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First Generation Synchrony of Isolated *Hyphomicrobium* Swarmer Populations[1]

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A method is described for obtaining synchronously growing swarmer cell populations of *Hyphomicrobium* sp. strain B-522. This was accomplished by isolating young swarmers from random cultures by centrifugation and filtration. Cell multiplication occurred during 38% of the growth cycle in populations synchronized in this manner. Observations were made of the changes in cellular morphology which occurred during the growth cycle. Of the 14.25 h required for the doubling in cell numbers, an average of 5 h passed before the swarmer cells began to develop their hyphae. This time varied over a range of 10 h. The time interval between the beginning of hyphal development and the beginning of bud formation was 3.5 to 4.5 h. The maturation of the first buds and their separation from the mother cells were completed in 5.5 h. The duration of these steps is compared to those measured previously in agar slide cultures.

During the reproductive cycle of *Hyphomicrobium* sp. strain B-522, a swelling at the tip of the hypha begins the process of visible bud formation (5, 11). The bud continues to increase in size and at some point before its completion, as yet uncertain, develops one, or sometimes two, subpolar flagella. A septum is formed in the hypha just below the bud, and vigorous movements of the bud eventually cause it to become detached from the hypha of its mother cell. The mother cell can continue to produce a number of daughter cells in this fashion. The liberated bud, still immature, is now referred to as a swarmer. After a period of morphological changes, the swarmer produces a hypha from the pole of the cell opposite to where it was formerly attached to the mother hypha. At some time during this process, the flagellum is lost. The new hypha may form a bud as before. The time required for swarmer maturation, hyphal growth, new bud formation, and the production of a separate daughter cell is referred to as the "mother generation time" (Hirsch and Jones, Bacteriol. Proc., p. 44, 1968).

In actively growing cultures, *Hyphomicrobium* B-522 shows very little tendency to form branched hyphae, although as many as 5% of the cells may possess hyphae with a single, short branch near the mother cell at right angles to the main hypha. However, under poor growth conditions, hyphae branch extensively (2, 3). This was also observed with other hyphomicrobia (2) and *Pedomicrobium* spp. (10).

A detailed study of the rather elaborate life cycle of *Hyphomicrobium* is difficult to accomplish with ordinary, random cultures because two processes, the formation of mother cells and the formation of daughter cells, occur at the same time. Studies of individual cells on agar slide cultures are helpful but are limited in their use for making biochemical measurements (3, 4; Hirsch and Jones, Bacteriol. Proc., p. 44, 1968). The availability of synchronously growing cells would serve to overcome these problems. A method for obtaining such cultures is described in the present investigation. It is used to obtain information on the morphological changes which occur during the life cycle of *Hyphomicrobium* B-522.

MATERIALS AND METHODS

Hyphomicrobium sp. strain B-522 was grown in medium 337 a (2, 3) which consists of the following: 0.14% KH_2PO_4, 0.11% Na_2HPO_4, 0.05% $(NH_4)_2SO_4$, and 0.01% $MgSO_4 \cdot 7\ H_2O$, with 5 ml of 67.6% filter-sterilized methylamine hydrochloride per liter as the carbon source, and with 2 ml of trace element solution per liter containing 100 mg% $FeSO_4 \cdot 7\ H_2O$, 150 mg% $CaCl_2$, 35 mg% $MnSO_4 \cdot H_2O$, and 50 mg% $Na_2MoO_4 \cdot 2H_2O$. The pH of the medium was adjusted to

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7.5 before autoclaving. The cells were grown at 30 C in 250-ml Bellco sidearm flasks in a shaking water bath.

Turbidity measurements were made on a Klett-Summerson colorimeter fitted with a no. 66 red filter. Viable counts were obtained by spreading appropriate dilutions of the cultures onto medium 337a solidified with 0.8% Noble Agar (Difco Laboratories, Detroit, Mich.). For direct cell counts, a Petroff-Hauser counting chamber was employed, and sodium azide (0.1 M final concentration) was added to the culture samples to stop growth. All counts were done in triplicate.

Cell size measurements were made on photomicrographs with a final magnification of ×1,280 (taken on Kodak Plus X film with a Zeiss Photomicroscope II) or on electron micrographs of ×8,050 to ×20,500 magnification (taken with a Philips EM-300 electron microscope at 60 kV). In the latter case, cells on Formvar-coated grids were negatively stained with 1% phosphotungstic acid (pH 7.2) containing 0.2% bovine serum albumin.

