# Morphogenesis and Differentiation in *Rhodomicrobium vannielii* and Other Budding and Prosthecate Bacteria

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## PREVIEW

This monograph is in two parts. Part A sets out the essential nature of budding and prosthecate bacteria in terms of their cell cycle and reproductive processes and contrasts them with other eubacteria such as *Escherichia coli*. Advantages budding bacteria may possess as models for the study of development in procaryotes are highlighted in this comparative treatment, which also underlines the meaning of morphogenesis and differentiation when used in a procaryote context.

*Rhodomicrobium vannielii* seems the most promising of the budding bacteria for the study of morphogenesis and differentiation and is the

central subject of this monograph. Certain sections of Part A draw upon information in Part B, which begins with a brief review of published information on *R. vannielii* but is principally an account of our studies on the biology of the cell cycles of this organism, with particular reference to morphogenesis and differentiation.

## PART A – INTRODUCTION

Growth and development of higher plants and animals is a composite expression of the growth and differentiation of their component cells. Consequently, it has been argued that a study of molecular events taking place during the cell cycle, especially those events related to differentiation and morphogenesis, is directly relevant to the understanding of growth and development of the whole organism.

Eucaryotic cells, obviously, are the cells of choice in which to explore these events. However, so much more is known about molecular processes and their genetic control in procaryotes that it is plausible to suppose that pertinent information about the basic principles, if not the precise processes, of morphogenesis and differentiation in higher organisms might be unraveled more rapidly by studying procaryotes.

The ideal choice of bacterial species for such studies would be *Escherichia coli,* simply because of the vast bank of genetic and biochemical information that already exists about this species. However, *E. coli* lacks significant differentiation; it has a simple morphogenetic cell cycle, cell division being the only clearly microscopically observable "landmark" event. Procaryotes with complex fine structures and morphologically distinctive cell cycles would seem better choices and have proved to be so.

Procaryotes that offer possibilities for such exploitation include species of *Bacillus, Streptomyces, Arthrobacter, Myxobacter,* the prosthecate bacteria (including *Caulobacter* and the budding bacteria), and filamentous cyanobacteria (blue-green bacteria). Some of these organisms, of course, have already been studied in this context. Endosporeforming *Bacillus* species (26, 30, 49, 82) are the most prominent examples, followed more recently by *Caulobacter* (43, 74, 76).

Before pursuing the discussion on morphogenesis, differentiation, and development in budding bacteria, we think it necessary to attempt to be more precise than is presently the case about the meaning and use of these terms as applied to procaryotes. We spell out below our notions of these terms and then use them in further descriptions of categories of cell cycles,

intercellular relationships, and levels of organization of cells.

### Cellular Events

*Morphogenesis* – changes in external morphology of the cell and in internal architecture during the cell cycle.

*Differentiation* – events initiated by a "switch" in the cell cycle leading to the formation of a new type of cell. Such differentiated cells may revert to the original form (e.g., spore germination), or the differentiation event may be permanent (e.g., heterocysts of cyanobacteria and cells of certain methane oxidizers that have budded off spores [95]).

*Development* – a composite event involving morphogenesis and differentiation under intercellular influence, as in some myxobacters and cyanobacteria when cells are modified to perform a particular function necessary to the activity of the multicellular complex.

### Cell Cycle Types

*Monomorphic vegetative cell cycle type* – this phrase describes species of procaryote existing always as vegetative cells and in which there is only one distinct morphological type of cell formed under what are considered to be normal nutrient and physiological regimes (e.g., *E. coli*).

*Dimorphic cell cycle types* – this phrase describes those procaryotes which, at division, form two cells that differ from each other in shape (e.g., *Caulobacter*), or in size (e.g., certain filamentous blue-green bacteria), or in the possession by one of the cells of an appendage (e.g., the swarm cell of *Rhodopseudomonas acidophila* possesses a flagellum not possessed by the mother cell), or in a combination of these features.

*Polymorphic vegetative cell cycle types* – this phrase describes species that may produce under different nutrient and physiological regimes (but not so bizarre as to cause malfunction in the cell cycle) two or more morphologically distinct types of cell, each of which undergoes a distinctive and constant cell cycle (e.g., *Arthrobacter* species [24, 42], *Sphaerotilus* species [54, 66] certain cyanobacteria [9, 51], *Geodermatophilus* [36], *Hyphomicrobium* species [88], and *Rhodomicrobium* species; all these have two or more vegetative cell cycles). Cell cycle changes in response to nutrient conditions in *Geodermatophilus* are shown in Fig. 1. Two other species with such cell cycles have been studied by us, a *Hyphomicrobium* species (3) (Fig. 2 and 3) and a prosthecate bacterium (Fig. 4 and 5).
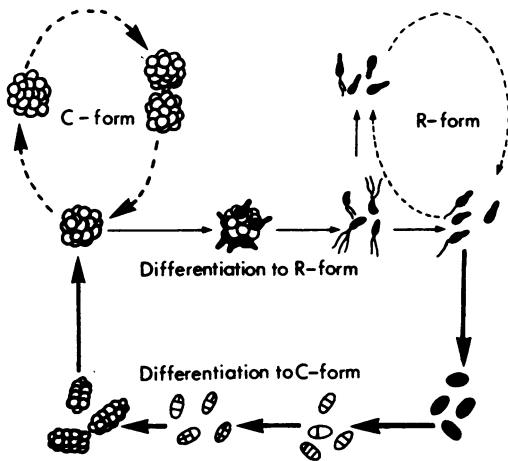
FIG. 1. *The polymorphic growth cycle of Geodermatophilus strain 22-68 (after Ishiguro and Wolfe [36]). Growth and division in the C-form requires the presence of a factor found in tryptose. Absence of this factor induces differentiation to the R-form. Readdition of the tryptose will induce differentiation from the R-form to the C-form.*
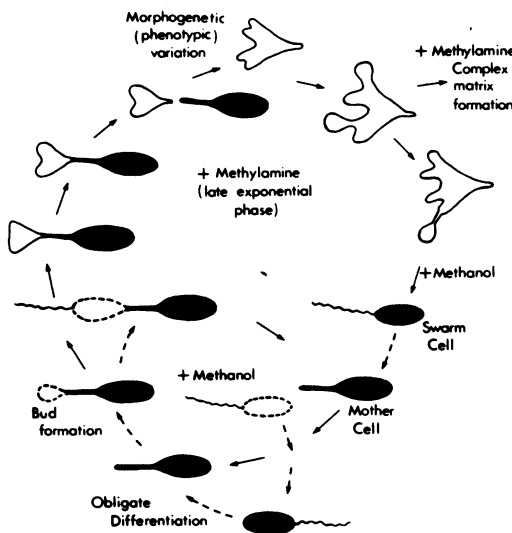


FIG. 2. *Polymorphic variation evident in Hyphomicrobium species with substitution of the carbon source. The basic medium contained, per liter: 20 ml of mineral base (NH₄Cl, 0.5 g; MgSO₄·7H₂O, 0.4 g; NaCl, 0.1 g; CaCl₂·2H₂O, 0.05 g; trace elements, 10 ml [61]), distilled water to 1 liter, 10 ml of 0.01 M KH₂PO₄, 100 mM methanol, and/or 100 mM methylamine. Cultures were incubated aerobically at 30°C.*

*Spore and cyst cycles* — this describes species that may undergo a cell cycle in addition to the vegetative cell cycle. Such species may be of the monomorphic vegetative cell cycle variety (e.g., *Bacillus* and *Methylosinus* species) or of

the polymorphic cell cycle type (e.g., *Rhodomicrobium* species, as described in Part B).

## Levels of Organization

*Unicellular* — the organism is normally independent of its fellows in terms of function and reproduction.

*Occasional intercellular cooperation* — describes bacteria able to lead a unicellular existence but which may express intercellular cooperation to form a multicellular structure (e.g., fruiting-body formation by some species of *Myxobacter* [23]).

*Multicellular procaryotes* — this expression describes those procaryotes with the capacity (not necessarily obligate) to differentiate and develop into a multicellular complex that clearly has (i) survival potential superior to that of a single cell of that species in the same environment, and (ii) an intercellular activity that results in selected cells of that complex undergoing differentiation.

Conventional wisdom holds that eubacteria are unicellular entities that do not form differentiated complexes, as is the case with many types of eucaryotic cells. That there are instances of cellular interdependence in procaryotes has been recognized, as in the case of myxobacter and related organisms, but what seems to have escaped general recognition is the existence of multicellular units of cyanobacteria (Fig. 6) where the evidence (97, 98) indicates to us that the filament of cells should be regarded as a whole organism. That the cells are interdependent, in the sense that differentiation (the formation of heterocysts, end cells, and akinetes) is a function of cell-cell relationships, is now clearly established (9, 51). The advantage of such a multicelled organism over a single-celled cyanobacterium of the same species is indicated by the gliding movements of the trichome, the N₂-fixing powers under aerobic conditions of the heterocyst, and the formation of one resting cell per filament (9). Obviously, a multicellular organism made up of procaryotic cells is potentially an excellent model for studies on differentiation and morphogenesis.

In addition, studies with the cyanobacterium *Chlorogloea fritschii* (25) have shown that complex but predictable morphological variations can be induced by a variety of environmental stimuli (Fig. 7).

## Budding — What It Means in Bacteria

Before we can examine the proposition that budding bacteria can serve as good models for the study of cell development, it is necessary to
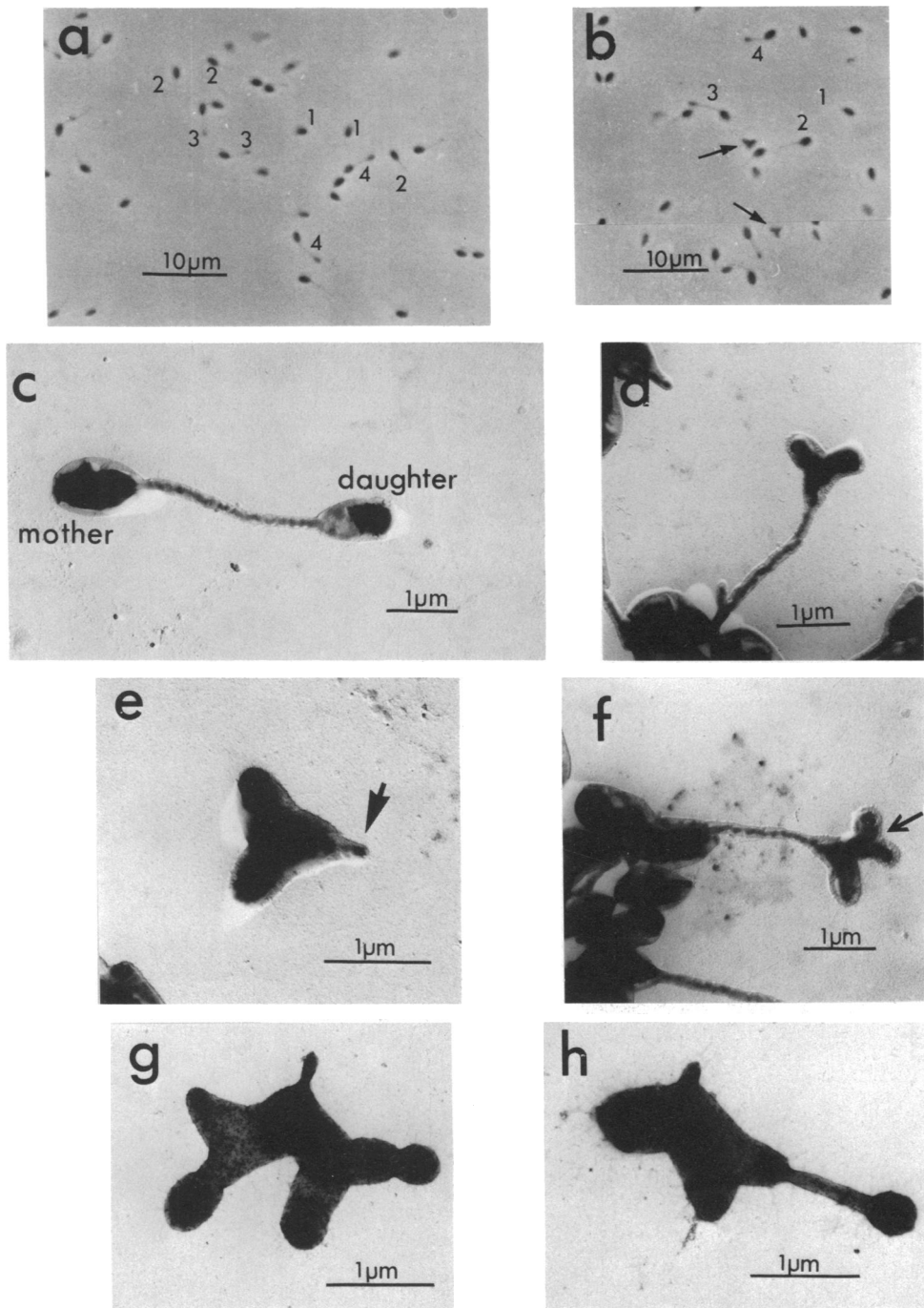
FIG. 3. *Phase-contrast photomicrographs. (a) Aerobic growth on methanol. Characteristic Hyphomicrobium cell cycle (33, 45). (b) Induction of Y-cell formation (arrowed) on substitution of methanol by methylamine. 1, Swarm cell; 2, stalk development; 3 and 4, daughter cell formation. Gold–palladium-shadowed electron micrographs. (c) Normal daughter cell development in Hyphomicrobium strain C (3). (d) Y-cell formation. (e) Released Y-shaped cell showing retention of a portion of the mother cell filament (arrow). (f) Dichotomous branching (arrow). Negatively stained (1% [wt/vol] phosphotungstic acid) electron micrographs. (g) Development of a Y-shaped cell grown on methylamine as the sole carbon source. Dichotomous branching yields one cell that "buds off" a daughter cell, whereas the other continues the branching process. (h) On readdition of methanol, Y-shaped cells produce filaments of "normal" dimensions, which subsequently give rise to "normal" motile cells. It should be noted, however, that recent studies (A. Lawrence, unpublished data, this laboratory) have shown that similar morphogenetic variations can be induced by altering the intracellular ionic environment. A similar dependence of morphogenetic expression on the intracellular ionic environment has been recorded for Geodermatophilus species (37). (Media and growth conditions as described in Fig. 2.)*
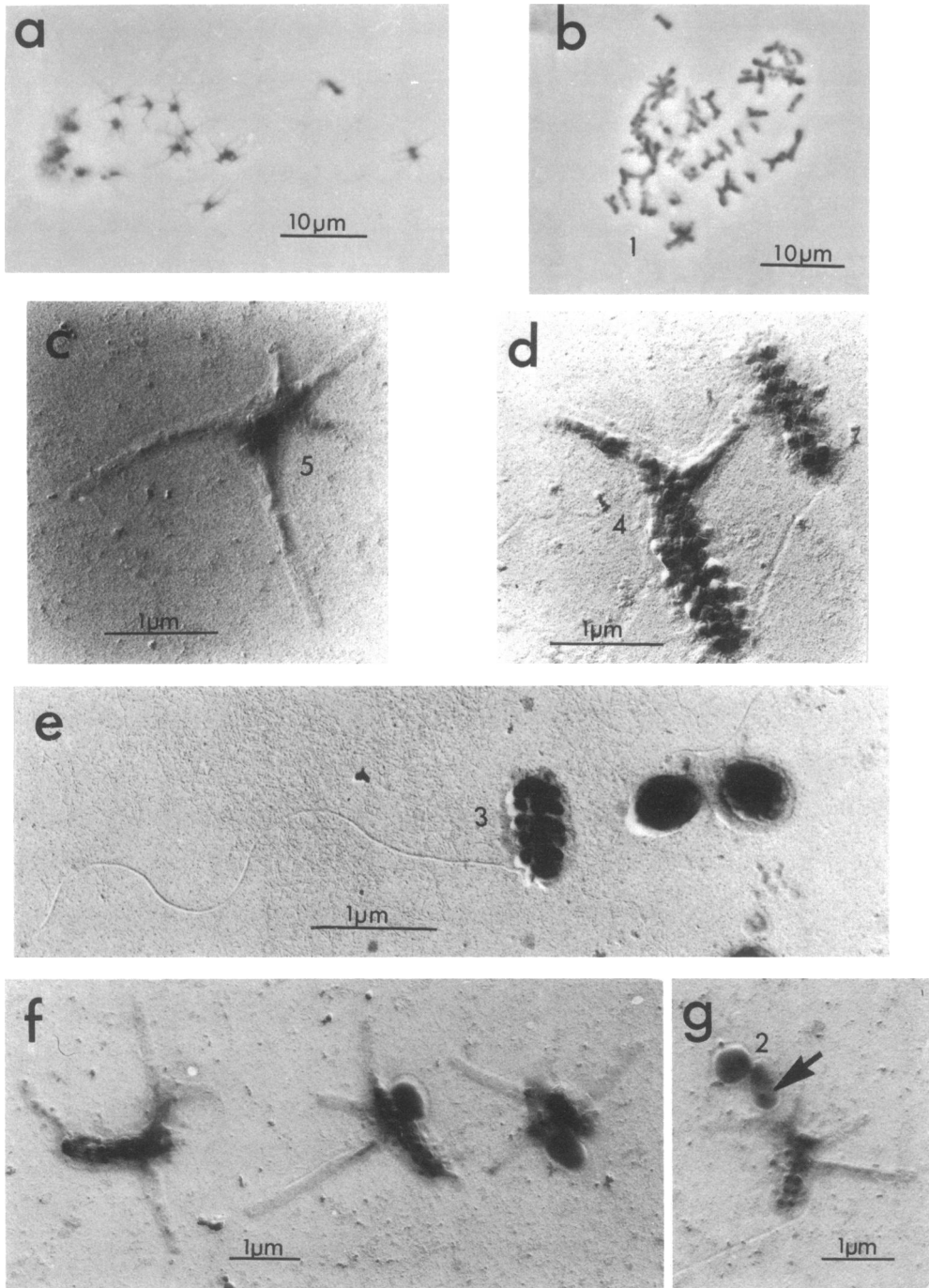
FIG. 4. A prosthecate bacterium, isolated from freshwater, which responds to environmental stimuli by the induction or repression of the formation of cellular appendages (prosthecae) and by marked phenotypic changes. Phase-contrast photomicrographs. (a) Expression of multiple cellular appendages when grown at 25°C under dilute organic nutrient conditions, i.e., 50 μg of peptone per ml in distilled water. This phenotype is analogous to that previously described for Ancalomicrobium species (79). Growth is unidirectional and polar. Division is by binary fission. (b) When cultured at 25°C under high nutrient conditions, i.e., 175 μg of peptone per ml in distilled water, the cells become nonappendaged and assume an alternative distinctive morphology. Gold–palladium-shadowed electron micrographs. (c) Multiappendaged cell, which is character-istic of cultures grown in dilute medium as described in (a). Considerable phenotypic variation is shown in cultures grown at 25°C on 75 to 100 μg of peptone per ml. The majority of cells have a very characteristic knobbed appearance and may or may not be appendaged (d). In addition, nonappendaged motile cells (e), which have a similar knobbed wall structure, are apparent. There are also small, nonmotile, ovoid cells present, which lack the knobbed wall (e and f) but which can be found in the process of its formation (g). Numbers on figures refer to phenotypes shown in Fig. 5.
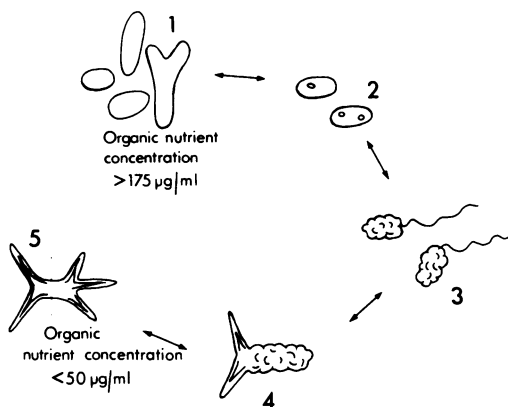
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FIG. 5. *Summary of the phenotypic variations induced in a freshwater prosthecate bacterium by variation of the organic nutrient concentration. The cell types have been correlated with those in Fig. 4.*
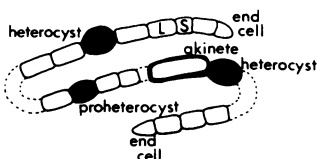


FIG. 6. *Diagrammatic representation of an Anabaena cylindrica filament showing the constituent cell types. Asymmetric division of the vegetative cells yield a large (L) and a small (S) cell, which differ in division time (51).*

define what budding really means in the context of bacterial reproductive processes.

Budding bacteria are currently assumed to differ fundamentally from other bacteria in their mode of reproduction. Such an assumption stems from a comparison of their cell cycles with those of rod-shaped bacteria such as *E. coli* (Fig. 8). In budding bacteria, cell separation usually results in two asymmetric cells (one cell with a filament, one without) which are not siblings, whereas in *E. coli* division usually results in two symmetrical siblings. Division processes, however—binary in both cases—are fundamentally similar.

A second point of significance concerns a characteristic that has been consistently overlooked and is probably the most important single feature characterizing budding bacteria: wall growth is always polar whatever the medium in which the organism is grown. Wall growth, or cell extension, may be either unidirectional, from only one pole (always the same pole) as in *Rhodopseudomonas palustris* (96), or unidirectional but from either pole, e.g., *Hyphomicrobium* species (A. Lawrence, unpublished data, this laboratory), or bidirectional, that is to say, both poles of the bacterium (e.g.,

*R. vannielii* [see Part B]) may give rise to cells. *E. coli* and other rod-shaped bacteria, on the other hand, usually grow from multiple growth points along the envelope approximating to intercalary growth (4, 5, 17).

That even this process is not always the case with rod-shaped cells has been demonstrated with *E. coli* (17, 18). In minimal medium with a generation time of 60 min, *E. coli* will extend only from one pole. The only difference between budding bacteria and *E. coli* in this respect is that in budding bacteria division is asymmetric (Fig. 8). Consequently, no fundamentally im-
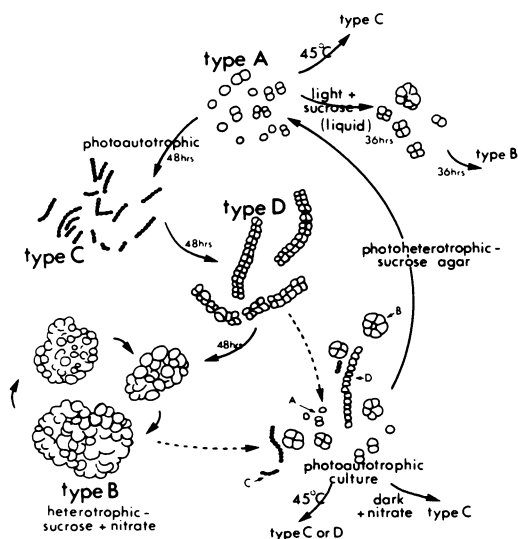


FIG. 7. *Diagrammatic summary of the morphological variations induced in Chlorogloea fritschii by different environmental stimuli (after E. H. Evans, I. Foulds, and N. G. Carr [25]). Morphological cell types. Type A — large (2 by 3 µm) granulated cells existing either singly or as clumps containing two or more cells which arise from division in up to three planes. Type B — found in clumps which combine larger groups of cells surrounded by a mucilaginous sheath, which is termed "aseriate" (44). Type C — small (1 µm) cells found in short filaments. Type D — larger than type C (1.5 µm) and found in filaments in the process of dividing.*



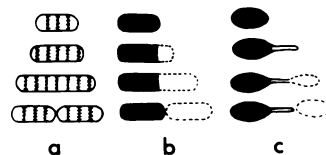FIG. 8. *Diagrammatic representation of: (a) intercalary growth of Escherichia coli with a generation time of less than 40 min (4, 17); (b) polar growth shown by E. coli with a generation time in excess of 60 min (17); (c) obligate polar growth of a budding bacterium, e.g., Hyphomicrobium species (33, 45).*

portant feature in cell growth and division processes can be singled out which separates absolutely the budding bacteria from the rod-shaped bacteria.

By adopting this mode of envelope growth as a basis of distinction and disregarding the term budding, more useful concepts can be evolved about cell growth and development in budding bacteria. Once bacteria have adopted a "unipoint" mode of growth, certain constraints as regards shape and internal architecture which govern such bacteria as $E.$ $coli$ appear no longer to apply, and it is possible for morphogenetic evolution to take place. That such an idea may be reasonable is illustrated in Fig. 9, in which some of the budding bacteria are classified according to degree of morphological complexity.

Three major features of this particular example of morphogenetic evolution, pertinent to $R.$ $vannielii$ in particular, are worth emphasizing. (i) The membranous unit of lamellar structure is no longer symmetrical. A "horseshoe" shape has evolved (e.g., $Nitrobacter$ $winogradsky$ and $R.$ $palustris$ [56, 93, 96]), which does not give rise to complications at division, since the fission point is beyond the "horseshoe" structure,
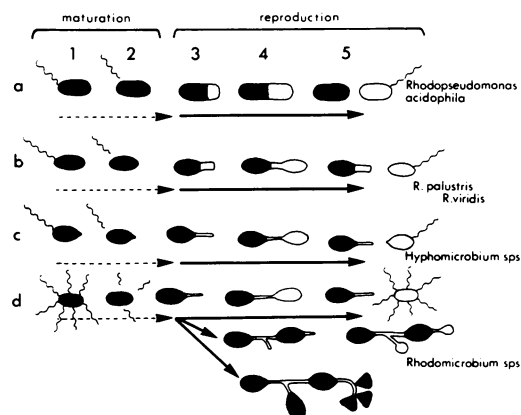


FIG. 9. Budding bacteria ordered in degrees of morphological/cell cycle complexity. In all cases motility (1), flagella shedding (2), maturation of the cell (3), daughter cell synthesis by obligate polar growth (4), and asymmetric cell division (5) give rise to an immature daughter cell and a mature mother cell. (a) Rhodopseudomonas acidophila (60). Simple dimorphic life cycle not involving tube/filament synthesis. (b) Rhodopseudomonas palustris and R. viridis (96). Involvement of tube/filament synthesis prior to daughter cell formation. (c) Hyphomicrobium species (33, 45). Reduction in width coupled with an increase in length of the tube/filament compared with R. palustris. (d) Rhodomicrobium species (see Part B). Environmentally induced life cycle variations ranging from a simple dimorphic cell cycle through complex matrix formation to exospore production.

which is formed de novo in the daughter cell. (ii) Multicellular arrays are formed whereby cells remain permanently linked to one another. (iii) A second mode of cell separation other than binary fission has evolved in $R.$ $vannielii$, the plugging of filaments or the laying down of cross walls in the filaments. These separation sites, part way along the filament, are not those at which binary fission would have taken place; fission sites are directly adjacent to the daughter cells.

