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Association of Microcyst Formation in *Spirillum itersonii* with the Spontaneous Induction of a Defective Bacteriophage

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Mitomycin C and ultraviolet light were found to induce the formation of microcysts in *Spirillum itersonii*. These forms, as well as spontaneously occurring microcysts in this species, were found to contain phage tail parts, rhapidosomes, and a granular substance not seen in normal cells. It is suggested that microcysts are formed as the result of the induction of a defective phage. The production of phage lysozyme within the cell could lead to the formation of spherical forms as the cells lose their structural mucopeptide layer. Complete virus particles were not seen, nor was any biological activity demonstrated when the induced cultures were tested against two other strains of *S. itersonii*. The other strains of this bacterium also formed microcysts and phage tail parts when induced with mitomycin. Attempts to isolate an organism lacking the defective phage have been unsuccessful.

In the course of studies on cytochrome formation in *Spirillum itersonii* (2), it was thought worthwhile to look for a transducing phage. The initial experiment towards this end involved the use of mitomycin C in an attempt to induce temperate phages (9). Although no lysis of the treated culture was apparent from the growth curve, examination of the culture by light microscopy revealed that a large number of the cells had formed microcysts. The spontaneous formation of spherical cells or microcysts by members of the genus *Spirillum* has previously been described (13, 14), and, as these shapes appeared more often in older cultures, they were thought to be a resting stage. However, in view of the observation that mitomycin caused the formation of large numbers of microcysts, a further examination of this problem was undertaken with the aid of an electron microscope. This communication reports that spontaneously occurring microcysts in *S. itersonii*, and those artificially induced by mitomycin or ultraviolet light, contain phage tails, rhapidosomes, and a granular substance not seen in normal cells.

MATERIALS AND METHODS

Bacterial cultures. The present study was carried out with the strain of *S. itersonii* used previously (2), which was originally obtained from R. Martinez in this department. Two other strains, ATCC 12639 and ATCC 11331 subspecies *vulgatum*, were obtained from the American Type Culture Collection. These strains were reisolated before use.

Media. A tris(hydroxymethyl)aminomethane (Tris)-asparagine (TA) medium was used for liquid cultures. It contained, per liter: L-asparagine·H_2O, 3 g; $K_2HPO_4 \cdot 3H_2O$, 1.0 g; KH_2PO_4, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.025 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; and ferric citrate·$3H_2O$, 0.0054 g. TA medium was made up in 50 mM Tris-chloride (*p*H 7.0), and the final *p*H of the medium was 7.0. A glutamate-malate medium (6) supplemented with 0.1% yeast extract (YEMG) and containing 1.5% agar was used for plates.

Growth. Erlenmeyer flasks (125 ml) containing 50 ml of TA medium were inoculated with 0.5% of an overnight culture. The flasks were shaken at 200 rev/min on a gyratory shaker at 30 C, and 1-ml samples were taken at appropriate intervals for determinations of optical density at 540 nm. An optical density of 1.0 at this wavelength is equivalent to 260 μg of protein/ml with bovine serum albumin as a standard.

Mitomycin treatment. Mitomycin, to a final concentration of 0.5 μg/ml, was added to logarithmically growing cells when the optical density of the culture was approximately 0.3.

Ultraviolet treatment. A logarithmically growing culture with an optical density of 0.3 was exposed to 600 ergs/mm² of ultraviolet radiation from a 15-w G.E. germicidal lamp. After irradiation, both the test and control cultures were supplemented with 0.1% yeast extract to aid the recovery of the treated cells; the irradiated culture was protected from the light.

Spontaneously occurring microcysts. Cultures of *S.*

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itersonii were incubated at 30 C in TA medium for 3 to 4 days. Microcysts generally occur in these old cultures.

Purification of phage tails. A large volume of culture (800 ml) was treated with mitomycin and incubated for a further 8 hr. After this time, 1 ml of chloroform and 8 g of polyethylene glycol 6000 were added, and the culture was allowed to stand overnight at 2 C. It has been empirically found that the addition of polyethylene glycol aids in the dissolution of the spherical cells and the liberation of phage tails. The culture was centrifuged at 8,000 × *g* for 10 min to remove unlysed cells and major debris. The supernatant liquid was then poured into fresh tubes and underlayered with 1 ml of sucrose solution (100 g of sucrose made to 100 ml with 100 mM potassium phosphate buffer, *p*H 7.0). The phage tails and small particles were collected on the sucrose cushion by centrifugation at 27,000 × *g* for 1 hr. After careful removal of the supernatant liquid, the sucrose suspension of particles was dialyzed against 50 mM potassium phosphate buffer (*p*H 7.0) to remove excess sucrose. The particle suspension was then sedimented through a 5 to 20% sucrose gradient containing 50 mM phosphate buffer. After 90 min at 25,000 rev/min in an SW25 Spinco rotor, 4-ml fractions were collected and examined in an electron microscope for phage tails. The fraction containing the greatest number of phage tails was the second from the top, and this fraction was dialyzed against the phosphate buffer for subsequent examination in an electron microscope.