A culture of cells in the late log phase of growth with a turbidity of 120 to 140 Klett units was centrifuged at 3,000 × *g* for 3 min. The supernatant fluid was filtered with very gentle suction through a 47-mm diameter nitrocellulose membrane filter (Millipore Filter Corp., Bedford, Mass.) with a pore size of 3 μm. A single filter could be used for up to 40 ml of culture. The swarmers in 8 ml of filtrate were then collected on a Millipore nitrocellulose membrane filter (pore size 0.22 μm) and transferred on the filter to 30 ml of medium which was prewarmed to 30 C. Care was taken to prevent contamination of the cultures during the various operations. The cultures were incubated in a shaking water bath at 30 C. The agitation of the medium quickly washed the cells from the filter which was removed after 5 min. This procedure yielded a density of approximately 2×10^7 swarmers per ml.

RESULTS

Poindexter (7) was able to obtain synchronously dividing cell populations of *Caulobacter crescentus* with swarmers isolated from a random culture as the starting inoculum. These cells produced synchronous growth because they represented the younger cells of the culture and had a very restricted range of age. A homogenous swarmer population was obtained by centrifugation of an exponentially growing culture. The upper layer of the resulting cell pellet contained mainly stalked cells, while the lower consisted of swarmers. A centrifuged culture of *Hyphomicrobium* sp. B-522 yielded just the opposite result. The swarmers were found in the upper layer of the pellet, and the mother cells were found with hyphae in the lower layer. By regulating the time and speed of centrifugation, a large proportion of mother cells could be pelleted, while most of the swarmers remained suspended in the medium. However, complete separation of the two cell types could not be accomplished in this manner.

Swarmers of *C. crescentus* have also been separated from the stalked mother cells by filtration (9). This method proved useful with *Hyphomicrobium* B-522 but suffered from the tendency of even large filters to clog when enough culture was used to obtain a desirable quantity of swarmers. The best and most rapid method to obtain large quantities of *Hyphomicrobium* swarmers was found to be a combination of centrifugation and filtration as described in Materials and Methods. The contamination of swarmer populations with cells already bearing hyphae ranged from 1 to 6% in the various synchrony experiments. It is likely that these cells represent older swarmers which have recently begun hyphal growth. Cells with buds were rarely seen.

Three representative cell types from a random culture of *Hyphomicrobium* sp. B-522 are shown in Fig. 1. The polarity of such cells was recognized by the small projection from the more pointed end which was originally a part of the mother hypha. In most cells the first hypha originated from the blunt end. Due to the ease with which the projections of the swarmer cell could have been confused with the beginning stages of new hyphal growth in cells with two nearly pointed ends, the stated percentages of "cells with hyphae" present in isolated swarmer populations may represent maximal values. The size of the mother cell body remained fairly constant after the outgrowth of a hypha. Overall differences in length resulted from the presence or absence of hyphae, or buds, or both.

The more uniform appearance of swarmers isolated from a random culture is seen in Fig. 2. During the process of negative staining, flagella were lost from all of the cells. The mean length of 412 individual cells of the swarmer population was 2.1 μm with a range of 1.8 and a standard deviation of ±0.25. The average width of such swarmer cells was 0.73 μm. Measurements from light micrographs on 200 cells of the random parent culture, performed at the time of swarmer isolation, gave a mean length of 5.2 μm with a range of 11.8.

In Fig. 3, a comparison of the frequency of various size classes is made between the isolated swarmers and cells from the random parent culture. Although the swarmer isolation procedure has effectively reduced the presence of larger size classes, little difference exists between the swarmers in the random population and the isolated swarmer population. Forty percent of the cells in the parent culture were swarmers. Approximately 80% of these could be