The significance of the adoption by a bacterium of a polar or unipoint mode of growth as opposed to multipoint, or what approximates to an intercalary mode of growth, is far-reaching. The dramatic consequences of such as apparently simple change in growth style are outlined below in a comparison of intercalary with obligate polar growth processes.

**Intercalation.** If a rod-shaped bacterium growing exponentially or in a steady state in continuous culture lengthens by ideal intercalary processes (i.e., old and new structures and cytoplasmic material are evenly distributed throughout the expanding cell), then at division the two siblings will be both $quantitatively$ and $qualitatively$ similar to each other. Not only will the siblings be to all intents and purposes indistinguishable, but they will also be similar physiologically and structurally (i.e., in the distribution and ratio of old to new components) to the cell that gave rise to them at the moment it was formed by division. From this description of events, four major features emerge which characterize the ideal intercalary-type cell: (i) such cells are potentially immortal; (ii) the cells have an age span that does not exceed the division time; (iii) at division, siblings are equivalent in all respects; (iv) internal structures, such as membranous complexes, are likely to be symmetrical (for uniform distribution at division between the two sibling cells) or to be small structures, such as mesosomes, which do not interfere with division.

**Polar growth.** Taking a $Hyphomicrobium$ species as a typical example of a bacterium growing by polar processes (Fig. 8 and 9), the following features characterize its growth cycle, observed in ideal circumstances similar to the previous example of an intercalary growing cell.

At division, the cell divides asymmetrically, yielding a mother cell and a daughter cell. The mother cell is a filamented cell composed of structural material present in the cell when it was formed at the previous division (this material will be confined mainly to the nonfilamented part of the cell), and of structural mate-

rial synthesized during the recent cell cycle (this will be restricted to the budding filament). The flagellated daughter will be composed mainly of new material synthesized in the preceding cell cycle.

In subsequent rounds of division, little if any new structural material will be added to the mother cell: in this sense the mother cell ages.

The implications of polar growth in contrast to intercalary growth are as follows.

(i) Aging is a real process that extends through all the division cycles of a particular mother cell's life-span. At division, the swarmer (daughter cell) is structurally younger than the mother cell.

The aging concept is best illustrated at present in procaryotes by the *Caulobacter* stalked cells, which have been reported to acquire an additional cross band in the stalk after each division (80), a possible consequence of a new piece of stalk being synthesized in each division cycle. However, recent evidence summarized below suggests that this interpretation of events may need modifying (U. Retnasabapathy, unpublished data, this laboratory). A synchronized *Caulobacter* (NCIB 9083) swarm cell population was gently filtered onto a cellulose triacetate membrane, the membrane was inverted, and preconditioned medium was passed through at a flow rate of 2 ml/min. As the attached population grew and differentiated (Fig. 10a), the effluent was monitored for total cell numbers against time. The data obtained (Fig. 10b) quantified the number of swarm cells produced per attached mother cell and gave the time required for each turn of the cycle; i.e., the precise history of the attached, synchronized mother cell population was known.

Electron microscopic examination of the attached mother cells at various times showed that in *Caulobacter* (NCIB 9083) cross bands did not appear until after the third or fourth generation and that there was no subsequent correlation between swarm cell production and cross band formation.

(ii) Cells are probably mortal; they can undergo only a limited number of reproductive cycles (e.g., *R. vannielii* in one phenotypic form [see Part B] only gives rise to four daughter cells).

(iii) Cell division may be asymmetrical.

(iv) Complex internal membranous bodies of obvious asymmetry may be formed, as constraints on pattern are not imposed by intercalary growth and subsequent division processes (e.g., *Nitrobacter agilis* [62] and *Rhodopseudomonas palustris* [21]). These membranous bodies are formed de novo in the swarmer or daughter cell.
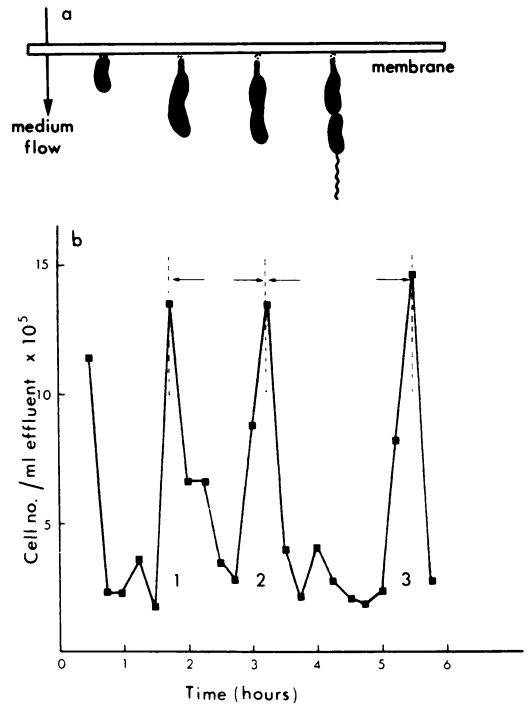


FIG. 10. *(a) Schematic illustration of the successive stages in the life cycle of an individual Caulobacter cell attached to a cellulose triacetate membrane, leading to the elution of the daughter cell. With a synchronized population, sequential waves of swarm cells appear in the effluent. (b) Cell number per milliliter of effluent plotted against time. The interpeak distances indicate the time taken for successive waves of swarm cell production. The number of peaks gives the number of swarm cells produced per mother cell (U. Retnasabapathy, unpublished, this laboratory).*

(v) The swarmer or daughter cell, at division, is *immature;* it has to form internal structures and filament or budding structures to achieve physiological and structural parity with the mother cell that gave rise to it in the first place. The term "siblings" for such cells is seen to be inappropriate. The parent-offspring terminology used is considered to reflect more accurately relationships of the cells at division. With such bacteria, the term sibling now describes the relationship between the daughter cells produced by one mother cell.

(vi) The adoption of a polar growth mechanism means that the cell is polarized. In other words, such bacteria now have a "front" end and a "back" end. This orientation can be plainly discerned by looking at an *R. palustris* mother and daughter cell (Fig. 9). Reproduction in this instance always takes place from the one (anterior) pole; holdfast and flagellum are al-

ways at the posterior pole, and each daughter cell formed proves to be a mirror image of the mother cell — in other words, the pole of the daughter cell adjacent to the mother cell's reproductive pole is the pole that eventually gives rise to that cell's first daughter cell.

Organisms growing by obligate polar processes clearly have a potential for morphogenetic evolution not afforded to cells that grow, for the most part, by intercalary processes. This can be claimed to have led to the evolution of a complex morphogenesis in some bacteria (an increasing pattern of morphological complexity in budding bacteria is outlined in Fig. 9). *R. vannielii* (see Part B) is probably the most specialized example of this form of morphological development.

## Budding Bacteria as Models for the Study of Morphogenesis and Differentiation

All of these features, polar growth, polarization of the cell, and the mother/daughter cell cycles, involve the regulation of temporally related sequences of morphogenetic events, thus underlining the suitability of polarly growing bacteria as models for studies on morphogenesis and differentiation.

Dimorphic bacteria, such as *Caulobacter*, are already being explored as models of differentiation and morphogenesis because they undergo well-defined, microscopically distinct events throughout the cell cycle (Fig. 11 and 12) which can be linked with distinct molecular events and control systems relevant to morphogenesis and differentiation (15, 16, 43, 57, 74). Various marker systems have already been employed to follow these events in *Caulobacter*, e.g., flagella shedding and bacteriophage adsorption, both of which accurately mark stages in the cell cycle (1, 75, 77). Budding bacteria offer similar possibilities for study. However, they have several unique advantages, and these we wish to emphasize in this monograph with particular reference to *R. vannielii*.

## PART B — BIOLOGY OF THE CELL CYCLES OF *R. VANNIELII*

### Review of Published Information on *R. vannielii*

A brief synopsis is given of the information accumulated on *R. vannielii* since its isolation by Duchow and Douglas (22). As will be evident, most of the information refers to physiological and biochemical activities and not to properties directly relevant to development.

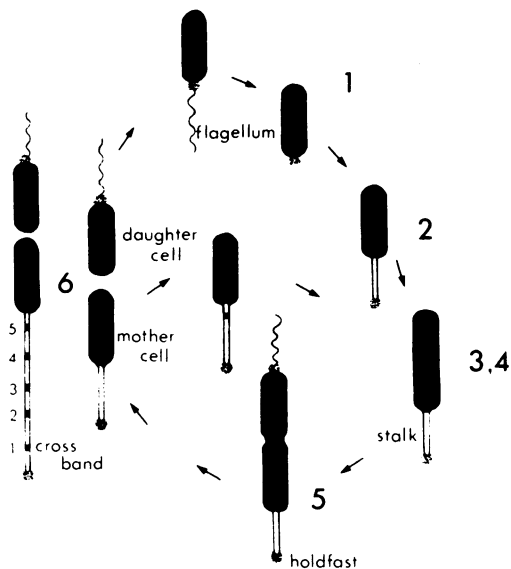*R. vannielii* was shown to be a photoheterotrophic bacterium of unusual morphology con-



FIG. 11. *Diagrammatic representation of the cell cycle of Caulobacter species. Starting from the motile swarm cell stage, the temporal sequence of morphological events is as follows. Maturation — (1) Loss of motility, flagella shedding (77); (2) stalk synthesis; (3) swarm cell formation; (4) formation of the membranous organelle at the pole opposite the stalk. Daughter cell synthesis — (5) flagella and holdfast synthesis; (6) cell division to yield a stalked mother cell and a motile swarm cell. On release, the swarm cell follows the above cycle, while the stalked mother immediately initiates daughter cell formation; i.e., there is no requirement for the maturation phase. It has recently been reported (80) that the mother cell synthesizes a cross band after each cycle of replication, cross band formation acting, therefore, as a generation marker.*

sisting of ovoid cells (2 to 3 $\mu$m by 1 $\mu$m) joined by filaments (0.3 $\mu$m by 2 to >30 $\mu$m), which may be branched, thus forming ramifying groups of cells. Subsequently, Murray and Douglas (55) revealed that reproduction was by a system that resembled budding, the cells being separated from one another by a cross wall laid down in the filament. Nonstalked, peritrichously flagellated cells have been reported to occur in early exponential cultures (20), but their mode of formation and subsequent development was not reported.

More recently, angular, heat-resistant bodies (termed, by us, "exospores") have been observed in several freshwater isolates (27). Processes leading to their formation and germination have not been documented.

The biochemical and physiological properties of *R. vannielii* are given in Tables 1 to 6. The resulting impression is that this organism is biochemically similar in many respects to other

photoheterotrophs. The obvious gap in information concerns the biology of the cell cycle, which is the subject of our studies described below.

### Enrichment, Isolation, and Cultivation

In devising growth conditions and isolation procedures for strains of *R. vannielii,* properties consistent with the use of the species as a model for morphogenesis and differentiation

FIG. 12. *Gold/palladium-shadowed electron micrograph of a Caulobacter species.*

were taken into account. This meant that growth on defined media was considered to be essential and that the carbon source also had to be the energy source when the organism was to be grown chemoorganotrophically under aerobic conditions. Selective enrichment by substrate variation (90) was employed and, of the various enrichment media devised, malate salts medium ($NH_4Cl$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; NaCl, 0.4 g; sodium hydrogen malate, 1.5 g per liter of distilled water, adjusted with KOH; and 50 ml of 0.1 M phosphate buffer per liter, added aseptically) at pH 5.5 or 6.0 was the most successful. Incubation was in glass bottles (100 ml) at 30°C with an incident light intensity of 1,000 lx (94); 50 ml of medium was added, and the gas atmosphere was dinitrogen (oxygen free). Approximately
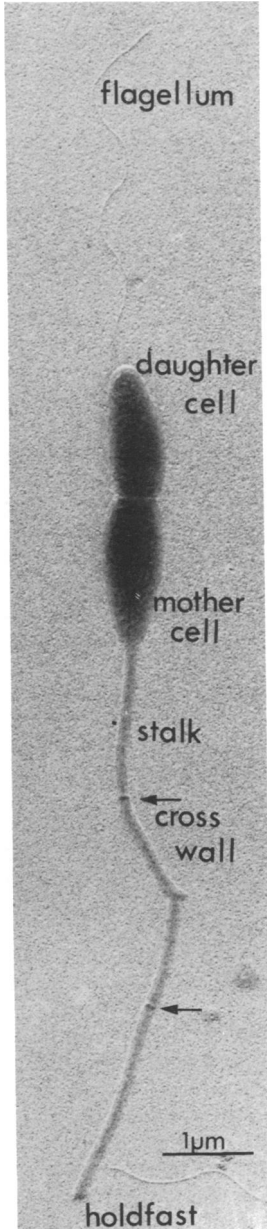
TABLE 1. *Cytochromes of Rhodomicrobium vannielii (53)*

| Cytochrome | Reduced vs oxidized (nm)[a] | | |
|---|---|---|---|
| Cytochrome | | | |
| b | 563 | 530 | 423 |
| c | 553 | 521 | 423 |
| $c_2$ | 550 | 522 | 414 |
| CO-binding heme protein | 570[b] | 538–543[b] | 414[b] |

[a] Numbers across are the three peaks of each cytochrome.

[b] CO red versus red.

TABLE 2. *Enzymes/coenzymes of Rhodomicrobium vannielii*

| Enzyme/coenzyme | Remarks |
|---|---|
| Alcohol dehydrogenase (70) | All lower, straight-chain primary alcohols tested, except methanol, served as substrates; enzyme was constitutive, required NADP as coenzyme with a pH optimum of 10.2; it has since been shown that methanol can be utilized by a few *R. vannielii* strains (65) |
| Glyceraldehyde 3-phosphate dehydrogenase (78) | NAD dependent |
| *N*-acetylglutamate phosphokinase (64) L-Glutamate-specific transacetylase (65) | Specifically inhibited by arginine |
| Hydrogenase (99) | Reduces methylene blue but not inorganic compounds |
| Ubiquinone Q10 (10) Coenzyme Q9 (59) | |

TABLE 3. *Lipid composition of Rhodomicrobium vannielii (58, 59, 70)*

| Compound | % of dry wt in: | |
|---|---|---|
| | Cells | P-lipids |
| Total P-lipids | 4.2 | |
| Sulfolipids | 0.01 | |
| Steroids | ND[a] | |
| Lipo amino acid (o-ornithine ester of phosphatidylglycerol) | | 46.5 |
| Phosphatidylcholine | | 26.5 |
| Phosphatidylglycerol | | 9.7 |
| bis-Phosphatidic acid | | 6.7 |
| Phosphatidic acid | | 1.8 |
| Phosphatidylethanolamine | | 4.5 |
| Lysophosphatidyl glycerol-o-ornithine ester | | 3.2 |
| Phosphatidyl-N-methyl-ethanolamine | | ND |
| Phosphatidyl-N,N'-dimethyl-ethanolamine | | ND |
| Cardiolipin | | ND |
| N,N-ornithine amide of an unidentified fatty acid | | 0.95 |

[a] ND, Not detected.

TABLE 4. *Fatty acids of Rhodomicrobium vannielii (59)*

| Fatty acid | Remarks |
|---|---|
| Vaccenic acid (11-octadecenoic acid) | Largest single component (90%) of both simple and complex lipids |
| Extractable lipids (room temperature, organic solvents) | Only straight-chain saturated and unsaturated even-numbered fatty acids |
| Nonextractable bound lipids | Even- and odd-numbered saturated and unsaturated straight-chain fatty acids, cyclopropane-, branched-, and α- and β-hydroxy fatty acids |

50% of all enrichment cultures inoculated with freshwater sediment (surface layer) proved to contain *Rhodomicrobium* species (detectable within 1 to 2 weeks). Subsequent isolation and purification was by dilution of the enrichment medium into agar deeps.

The routine culture medium was the enrichment medium plus 1.5 g (wt/vol) sodium pyruvate, the pH being adjusted to 6.8. This medium was chosen on the basis of growth rate and cell density achieved. Of the various carbon sources, tested individually and as mixtures, malate and pyruvate were the best individually, whereas a mixture of the two surpassed either singly (Fig. 13).

Pure cultures were grown in a variety of glass vessels, which were sealed with rubber serum caps (Suba-Seals, William Freeman and Co. Ltd., Barnsley, Yorkshire) flushed with oxygen-free nitrogen and incubated with shaking at 30°C under constant illumination from tungsten lamps (incident light intensity of 2,000 lx). The gaseous environment was assayed chromatographically (32).

Several of the isolated strains were compared with the neotype culture (ATCC 17100) both directly and with published results. *Rhodomicrobium* strain 5 (Rm 5) was used in all subsequent experiments.

## Physiological and Biochemical Properties

**Characterization of DNA.** Determination of the guanine-plus-cytosine mole percents by analytical ultracentrifuge techniques using neutral CsCl density gradients (48) gave results of 62.5% for strain Rm 5 and 63% for *R. vannielii* ATCC 17100. Thermal denaturation curves (47) were characteristic of a single deoxyribonucleic acid (DNA) population and gave results of 62.5% in both cases. No plasmid or other satellite DNA was detected.

**Photopigments.** In the exponential growth phase, all strains had similar absorption characteristics (see Fig. 40). Isolation and analysis of the photopigments confirmed the presence of bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series, with rhodopin as the major constituent. β-Carotene is also present in considerable quantities (11, 39, 46, 69, 92).

**Cytochromes.** The cytochrome content of both strains (strain Rm 5 and *R. vannielii* ATCC 17100) grown photosynthetically was identical and correlated closely with published observations (Table 1).

**Growth physiology.** *Rhodomicrobium* species growing photosynthetically require an electron donor, a terminal electron acceptor, and a source of cell carbon. Table 5 lists the substrates used by strain Rm 5. There was no requirement for growth factors. However, riboflavin has been reported to be a growth stimulant (86).

The addition of $CO_2$, furnished as bicarbonate, to the culture medium is only essential in those instances where $CO_2$ cannot be generated from the organic electron donor; e.g., malate and pyruvate yield considerable amounts of $CO_2$ during growth and, consequently, the addition of bicarbonate is unnecessary. However, if $CO_2$ is removed from such a culture by vigorous gassing with oxygen-free $N_2$, growth only proceeds when carbon dioxide is also added; this was shown not to be a pH effect.

TABLE 5. *Growth substrates tested with Rhodomicrobium vannielii*

| Substrate (reference no.) | Utilized + or not utilized − | Substrate (reference no.) | Utilized + or not utilized − |
|---|---|---|---|
| Tricarboxylic acid cycle intermediates | | Alcohols | |
| Pyruvate (60) | + | Methanol (8, 65) | + |
| Citrate | −[a] | Ethanol (22) | + |
| Isocitrate | (+)[a] | Propanol (22) | + |
| α-Ketoglutarate | −[a] | Butanol (22) | + |
| Succinate (60) | + | | |
| Fumarate (60) | + | Carbohydrates | |
| Malate (22) | + | Glucose (22) | − |
| Oxalacetate | +[a] | Fructose (22) | − |
| Acetate (22) | + | Mannose (22) | − |
| | | Sorbitol (22) | − |
| Organic acids | | Mannitol (22) | − |
| Butyrate (22) | + | | |
| β-Hydroxy-butyrate (35) | + | Inorganic compounds | |
| Lactate (22) | + | $H_2$ (60) | + |
| Formate (22) | ± | $S_2O_3^{2-}$ (22) | − |
| Propionate (22) | + | Sulfide (22) | + |
| Malonate (60) | + | | |
| Tartrate (22) | − | | |
| Glycerol | +[a] | | |
| Aspartate (34) | − | | |
| Pelargonate (34) | − | | |
| Glycolate (34) | − | | |
| Oxalate (34) | − | | |
| Benzoate (34) | − | | |
| Glutamate (34) | | | |
| Valerate (22) | + | | |
| Caproate (22) | + | | |
| Caprylate (60) | + | | |

[a] Additional substrate tested in this study. (+), Growth poor.

TABLE 6. *Nitrogen sources of Rhodomicrobium vannielii*

| Compound | Assimilation | Remarks |
|---|---|---|
| $NH_4Cl$ | + | |
| $KNO_3$ | (+)[a] | |
| $NaNO_3$ | (+) | |
| Casamino Acids | + | An amino acid concentration of 0.01% inhibits growth (22) |
| Urea | (+) | |
| $N_2$ | + | |

[a] (+), Growth poor.

Ammonium chloride was used as a nitrogen source. $N_2$ is fixed actively in the absence of other nitrogenous sources, as judged by growth density on $N_2$ gas and the consequent induction of acetylene to ethylene reducing activity.

## Cell Cycle Morphology of *R. vannielii*

Microscopic preparations of strain Rm 5 grown in batch culture reveal (i) complexes of cells linked by filaments (multicellular arrays), (ii) unattached motile cells (swarmers), and (iii) nonmotile angular cells (exospores; Fig. 14).
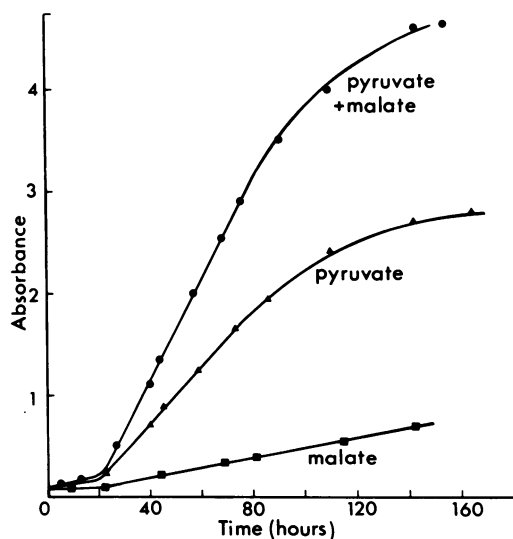
FIG. 13. *Growth curves for strain Rm 5, growing at 30°C with an incident light intensity of 1,500 lx, on malate (■), pyruvate (▲), and malate plus pyruvate (●). Each carbon source was at a concentration of 1.5 g/liter (wt/vol) in mineral salts medium (previously described). Growth was measured as absorbance at 540 nm.*
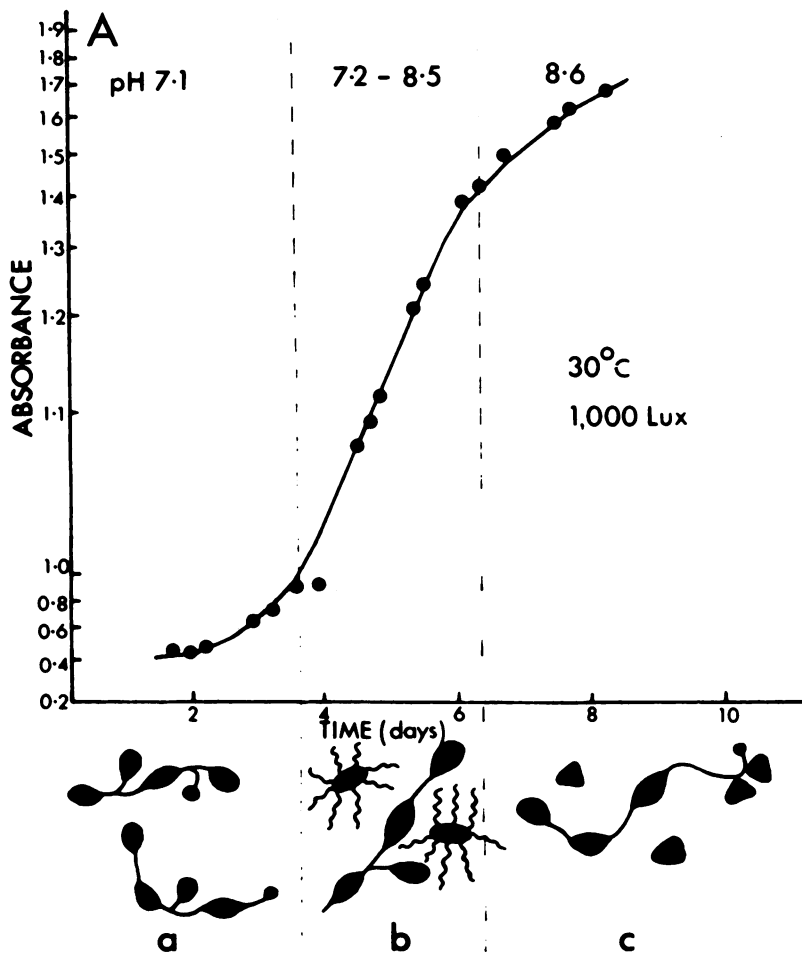
FIG. 14. *(A) Growth curve of strain Rm 5 grown on malate medium at 30°C with an incident light intensity of 1,000 lx in a 15-liter batch fermentor. (B) Phase-contrast photomicrographs of cellular expression during batch culture growth of strain Rm 5. (a) Lag and early exponential phases are characterized by the presence of complexes of cells linked by filaments. No motile cells are apparent. (b) As growth proceeds, nonstalked motile cells are produced. (c) With the onset of the stationary phase, angular exospores are produced in considerable numbers.*

To confirm the sequence of events leading to the formation of these cell forms, it was necessary to devise means of separating and synchronizing the swarm cells which in turn give rise to all the other morphological types observed.

**Synchronous cultures.** Experimental analysis of the cell types of this organism is only possible when a morphogenetically uniform collection of cells is used as a starting point. Of the two major types of synchrony, induction and selection, the latter is preferable to the former in that physiological disturbances of the population can be minimized and perturbances in the subsequent round of growth can be kept to a minimum. However, a problem accompanying the techniques of selective synchronization is

that of small yield. By exploiting the morphological differences between cell types, a method was devised which permitted the selective synchronization, in high yield, of the swarm cell fraction of *Rhodomicrobium* strain Rm 5.