Biological activity. Induced cultures of all three strains and the purified fraction of phage tails were tested for biological activity, after the addition of a small amount of chloroform, by spotting onto lawns of the three strains. A lawn was prepared by overlaying YEMG plates with 5 ml of semisolid YEMG agar (0.6%) containing approximately 10^8 cells/ml. The tests were repeated with the use of TA medium in place of the YEMG.

Curing. Treatment of the cells with acridine orange was based on the method of Hirota (4). Cells were grown through approximately twelve generations in TA medium supplemented with 0.2% yeast extract and containing 50 μg of acridine orange per ml. The initial *p*H of the medium was around 7.0 and rose to 7.5 at the end of growth. Suitable dilutions were plated onto YEMG medium containing 0.05, 0.1, and 0.2 μg of mitomycin per ml. Large colonies which grew in the presence of 0.1 and 0.2 μg of mitomycin per ml were subcultured into TA medium and were subsequently treated with 0.5 μg of mitomycin per ml. The treated cultures were examined for the loss of microcyst formation in a light microscope and for the loss of phage tails in an electron microscope.

Test for acridine orange resistance. Drops of acridine orange (25 mg/ml) were placed onto YEMG plates, and the drops were allowed to diffuse into the agar overnight. Cultures were tested for acridine orange resistance by streaking with a needle once across the drop. A similar number of control colonies were streaked at right angles to the test cultures.

Electron microscopy. Samples to be examined were placed on a thin carbon film supported on 400-mesh grids. The original parlodion film supporting the carbon layer was removed by leaving the grids overnight on filter paper saturated with amyl acetate. Samples were stained with 1% neutral potassium phosphotungstate or 1% uranyl acetate (UA) after allowing 1 min for adhesion to the carbon film. Preparations containing phosphate buffer were rinsed on the grid with distilled water before the application of uranyl acetate. Electron microscopy was carried out with a Hitachi HUIIA electron microscope.

Chemicals. Mitomycin C (Nutritional Biochemicals Corp., Cleveland, Ohio) was made to 0.5 mg/ml in sterile distilled water and was protected from light. Acridine orange (*N*,*N*-tetramethyl-3,6-diaminoacridine; Eastman Organic Chemicals, Rochester, N.Y.) was made to a concentration of 25 mg/ml in sterile distilled water.

RESULTS

The growth of *S. itersonii* upon treatment with mitomycin C and with ultraviolet light is shown in Fig. 1. The mitomycin-treated culture continued to grow for a further 5 hr, and thereafter the optical density remained constant. The ultraviolet-treated culture continued to grow slowly. Both cultures contained an occasional spherical cell after 2 hr of incubation and considerable numbers of spherical cells after 4 hr, whereas the

