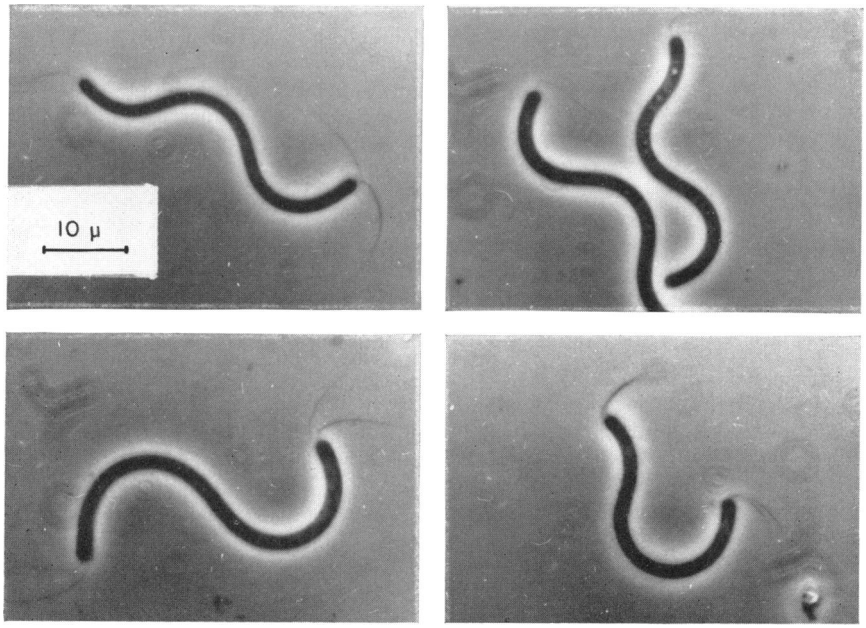

FIG. 1. *Living cells of Spirillum volutans as seen by phase contrast.*

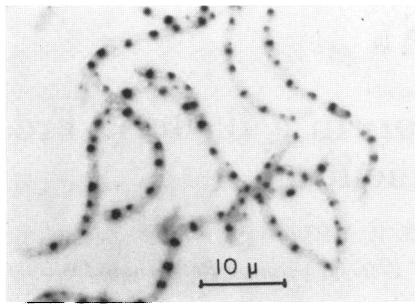

FIG. 2. *Volutin granules of Spirillum volutans (Ponder's stain).*

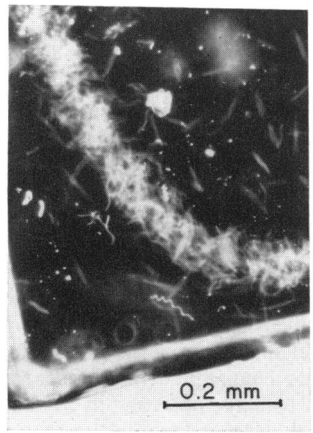

FIG. 3. *Portion of the narrow band of Spirillum volutans in a covered wet mount made from a sac culture (dark-field illumination). A corner of the cover glass can be seen, with rounding off of the band.*

plished by the following procedure. Side-arm flasks (250 ml) containing 75 ml of nutrient broth were employed. The mouths were closed with vaccine stoppers, the side arms were plugged with cotton, and the flasks were autoclaved. After cooling, the flasks were evacuated, the air removed was replaced with nitrogen, and the flasks were sealed. Inoculation with 0.5 ml of sac culture was done with a syringe. When flasks containing atmospheres of 3, 6, 9, 12, and 21% oxygen were prepared, faint turbidity appeared in 3 days at the 3, 6, and 9% levels only. Microscopic observation indicated large numbers of spirilla in these flasks, but not in the others, except for a few nonmotile forms. Serial transfer to fresh 3% flasks were made, and one of our isolates has been so maintained for 51 transfers (transfers being made every 2 to 3 days). Subculture to nutrient broth from sac culture has been accomplished on three separate occasions, each series being transferred at least 10 times before being discontinued. It is essential that the air evacuation and nitrogen replacement be done as soon as possible after autoclaving and cooling. This is of particular importance during the first few transfers from sac culture to nutrient broth.

TABLE 1. *Effect of oxygen concentration on growth of Spirillum volutans*

Conditions*	Cells/ml after 3 days at 30 C†
Medium reduced for resazurin, 100% nitrogen atmosphere	$<10^4$
Oxygen atmosphere, 1%	3.2×10^5
Oxygen atmosphere, 3%	3.5×10^5
Oxygen atmosphere, 6%	3.8×10^5
Oxygen atmosphere, 9%	3.9×10^5
Oxygen atmosphere, 12%	$<10^4$
Oxygen atmosphere, 21%, cotton-stoppered flasks	$<10^4$

* Medium = nutrient broth + 100 mg of cysteine hydrochloride + 1 mg of resazurin per liter; inoculum per flask = 100,000 spirilla in 0.25 ml of culture.

† By hemocytometer count.

In determining the upper and lower limits of the oxygen requirement, ordinary nutrient broth could not be made highly anaerobic without very prolonged boiling; therefore, 100 mg of cysteine hydrochloride and 1 mg of resazurin per liter were added. Flasks were then prepared as above, autoclaved, and immediately boiled until the resazurin was decolorized; they were then sealed tightly. The flasks were cooled and evacuated, and oxygen-free nitrogen was introduced, followed by various amounts of air. Table 1 indicates the growth response to a range of oxygen concentrations of inocula containing 100,000 spirilla. The organism is a true microaerophile, failing to grow under highly reduced conditions or at oxygen concentrations of 12% or above; however, the size of the inoculum is important at the 12% level because a larger one can initiate growth at this level.

Because of the correspondence of our organism with the description of *S. volutans* by Williams and Rittenberg and the failure of our organism to grow on liquid or solid media aerobically (even enriched media such as yeast extract-nutrient agar), we believe that we have isolated *S. volutans* in a completely bacteria-free environment for the first time. The previously observed dependence of *S. volutans* on other bacteria outside the dialysis sac would seem to be a function of the removal of dissolved oxygen by these other bacteria, rather than dependence of the spirilla on a nutritional factor.

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