**Synchrony by filtration.** This technique exploited the cellular asymmetry observed in exponentially growing cultures. Motile, unappendaged daughter or swarmer cells were unhindered in their passage down a glass wool column (Fig. 15). However, nonmotile appendaged mother cells and cells connected by filaments were impeded and remained in the column. The major operational points are: (i) filtration is through a glass wool-Ballotini bead system; (ii) the whole apparatus can be sterilized and
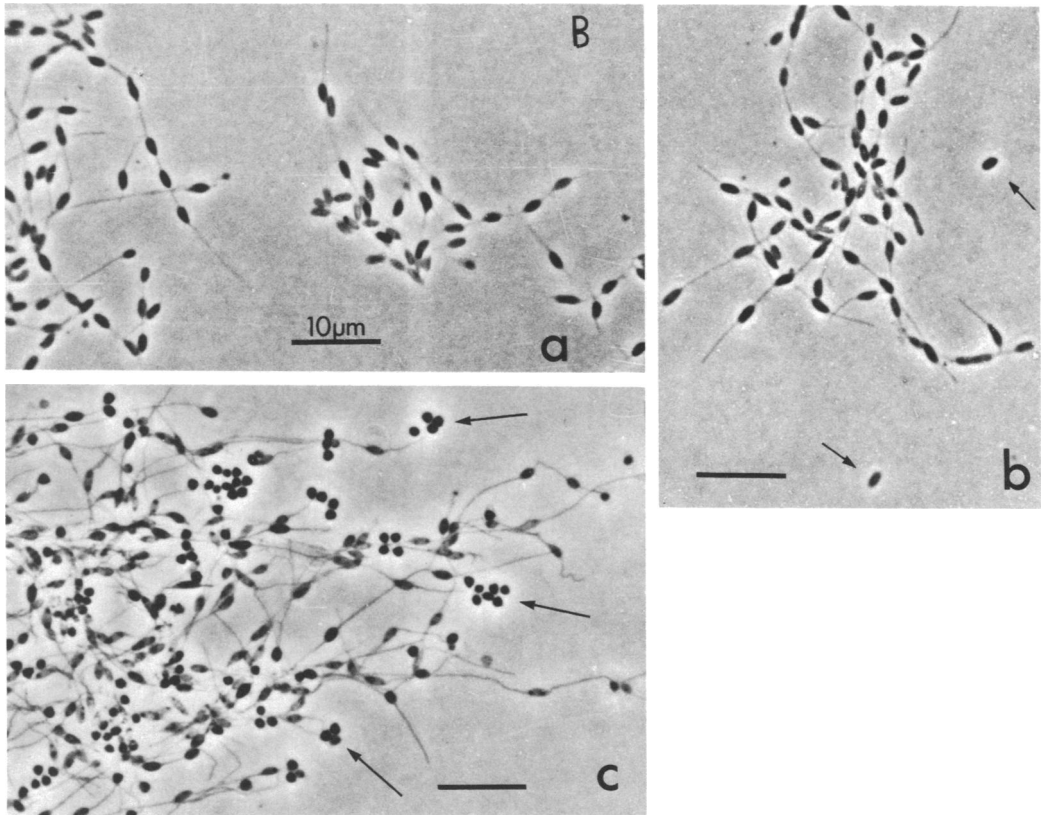
FIG. 14 B

operated aseptically; (iii) physiological conditions can be controlled to ensure a minimum of environmental stress (i.e., the temperature and gaseous atmosphere can be adjusted to that of the culture being synchronized); (iv) the whole operation can be completed within a few minutes; (v) the apparatus can be adapted to take large quantities of culture (i.e., the column shown can be substituted by a wide-bore column, thus allowing synchronization of cultures in excess of 15 liters); (vi) sufficient quantities of cells, selectively synchronized, can be collected for quantitative biochemical investigation. For instance, 5- to 10-liter quantities of strain Rm 5 swarm cells of up to $3 \times 10^7$ cells/ml (viable count) can be collected within 10 to 15 min, a much greater quantity of selectively synchronized cells than has been obtained by previously described methods (Table 7).

A disadvantage of the system is that swarm cells are the only components of the vegetative population that can be synchronized in this manner. Cells trapped within the glass wool column are a heterogeneous collection of cells at all stages of the cycle.

## Synchronous Growth of *Rhodomicrobium* Swarm Cells

**Assay of "landmark" events in liquid culture.** Synchronous cultures for experimental work were obtained from exponentially growing populations cultured at 30°C with an incident light intensity of 1,500 lx.

Initial experiments indicated that no detectable lag phase developed in swarmer cultures. Optical extinction curves of swarm cells in batch culture increased at a constant rate over three or four generations and at the rate observed in untreated, nonsynchronized cultures growing under the same conditions. We therefore assumed that a minimum of physiological perturbation had taken place in the selection process.

Over a generation period of 6.5 h, a number of temporally related "landmark" events were observed in the cell cycle (Fig. 16; see also Fig. 59). All of these could be used in assessing synchrony. However, only the most convenient of these, in terms of technique, were routinely used. They included the following: (i) the onset
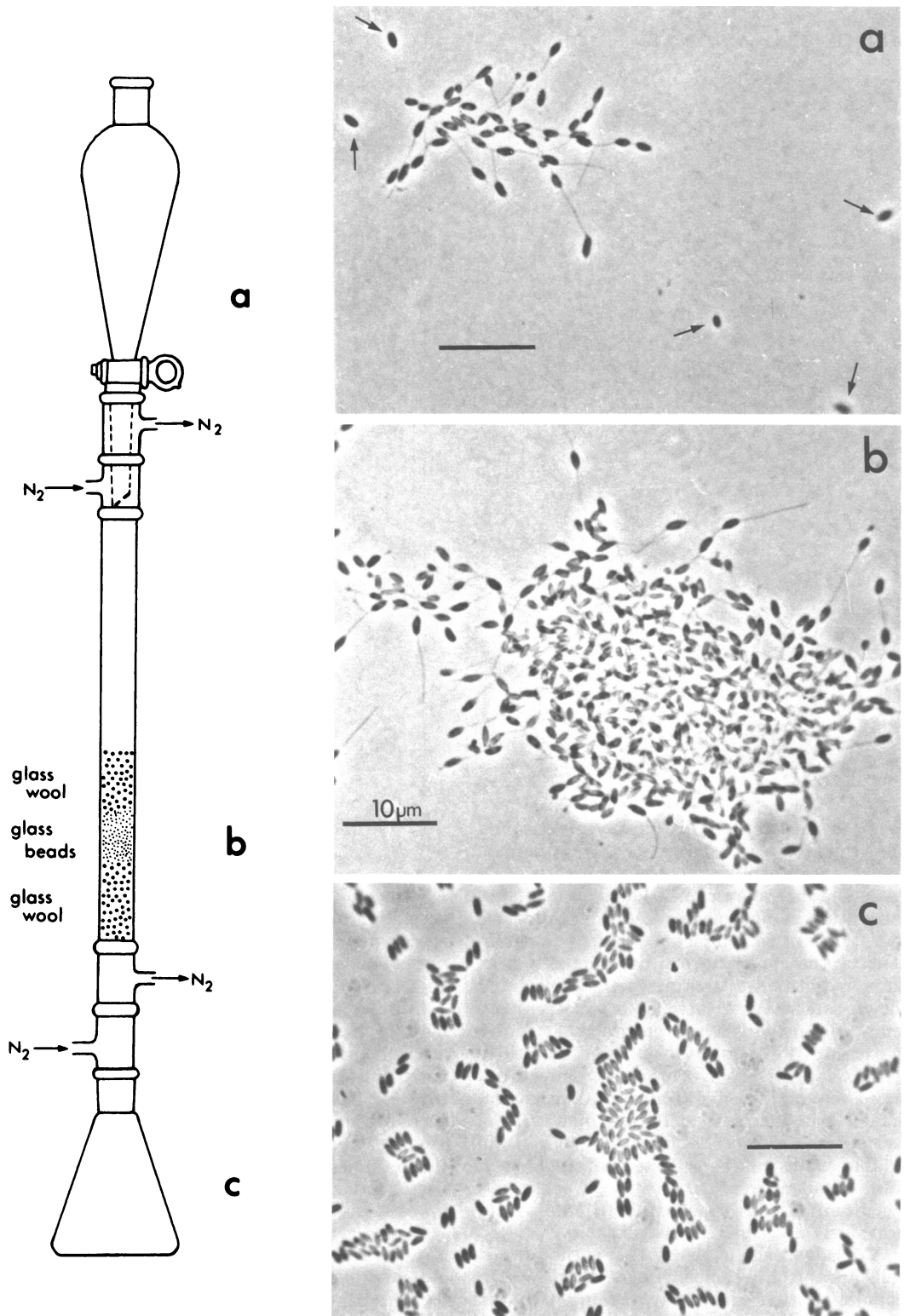
F<small>IG</small>. 15. *Apparatus used for the selective synchronization of strain Rm 5 consisted of the following: a 500-ml dropping funnel (Jobling D2), socket adapters (Jobling MF18), a glass condenser (Jobling C2), and 250-ml Erlenmeyer flasks (Jobling FE 250), all of which were sterilized by dry heat. Before use the column was washed with 250 ml of sterile medium and gassed with oxygen-free N$_2$. (a) Mid-exponential-phase cultures grown on pyruvate-malate medium at 30°C and with an incident light intensity of 1,500 lx were used in all synchronization experiments. (b) Stalked and chain complexes retained on the column. (c) Selectively synchronized population of swarmer cells.*

TABLE 7. *Cell yields by selective synchronization techniques*

| Organism | Synchronization procedure | Volume of synchronized culture | Viable cells/ml | Reference |
|---|---|---|---|---|
| *Caulobacter* | (i) Filtration | Not given | | 72 |
| | (ii) Repeated centrifugation | 2–3 ml | Not given | 81 |
| | (iii) Adhesion of "mother" cell | 25 ml | $1 \times 10^7$–$3 \times 10^7$ | 15 |
| | (iv) Adhesion of "mother" cell | 150 ml | $5.9 \times 10^6$ | 80 |
| *Hyphomicrobium* | Centrifugation and filtration | 30 ml | $2 \times 10^7$ | 52 |
| *Rhodopseudomonas palustris* | Density gradient centrifugation | 2–3 ml | $1.4 \times 10^9$ | 93 |
| *Rhodomicrobium* | Filtration | 100 ml–15 liters | $1 \times 10^7$–$2.5 \times 10^7$ | |
| *Bacillus subtilis* | Filtration | | 1–2% of initial population | 71 |
| *Escherichia coli* | Density gradient centrifugation | 1–2 ml from a 15-ml gradient | $4 \times 10^8$ | 50 |

and period of tube formation as reflected in a change of slope when following the absorption characteristics of a synchronized population with time (Fig. 16). (ii) The constant pattern of events recorded in Fig. 16 were confirmed by plotting histograms of cell shape distribution in a large number of microscopic fields during synchronous growth (Fig. 17). (iii) Cell volume changes during synchronous growth added further confirmation to the above (Fig. 18). One-step growth curves cannot be used with cultures in this morphogenetic form, since daughter cells are not released at division. Viable counts, consequently, do not double at cell division.

## Vegetative Cell Cycle

Swarm cell growth leading to the formation of a first-generation cell was classified into two major sequences — maturation and reproduction.

**Maturation sequence.** This occurred (under the growth conditions described) over a period of 130 to 150 min and is subdivided into three distinctive phases.

(i) **Motile period.** This period lasts for 20 to 30 min. The swarm cell is peritrichously flagellated and has dimensions of 1.5 by 0.8 $\mu$m (Fig. 19a).

(ii) **Flagella shedding period.** The flagella are lost over a 15-min period, and all cells become permanently immotile. Flagella are shed intact; i.e., they retain the flagella hook (Fig. 19c). Only newly formed daughter cells bear flagella (Fig. 19d). When cells are grown in slide cultures, flagella shedding can lead to the appearance of flagella bundles (Fig. 19b) analogous to those described for *Vibrio alginolyticus* (89).

(iii) **Terminal maturation period.** During

this phase, no obvious external morphological changes take place. This period lasts for a further 100 to 120 min.

**Reproductive sequence.** This takes place over 200 to 240 min (Fig. 16 and 21 to 24) and is divisible into three phases.

(i) **Filament formation phase.** During this phase, filament synthesis occurs at one or, occasionally, at both poles of the cell and requires 40 to 50 min for completion (Fig. 16, 21, 22, 24).

Both filament length and the proportion of time of the cell cycle occupied in filament synthesis can be lengthened or shortened by changes in the nutrient status of the medium. This effect is particularly obvious when phosphate is limiting. Medium that has no added phosphate results in intercell distances in excess of 30 $\mu$m with a cell generation time of 18 to 20 h. Subsequent addition of phosphate (10 mM) to such cultures results in a burst of cell synthesis and replication with intercell distances drastically reduced (2 to 3 $\mu$m) and a reduced generation time of 5 to 6 h (Fig. 20).

(ii) **Bud synthesis.** After 220 to 250 min from initiation of the cycle, bud synthesis begins. The distal end of the filament begins to swell detectably, and the process lasts for 150 to 180 min. Only one bud is formed at a time, irrespective of the number of filaments possessed by the cell.

(iii) **Daughter cell completion.** This is the period when no increase in daughter cell size takes place, but physiological separation from the mother cell occurs. This may be either by binary fission at the junction of filament and daughter cell, or by the synthesis of a plug within the filament a short distance from the daughter cell (Fig. 21i and j, and 25). This distance is reasonably constant and is not a function of overall filament length (Fig. 26).
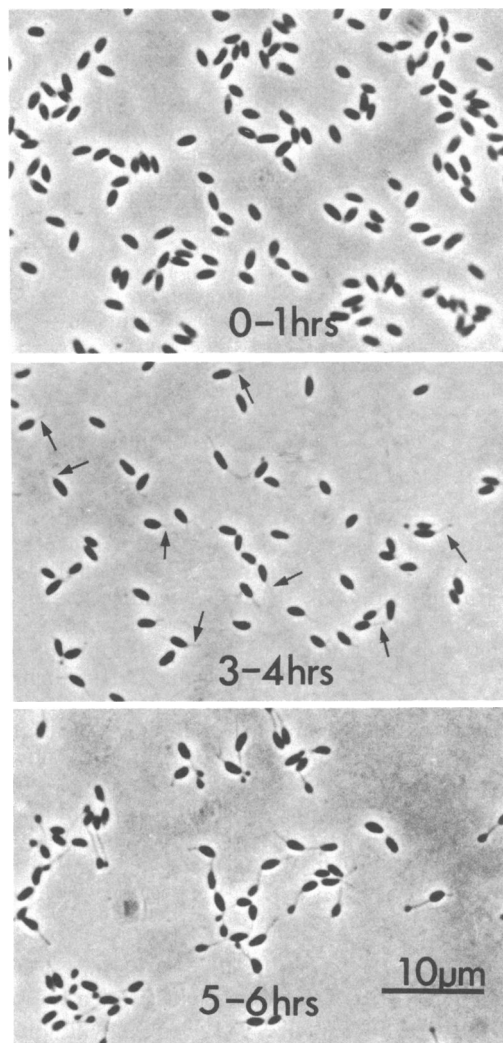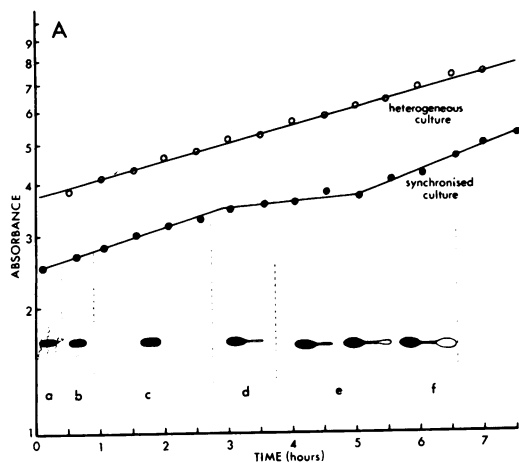
FIG. 16. (A) Extinction changes ($E_{540}$) of a synchronized Rhodomicrobium Rm 5 culture growing at 30°C with an incident light intensity of 1,500 lx. (B) Temporal sequence of changes in cellular morphology as shown by phase-contrast microscopy.

Consequently, it is assumed that plug synthesis is under the regulatory control of the daughter cell. Separation by plug formation is a prerequisite for chain formation. In division by binary fission, the motile daughter cells retain no part of the mother cell filament.

**Subsequent development. (i) Swarmer sibling formation.** When the mother cell releases a swarmer sibling, then the pole of the filament is available as a site for a second round of reproduction. No branching occurs and only one filament is synthesized. In this instance, the growth sequence is similar to that of the other photosynthetic budding bacteria such as R. palustris, R. viridis (96, 93), and R. acido-

phila (60, 84) and also to the non-photosynthetic Hyphomicrobium species (33). Such a cell cycle is established by strain Rm 5 (Fig. 27 and 28) growing in the presence of high $CO_2$ concentrations ($\sim 2.5 \times 10^4$ $\mu$l/liter) (A. France and C. S. Dow, unpublished data, this laboratory). When plated onto solid medium and grown photosynthetically, cells with this "simplified cycle" give rise to a markedly different colony type from that characteristic of the "chain cycle" (Fig. 27). In batch culture, swarmer siblings are formed in the early- and mid-exponential-growth phases only from multicellular arrays (Fig. 29). When isolated by the glass wool column technique, these swarm cells

reproduce by chain formation; i.e., they give rise to rough colonies. However, a low percentage (1 to 2%) of swarmer siblings isolated from the late-exponential-growth phase, when the $CO_2$ concentration is highest ($2.5 \times 10^4$ µl/liter), follow a "simplified cycle"; i.e., they give rise to "smooth" colonies.

(ii) **Bipolar extension.** In this instance, the mother cell initiates bud formation on the filament at the opposite pole to that at which the first sibling was formed (Fig. 21, 22, 24). Siblings are formed alternatively at both poles to create a chain identical in appearance to that occurring when growth is unidirectional. Only one daughter cell at a time is ever formed by a mother cell.

(iii) **Uni- and bipolar branch development.** This form of development is the most complex and leads to the tangled mutlicellular arrays characteristic of this species. The initial sibling production is as described above. If growth is unipolar, then the second daughter cell is formed on a filament branching from the first filament. A third daughter cell develops on a branch filament arising from the second filament, and a fourth develops on a branch filament arising from the third filament; i.e., *a branch filament can only be formed on the most recently synthesized filament* (Fig. 22 to 24). It is an obligately progressive sequence. Each completed daughter cell is accompanied by plug formation in its filament *before* that filament branches to form the next daughter sibling. Should growth be bipolar (only observed with swarm cells), then branch formation is as already described. However, *in all cases no more than four daughter cells are ever formed by one mother cell* (Fig. 24). Growth conditions appear not to be a cause; this phenomenon occurs whatever the nutrient situation. Daughter
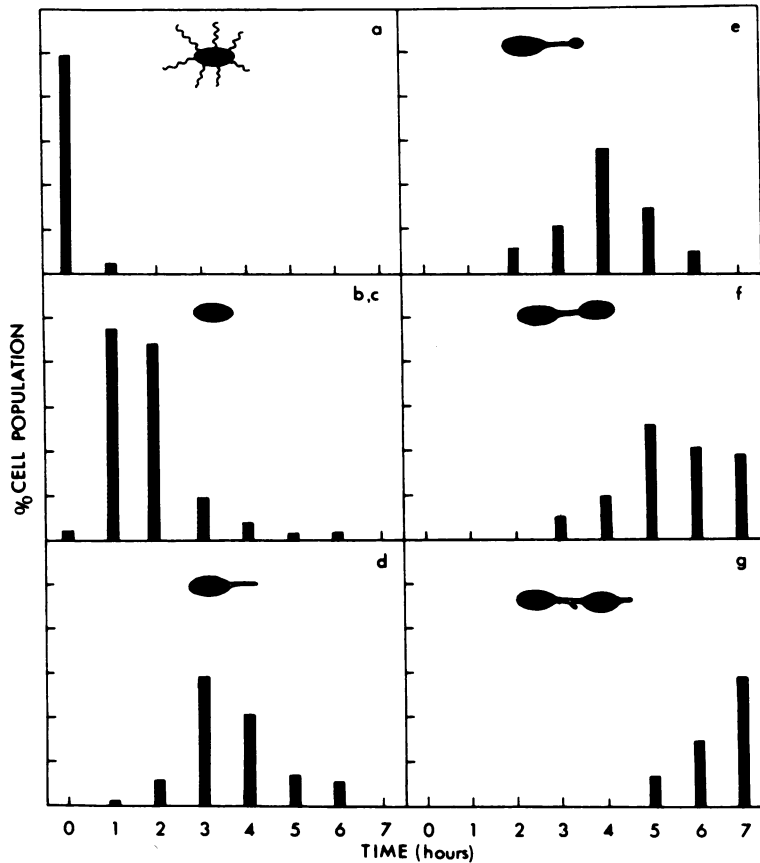


FIG. 17. *Synchronized population examined microscopically with time and the changes in cellular morphology scored as a percentage of the total cell population. The initial population was in the region of 98 to 99% swarm cells. However, with time, the randomization of cell types increased and resulted in broader peaks. This was reflected not only in the histogram but also, and more accurately, in the Coulter Counter experiments (Fig. 18).*
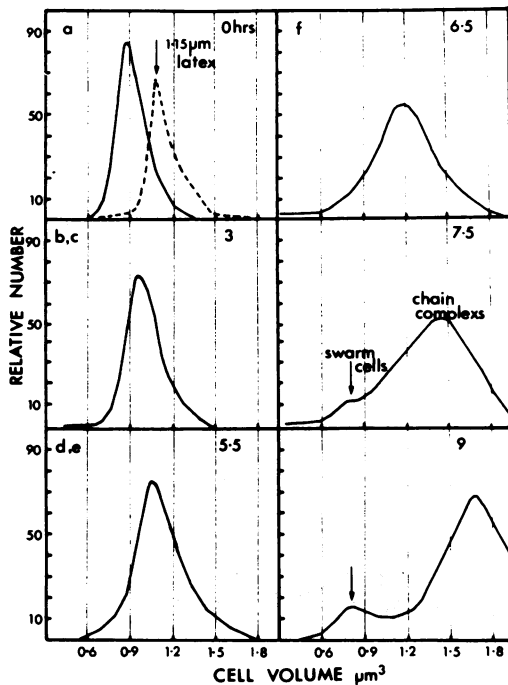
FIG. 18. *Changes in the cell volume of a synchronized population as detected by a Coulter Counter (ZB1)/Channelyzer (C1000) system. The experimental procedure was as follows. One-tenth milliliter of sample was diluted in 19.9 ml of filtered (0.22 μm) electrolyte and analyzed by using a 20-μm orifice. The system permits analysis of particles having a spherical diameter of ≥0.5 μm. Each stage of the cell cycle can be monitored precisely, i.e., swarm cells (0 h) to mature and stalked cells (3 h) to bud formation (3.5 to 5 h) to mature mother and daughter (6.5 h) to complex chain formation and the reappearance of the swarm cells (7.5+ h).*

cells and their progeny proceed to multiply in cultures containing the original mother cell, which has ceased to form new daughter cells. What dictates that this finite number of cells should be synthesized per mother cell is not known.

## Other Cell Types Formed

**"Double-yolk" cells.** In some microscopic preparations, unusual constricted cells are apparent (Fig. 30a). These are suggestive of an aberrant growth and division process. However, they remain viable and behave as one cell (Fig. 23h).

**Exospore formation.** In batch culture, strain Rm 5 frequently sporulates, forming up to four exospores per cell (Fig. 32). Exospore formation, germination, physiological properties, and fine structure are described in the following section. However, two points need to

be made here. (i) Since four exospores are formed from the one cell, the process can be interpreted as a further mechanism of reproduction, rather than simply the production of a resting stage, as in the case of organisms forming one spore or cyst per cell. (ii) A cell either gives rise to exospores *or* vegetative cells, not a mixture of both. Exospore formation apparently reflects an early and irreversible event in the mother cell.

It has, as yet, not been possible to induce exospore formation in cultures of strain Rm 5 growing as the "simplified" cycle. However, when such cultures are in the late-exponential-stationary-growth phase, small angular cells are formed, initially in pairs (Fig. 30b) and then singly (Fig. 30c) (A. France, unpublished data, this laboratory). The nature of these cells has still to be determined.

## Exospores

Strains of the photosynthetic bacterium *R. vannielii* in batch culture occasionally form angular cells that are quite distinct from vegetative cells in resistance properties, mode of formation, morphology, and fine structure. They were first described by Gorlenko (27, 28). He considered them to be spores and described some of their resistance properties and deduced something about their formation and germination from microscopic observations of batch cultures.

Similar structures were detected by us, both in the neotype culture ATCC 17100 and in strains isolated during this investigation. They are termed "exospores" to distinguish them from bacterial endospores. The following account of their formation and germination in slide and batch culture, their resistance properties, and fine structure confirms and extends the findings of Gorlenko.

**Occurrence and collection.** Exospores were formed profusely in malate medium (described previously) after several days of incubation at 30°C with an incident light intensity of 1,000 lx. The carbon source had some importance in these events, since it was noted that cultures ceased to sporulate after two or three serial subcultures in pyruvate/malate medium. As yet, we have no firm evidence about the particular conditions that initiate sporulation.

Preparations of exospores, free from vegetative cells, were obtained by filtering through a glass wool column (Fig. 15). Washed exospores suspended in 0.01 M phosphate buffer (pH 6.8) were used in all subsequent experiments.