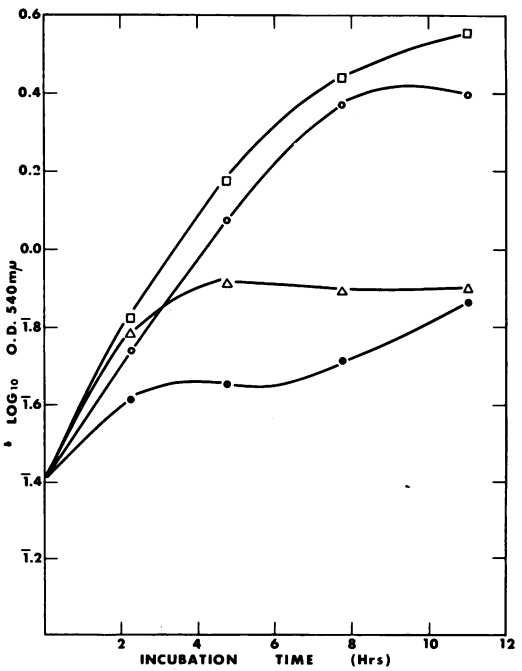
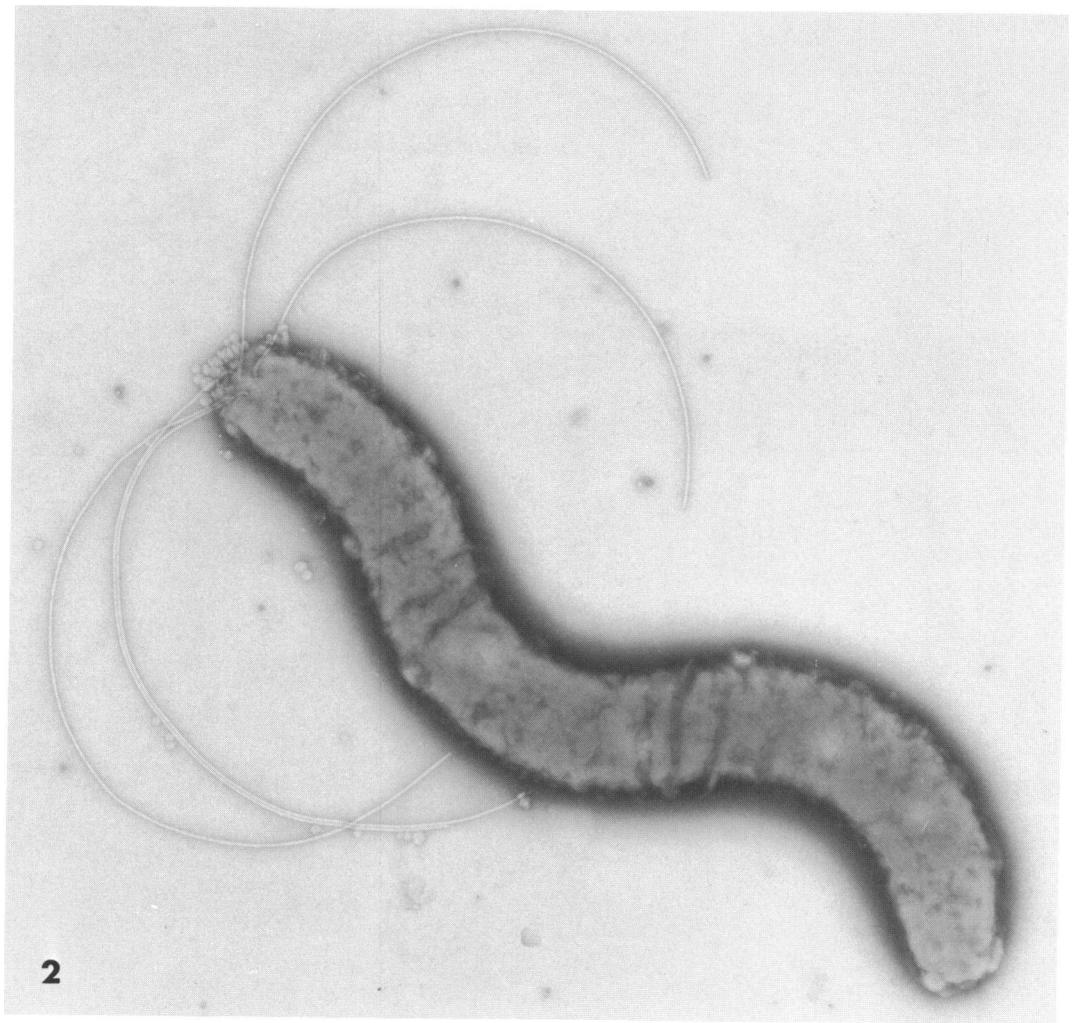


FIG. 1. *Effect of inducing agents on the growth of S. itersonii. Symbols:* ○, *control, no additions;* △, *0.5 μg of mitomycin per ml;* □, *control, plus 0.1% yeast extract;* ●, *600 ergs/mm² of ultraviolet radiation, plus 0.1% yeast extract.*



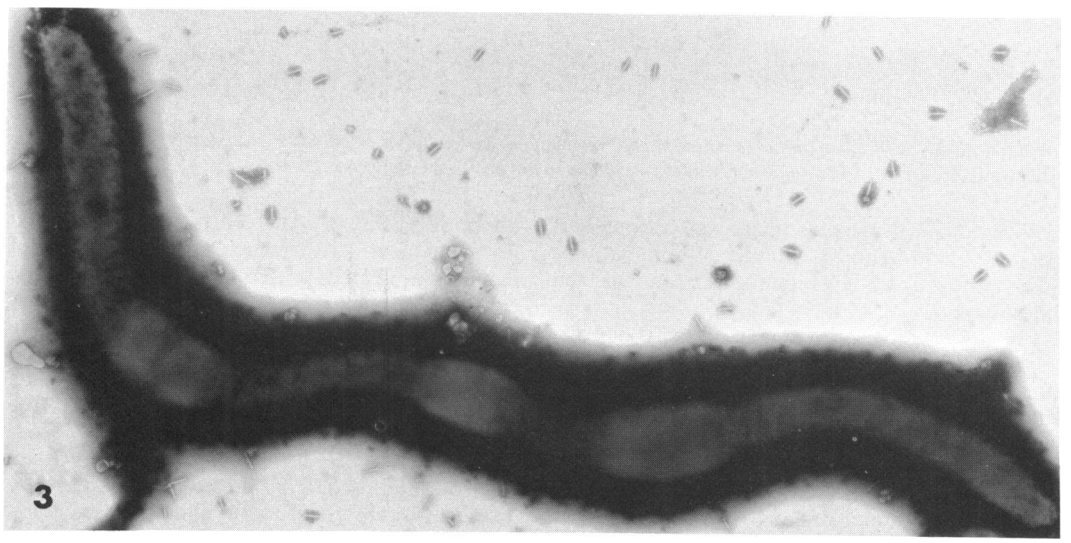


FIG. 2. *Normal cell of S. itersonii. Potassium phosphotungstate stain.* × *33,600.*