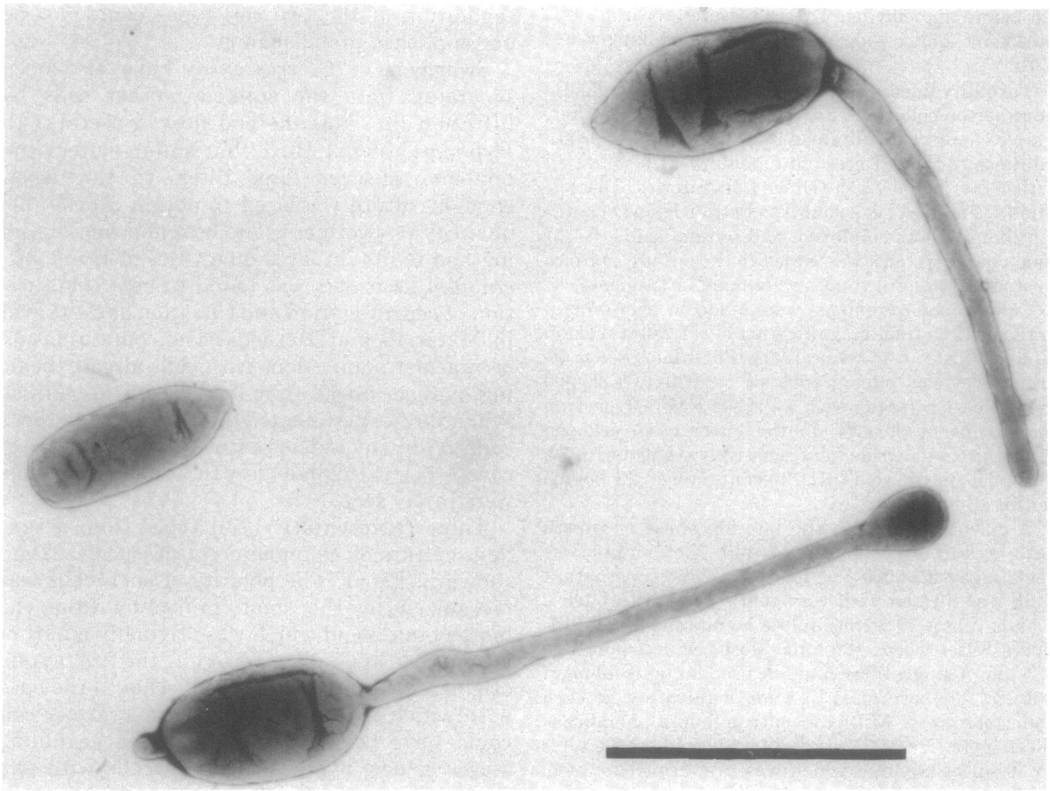

FIG. 1. *Three representative cell types of a random culture of Hyphomicrobium sp., strain B-522. Note short processes on two of the three cells, thought to be remnants of the former mother hypha. Phosphotungstic acid negative stain. The scale marker represents 2 μm.*

isolated by the method described above.

The distribution of sizes in the parent culture is not completely random. There is a higher frequency at cell lengths of 2, 5, 8, and 10 μm. Previous observations of individual cells growing on agar slides indicated that hyphal growth stopped when the budding process began and that it was resumed again soon after the bud had separated from the mother cell. Hyphal growth and bud growth thus were mutually exclusive processes. The four predominant cell lengths observed here may represent such discrete stages of the growth cycle.

A swarmer population, prepared as described above, was examined for the distribution of cell lengths after various periods of growth (Fig. 4). The small, but definite, increase in length that had occurred after 4 h can be seen from a comparison with Fig. 3. By 8 h, approximately 60% of the cells were distributed in size classes larger than those expected for swarmers. After 10 h of incubation, the size frequency more closely resembled that of the parent culture except for a much smaller proportion of cells with lengths greater than 6 μm and less than 2 μm.

The increase in mean cell length during the growth of this population of cells is shown in Fig. 5. After 8 h of incubation, the rate of increase in cell length is 7.5 times greater than during the first 5 h. Under conditions of perfect synchrony, an even greater increase in the rate of cell elongation would be expected.