**Morphology and formation.** The distinctive shape of the exospores and their overall dimensions, in comparison with vegetative cells, were
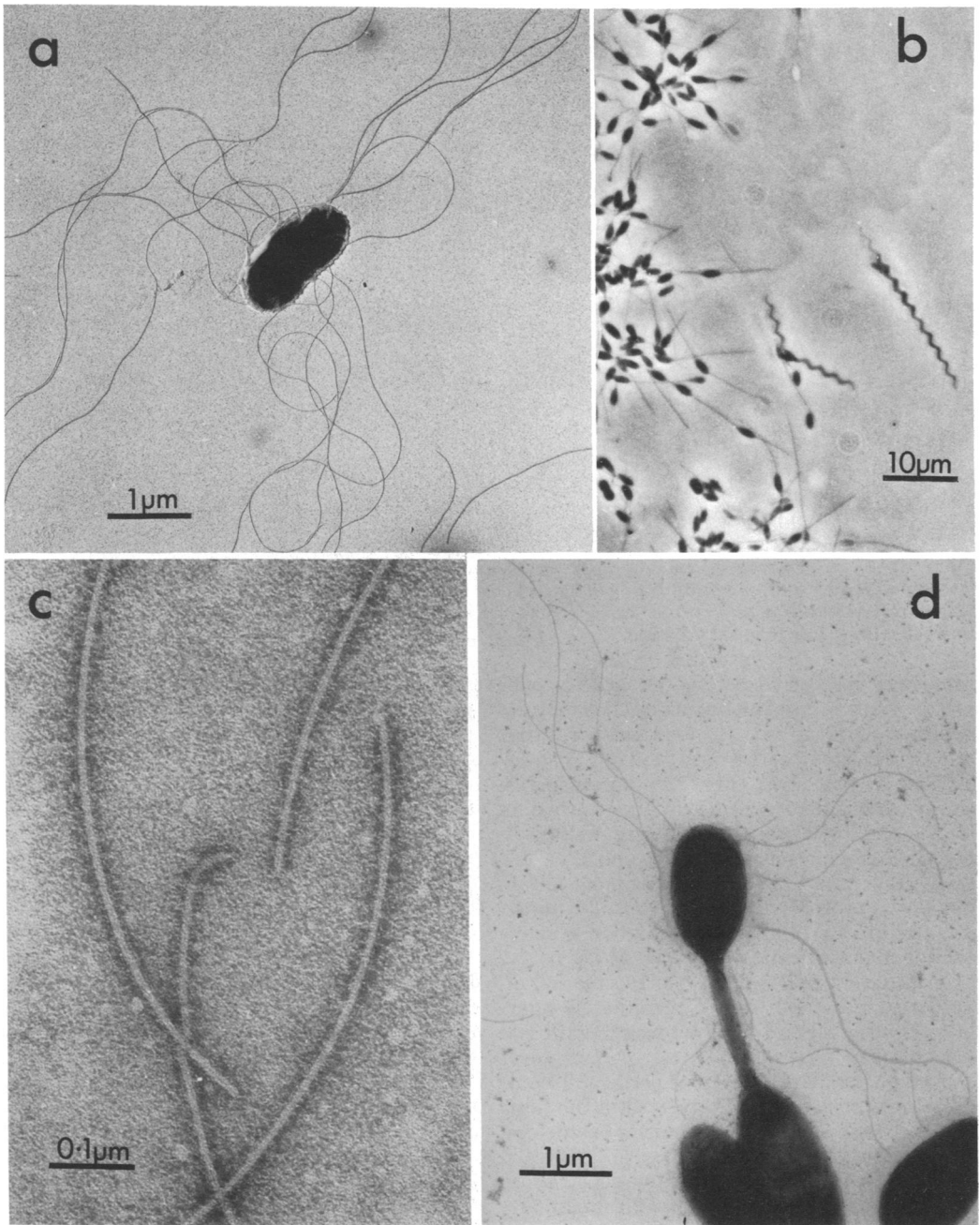
FIG. 19. *(a) Strain Rm 5 swarm cell. Gold-palladium shadow. (b) Phase-contrast photomicrograph of a strain Rm 5 slide culture showing spirillar flagella bundles. (c) Electron micrograph of shed flagella showing an intact flagella hook (1% [wt/vol] phosphotungstic acid, negative stain). (d) Swarm cell formation (1% [wt/vol] uranyl acetate, negative stain).*

observed by phase and electron microscopy (Fig. 31 and 32). These photomicrographs show the asymmetry and angularity of the exospores, which, in addition, are frequently sur-

rounded by a fibrillar capsular material (Fig. 36).

Once sporulation is initiated, vegetative cell synthesis ceases. Up to four exospores are
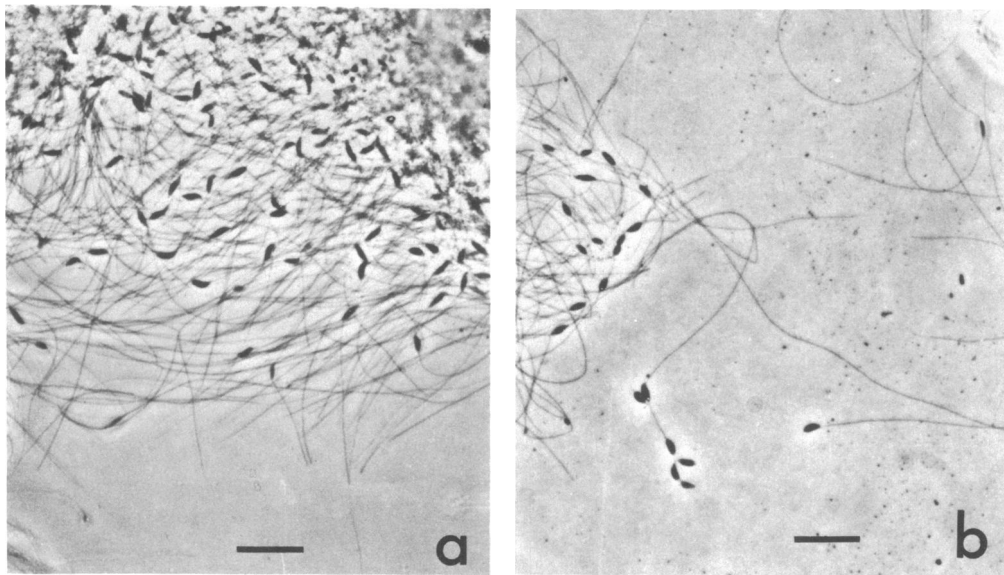
FIG. 20. *Phase-contrast photomicrographs. (a) Strain Rm 5 grown under phosphate limitation (no added phosphate). (b) Strain Rm 5 initially grown under phosphate limitation followed by addition of phosphate (10 mM) to the system. The intercellular distances are considerably reduced, with a corresponding decrease in generation time. Bar represents 10 μm.*

formed per vegetative mother cell, and the pattern of events is best illustrated by slide culture observations (Fig. 32). All four exospores are formed terminally on the one filament (Fig. 32 and 33). At first sight (Fig. 32), the exospores appear to be in an organized cluster attached to the filament, but electron micrographs reveal that they are formed terminally and then released (Fig. 33). Subsequent attachment to the filament is probably due to the capsular material. The process of exospore formation is summarized diagrammatically in Fig. 34.

**Germination and outgrowth.** Inoculation of exospores into fresh liquid medium or agar slide cultures induced their outgrowth within 3 to 4 h. Loss of refractility, loss of resistance properties, and development of permeability to dyes occurs coincidentally and within the first 60 min. However, the most unusual events observed relate to outgrowth.

Exospores are subject to the same constraints on growth as are vegetative multicellular arrays; i.e., on filament is synthesized at a time and only one vegetative daughter cell is formed at a time; that is, the next filament and daughter cell are not morphologically apparent until the previous daughter cell is sealed off by a plug in the filament (Fig. 36 and 37). Exospore outgrowth can follow one of two mutually exclusive patterns. (i) Up to four filaments are formed sequentially, under normal growth conditions (anaerobic and illuminated), from four

specific sites on the cell—the points of the angles (Fig. 35f'-k'; 36e'-f'; 37). (ii) One filament only emerges from the exospore. Subsequent daughter cells are formed on branch filaments (Fig. 35 to 37). Time lapse photographs of sibling production (Fig. 35 and 36) reveal the same constraints and variations in pattern as occurs in the formation of multicellular arrays.

Exospores also germinate under dark, aerobic conditions in the absence of exogenous energy sources. Outgrowth of filaments occurs, and daughter cell synthesis may be initiated. However, growth ceases before completion of the daughter cell. All of these changes appear to be driven by endogenous energy substrates mobilized in the absence of light.

**Physiological properties.** *Desiccation.* Exospores, dried onto microscope slides and held in a dry atmosphere for 3 days at 30°C, germinated on inoculation into fresh medium or on agar slide cultures. Vegetative cells were killed by the same drying process.

*Heat resistance.* In contrast to the vegetative cells, exospores were tolerant of high temperatures. They will survive treatment at 100°C for several minutes, whereas the vegetative cells are unable to survive 50°C for 20 min. Survival curves of exospores at 50, 60, and 80°C (Fig. 38) characteristically show a plateauing effect. It is probable that the initial steep fall in viability reflects the high proportion of partially formed/germinated exospores in the preparation.
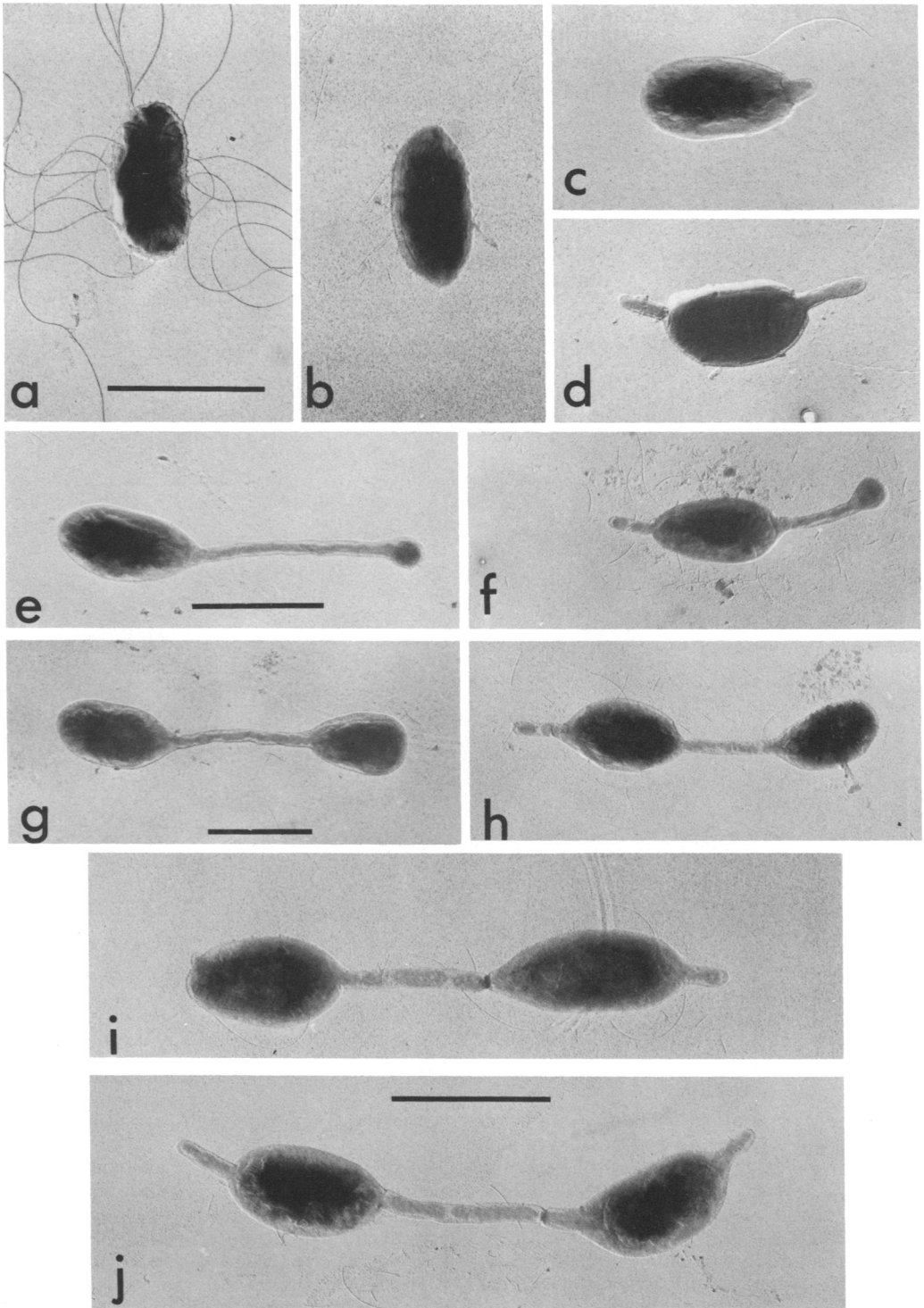
FIG. 21. *Gold/palladium-shadowed electron micrographs of swarm cell development in strain Rm 5. (a) Peritrichously flagellated swarm cell; (b) swarm cell after flagella have been shed; (c) filament formation from one pole; (d) bipolar filament formation; (e–h) daughter cell synthesis — note continuity of cytoplasm; (i and j) plug formation — note the compartmentalization within the filament; (k and l) subsequent cell synthesis, matrix formation. Bar represents 2 µm.*
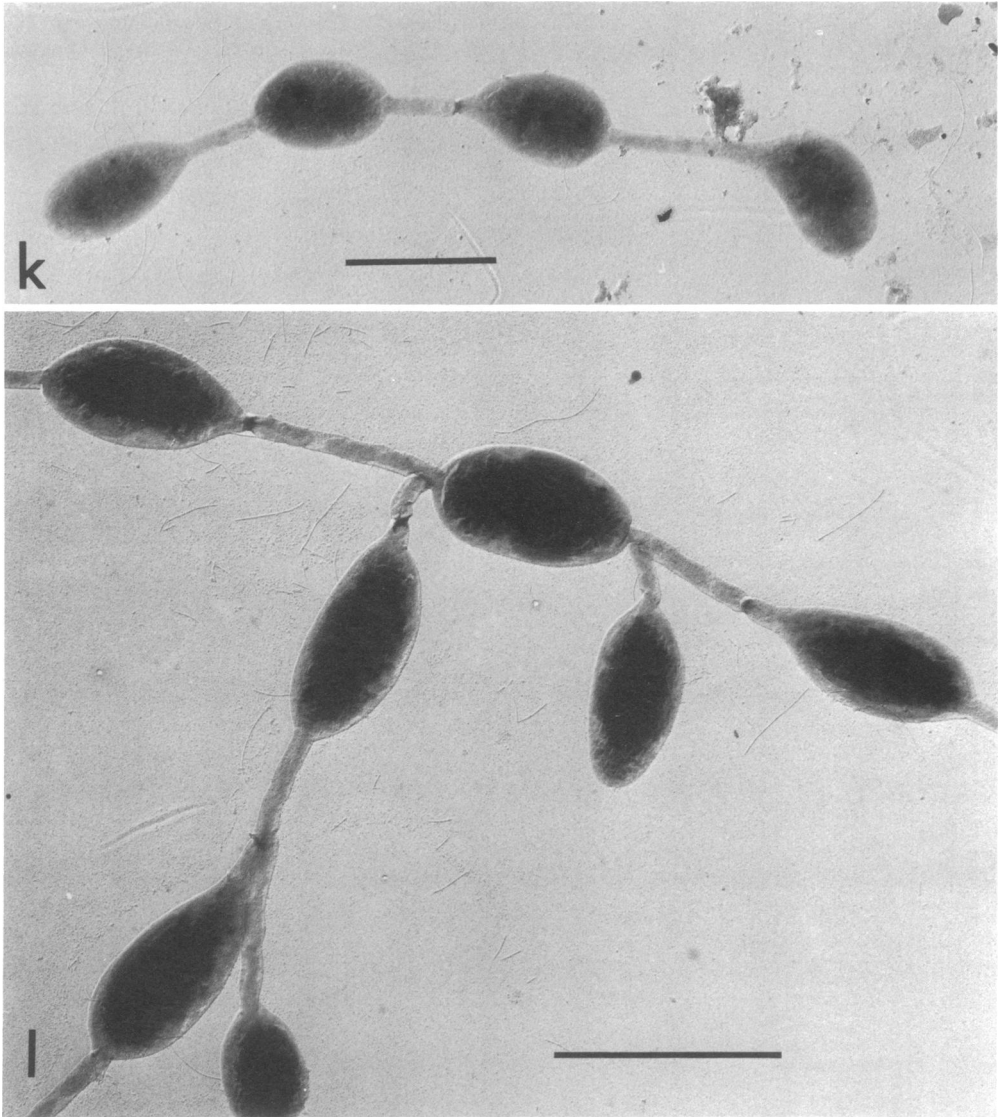
FIG. 21 k-l

*Ultraviolet irradiation.* The difference in resistance between exospores and vegetative cells was relatively slight; 30% more energy was required to kill the same proportion of exospores as vegetative cells in a population over a fixed period of time. Survival curves of exospore preparations are nonlinear (Fig. 39), which again suggests that a substantial proportion of the exospores were partially formed/germinated and that the lower part of the curve more accurately reflects mature exospore resistance to ultraviolet irradiation.

*Lysozyme resistance.* In contrast to endospores (85), exospores and vegetative cells of strain Rm 5 appear equally sensitive to lysozyme.

*Stainability.* A number of exospore preparations, harvested at various times, were treated with malachite green (2) and other dyes such as crystal violet and dilute fuchsin. The proportion of exospores resistant to staining in a given population was similar to that proportion seen to be refractile under the phase-contrast microscope. At the most, 25% were resistant to staining. Whether or not germination is initiated before or during harvesting, or whether a major part of the population never becomes refractile, remains to be determined. These observations
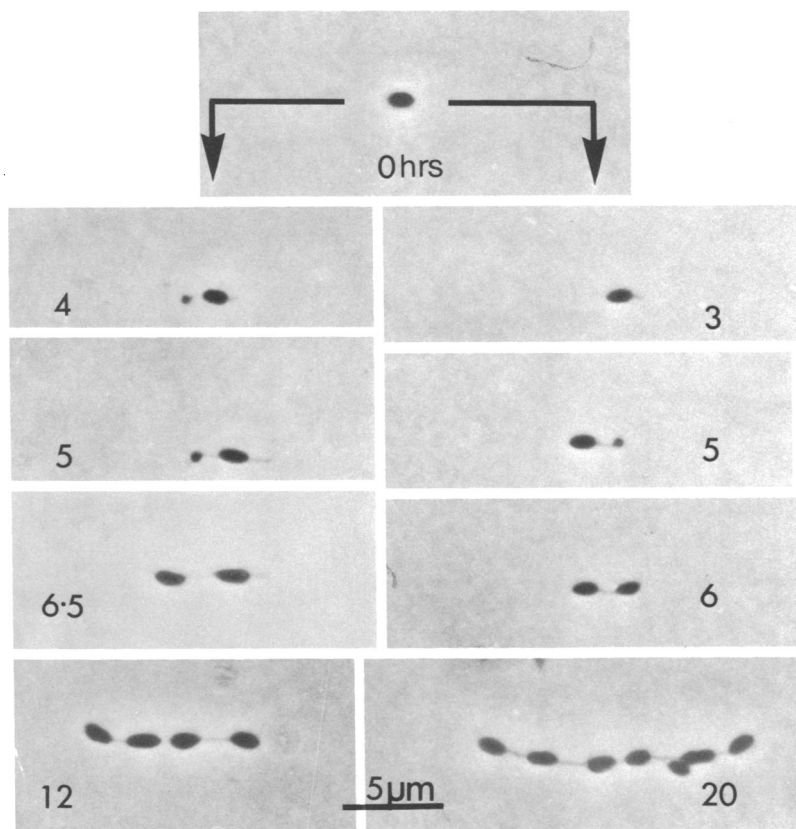
FIG. 22. *Swarm cell development.*

also support the interpretation of the nonlinear graphs obtained for ultraviolet irradiation and heat resistance.

*Dipicolinic acid content.* In contrast to endospores, and as in methane-oxidizing bacteria (95), no dipicolinate could be detected in exospore preparations.

*Photopigments.* Absorbance spectra for purified exospore preparations show a marked decrease in both chlorophyll and carotenoid content in comparison to that found in vegetative cells from the same culture (Fig. 40).

### Fine Structure of Vegetative Cells

**Fine structure.** The membrane complex present in strain Rm 5 is similar to that previously described (12, 87, 91) in other *Rhodomicrobium* strains. Membrane content (as judged by thin sections) varied inversely with light intensity; grown at 500 lx (incident light) or less the bacteria were packed with multiple layers of double membranes (Fig. 41), whereas at 2,000 lx or higher only one layer of double membrane (in addition to the cytoplasmic mem-

brane) was usually observed (Fig. 42 and 43), an effect of light intensity described previously (87). Grown in continuous culture in malate-pyruvate medium with an incident light intensity of 1,500 lx at 30°C (steady-state optical density, 1.52; dilution rate, 0.026) cells were found to contain a considerable number of cellular inclusions (polyhedral bodies) (Fig. 44), which were similar in appearance to the carboxysomes of *Thiobacillus intermedius* (63).

Questions unanswered in the past concern the point of origin of the double membrane layers and the three-dimensional architecture of cells containing these lamellae. This latter point has a bearing on the interpretation of evidence concerning the process of reproduction discussed later.

Electron micrographs provided the information used in the construction of a three-dimensional model (Fig. 45), which was subsequently sectioned in various dimensions and found to account for the different membrane arrangements seen in thin sections (Fig. 41 to 43).