FIG. 3. *S. itersonii after mitomycin treatment, showing initial stage of microcyst formation characterized by regional swellings. Potassium phosphotungstate stain.* × *16,600.*

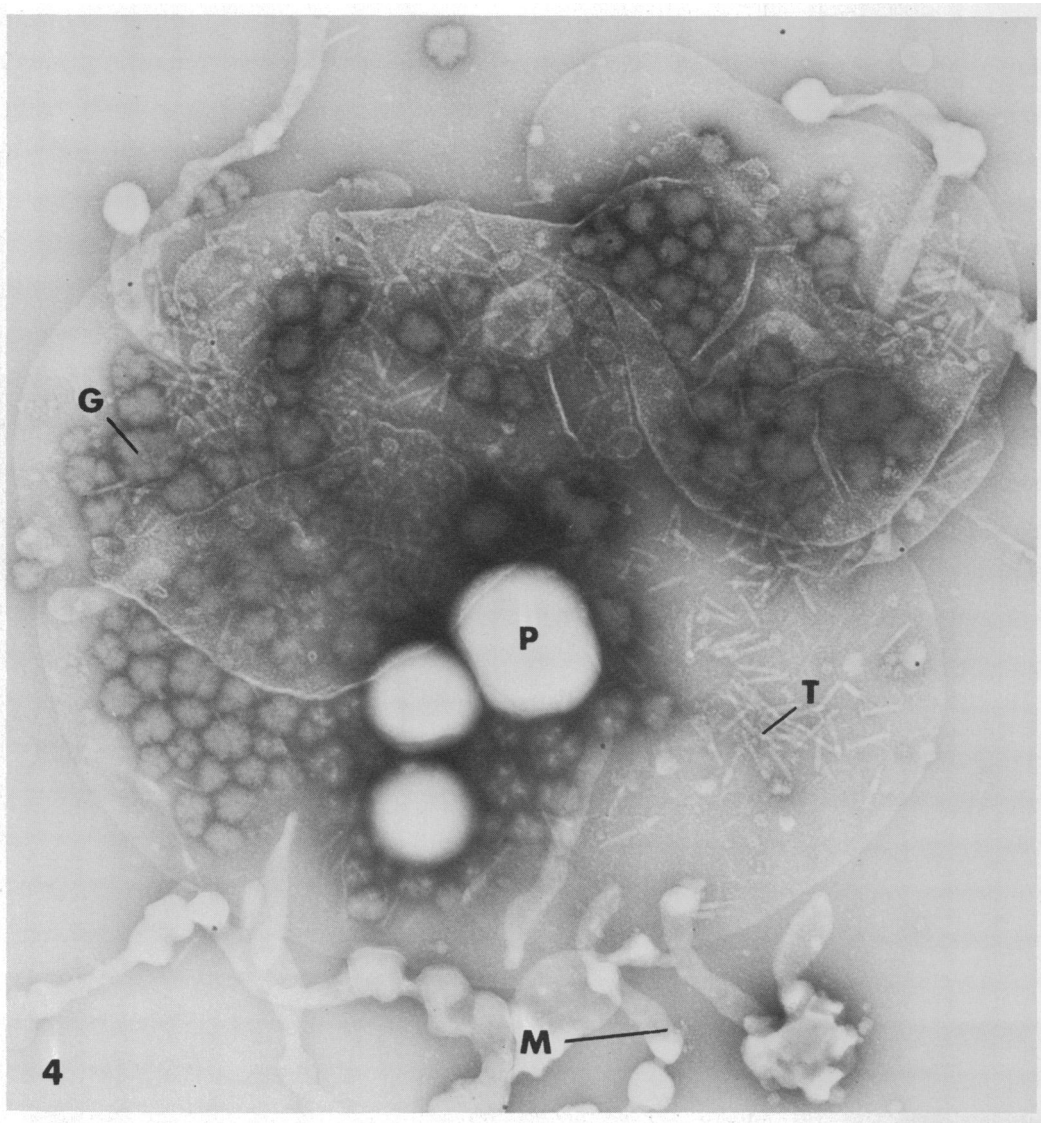


FIG. 4. *Lysed cell after mitomycin treatment, showing β-hydroxybutyrate polymer granules (P), tubular membrane (M), phage tails (T), and unidentified granular material (G). Uranyl acetate stain. × 45,200.*

controls did not produce microcysts throughout the experiment. At 6 hr, samples of both cultures were removed for examination in an electron microscope.

For comparison, the appearance of a normal cell of *S. itersonii* is presented in Fig. 2. The general undulating shape characteristic of spirilla is apparent together with polar flagella, which in actively growing cultures of this strain are seen only at one end of the cell. After mitomycin treatment of a growing culture, the first apparent morphological change was a regional swelling, generally at the end of the cell but sometimes occurring in the middle (Fig. 3). The cell then lost all helical form and assumed a spherical shape. After 6 hr of incubation in the presence of mitomycin, the culture contained mainly a mixture of sluggishly motile elongated helical cells or nonmotile spherical cells of various sizes, although a few highly motile small cells were still present. When viewed in a phase-contrast microscope, the spherical cells did not show any refraction.