The proportion of morphologically distinct cell types present in the culture after various periods of incubation was determined by direct microscope observation (Fig. 6). An average of 800 cells was examined at each time period. A small proportion of swarmers already had short hyphal appendages at the beginning of the observations. Their number began to increase immediately. Within 5 h, 50% of the cells were of this type, and after 9.25 h 90% of the cells had hyphae. This value then leveled off before reaching 100% because of the introduction of new swarmers into the population, or because some swarmers were incapable of growing hy-

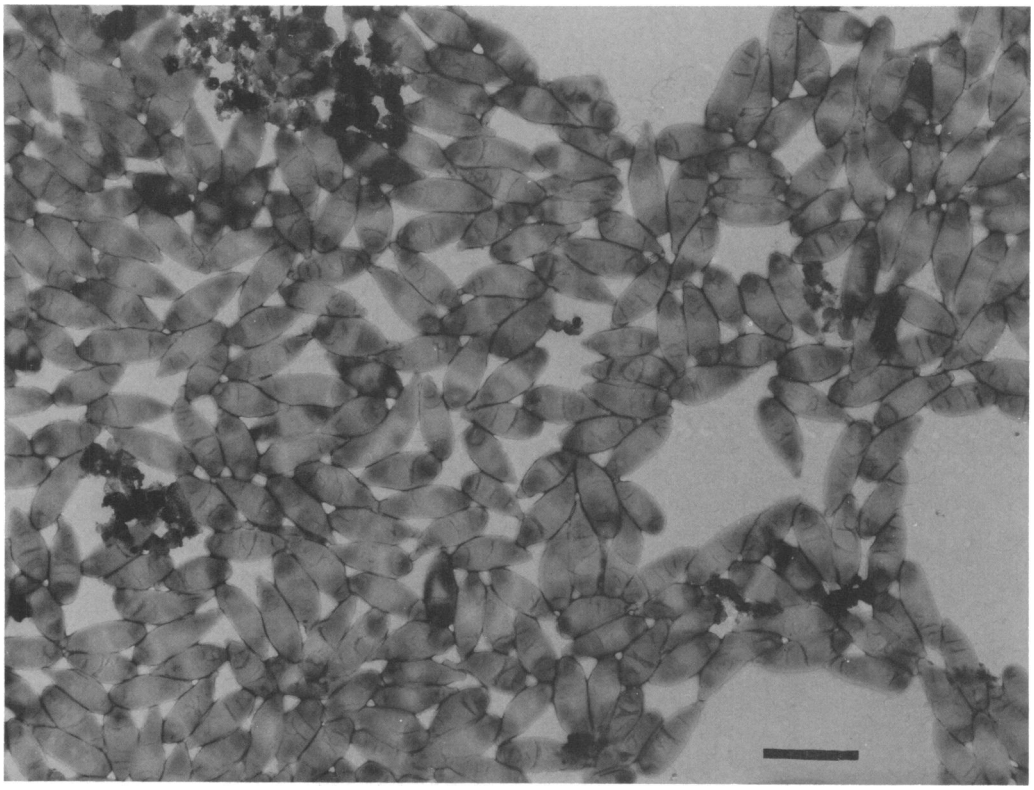

FIG. 2. *Hyphomicrobium B-522 swarmer population as isolated from a random culture. Note the uniform appearance of cell morphology. Phosphotungstic acid negative stain. The scale marker represents 2 μm.*

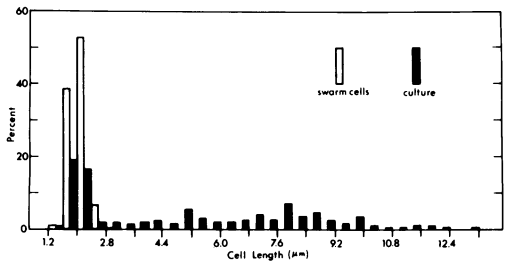

FIG. 3. *Comparison of the frequency of various size classes between isolated swarmer cells of Hyphomicrobium B-522 and their random parent culture of exponential growth phase. Each bar represents a range in size of 0.4 μm.*

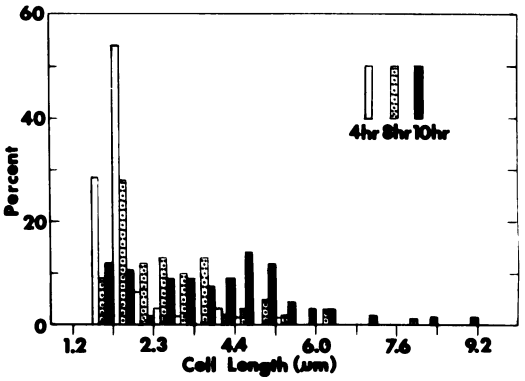

FIG. 4. *Distribution of cell lengths of a swarmer population isolated as described in the text. Measurements after 4, 8, and 10 h, respectively. Each bar represents a range in size of 0.4 μm.*