A key event in development of lamellae appears to be the circumpolar invagination of the
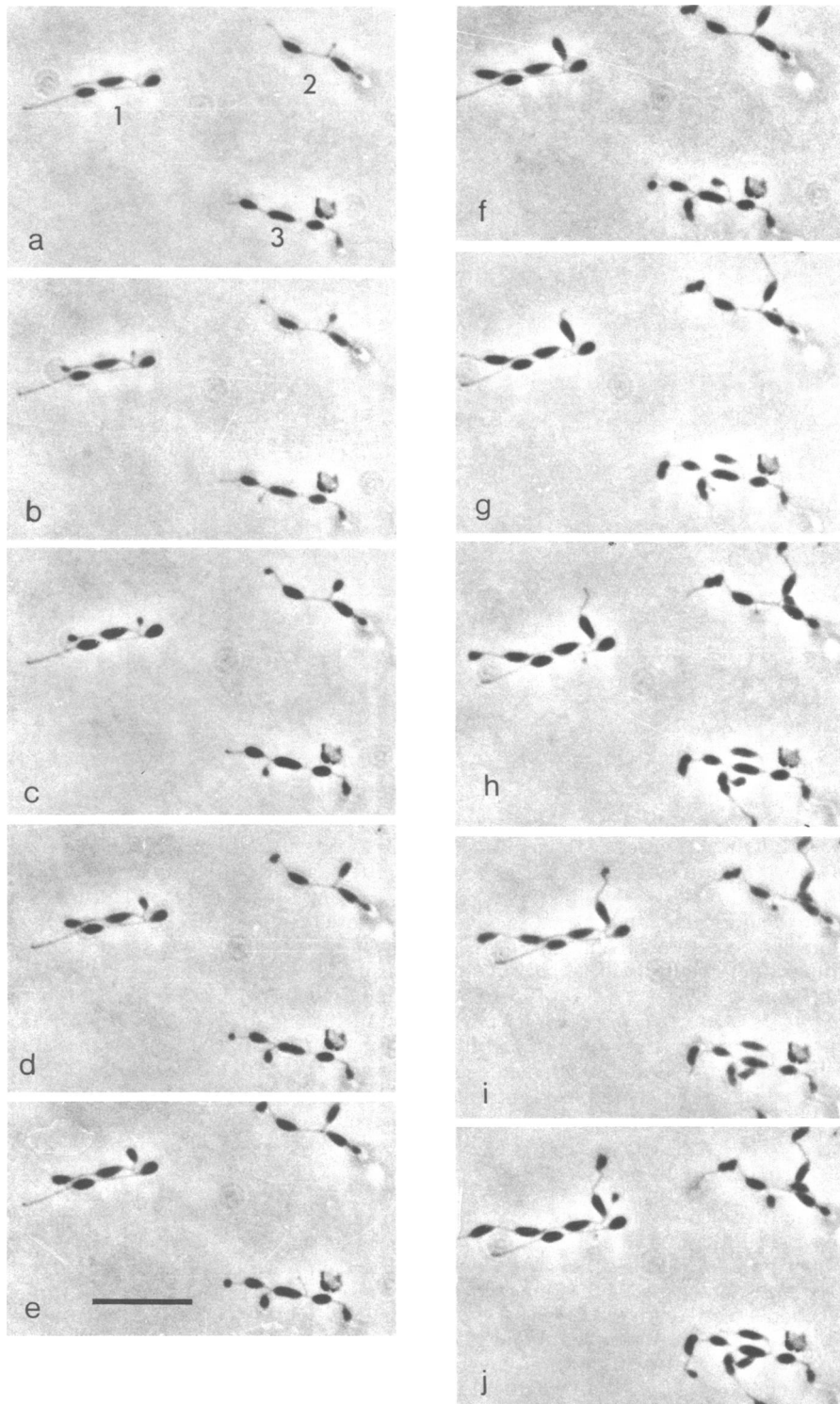
FIG. 23. *Sequential phase-contrast photomicrographs of a slide culture of strain Rm 5 vegetative multicellular arrays. Bar represents 10 μm.*
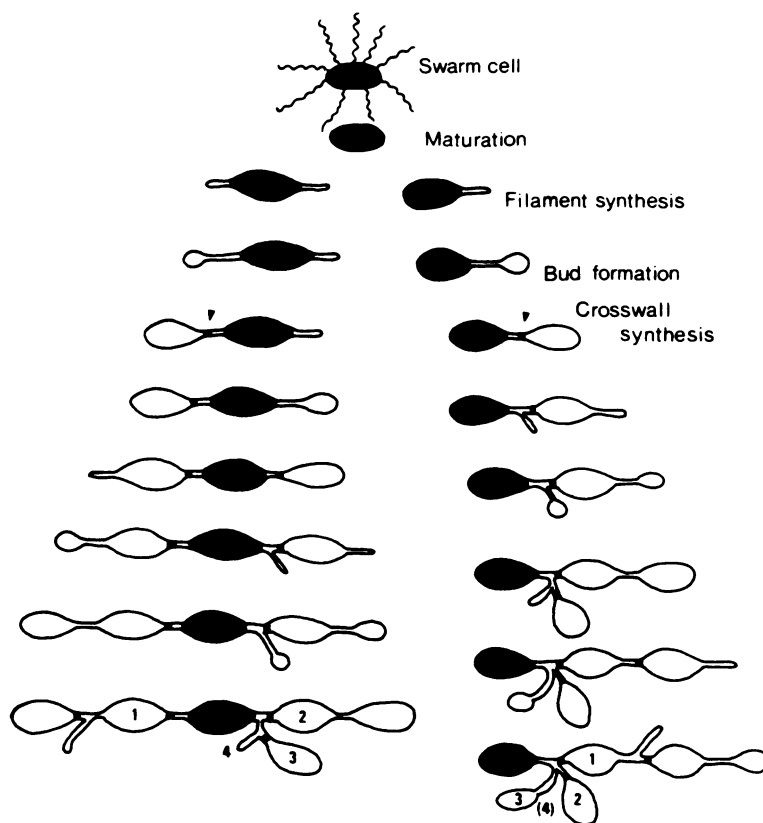
FIG. 24. *Diagrammatic representation of vegetative growth and development of batch culture swarm cells of Rhodomicrobium strain Rm 5.*
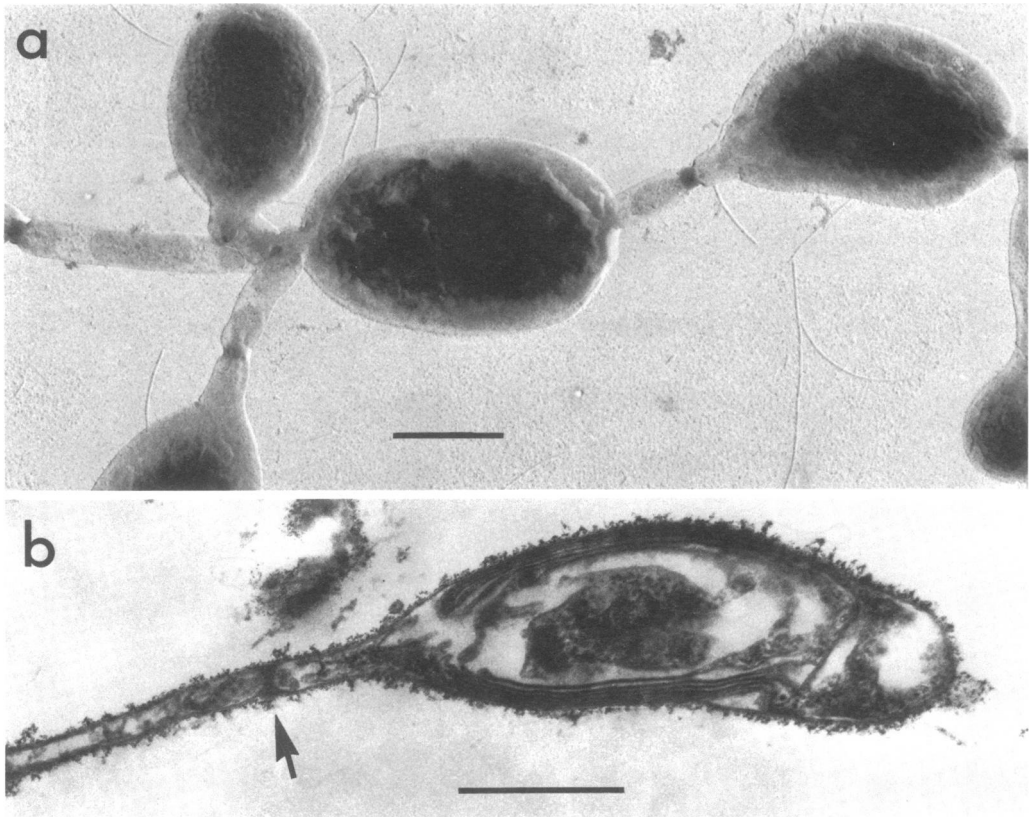
FIG. 25. (a) Gold/palladium-shadowed electron micrographs showing plug/cross wall formation in the filament of strain Rm 5 matrices. (b) Thin section of a plug/cross wall (prepared by the method of Ryter and Kellenberger [68]). Bar represents 0.5 μm.
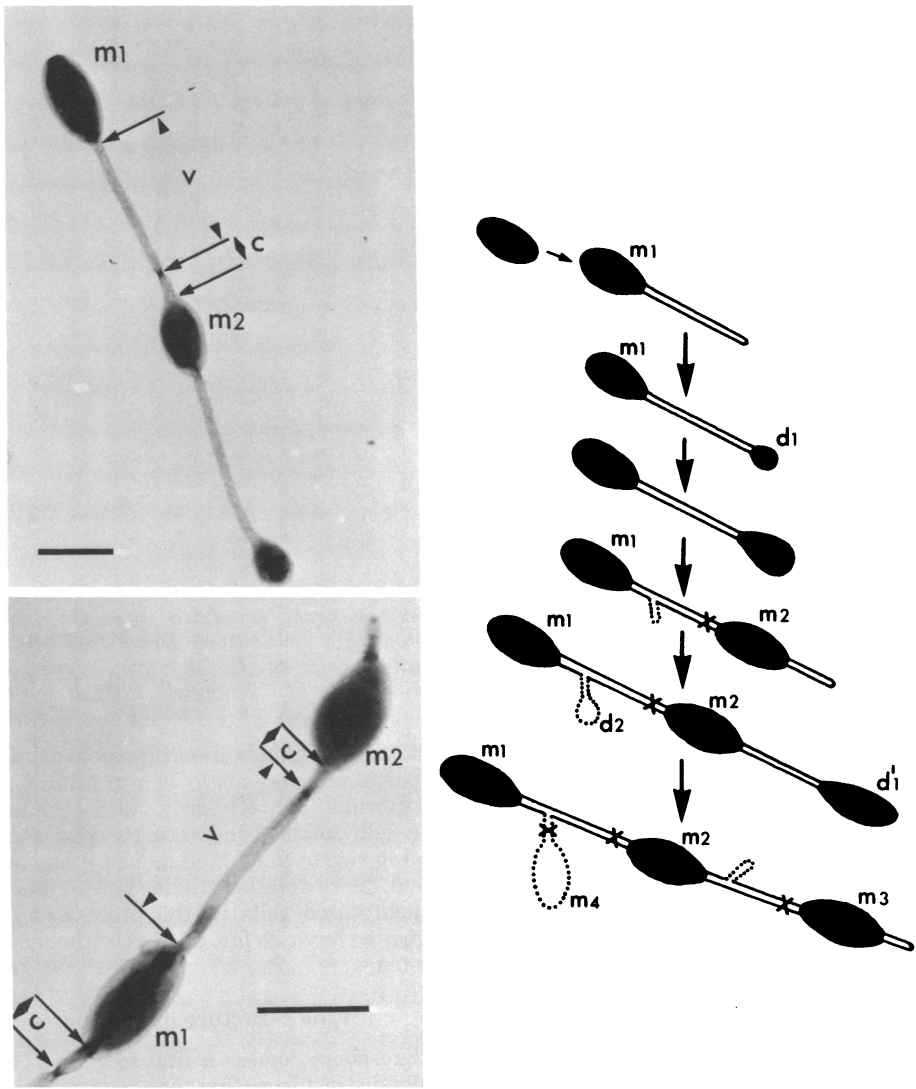
FIG. 26. *Gold/palladium-shadowed electron micrographs showing sites of plug formation. The distance from the mother cell (m) to the plug is variable and dependent on the growth rate. The distance from the completed daughter cell to the plug is relatively constant (c). The sequence of events leading to the formation of cellular matrices is shown diagrammatically. Bar represents 2.5 μm.*
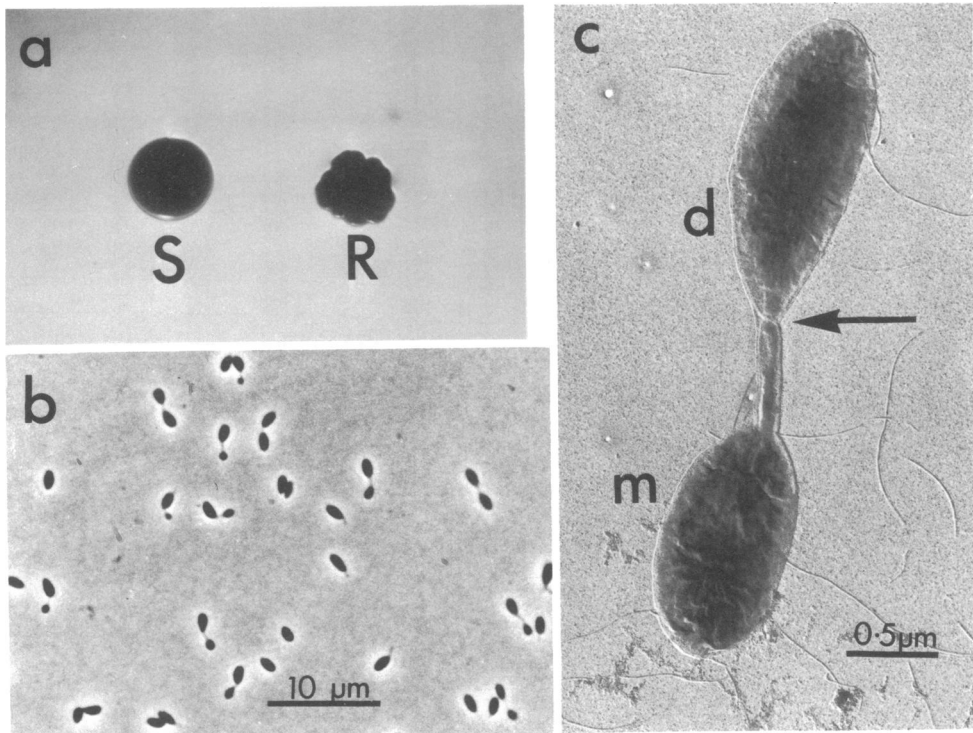
FIG. 27. *(a) S, "Smooth" colony formed by strain Rm 5 expressing the "simplified" vegetative cell cycle. R, "Rough" colony formation by strain Rm 5 vegetative cells growing as multicellular arrays. (b) Phase-contrast photomicrograph of an exponential culture of strain Rm 5 expressing the "simplified" cycle. (c) Gold/ palladium-shadowed electron micrograph showing cell division in the "simplified" cycle giving rise to a stalked mother cell (m) and a nonstalked daughter (d).*
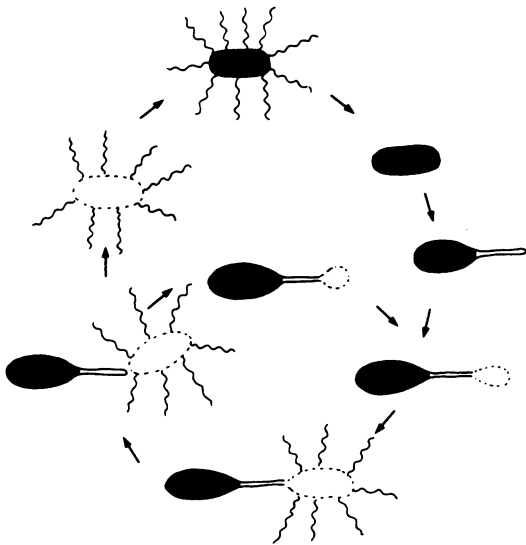


FIG. 28. *Digrammatic representation of the "simplified" cell cycle of strain Rm 5. It should be noted that filament synthesis precedes the formation of each and every daughter cell; i.e., the mother cell synthesizes a "new" piece of filament during each round of the mother cell cycle.*

cytoplasmic membrane at the pole distal to cell separation (Fig. 46a). The resultant loop of membrane (Fig. 46b) grows along the length of the cell, infolds, and returns (Fig. 46a), thus giving rise to the lamellae membrane arrangement. More detailed studies now in progress on synchronized cells in the process of forming lamellae have, so far, confirmed the model suggested.

## Fine Structure of Exospores

Exospores possess a fine structure (Fig. 47) quite distinct from that seen in vegetative cells. Thin sections of mature exospores reveal the presence of a very thick wall layer and a poorly defined cytoplasmic region. On germination, the occluding material in the thick wall disappears to reveal the existence of a layer of concentric membranes. The formation of these layers of membrane has yet to be followed in developing exospores; however, the overall appearance of the arrangement in mature exospores is distinctive and not identical to that seen in vegetative cells. The difference in appearance of the membranes between the two types of cells is probably a reflection of the geometry of
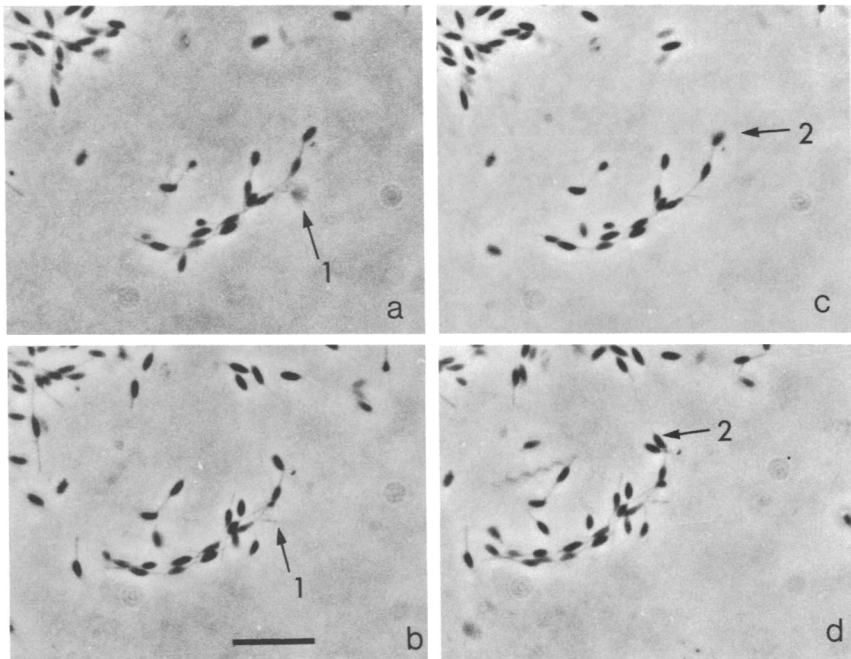
FIG. 29. *Phase-contrast photomicrographs of swarm cell formation from multicellular arrays. (a) Swarm cell (1) just prior to release (highly motile). (b) Swarm cell (1) released. (c) Swarm cell (2) becomes motile. (d) Swarm cell (2) retained. Bar represents 10 μm.*
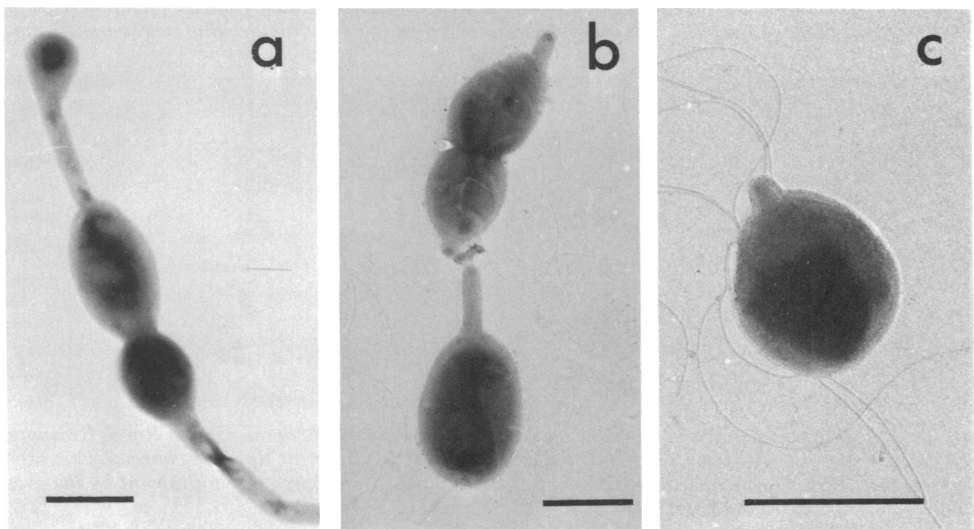


FIG. 30. *Gold/palladium-shadowed electron micrographs. (a) Constricted vegetative cell. (b) Small, angular cells produced by strain Rm 5, growing as the "simplified" cycle, on reaching the stationary phase. (c) Small angular cell. The flagella in the background are not attached to the cell. Bar represents 1 μm.*
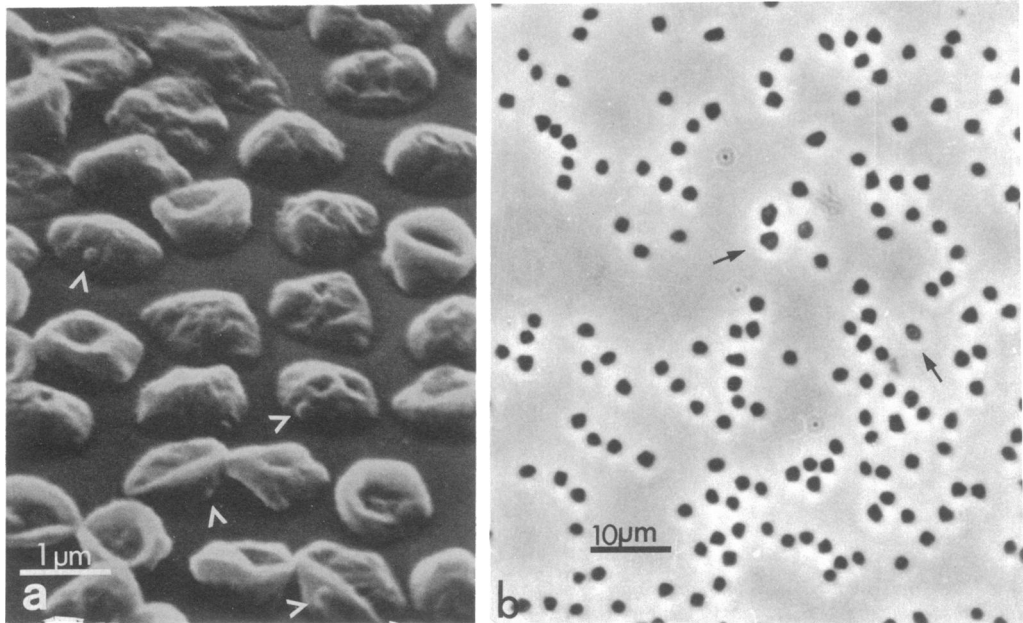
FIG. 31. (a) Scanning electron micrograph of a purified exospore preparation. Note the formation scar, which denotes the point of separation from the mother cell (arrowed). (b) Phase-contrast photomicrograph of a purified exospore preparation. The mature exospores are only slightly refractile. Bar represents 10 μm.
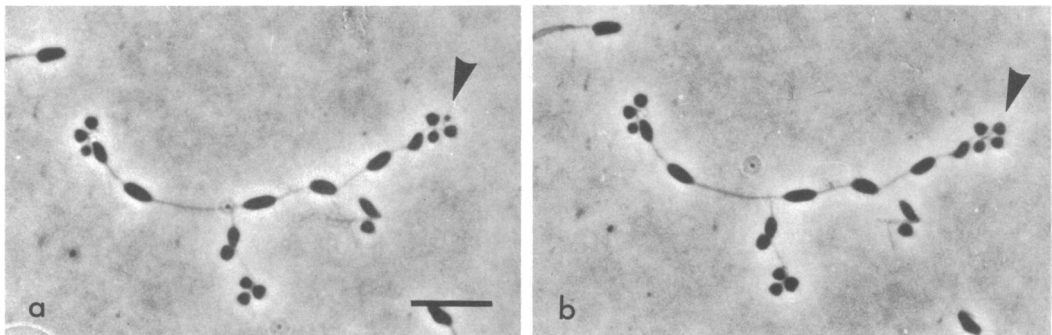


FIG. 32. Phase-contrast photomicrographs of exospore formation in Rhodomicrobium Rm 5. Exospores are formed (as are swarm cells) terminally and sequentially from the filament tip. The characteristic exospore clusters presumably arise because of attachment of the completed exospores to the filament by the exospore capsular material. Bar represents 10 μm.
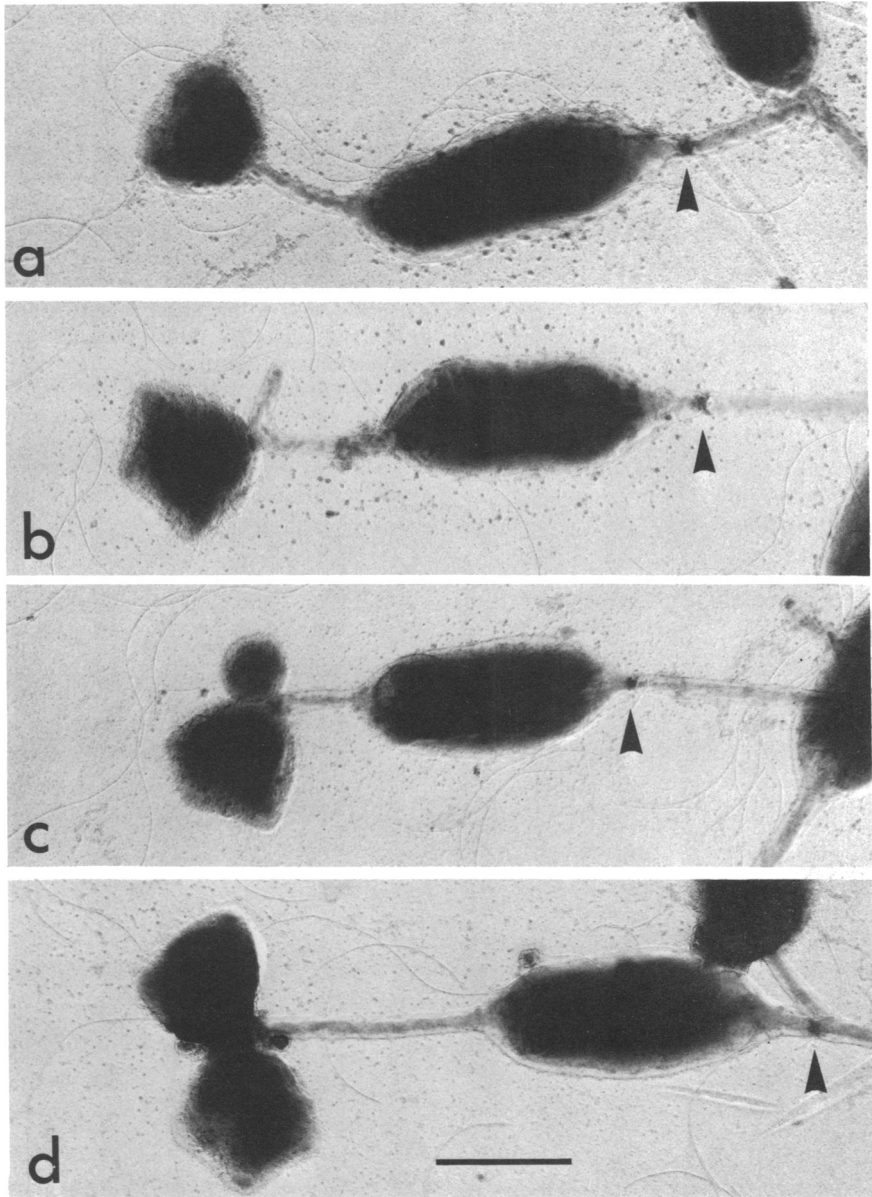
FIG. 33. *Gold/palladium-shadowed electron micrographs showing exospore formation in strain Rm 5. (a) Synthesis of first exospore. (b) Completion of exospore synthesis followed by filament elongation. (c) Formation of second exospore. (d) Completion of second exospore. Note that each mother cell involved in exospore formation is separated from the vegetative cellular array by a filament plug. Bar represents 1 μm.*
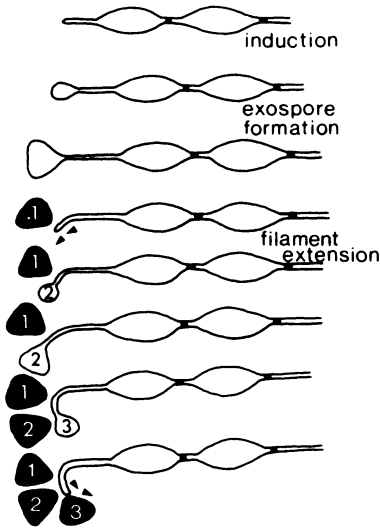
FIG. 34. *Diagrammatic representation of exospore formation in R. vannielii.*

the two types rather than an indication of a difference in how the layers are formed. Photopigment content (Fig. 40) of the mature exospores appears to be much less than that found in vegetative cells.