The appearance of the spherical cells in an electron microscope is presented in Fig. 4–6. These cells are fragile and undergo lysis upon contact with the grid, allowing the stain to pene-

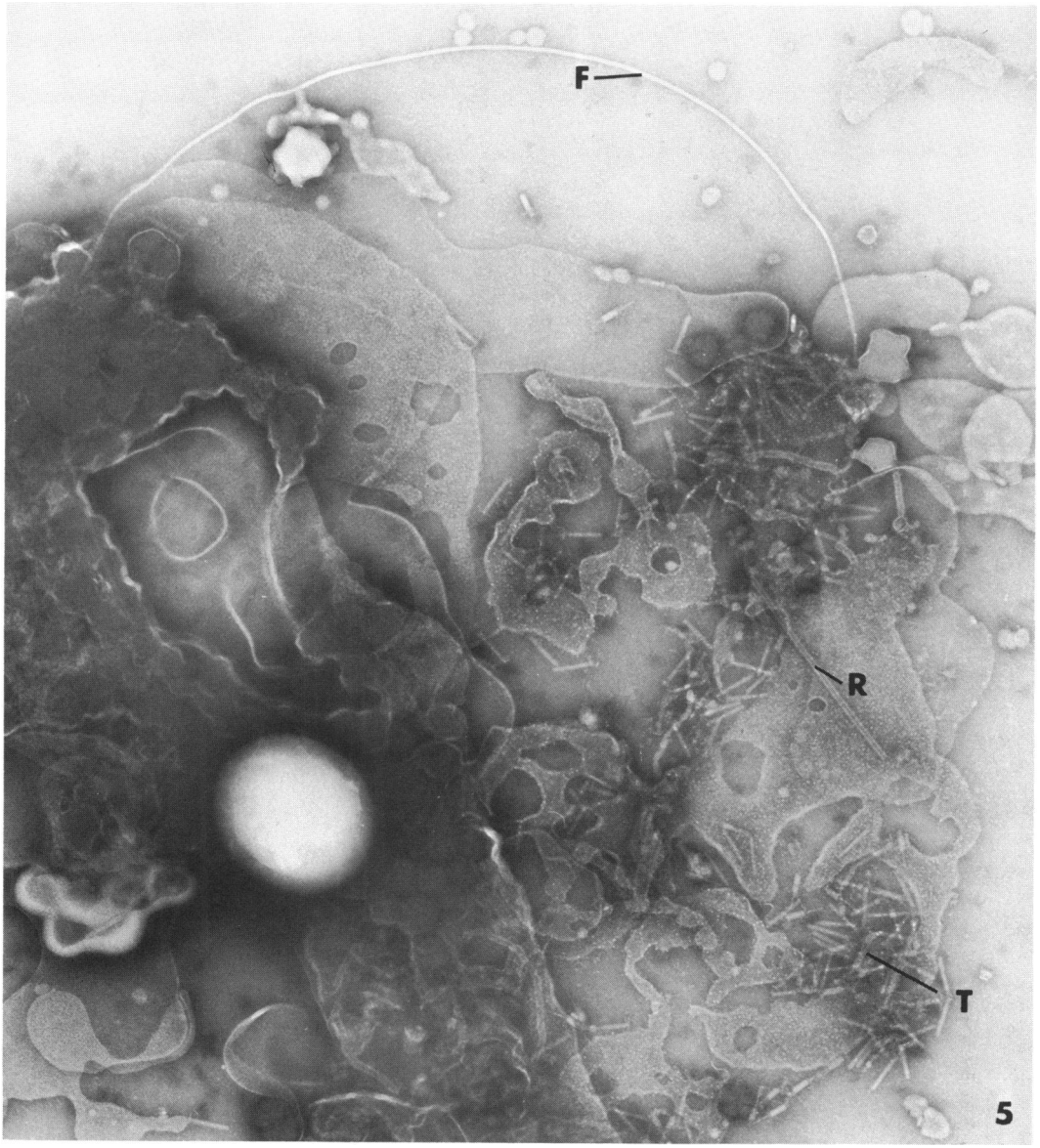


FIG. 5. *Lysed cell after mitomycin treatment, showing a flagellum (F), phage tails (T), and a rhapidosome (R). Potassium phosphotungstate stain. × 45,200.*

trate and reveal internal structures. Figure 4 shows a lysed cell which contains polymer granules composed of β-hydroxybutyrate (8) and tubular membrane fragments. Numerous phage tails and unidentified granular material are also located within the cell. Phage tails are again visible in Fig. 5, together with a flagellum and a microtubule or rhapidosome. Figure 6 illustrates the occurrence of four rhapidosomes in a single cell; however, this number of rhapidosomes is infrequently seen in mitomycin-induced cells. Although not illustrated here, ultraviolet-induced spherical cells contained the same three unusual elements but generally in less abundance. Nearly all microcysts from the artificially induced cultures, which were identifiable in an electron microscope, contained phage tails. On the other hand, spontaneously occurring microcysts rarely