phae, or both. The rate of appearance of cells with a hypha and bud tended to parallel that of cells with a hypha only. There was at first a 3.5- and later a 4.5-h difference between the two stages. This period represented the time interval between the beginning of hyphal growth and the beginning of visible bud formation. The total number of cells remained constant for the first 8 h of the experiment and then began to increase. Since the first visible buds had appeared after 2.5 h, it follows that the time required for the first buds to mature and to separate was 5.5 h. Although the determinations were made only for the first 10 h of this

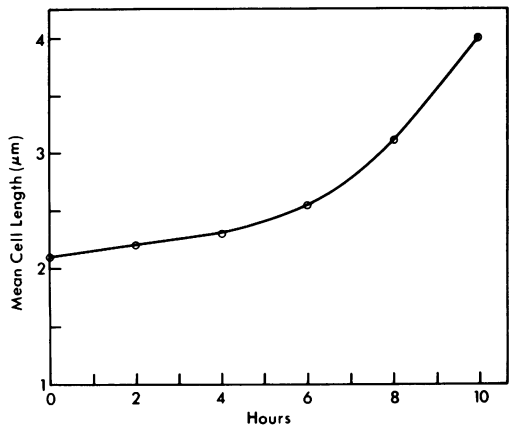

FIG. 5. *Mean cell length of Hyphomicrobium sp. strain B-522 at various times during synchronous growth. The measurements were made from photomicrographs.*

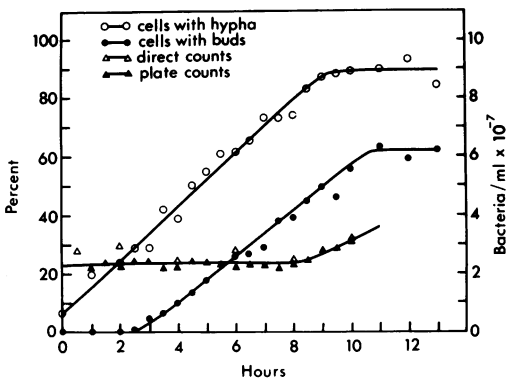

FIG. 6. *Proportions of morphologically different cell types at various times during synchronous growth of a swarmer population of Hyphomicrobium sp. strain B-522. The cell numbers at various times are also shown.*

experiment, it can be seen that the number of cells would have doubled at 13.5 h. The time for hyphal development of the 6% of cells with hyphae at the beginning of the experiment was estimated from Fig. 6 to be 0.75 h. Thus, the total doubling time for the swarmer population would be 14.25 h. This value is consistent with that observed in repeated runs of similar experiments in this study and those described in a related publication on the nuclear apparatus (R. L. Moore and P. Hirsch, J. Bacteriol., submitted for publication). Actual cell multiplication occurred for 5.5 h or 38% of this doubling time.

DISCUSSION

A starting inoculum of cells with a very restricted age distribution is required to obtain an unstressed population of synchronously dividing cells. Microorganisms which reproduce by budding are well suited for this purpose because of the large differences in size between the younger and older cells (1, 5, 6, 8, 11). Advantage has been taken of this fact to obtain a high degree of synchronous first-generation cell development and multiplication of *Hyphomicrobium* sp. B-522. By combining a centrifugation procedure with filtration steps, a useful quantity of the swarmer cells of this bacterium was easily isolated. However, results show that initiation of hyphal growth and bud formation still occurred over a wide time interval when this mechanical separation method was used. Procedures to improve synchrony which involved heat shock or chilling were unsuccessful. It seems likely that the selection of an even narrower size range of swarmers for the starting inoculum would improve physiological synchrony.