Observations on budding tube outgrowth of germinating exospores (Fig. 36) and their complex internal architecture indicate novel mechanisms of outgrowth. Thin sections (Fig. 48) illustrate the early stages of outgrowth and confirm the complexities of the process. A membranous tube, probably originating from the cytoplasmic membrane, grows through the gap in the concentric membrane layer and along the area between these membranes and the exospore wall to an angle where cellular outgrowth is initiated. More detailed studies on synchronously germinating exospores (in progress) are necessary to give structural details of this process throughout the germination phase. What is evident at this time is that outgrowth is a complex process and raises many questions about regulation of structural development,
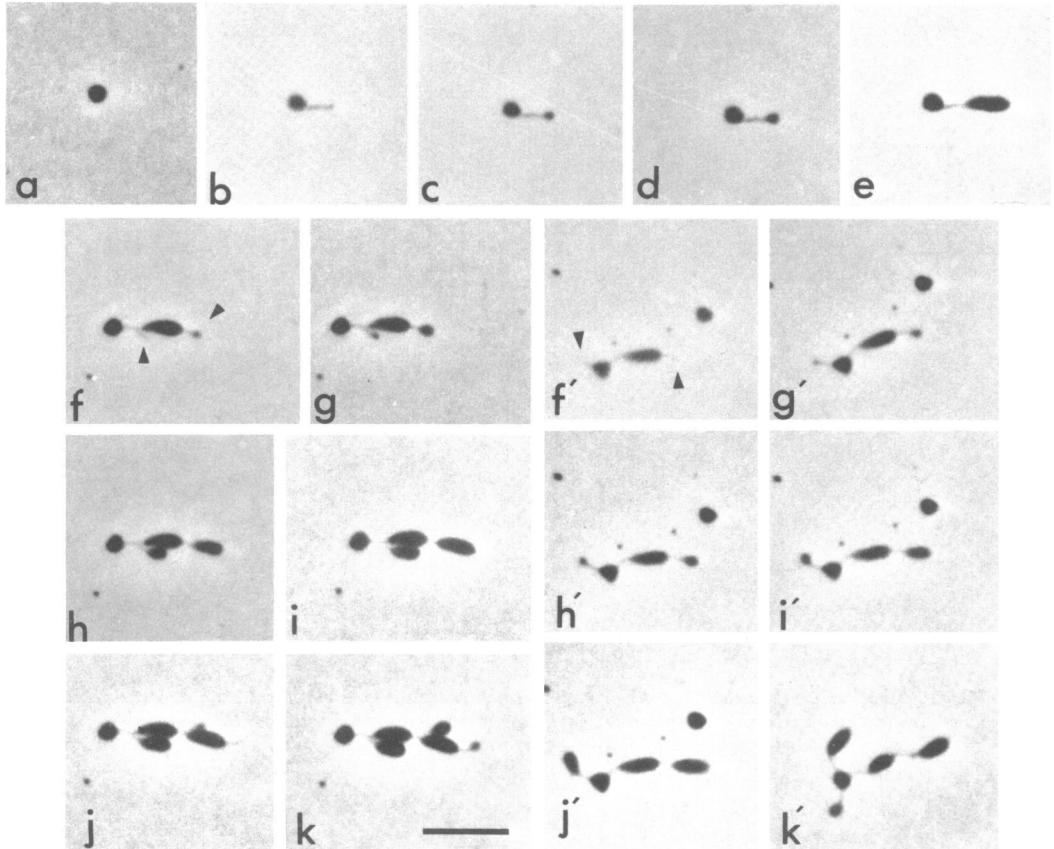


FIG. 35. *Time lapse phase-contrast photomicrographs of exospore outgrowth. (a–e) Formation of the first daughter cell. (f–k) Subsequent daughter formation by filament branching. (f'–k') Sequential filament and daughter synthesis from each apex of the angular exospore. Bar represents 10 μm.*
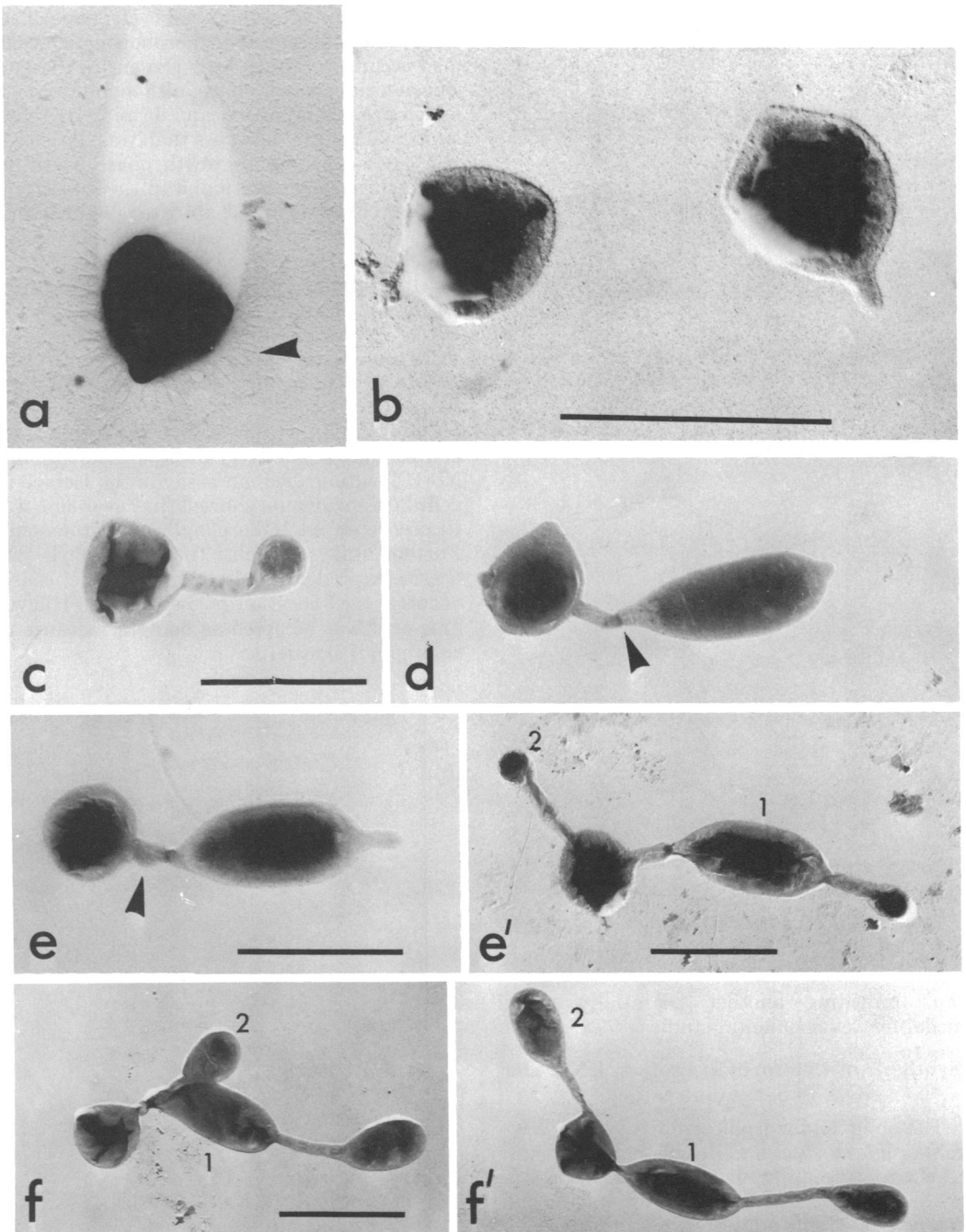
FIG. 36. *Gold/palladium-shadowed electron micrographs of exospore outgrowth. (a) Mature exospore. Note the fibrillar capsular material. (b) Filament formation. (c) Daughter cell formation. (d) Completion of daughter cell as indicated by plug formation in the filament (arrow). (e) Branch formation. (e') Filament and daughter cell formation from the second apex. (f and f') Formation of the second daughter cell. Bar represents 2 μm.*
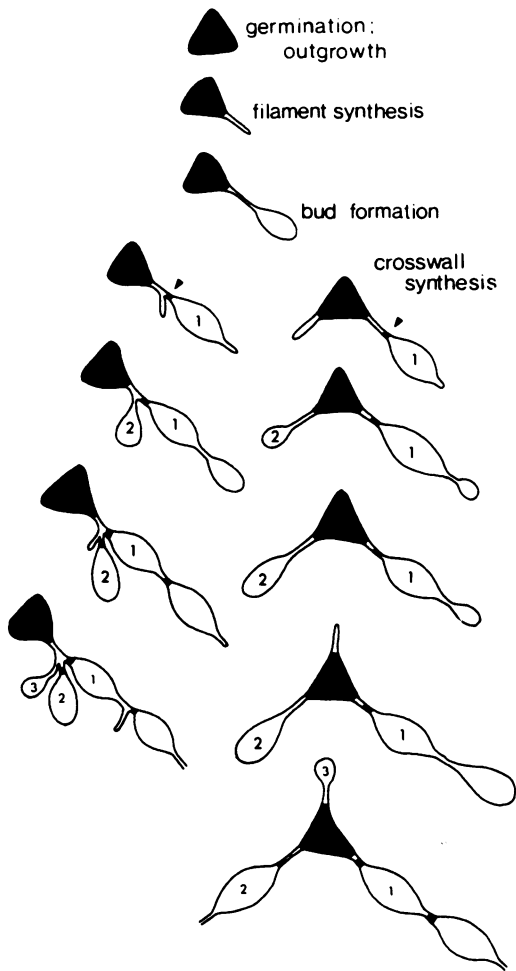
FIG. 37. *Diagrammatic representation of exospore outgrowth in R. vannielii.*

thus providing another potentially useful model for developmental studies.

## Synthesis of Chlorophyll, Protein, DNA, and RNA in Synchronized Cells

The obligate dimorphic growth cycle of *Rhodomicrobium* swarm cells means that points reached in the differentiation sequence can be precisely determined simply by microscopic and/or cell volume analysis; i.e., cellular differentiation can be subdivided and associated with *specific* and *exclusive* morphological forms. It is therefore possible to associate physiological and biochemical events with distinct cellular (morphological) parameters.

All these studies were carried out on swarm cells harvested (Fig. 15) during the early- to mid-exponential phase. Cells so obtained were

at the beginning of their cell cycle; none of the assayable differential or morphogenetic events had occurred. Swarm cells present in the late-exponential-growth phase, although present in much greater numbers, and forming a greater proportion of the cell types than was the case in the early-exponential-growth phase, were not used, since they were found in the majority of cases to have acquired an extensive membranous complex and consequently to have completed initial developmental stages.

All studies on synchronized populations were performed on cultures grown in 250-ml Quickfit flasks containing 100-ml cultures. The flasks were sealed, gassed with oxygen-free nitrogen, and incubated at 30°C with an incident light intensity of 1,500 lx.

**Chlorophyll synthesis.** Synchronous swarm cell populations actively incorporated 5-amino-$[G\text{-}^3H]$levulinic acid; an assay of the isotope in cellular components showed that over 80% was incorporated into chlorophyll and carotenoids. Further assays revealed that chlorophyll synthesis paralleled levulinic acid uptake; consequently, incorporation of 5-amino-$[G\text{-}^3H]$levulinic acid was accepted as being a measure of chlorophyll synthesis.
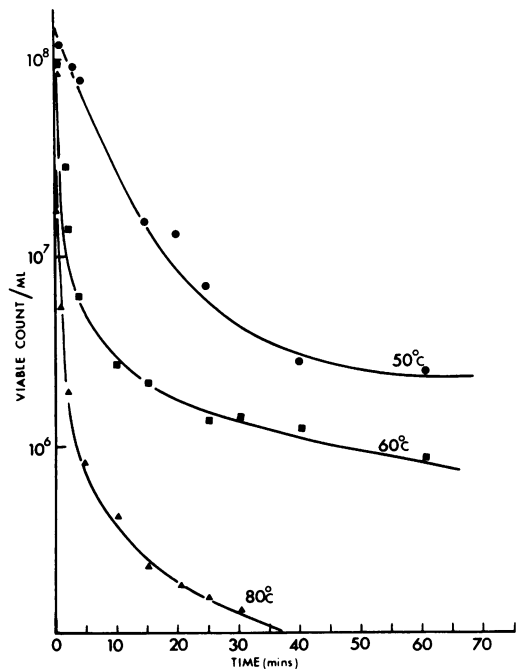


FIG. 38. *Survival curves at 50, 60, and 80°C for a purified exospore preparation. Samples were heat treated in 2-ml thin-walled glass tubes, which were totally immersed. Samples, 0.1 ml, were removed at intervals for a viable count.*
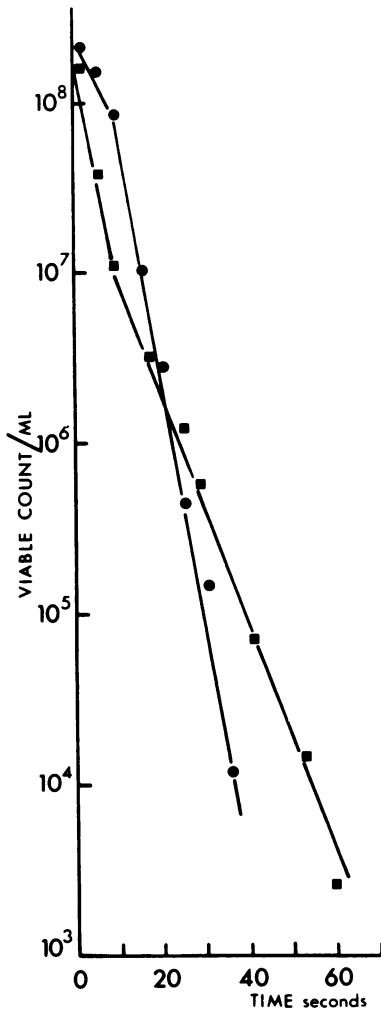
FIG. 39. *Ultraviolet irradiation survival curves for strain Rm 5 exospores (■) and Escherichia coli (●).*

Chlorophyll synthesis was found to occur only at one stage in the cell cycle—the early phase of swarm cell maturation (Fig. 49), when the membranous complex was being formed. Swarm cells that had undergone membrane development but had not yet initiated budding tube synthesis (late-exponential-growth phase) showed very little, if any, uptake of the isotope. Chlorophyll synthesis, therefore, appears only to occur when membranes are being synthesized in the early development stage of the swarm cells.

This method of assaying chlorophyll synthesis throughout the cell cycle, starting with synchronous swarm cell populations, appears to be a novel system for studying regulatory events

and environmental conditions that affect chlorophyll synthesis in photosynthetic bacteria. A further refinement is possible. By using the cells from the "simplified cell cycle" (Fig. 27 and 28), not only can chlorophyll synthesis be studied throughout the cell cycle, but the chlorophyll content of the daughter cells arising from that cycle can be assayed and compared with that of the mother cell—simply by separating new daughter cells from the mother cells on a sucrose gradient. Asymmetric growth in this instance permits not only the study of the individual, but also the study of that individual's progeny—a situation without parallel in microbes that divide symmetrically.

**Protein synthesis and inhibition.** Protein synthesis in synchronized swarm cell cultures, as determined by the incorporation of L-[4,5-$^3$H]leucine (Fig. 50) into cellular material, changed during the cell cycle. The change, a decrease, correlated with the onset of filament formation. Once tube synthesis was complete, the uptake of leucine continued at a level similar to that observed in heterogeneous cultures.

Studies with chloramphenicol revealed temporal variations in sensitivity during the cell cycle. These were demonstrated by incubating synchronized populations in the presence of varying concentrations of inhibitor for 15 h and then subjecting them to "end-point" analysis by microscopy, cell volume assay (Fig. 51), and incorporation of L-[*methyl*-$^3$H]methionine into cellular protein (Fig. 52).

From these observations, we concluded that there were three major steps in swarm cell morphogenesis that differed in chloramphenicol sensitivity. These were: (i) the initiation of filament formation (30 μg/ml); (ii) the initiation of bud or daughter cell formation (15 μg/
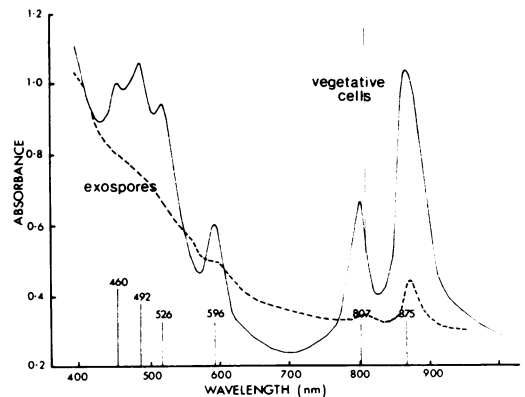


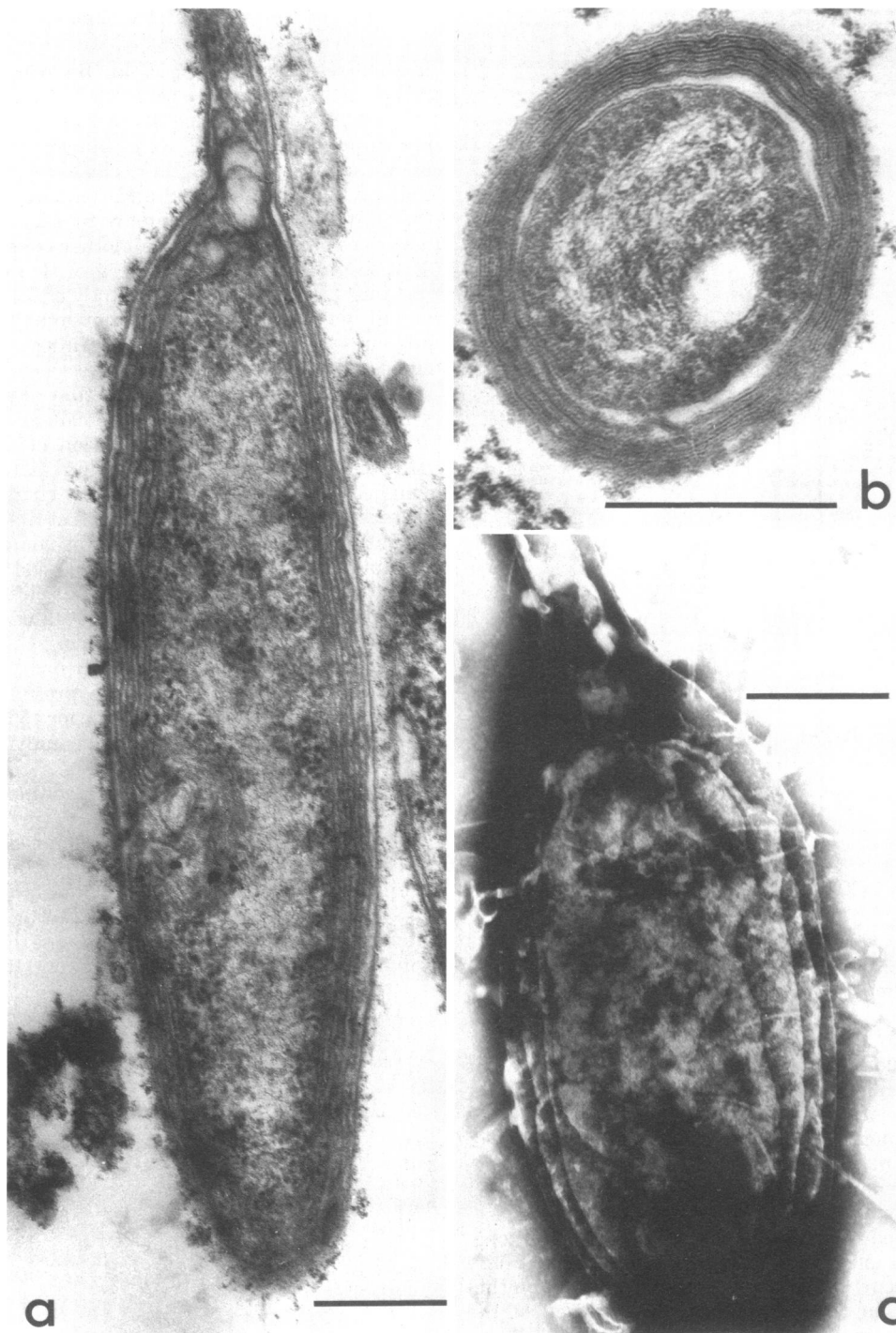FIG. 40. *Absorbance spectra for strain Rm 5 vegetative cells and exospores suspended in saturated sucrose solutions.*

FIG. 41. *Electron micrographs of Rhodomicrobium strain Rm 5 vegetative cells grown at 30°C with an incident light intensity of 500 lx. (a) Longitudinal section. (b) Transverse section. (c) Negatively stained preparation (phosphotungstic acid, 1% [wt/vol]) showing lamellar membrane "sheets." Bar represents 0.5 μm.*
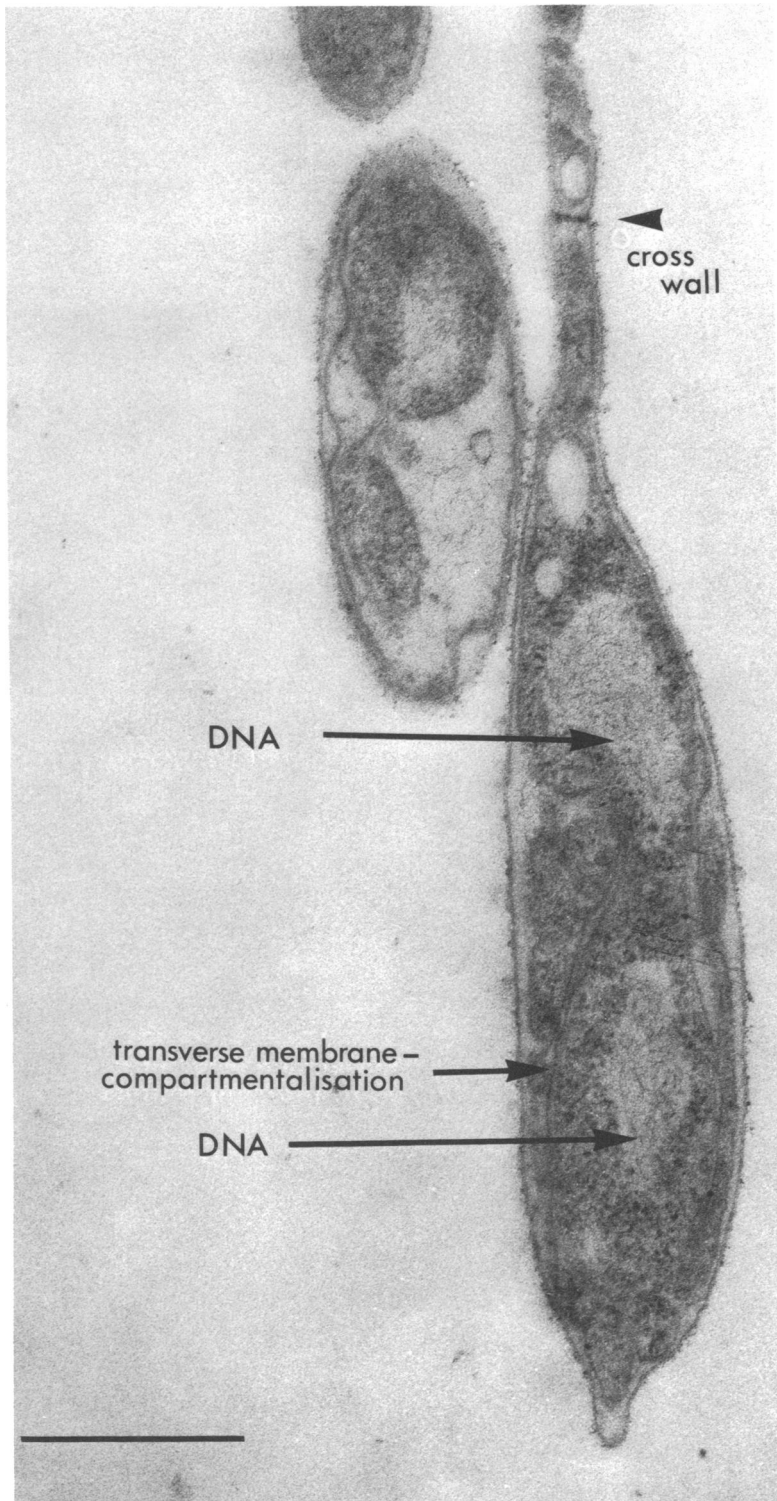
FIG. 42. *Longitudinal section of Rhodomicrobium strain Rm 5 vegetative cells grown at 30°C with an incident light intensity of 2,000 lx. Bar represents 0.5 μm.*
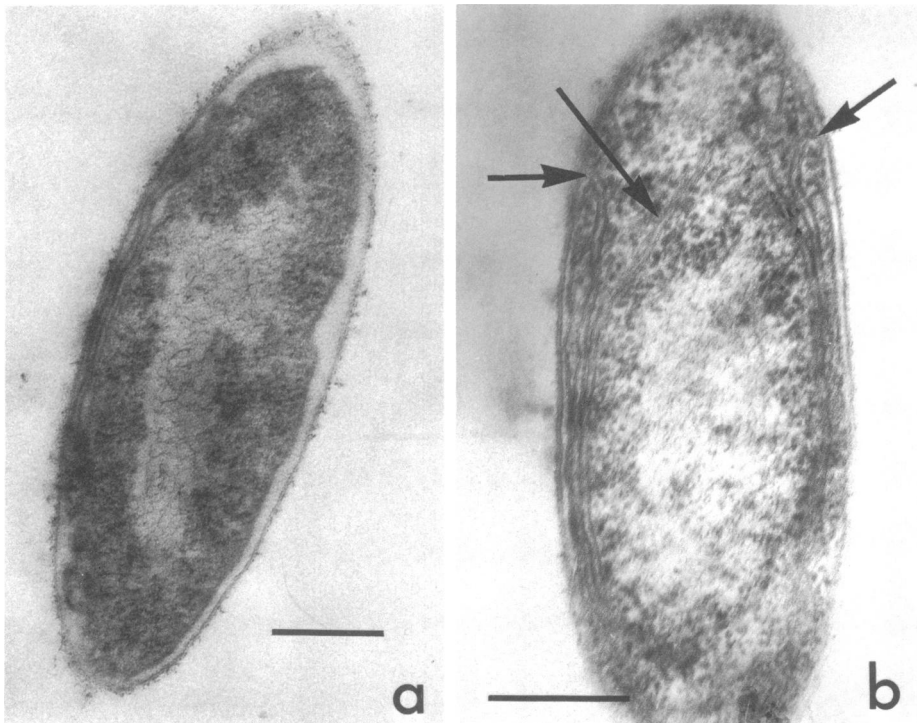
FIG. 43. *Longitudinal sections of immature swarm cells. (a) Swarm cell initiating lamellar membrane formation. (b) Swarm cell in the process of lamellar membrane synthesis. Note the invaginations of the cytoplasmic membrane and the transverse membrane. The latter can be interpreted as being the advancing front of the circumpolar invagination of the cytoplasmic membrane (Fig. 46). Bar represents 0.25 μm.*
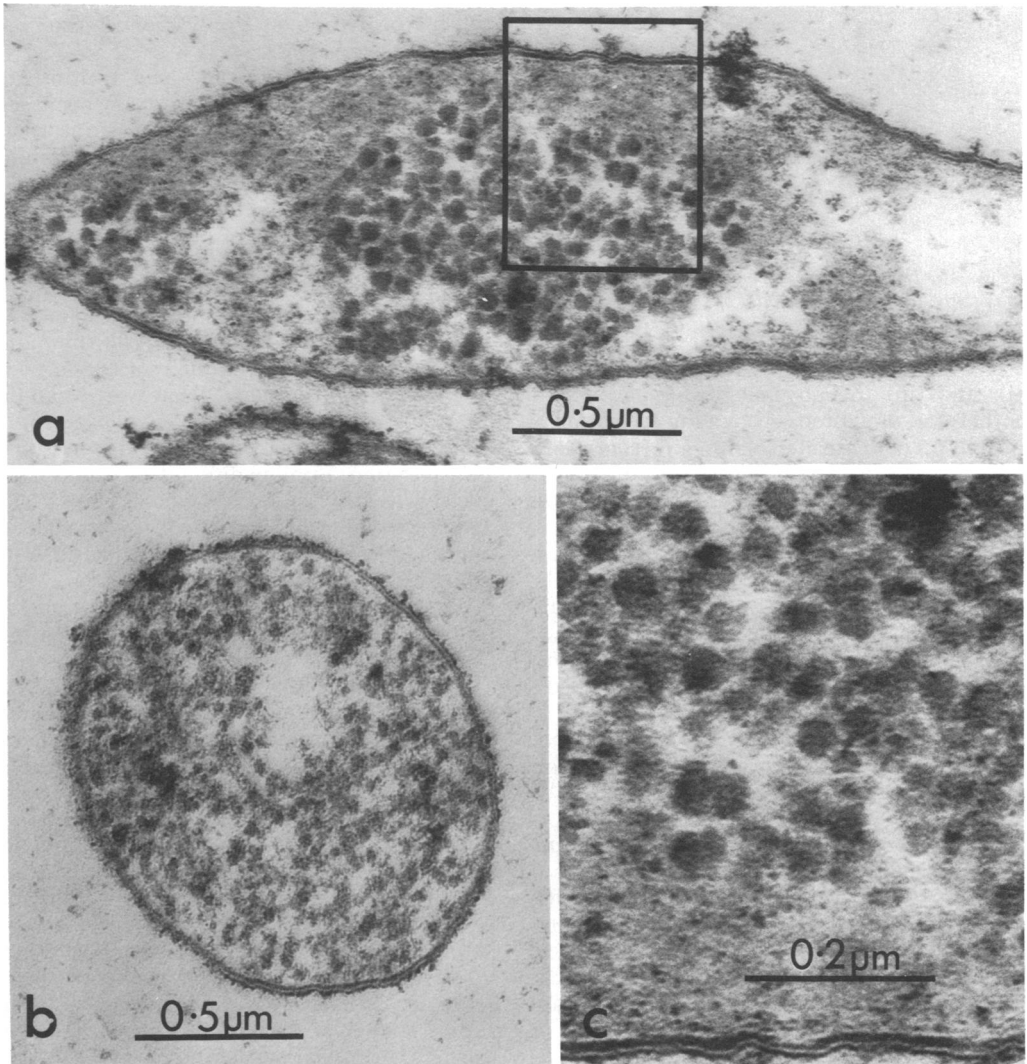
FIG. 44. *Polyhedral inclusions apparent in vegetative cells, growing as the "simplified cycle" in continuous culture with an incident light intensity of 1,500 lx. (a) Longitudinal section. (b) Transverse section. (c) Enlargement of the cell depicted in (a).*
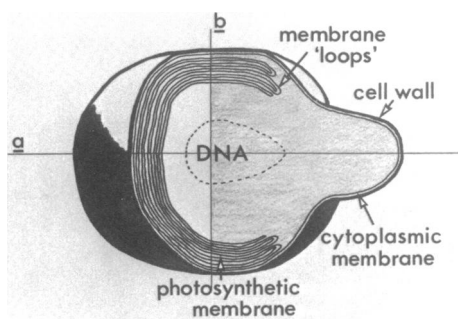
FIG. 45. *Diagrammatic representation of a cut-away three-dimensional model of a Rhodomicrobium vegetative cell grown under conditions of low light intensity (500 lx).*

ml); and (iii) the physiological separation of mother and daughter cell by plug formation (5 $\mu$g/ml), an obligate step before a further reproductive cycle. Electron microscopy of cells in the last category showed a total lack of cross wall synthesis.