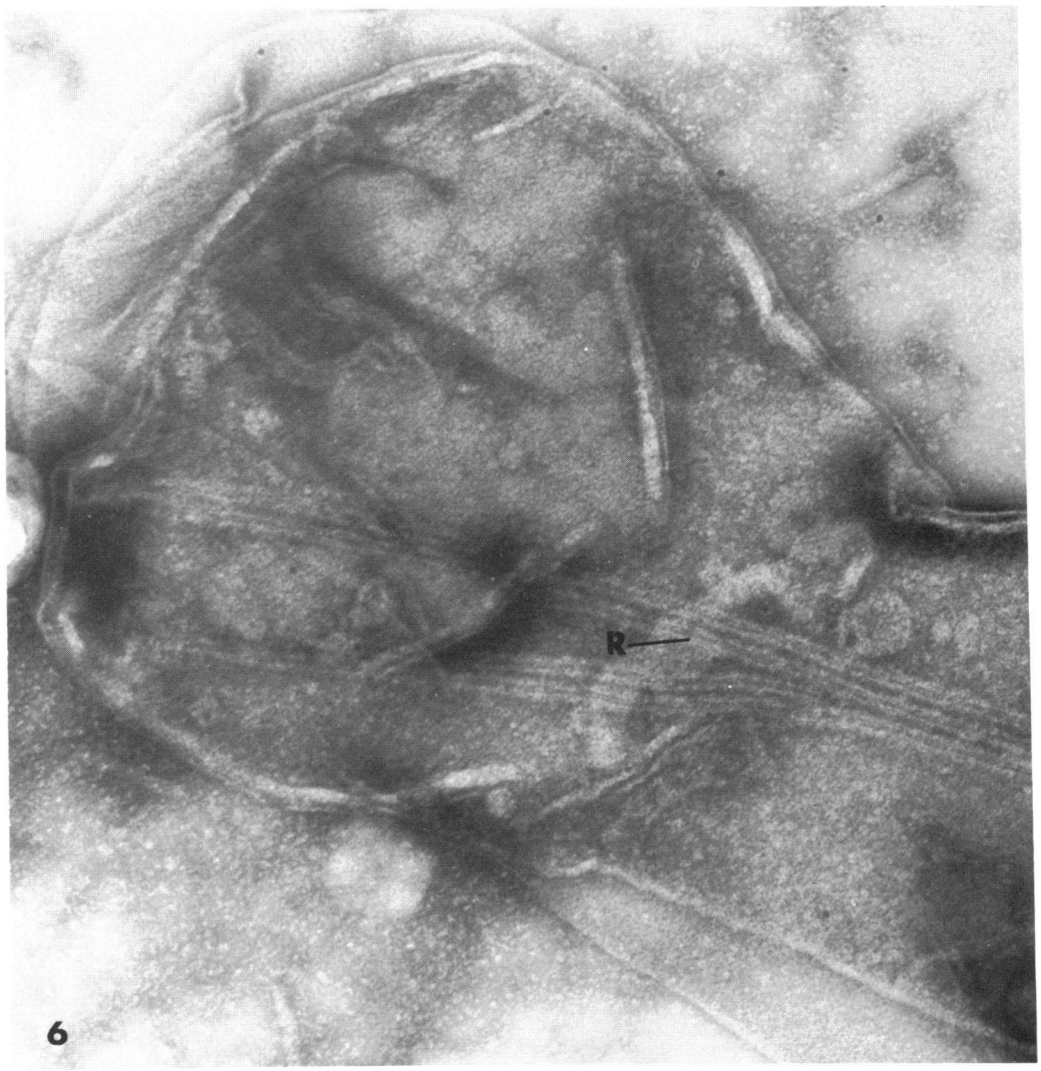


FIG. 6. *Lysed cell after mitomycin treatment, showing four rhapidosomes (R). Uranyl acetate stain.* × *108,000.*

contained phage tails or granular material; instead, many of these microcysts contained rhapidosomes.

Figure 7 shows a purified fraction of phage tails from a sucrose gradient centrifugation. About an equal number of phage tails with contracted sheaths are visible in this micrograph. The uncontracted tails are approximately 140 nm long and 20 nm wide, whereas the contracted sheaths measure approximately 60 nm long and 24 nm wide. The tails sometimes have small fibers at one end, as illustrated in Fig. 8.

The mitomycin- and ultraviolet-induced cultures and purified fraction of phage tails showed no biological activity when tested on the same strain or two different strains of *S. itersonii*. However, it is of interest to note that the two different strains also produced spherical cells and phage tails when induced with mitomycin.