In the swarmer population, hyphal outgrowth was initiated during the time from approximately 0.75 h before the beginning of the experiment's measurements to approximately 9.25 h afterwards, i.e., there was a spread of 10 h. Assuming that late initiation of hyphal outgrowth was due to young swarmer age at the onset of the experiment, swarmer "maturation" could have lasted as long as 10 h. The period of hyphal development required 3.5 to 4.5 h. Bud maturation and separation required an additional 5.5 h. Thus, the maximal obtainable mother generation time would be 10 + 4.5 + 5.5 = 20 h and the minimum, 0 + 3.5 + 5.5 = 9 h. This is similar to the values of 7.5 and 21 h observed by Hirsch and Jones (Bacteriol. Rev., p. 44, 1968) in previous studies accomplished with the use of agar slide cultures. The times required for the various stages of development observed in the present investigation compare favorably to those of the previous studies (Fig. 7). The similarity indicates that the harvesting procedure developed here was not too detrimental to the cells of strain B-522. Some dissimilarity in the results may be due to physical differences between the broth culture and the agar slide culture. In addition, the present study examined only the events leading to the production of the first daughter cell, while the earlier study was also concerned with the second and later generations of progeny. Since the later daughter cells are produced more rapidly (Hirsch, unpublished data), some differences in these results would be expected.

These results implicate the production of hyphae as an integral part of the reproductive process of this *Hyphomicrobium* strain. Bud-

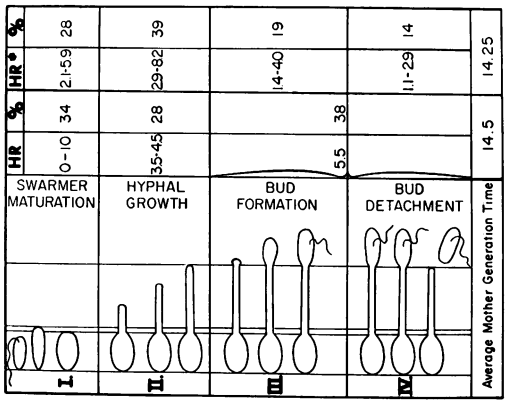

FIG. 7. *Time course of the developmental stages of Hyphomicrobium sp. strain B-522. The last two columns (marked with an asterisk) represent data obtained from agar slide cultures by P. Hirsch and H. E. Jones (Bacteriol. Proc., p. 44, 1968). The percentage figures are the average lengths of the individual stages with respect to the average mother generation time. The figures for bud formation and bud detachment are combined in this work (parenthesis).*

ding was not observed in mother cells without hyphae. This has also been noted by other investigators with *Rhodomicrobium vannielii* (6), *Hyphomicrobium neptunium* (5), and *Caulobacter* spp. (7). It suggests that a distance between the mother and daughter cell must be necessary for the proper functioning of certain cellular processes leading to multiplication. This poses an intriguing problem for future investigation.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Duchow, E., and H. C. Douglas. 1949. *Rhodomicrobium vannielii*, a new photoheterotrophic bacterium. J. Bacteriol. **58**:409-416.
2. Hirsch, P., and S. F. Conti. 1964. Biology of budding bacteria, I. Enrichment, isolation and morphology of *Hyphomicrobium* spp. Arch. Mikrobiol. **48**:339-357.
3. Hirsch, P., and S. F. Conti. 1964. Biology of budding bacteria, II. Growth and nutrition of *Hyphomicrobium* spp. Arch. Mikrobiol. **48**:358-367.
4. Jones, H. E., and P. Hirsch. 1968. Cell wall composition of *Hyphomicrobium* spp. J. Bacteriol. **96**:1037-1041.
5. Leifson, E. 1964. *Hyphomicrobium neptunium* sp.n. Antonie van Leeuwenhoek J. Microbiol. Serol. **30**:249-256.
6. Murray, R. G. E., and H. C. Douglas. 1950. The reproductive mechanism of *Rhodomicrobium vannielii* and the accompanying nuclear changes. J. Bacteriol. **59**:157-167.
7. Poindexter, J. S. 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28**:231-295.
8. Pongratz, E. 1957. D'une bacterie pediculee isolee d'un pus de sinus. Schweiz. Z. Allg. Pathol. Bakteriol. **20**:593-608.
9. Schmidt, J. M., and R. Y. Stanier. 1966. The development of cellular stalks in bacteria. J. Cell Biol. **28**:423-436.
10. Tyler, P. A., and K. C. Marshall. 1967. Pleomorphy in stalked, budding bacteria. J. Bacteriol. **93**:1132-1136.
11. Zavarzin, G. A. 1960. The life cycle and nuclear apparatus in *Hyphomicrobium vulgare* Stutzer and Hartleb. Mikrobiologiya **29**:38-42.