These observations have been further substantiated by subjecting synchronized populations to the critical chloramphenicol concentrations at differing times during the cycle; e.g., 5 $\mu$g of chloramphenicol per ml will inhibit cross wall formation (division) provided it is added a minimum of 30 to 40 min before the event. It would therefore appear that the data obtained by end-point analysis does not simply reflect that the higher the concentration of an inhibitor the sooner it exerts an effect; i.e., it is indicative of sequential changes in chloramphenicol sensitivity.

**RNA synthesis.** The incorporation of [$G$-$^3$H]uridine by swarm cells into cellular material was assumed to reflect ribonucleic acid (RNA) synthesis (Fig. 50) during the cell cycle; RNA synthesis appeared to be at a constant rate throughout the cycle.

The effect of different rifampin concentrations on swarm cell development was determined by using the same experimental protocol as was employed to study chloramphenicol sensitivity. The data (Fig. 53 and 54) indicated that there was a significant change in rifampin sensitivity between immature, nonreproductive swarm cells (50 $\mu$g/ml) and the mature reproductive mother cell (25 $\mu$g/ml). The distinct plateau occurring between 25 and 50 $\mu$g of rifampin per ml suggested that the RNA polymerase had been modified in some way. An interpretation is that the terminal event in the mat-

uration process is the modification of the RNA polymerase that subsequently permits recognition and transcription of those genes concerned with mother cell function, i.e., reproduction. Such a modified polymerase would be unable to transcribe those genes associated with swarm cell maturation, a once-only event in the life of each and every swarm cell.

**DNA synthesis.** Nucleic acid synthesis in synchronized *Rhodomicrobium* swarm cells was examined by: (i) radioisotope incorporation, (ii) fluorescence, (iii) Giemsa staining, and (iv) specific inhibitors.

**(i) Radioisotope incorporation.** Attempts to monitor deoxyribonucleic acid (DNA) synthesis by the uptake of [*methyl*-$^3$H]thymidine failed presumably because the thymidine was degraded to thymine by thymidine phosphorylase (6). It has been shown that in *E. coli* thymidine phosphorylase can be inhibited or repressed by the addition of uridine (7). However, when uridine (10 mM) was added to *Rhodomicrobium* cells, there was no significant change in isotope incorporation. Similar difficulties were experienced with thymidine 5'-monophosphate (73). There was no incorporation of deoxyguanosine and low levels of deoxyadenosine (0.1 mM) inhibited growth within 30 min.

Although we have used several mutation and isolation procedures, we have as yet been unable to isolate a thymine-requiring mutant.

**(ii) Fluorometric determination of DNA synthesis.** Taking advantage of the large quantities of synchronized cells we were able to prepare, by the filtration method described earlier, DNA synthesis (Fig. 55) was followed by using the fluorometric assay system proposed by Donkersloot et al. (19), which involves complexing the nucleic acid with ethidium bromide and measuring the resulting fluorescence. The major finding, when comparing synchronized and heterogeneous populations, was that DNA synthesis in the former was discontinuous, being repressed up to the midway point in the "maturation" period. A similar situation is known to exist for *Caulobacter* (15) and *R. palustris* swarm cells (93). As repression of DNA synthesis was observed only in "immature" swarm cells it was assumed that (i) mother cells synthesize DNA continuously, and (ii) first-generation swarm cells must pass part way through the maturation period before DNA synthesis is initiated. RNA synthesis in synchronously growing cells measured by this technique paralleled the curve recorded for uridine uptake (Fig. 50).

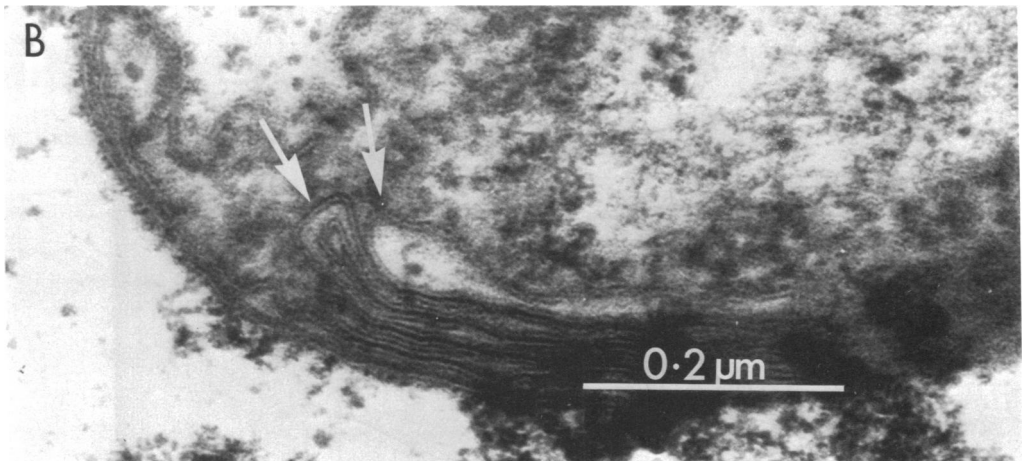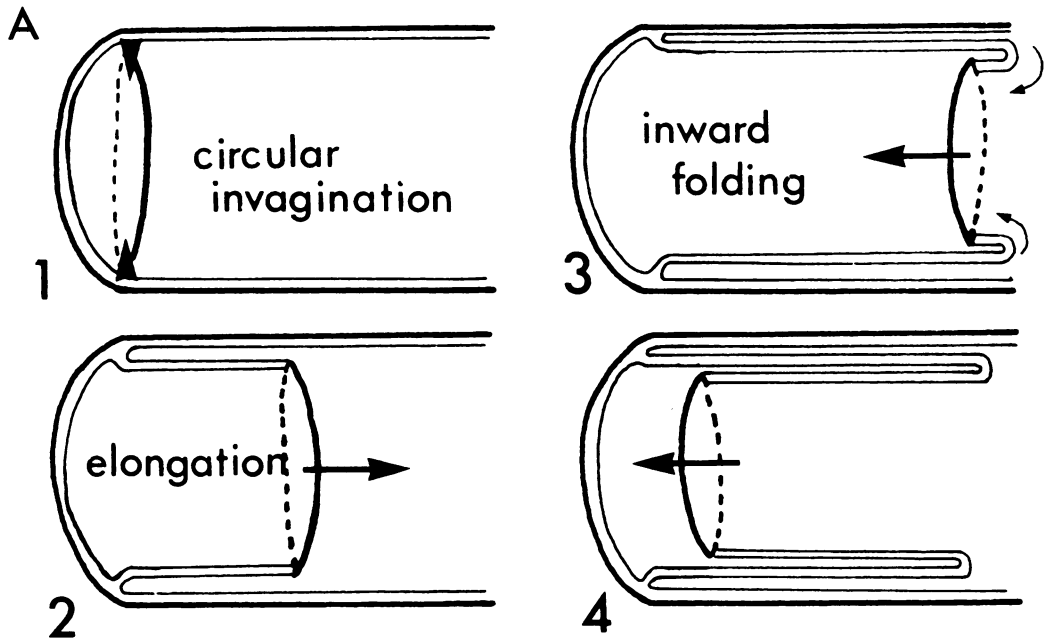**(iii) Giemsa staining.** Morphological changes, that is, broad changes in conformation

FIG. 46. *(A) Diagrammatic representation of lamellar membrane formation by circumpolar invagination of the cytoplasmic membrane at the pole distal to cell separation. (B) Longitudinal section of a vegetative cell showing a membrane loop and advancing "front."*
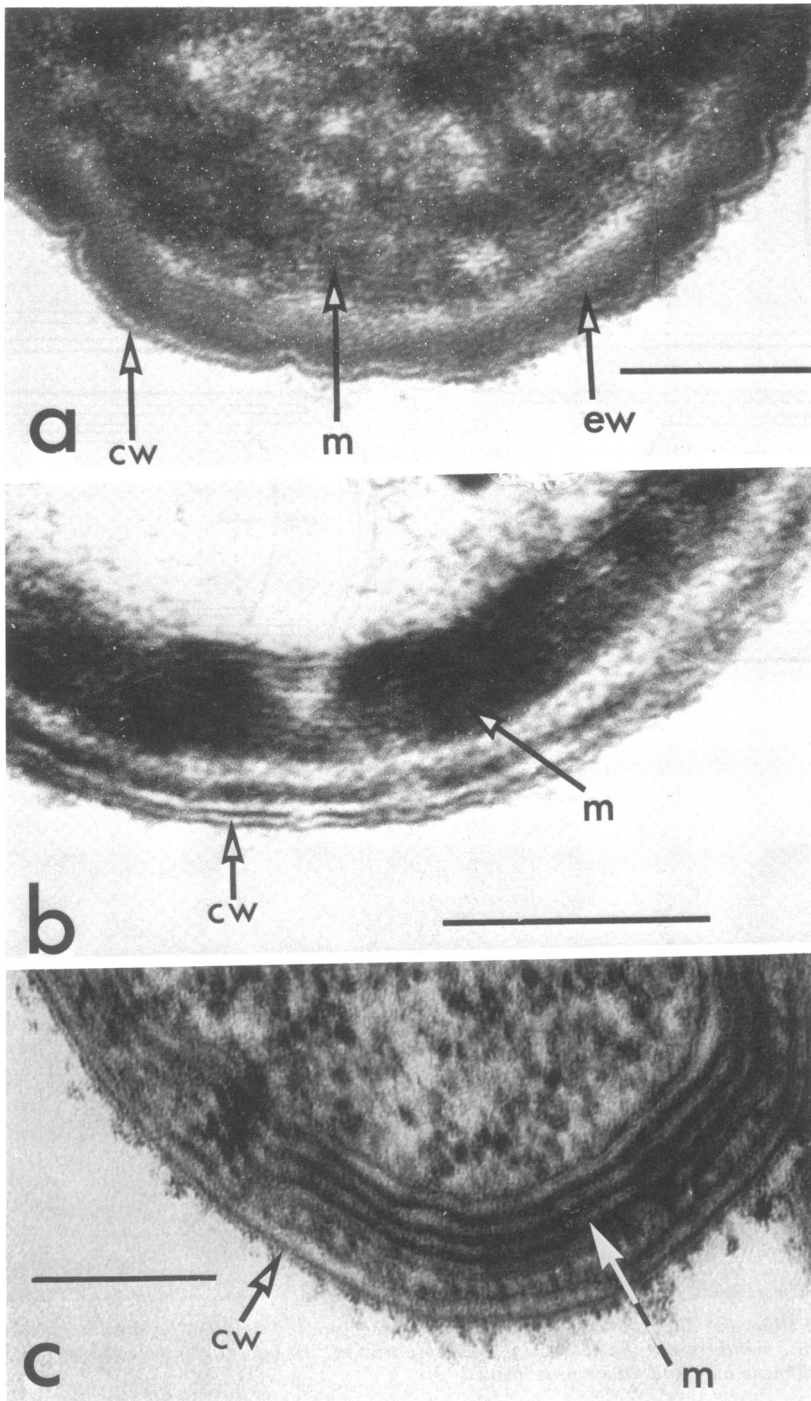
FIG. 47. *Ultrathin sections of: (a) mature exospore showing cell wall thickening, (b) germinating exospore, and (c) vegetative cell. m, Membrane; cw, cell wall; ew, exospore wall. Bar represents 0.1 µm.*
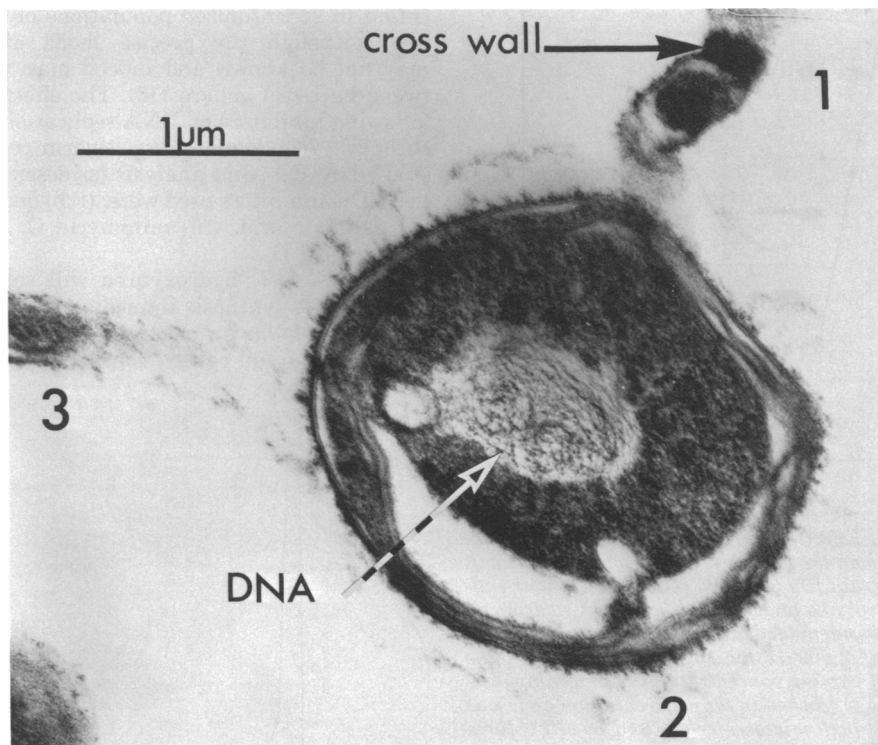
FIG. 48. *Ultrathin section of an exospore in the process of daughter vegetative cell formation. Bar represents 1 μm.*
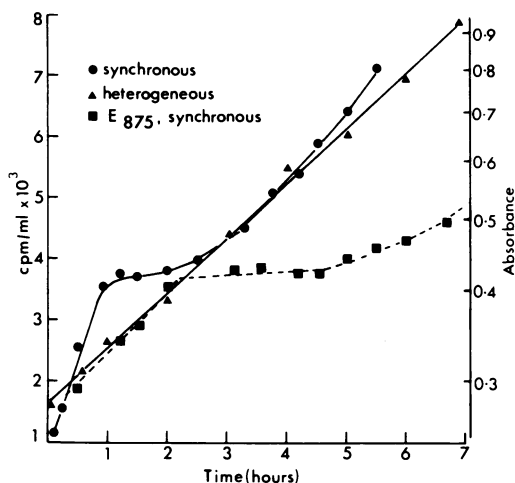


FIG. 49. *Uptake of 5-amino-[³H]levulinic acid by heterogeneous (▲) and synchronized (●) cell populations of Rhodomicrobium strain Rm 5. (■) $E_{875}$ absorption curve for the synchronized cell population.*



FIG. 50. *Uptake of L-[4,5-³H]leucine (synchronized population, ○; heterogeneous population, ●) and [G-³H]uridine (synchronized population, ▲) by vegetative cells of Rhodomicrobium strain Rm 5.*

and position of the nucleoid in the cells during the cell cycle of synchronized cultures, were followed by using the Giemsa staining technique (14) (Fig. 55). The main findings were:
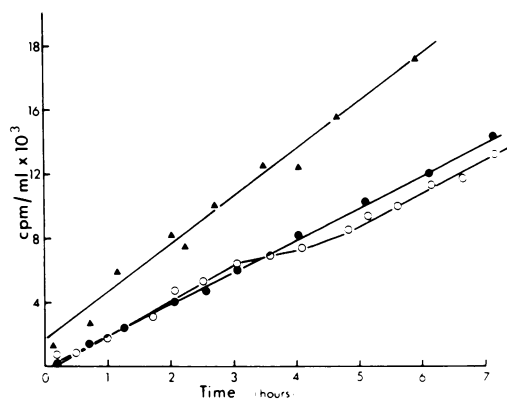
(i) that motile swarm cells possess small nucleoids positioned centrally; (ii) that at the stage in the cell cycle which corresponds to the initiation of DNA synthesis, the nucleoid assumes an ovoid shape and apparently increases in size; (iii) that just before filament formation the ovoid, elongated nucleoid moves to the pole
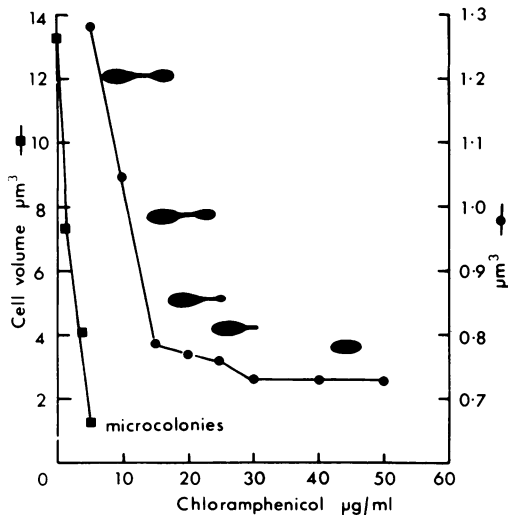
FIG. 51. *Effect of different chloramphenicol concentrations on the growth of synchronized Rhodomicrobium strain Rm 5 swarm cells. Fifteen-hour end-point cell volume analysis. An identical swarm cell preparation, incubated at 30°C with an incident light intensity of 2,500 lx for 15 h, was grown in the presence of varying concentrations of chloramphenicol. The stage reached in the differentiation cycle was determined both microscopically and by cell volume analysis (Fig. 18).*
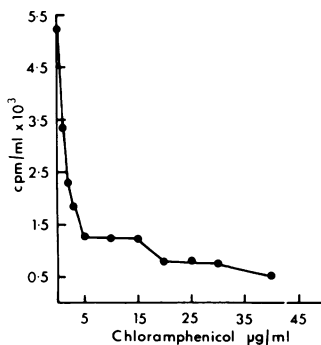


FIG. 52. *Incorporation of L-[methyl-$^3$H]methionine into cellular protein by synchronized Rhodomicrobium strain Rm 5 swarm cells in the presence of varying concentrations of chloramphenicol. Fifteen-hour end-point analysis.*

of the cell from which the filament emerges; (iv) that DNA is detectable in the bud at the time the new daughter cell is first discernible microscopically; and (v) the nucleoid in the mother cell moves back to a central position when the daughter cell is approximately half the size of the mother cell.

(iv) **Inhibition of DNA synthesis.** Selective and specific inhibitors of DNA synthesis have proved to be invaluable to studies of DNA repli-

cation in synchronized populations of bacteria (41), although the precise mode of action may not be known and indeed may vary between bacterial genera (16). The effects of the following inhibitors on DNA replication in synchronized *Rhodomicrobium* swarm cells were studied by end-point analysis (as described earlier). The inhibitors used were: (i) hydroxyurea, (ii) nalidixic acid, (iii) mitomycin C, and (iv) actinomycin D.

(i) In *E. coli*, hydroxyurea will selectively inhibit DNA synthesis immediately (67). With *Rhodomicrobium* swarm cells, concentrations of 0.1 to 15 µg/ml caused considerable cellular



FIG. 53. *Effect of varying rifampin concentrations on the growth and differentiation cycle of synchronized Rhodomicrobium strain Rm 5 swarm cells. Fifteen-hour end-point cell volume analysis.*
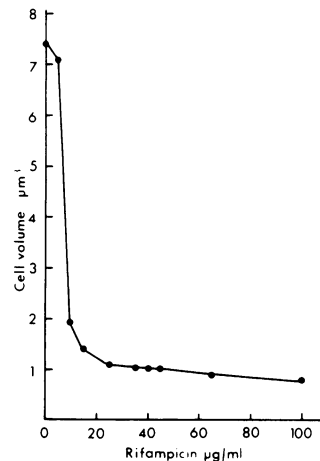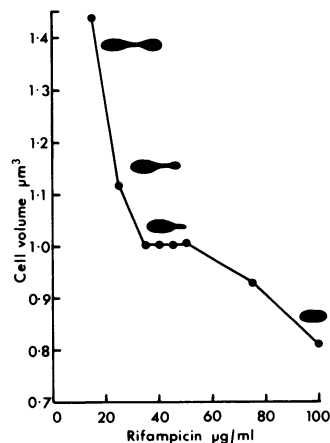


FIG. 54. *Effect of varying rifampin concentrations on the differentiation cycle of synchronized Rhodomicrobium strain Rm 5 swarm cells. Fifteen-hour end-point cell volume analysis.*
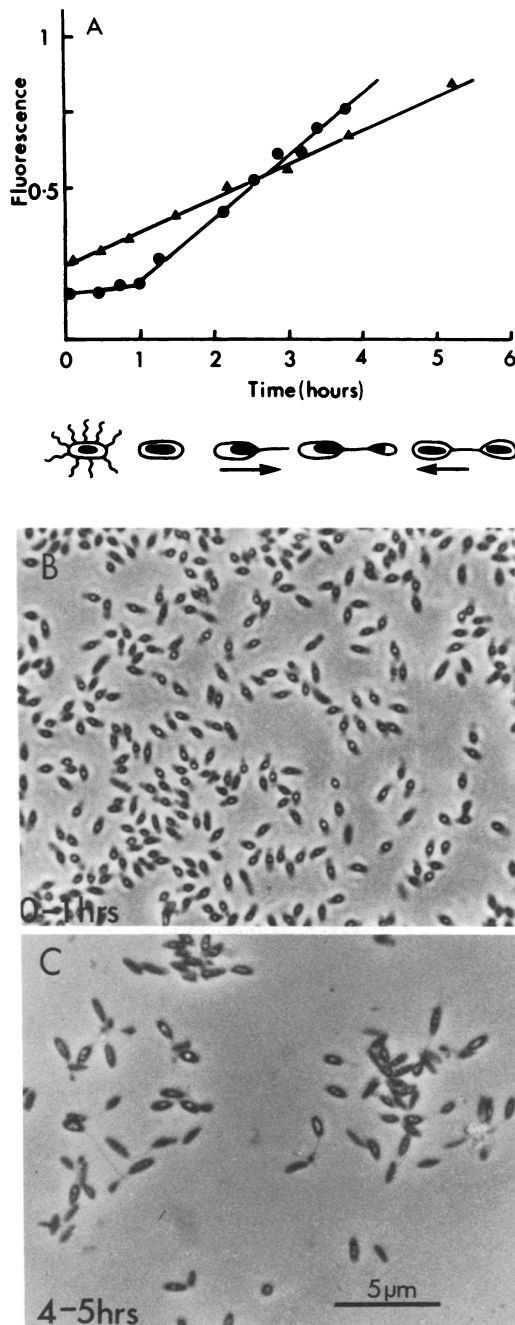
FIG. 55. *DNA synthesis in a heterogeneous (△) and a synchronized (●) swarm cell population of Rhodomicrobium strain Rm 5 as determined by the fluorometric method of Donkersloot et al. (19). The relationship between DNA synthesis and the cytological appearance of the nuclear apparatus is shown (A) diagrammatically and (B and C) by phase-contrast photomicrographs.*

elongation (Fig. 56) in subsequent generations; i.e., the overall developmental pattern was as normal and growth continued.