An attempt to cure the bacterium of the defective phage was undertaken with acridine orange according to the procedure of Hirota (4). After growth in the presence of acridine orange, cells were plated on medium containing various concentrations of mitomycin. This procedure was adopted for screening the treated culture, as it was reasoned that sublethal doses of mitomycin may cause cells lacking the defective phage to outgrow the original cells. Untreated cells grew poorly on YEMG medium containing 0.1 μg of mitomycin per ml and not at all on medium containing 0.2 μg/ml. After acridine orange treat-

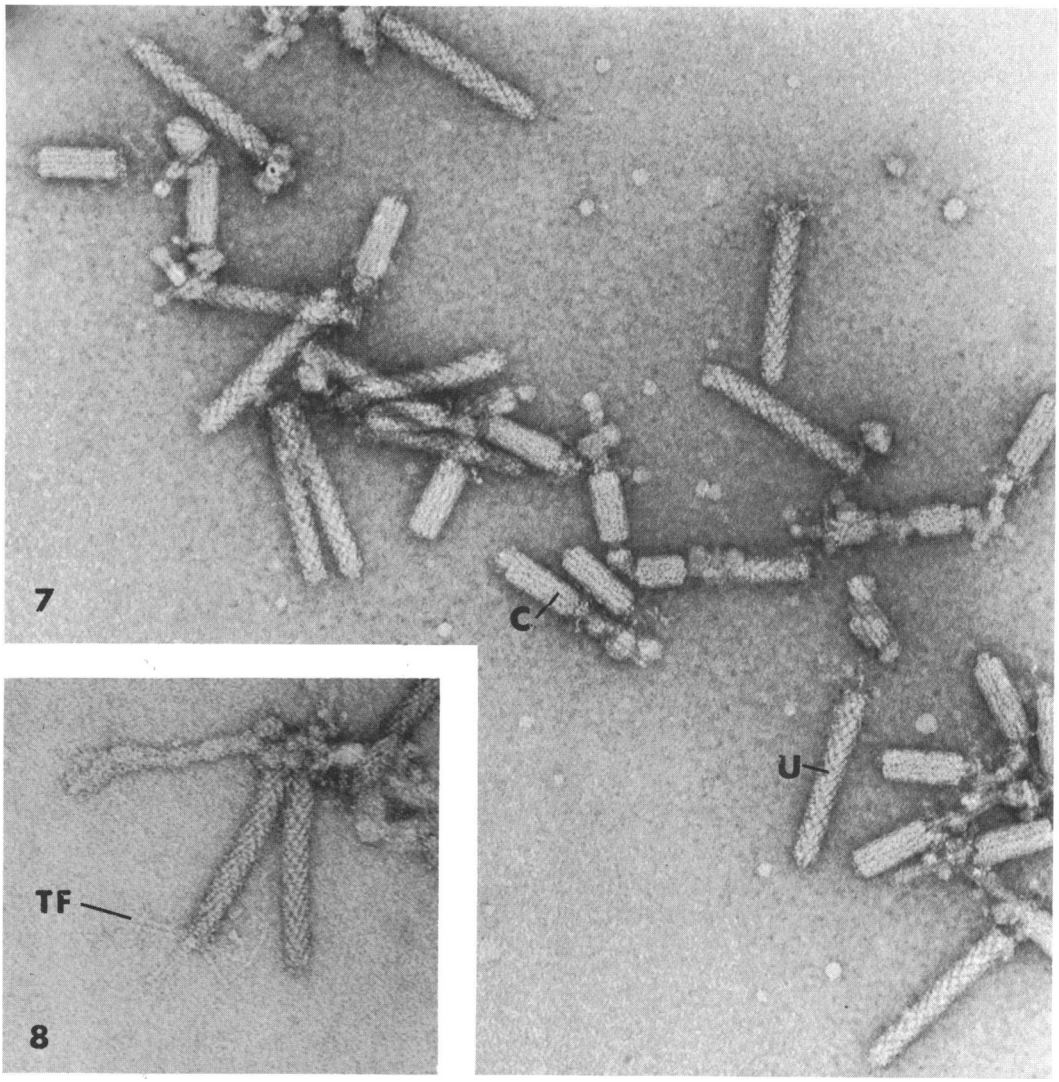


FIG. 7. *Fraction from a sucrose gradient centrifugation, showing contracted (C) and uncontracted (U) phage tails. Uranyl acetate stain. × 170,500.*

FIG. 8. *Uncontracted phage tail, showing fibers (TF). Uranyl acetate stain. × 182,000.*

ment, large colonies, in addition to small ones, were noted on 0.1 μg/ml plates, and a similar number of large colonies were found on 0.2 μg/ml plates. In duplicate experiments, these colonies occurred with a frequency of 0.3 and 1.5%. Thirty of these large colonies were grown in the presence of mitomycin. All colonies produced spherical cells, and when 15 of these cultures were examined in an electron microscope all contained phage tails. Streaking of these large colonies across acridine orange drops, compared with a similar number of small colonies streaked at right angles, revealed that the large colonies were acridine orange-resistant. The large colonies grew completely across the acridine spot, whereas the controls did not. It may be that resistance to acridine orange also leads to mitomycin resistance, but this possibility has not been investigated.

Colonies from plating of a culture grown for 24 hr in the presence of mitomycin were also examined for the loss of phage tails, as it has been reported (11) that mitomycin is effective for concentrating nonlysogenic cells in a lysogenic population. However, all 30 colonies tested from YEMG plates formed spherical cells when regrown in the presence of mitomycin, and when 10

of these cultures were examined in an electron microscope all contained phage tail parts.

DISCUSSION

The occurrence of spherical cells or microcysts in the genus *Spirillum* has been known for some time (13, 14). The present studies provide an explanation of how these structures could arise. It is thought that the spontaneous or artificial induction of a defective phage in *S. itersonii*, in addition to causing the formation of tail parts, also causes the production of phage lysozyme. The action of this enzyme from within could lead to the cells becoming spherical as they gradually lose their structural mucopeptide layer. However, some structural component may still persist in the cell envelope since the spherical cells do not lyse immediately, as judged from the growth curve, and since the spherical forms can remain in a culture for several days.

Complete virus particles or phage heads have not been observed in cultures of induced cells, nor has any biological activity been detected in induced cultures tested against other strains of *S. itersonii*. However, in appearance the phage tails seen in the present study certainly resemble the large bacteriocins which Bradley described in a recent review (1). The fact that the other strains of *S. itersonii* also contain a defective phage may account for the failure to demonstrate any bacteriocin activity. In this regard, attempts to obtain a sensitive strain lacking the defective phage, by procedures which others have used to eliminate plasmids, have been unsuccessful.

The presence of rhapidosomes and a granular substance in the spherical cells of *S. itersonii* is noteworthy, since both may be products of the defective phage genome. Several defects may have occurred in this genome, leading to the production of aberrant or excess components. Hence, the rhapidosomes could be a form of polysheath arising by a self-assembly of excess sheath subunits. Self-assembly of sheath subunits has been invoked to account for the formation of polysheath by coliphage T4 (5). Similar suggestions for the viral nature of rhapidosomes have been put forward recently (1, 12). However, different views have been presented as to the nature and origin of rhapidosomes since Lewin (3, 7) first coined the term for the rodlike structures found in spontaneous lysates of *Saprospira*. It has been variously suggested that rhapidosomes are possibly derived from the disintegration of the mesosome in *Chondrococcus* (10), or that they are normal components of several bacteria and are associated with the nucleoplasm in replication (15).

Further studies are contemplated to clarify the nature of rhapidosomes in *S. itersoni*.

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LITERATURE CITED

1. Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31:230–314.
2. Clark-Walker, G. D., B. Rittenberg, and J. Lascelles. 1967. Cytochrome synthesis and its regulation in *Spirillum itersonii*. J. Bacteriol. 94:1648–1655.
3. Correll, D. L., and R. A. Lewin. 1964. Rod shaped ribonucleoprotein particles in *Saprospira*. Can. J. Microbiol. 10:63–74.
4. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. 46: 57–64.
5. Kellenberger, E., and E. Boy de la Tour. 1964. On the fine structure of normal and polymerized tail sheath of phage T_4. J. Ultrastruct. Res. 11:545–563.
6. Lascelles, J. 1959. Adaptation to form bacteriochlorophyll in *Rhodopseudomonas spheroides*: changes in activity of enzymes concerned in pyrrole synthesis. Biochem. J. 72:508–518.
7. Lewin, R. A. 1963. Rod shaped particles in *Saprospira*. Nature 198:103–104.
8. Martinez, R. J. 1963. On the nature of the granules of the genus *Spirillum*. Arch. Mikrobiol. 44:334–343.
9. Otsuji, N., M. Sekiguchi, T. Iijima, and Y. Takagi. 1959. Induction of phage formation in the lysogenic *E. coli* K-12 by mitomycin C. Nature 184:1079–1080.
10. Pate, J. L., J. L. Johnson, and E. J. Ordal. 1967. The fine structure of *Chondrococcus columnaris*. II. Structure and formation of rhapidosomes. J. Cell Biol. 35:15–35.
11. Reich, E., A. J. Shatkin, and E. L. Tatum. 1961. Bacteriocidal action of mitomycin C. Biochim. Biophys. Acta 53: 132–149.
12. Reichenbach, H. 1967. Die wahre Nature der Myxobakterien 'rhapidosomen.' Arch. Mikrobiol. 56:371–383.
13. Williams, M. A., and S. C. Rittenberg. 1956. Microcyst formation and germination in *Spirillum lunatum*. J. Gen. Microbiol. 15:205–209.
14. Williams, M. A., and S. C. Rittenberg. 1957. A taxonomic study of the genus *Spirillum* Ehrenberg. Intern. Bull. Bacteriol. Nomen. Taxon. 7:49–110.
15. Yamamoto, T. 1967. Presence of rhapidosomes in various species of bacteria and their morphological characteristics. J. Bacteriol. 94:1746–1756.