(ii) Nalidixic acid selectively and reversibly inhibits DNA synthesis in a wide range of gram-negative bacteria (29). In *E. coli*, it stops DNA replication in vivo immediately on its addition, but it does not interfere with either RNA or protein synthesis. This is not the case with *Caulobacter* species where both DNA and RNA are inhibited (16). In strain Rm 5, concentrations of up to 50 $\mu$g/ml permitted the formation of multicellular arrays with few abnormalities; i.e., there were instances of cellular elongation at concentrations approaching 50 $\mu$g of nalidixic acid per ml, as was observed with hydroxyurea. At 50 to 75 $\mu$g/ml, however, events ceased after daughter cell formation (no binary fission or cross wall synthesis) in approximately 85% of the population. Above 100 $\mu$g/ml, swarm cell differentiation and development was completely arrested.

(iii) Mitomycin C has been reported to cause cross-linking of the complementary strands of DNA (83); i.e., effects are very similar to ultraviolet irradiation. Concentrations of 5 to 15 $\mu$g/ml inhibited growth of homogeneous swarm cell populations giving a "gradient of development" (Fig. 57 and Table 8), i.e., all events to daughter cell formation (no cross wall synthesis) at 5 $\mu$g/ml but only events leading to the initiation of filament synthesis at 15 $\mu$g/ml.

(iv) Actinomycin D in concentrations ranging from 10 to 100 $\mu$g/ml gave results similar to those obtained with mitomycin C (Fig. 58 and Table 9).

These inhibitors can be divided into two groups on the basis of their effect on the differentiation of synchronized swarm cell populations: (i) hydroxyurea, which causes cellular elongation but permits growth to continue; and (ii) nalidixic acid, mitomycin, and actinomycin D, all of which inhibit growth and give "gradients of development."

The initial interpretation of the latter result is that the cell cycle up to and including initiation of daughter cell synthesis is dependent upon the transcription of the mother cell genome; there is apparently no necessity for DNA synthesis during this sequence of events. However, completion of the cell cycle, i.e., physiological separation of mother and daughter and subsequent formation of a multicellular array, requires termination of chromosome replication.

More detailed studies, to determine the specificity of the inhibitors on RNA and DNA synthesis, on the reversibility of inhibitor action,
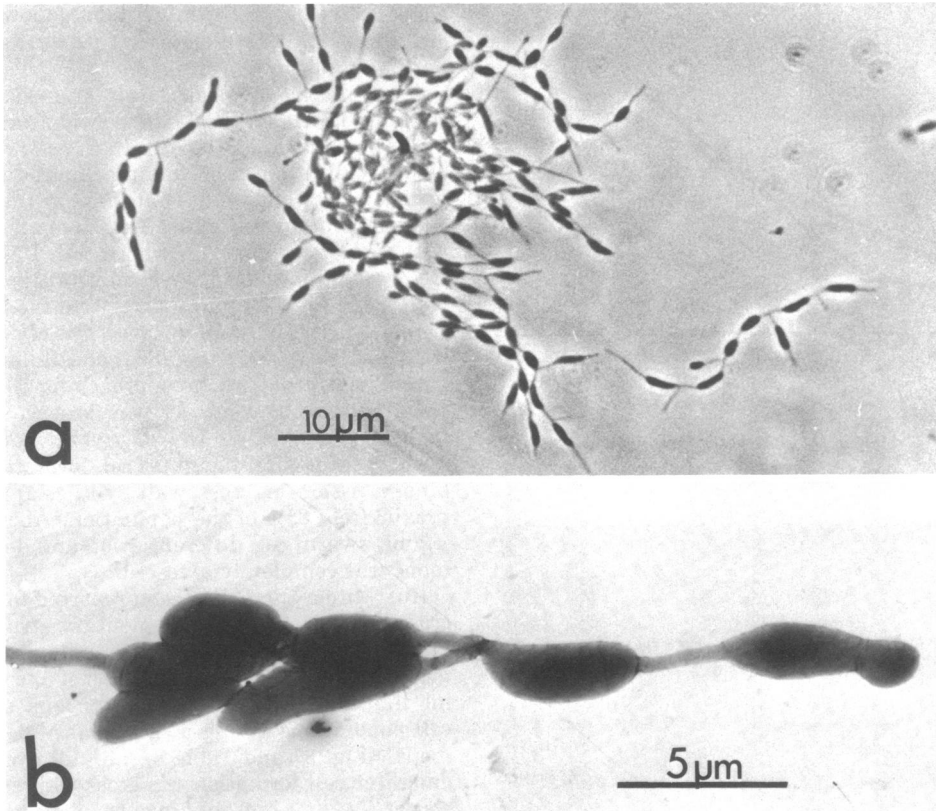
FIG. 56. *Cellular elongation induced by 0.1 to 15 µg of hydroxyurea per ml. (a) Phase-contrast photomicrograph; (b) uranyl acetate (0.5% [wt/vol])-stained electron micrograph.*
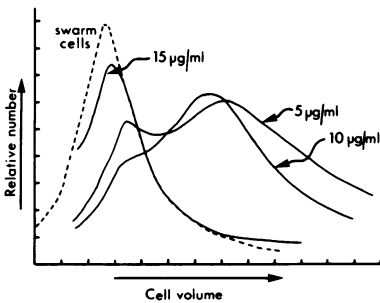


FIG. 57. *Effect of varying concentrations of mitomycin C on homogeneous Rhodomicrobium strain Rm 5 swarm cell populations. Fifteen-hour end-point cell volume analysis; direct traces from the Coulter Counter (ZB1)/Channelyzer (C1000) system.*

TABLE 8. *Mitomycin C*

| Inhibitor concn (µg/ml) | Cell expression | |
|---|---|---|
| 1 | Normal growth | |
| 2 | Normal growth | |
| 5 |  | Daughter cell formation — elongation |
| 10 |  | 99% Daughter cell formation |
| 15 |  | 99% No further development |

and on timed additions of inhibitors in relation to the initiation of DNA synthesis and subsequent morphological expression, should give insight into the correlation of DNA initiation and synthesis with cellular differentiation.

Morphological and physiological events in the cell cycle so far spatially identified are shown diagrammatically (Fig. 59).

## Reproduction

Reproductive processes of *Rhodomicrobium* can result in the formation of three cell types: (i) a vegetative daughter cell remaining linked to the mother cell but functionally separated by a wall or plug laid down part way along the filament (Fig. 26); (ii) a motile, vegetative (swarmer) daughter cell that separates by binary fission from the filamented mother cell at

the junction of the filament with the new daughter cell (Fig. 27 and 29); and (iii) angular, heat-resistant exospores that are formed sequentially and separate by binary fission from the filament of the mother cell (Fig. 32 and 33).

Reproduction in this organism is a more complex process than in, say, *E. coli,* particularly because daughter cells are formed at some distance from the main body of the mother cell where, we assume, cellular activities are centered.

The most complex reproductive situation, the formation of daughter cells in a branched sequence (Fig. 21, 23, 24) highlights the problem of proposing a single basic model that satisfies all the morphological variations seen in the reproduction of this organism. Questions which have to be answered by such a model are the following. (i) Where in the mother cell is DNA replicated? (ii) How is DNA transported to the daughter cell? (iii) What mechanism ensures that a genome is directed down the most recently synthesized branch of the budding tube to the youngest of the daughter cells? (iv) Why does a mother cell give rise to no more than four daughter cells or exospores in the branching situation? (v) Why is tube outgrowth restricted to the poles? (vi) When does the new daughter cell take over the regulation of its synthetic activities? (vii) Does the "spent mother" cell die



FIG. 58. *Effect of varying concentrations of actinomycin D on homogeneous Rhodomicrobium strain Rm 5 swarm cell populations. Fifteen-hour end-point cell volume analysis direct traces from the Coulter Counter (ZB1)/Channelyzer (C1000) system.*

or differentiate to perform a new function? That is, is the cell now analogous in any way to heterocysts in cyanobacteria?

The model proposed (Fig. 60) presupposes that DNA destined for the daughter cell is attached to the cytoplasmic membrane at the potential growth point of the cell. Subsequent outgrowth results in the formation of the filament, the growth area being confined to the filament tip. At some point in filament development, regulatory changes lead to the enlargement of the growth zone to form a new cell body (this event, the de novo formation of cell shape, is quite different, for instance, from the process in *E. coli* where "shaping" appears not to be a de novo activity; an extended cylinder is merely cut in half). Cell constituents (ribosomes and other cytoplasmic contents) presumably synthesized in the mother cell fill the new cellular areas (filament and developing daughter cell), as they are synthesized, except for the genome, which is actively transported into the new cell by the cytoplasmic membrane. The switch from filament synthesis to daughter cell formation is preceded by division of the growth point and of the DNA attachment point. One growth point remains active and gives rise to the daughter cell. The other remains inactive. Reactivation of this growth site (now situated part way down the filament) occurs on physiological separation of the mother and daughter cells. One mother-daughter unit can only have *one* active growth point. The first daughter cell becomes a separate entity when the cross wall is laid down in the filament. This event appears to be under the control of the daughter cell (Fig. 26). These assumptions seem to us necessary in proposing an explanation of subsequent events, i.e., the formation of a branch from the filament to lead to the formation of the second daughter cell (Fig. 21, 24). The formation of the second daughter cell begins with initiation of filament synthesis at a branch point on the filament, which led to the formation of the first daughter cell; subsequent events are identical in all respects to those that gave rise to the first daughter cell. The third and fourth daughter

TABLE 9. *Actinomycin D*

| Inhibitor concn ($\mu$g/ml) | Cell expression | | | | | |
|---|---|---|---|---|---|---|
| 10 | Normal growth | | | | | |
| 25 |  | 20% + |  | 50–60% + |  | |
| 50 |  | 15% + |  | 85–90% | | |
| 100 |  | 95% No development[a] | | | | |

[a] Although at 100 $\mu$g/ml there is no morphological development, the cells are considerably larger than batch swarm cells.
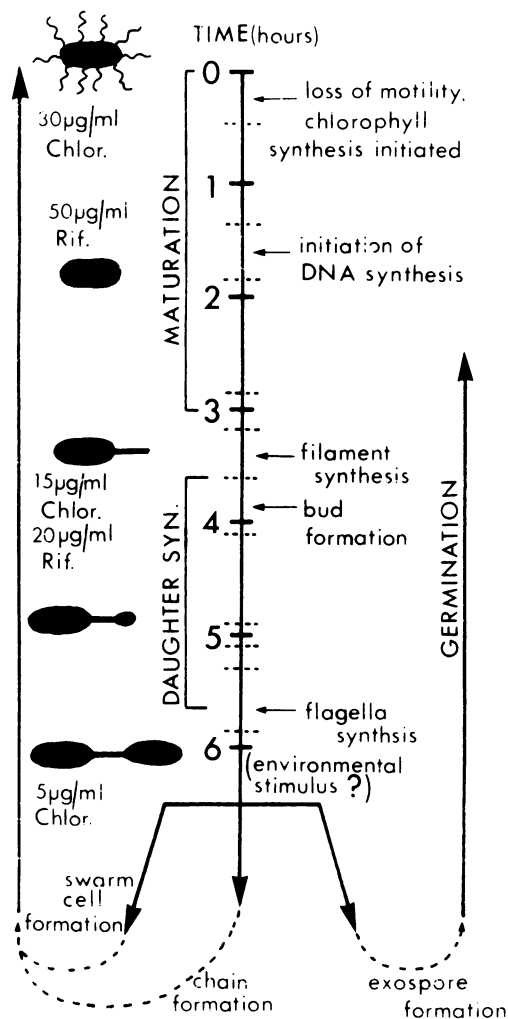
FIG. 59. *Diagrammatic representation of the spatial and sequential morphological and physiological events in the Rhodomicrobium cell cycle so far identified.*

cells are formed in a similar manner.

The same model applies to cells growing as the "simplified" cell cycle. The important point about this system is that each mother cell, before the initiation of daughter cell synthesis, undergoes an obligate period of filament synthesis; i.e., the nascent growth point remains in the filament at the cell division site, and the period of filament synthesis facilitates segregation of the DNA attachment sites.

The time spent in filament synthesis (as judged by filament length) can be explained as follows. There are two parallel sequences — protein synthesis and DNA replication, leading to initiation of "daughter" cell synthesis (division

of the growth-DNA attachment sites). The time taken to replicate the genome will most likely be constant (13, 31), whereas the time taken to pass through the specific and obligate protein synthesis sequences will be dependent on growth (environmental) conditions. Termina-
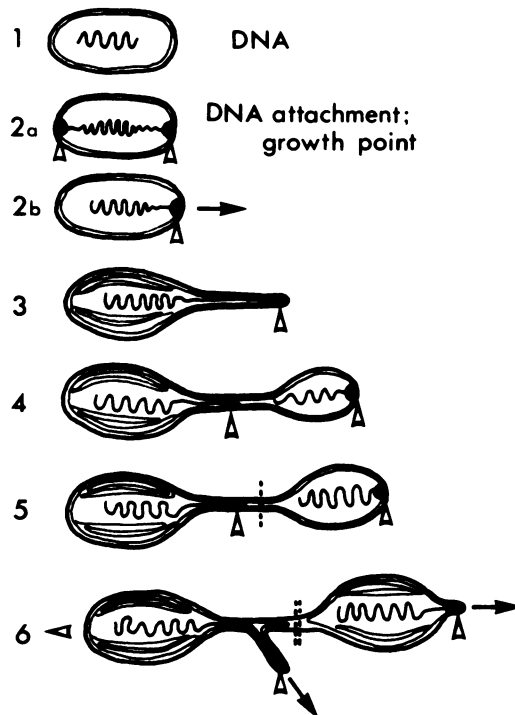


FIG. 60. *Diagrammatic representation of reproduction in Rhodomicrobium vannielii. (1) Swarm cell immediately after release from the mother cell. There is no DNA synthesis and therefore no requirement for membrane attachment (100). (2) Attachment of the DNA to the cytoplasmic membrane (38, 101) at the cell growth point (2b). If there are two growth points, one at either pole, then attachment will occur at both poles (2a) (bipolar cells, Fig. 21). (3) Filament synthesis. Filament length can be correlated with the nutrient level at that particular time (Fig. 61). (4) A point is reached when DNA synthesis/protein synthesis dictates the end of filament synthesis and the initiation of daughter cell formation. There is also division of the growth point/DNA attachment site. There can be only one active growth point per mother-daughter unit (restricted to synthesizing one cell at a time). The active growth point differentiates and gives rise to the daughter cell. DNA synthesis continues — divorced from the constraints imposed on cell growth points. (5) When daughter cell synthesis is complete, i.e., when cross wall or plug formation occurs, the growth site remaining in the filament is activated. (6) Filament synthesis occurs. The subsequent series of events are identical for second, third, and fourth daughter cell formation.*
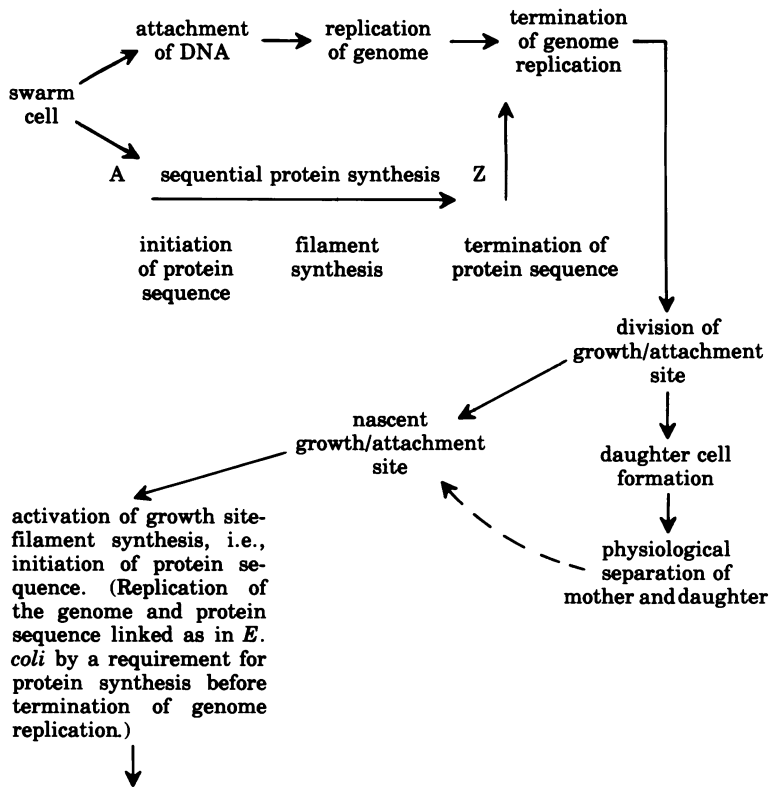
FIG. 61. *Control of swarm cell development by correlation of protein and DNA synthesis.*

tion of both pathways will be essential for initiation of daughter cell synthesis (Fig. 61). A somewhat similar model has been proposed to explain the control of replication and division in *E. coli* (40).

Obviously, the model proposed requires substantial experimental support before it can be claimed to be more than just a model; this we are now attempting to provide. Circumstantial evidence, in part answer to the original questions posed and in support of the proposed model, is as follows.

(i) Thin sections have only ever revealed a *single* cytoplasmic membrane in the filament (Fig. 41 and 42); in other words, DNA from the body of the mother cell is not transported to a second daughter cell by an outgrowth of an invagination of the cytoplasmic membrane from the mother cell.

(ii) The mother and daughter cells are always continuous up to the point of fission or plugging (Fig. 21).

(iii) Separation and fission processes appear to be under the control of the newly formed daughter cell. Alteration of filament length of

the mother cell by varying nutrient concentrations (Fig. 20) does not materially alter the point of fission or the distance of plug formation from the daughter cell.

(iv) The arrangement of lamellae, masking the cytoplasmic membrane from the DNA along the length of the cell, seems to be in part a reason why outgrowth only takes place from the poles of the cells where the DNA can make direct contact with the cytoplasmic membrane.

(v) That there is only a single growth point or growth zone is demonstrated by penicillin experiments (Fig. 62) and is further substantiated by autoradiographic experiments concerning the incorporation of 2,6-diamino-[$G$-$^3$H]pimelic acid by synchronous cultures of swarm cells. Pulse-chase experiments indicate that label is primarily incorporated at the filament tip and in the developing daughter cell at the pole distal to the mother cell and filament. That the active growth point is not located only at the pole of the mother cell is confirmed by branch growth.

(vi) Movement of DNA to new daughter cells by a process other than "membrane drag"
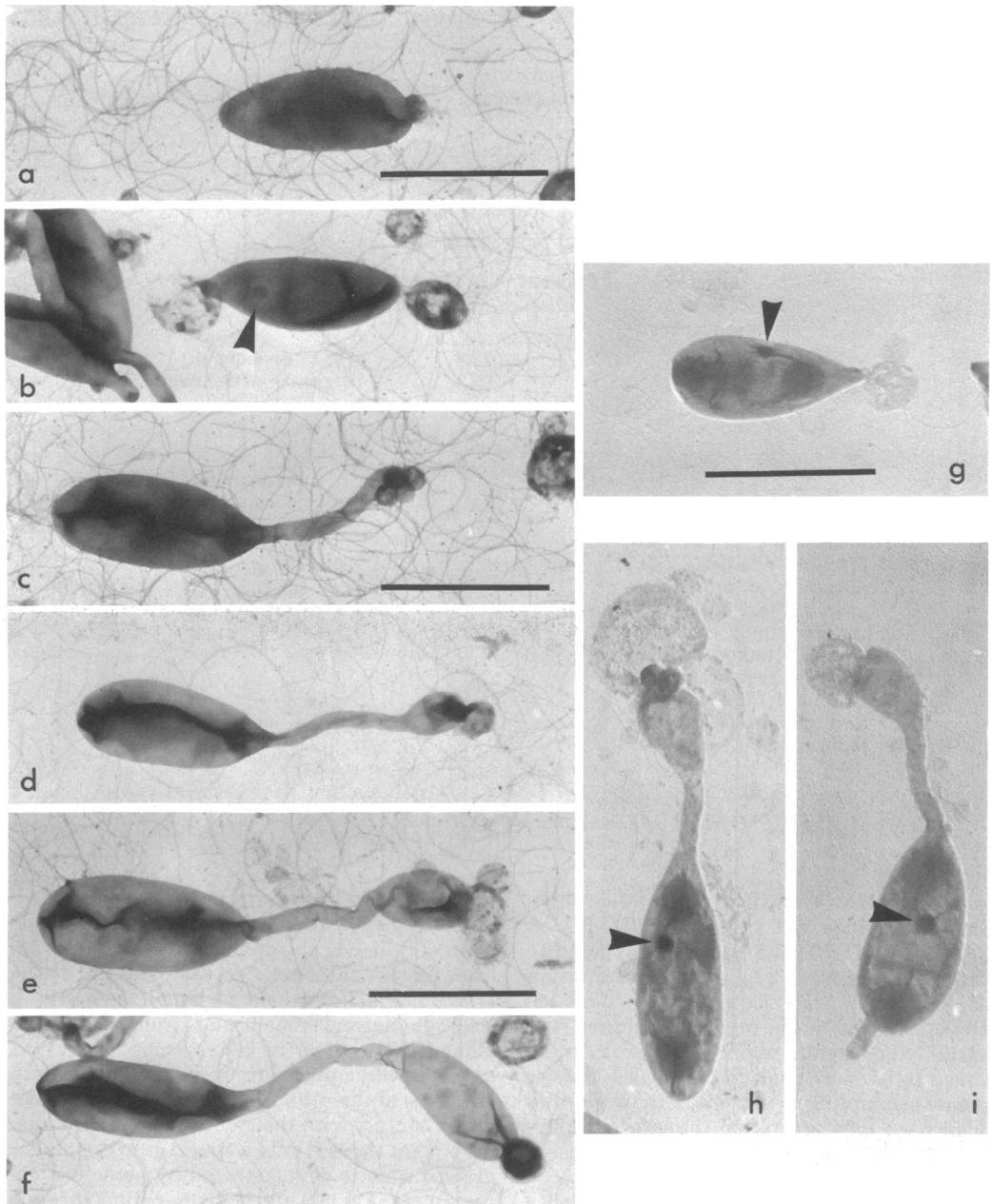
FIG. 62. *Samples taken periodically from a synchronized* Rhodomicrobium Rm 5 *population growing at 30°C with an incident light intensity of 2,000 lx in pyruvate/malate medium containing 8% (wt/vol) polyethylene glycol as an osmotic stabilizer were treated with 400 IU of penicillin G per ml and 0.1 µg of lysozyme per ml. Growth was allowed to continue; samples were taken from these preparations every few minutes, and 100 µg of magnesium chloride per ml was added to stop the reaction and stabilize the spheroplasts. The preparations were immediately washed and prepared for electron microscopy by staining (0.5% [wt/vol] uranyl acetate) or by metal shadowing (gold/palladium). Uranyl acetate (0.5% [wt/vol]) staining. (a) Swarm cell showing one polar growth point. (b) Swarm cell with a growth point at either pole. (c) The filament growth point is always at the tip. (d–f) Daughter cell formation. As with filament synthesis, the growth point for the daughter cell wall is always apical. Gold/palladium shadows. (g) Swarm cell which has just started filament formation. (h) Daughter cell formation, apical growth point. (i) Daughter cell formation. Note that the filament from the opposite pole is nongrowing; (i) i.e., after completion of bifilament synthesis only one growth point is active in daughter cell formation. Arrows indicate the spherical body, which is invariably found in these preparations. This structure correlates closely with that shown in Fig. 28. Bar represents 2 µm.*

seems most unlikely in view of daughter cell
formation when the tube is over 40 $\mu$m long
(phosphate limitation, Fig. 20) and when
branching is involved (Fig. 21 and 24).

(vii) Evidence for parallel pathways of DNA
and protein synthesis leading to daughter cell
development comes from inhibitor studies.
When DNA synthesis is specifically inhibited,
there is no physiological separation or binary
fission of mother and daughter cell. This sug-
gests that termination of genome replication is
essential to separation of cells (40). Similarly,
specific inhibition of protein synthesis and the
changes in sensitivity to chloramphenicol indi-
cate a temporally related sequence of protein
syntheses during differentiation, which termi-
nate in "division."

## CONCLUSION

Budding bacteria—unlike *E. coli* but like
*Caulobacter* species—possess dimorphic cell cy-
cles and, like *Caulobacter* species, should be
useful models for the study of morphogenesis
and differentiation in procaryotes. This possi-
bility has been explored by using *R. vannielii*.
To avoid confusion in comparing different ex-
amples of morphogenesis and differentiation as
expressed by different species of bacteria, we
have attempted to define and categorize such
events. *E. coli*, for instance, is seen to be a
morphologically simple organism with a simple
vegetative cell cycle in contrast to *Anabaena
cylindrica*, which we consider to be an example
of a multicellular procaryote, in which differen-
tiated cells of at least three types regularly
occur, differentiation reflecting intercellular
relationships.

The supposed differences between budding
and binary fission modes of reproduction were
shown to be more apparent than real. The key
feature distinguishing budding bacteria from
rod-shaped bacteria seemed to be the obligate
polar mode of growth by the former, leading
frequently to asymmetric cell division, and
more importantly to a mother-daughter cell cy-
cle rather than the sibling cell cycle as in *E.
coli*. Aging was shown to be a characteristic of
mother cells, and immaturity was seen to be
characteristic of daughter cells.

The uniqueness of the daughter cell—in com-
parison with a sibling cell formed by *E. coli*—is
that a series of events (both morphological and
molecular) occur which are not repeated in sub-
sequent rounds of cell cycles of that particular
cell. Such a feature, apparently restricted to
bacteria with dimorphic cell cycles, is clearly of
relevance to the study of morphogenesis and
differentiation in that it is an obligate event in
the cell cycle, not an occasional event, as is

spore formation and other systems presently
studied in this context.

*R. vannielii*, an exosporeforming, photosyn-
thetic bacterium possessing a complex internal
fine structure and external morphology, under-
goes complex cell cycles and proved to be a
useful example of a budding bacterium for ex-
ploring the nature of morphogenesis and differ-
entiation.

Selective synchronization—a prime requisite
for work on such organisms—was accomplished
rapidly and simply (by filtration through glass
wool) and resulted in yields of cells (daughter
cells at the beginning of the cell maturation
cycle) far in excess of previously recorded yields
for selective synchronization techniques. Such
yields meant that both quantitative and quali-
tative methods could be used in the investiga-
tions.

Changes occurring through the cell cycle
were explored. The pattern of events recorded
parallel, to some degree, those published for
*Caulobacter crescentus* but also included addi-
tional changes useful for further exploration of
the molecular biology of morphogenesis and dif-
ferentiation.

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