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Timing of Swarmer Cell Cycle Morphogenesis and Macromolecular Synthesis by *Hyphomicrobium neptunium* in Synchronous Culture

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The swarmer cycle of *Hyphomicrobium neptunium* consists of a temporal sequence of discrete developmental events. To time morphogenesis and to investigate modulations in macromolecular synthesis, we attempted methods for synchronous culture. During synchrony, swarmer maturation occurred over 32%, hyphal growth occurred over 36%, and bud maturation occurred over 32% of the time required to complete the swarmer cycle. Daughter cells were released after 265 min. Deoxyribonucleic acid replication was discontinuous, having a G_1 period of approximately 180 min. In addition, ribonucleic acid and protein syntheses were depressed during the earlier phases of development.

Hyphomicrobium neptunium is a member of a diverse group of prosthecate, budding bacteria that undergo a series of discrete cytological changes during their life cycles (4, 7, 8, 16; Fig. 1 and 2). Their relatively complex and unique morphogenesis, coupled with the observation that the genome must pass from mother cell to daughter cell through a hypha before division, has made them a focus for study of developmental biology (18).

Currently, there are four recognized genera of bacteria whose prosthecae have reproductive function, i.e., new cell formation by a budding process (6). These are *Hyphomicrobium, Hyphomonas, Pedomicrobium* (6), and *Rhodomicrobium* (17). However, the taxonomy of the budding bacteria remains in flux, and it has been suggested (6) that *H. neptunium* be reclassified formally as *Hyphomonas neptunium* based upon the pattern of amino acid utilization (5), DNA base sequence (11), and cell wall antigenic determinants (14).

Despite other fundamental differences, the budding bacteria share in common a diphasic mode of vegetative development (17). The timing of morphogenesis of the swarmer cycle (also termed daughter cycle [4]) has been elucidated in only two of the genera of these bacteria, represented by *Rhodomicrobium vannielii* (17) and *Hyphomicrobium* sp. strain B-522 (12). The former is shown to have approximately a 6.5-h cycle, and the latter has approximately a 14.5-h cycle.

We have extended these reports to another genus and here define *H. neptunium* as a model system, one which is easily manipulated (14) and has a comparatively rapid growth rate (5). We investigated the timing of swarmer cell morphogenesis and DNA replication for cells in synchronous culture. We found that the swarmer cycle of *H. neptunium* required approximately 4.4 h and that its modulation resembled that observed in *R. vannielii* and *Hyphomicrobium* sp. strain B-522 in some, but not all, respects.

MATERIALS AND METHODS

Measurement of cell growth, division, and viability. *H. neptunium* ATCC 15444 was cultivated in modified (11.22 g/liter [5]) marine broth 2216 (Difco [19]) at 36°C and checked for viability by plate count on marine agar (Difco [5]). Absorbance was determined at 550 nm (Spectronic 20; Bausch & Lomb, Inc.), and total cell numbers were determined in Petroff-Hausser chambers under dark phase-contrast optics (0.66 numerical aperature; Microstar microscope, American Optical Co.). Morphological observations and size determinations were made on wet mounts with a calibrated micrometer (numerical aperture 1.25, dark phase-contrast optics, American Optical Co.). Alternatively, cultures were placed on slides prepared with dried methyl cellulose and photographed (Kodak Pan-X film and Microdal-X developer) with a Zeiss II microscope equipped with a phase three objective (numerical aperture of 1.32).

Measurements of RNA, DNA, and protein synthesis. RNA and DNA were labeled with [2-^{14}C]adenine (ICN Pharmaceuticals, Irvine, Calif.; 40 to 60 mCi/mmol). The incorporation of the tracer into RNA and DNA was determined by a modification (16) of

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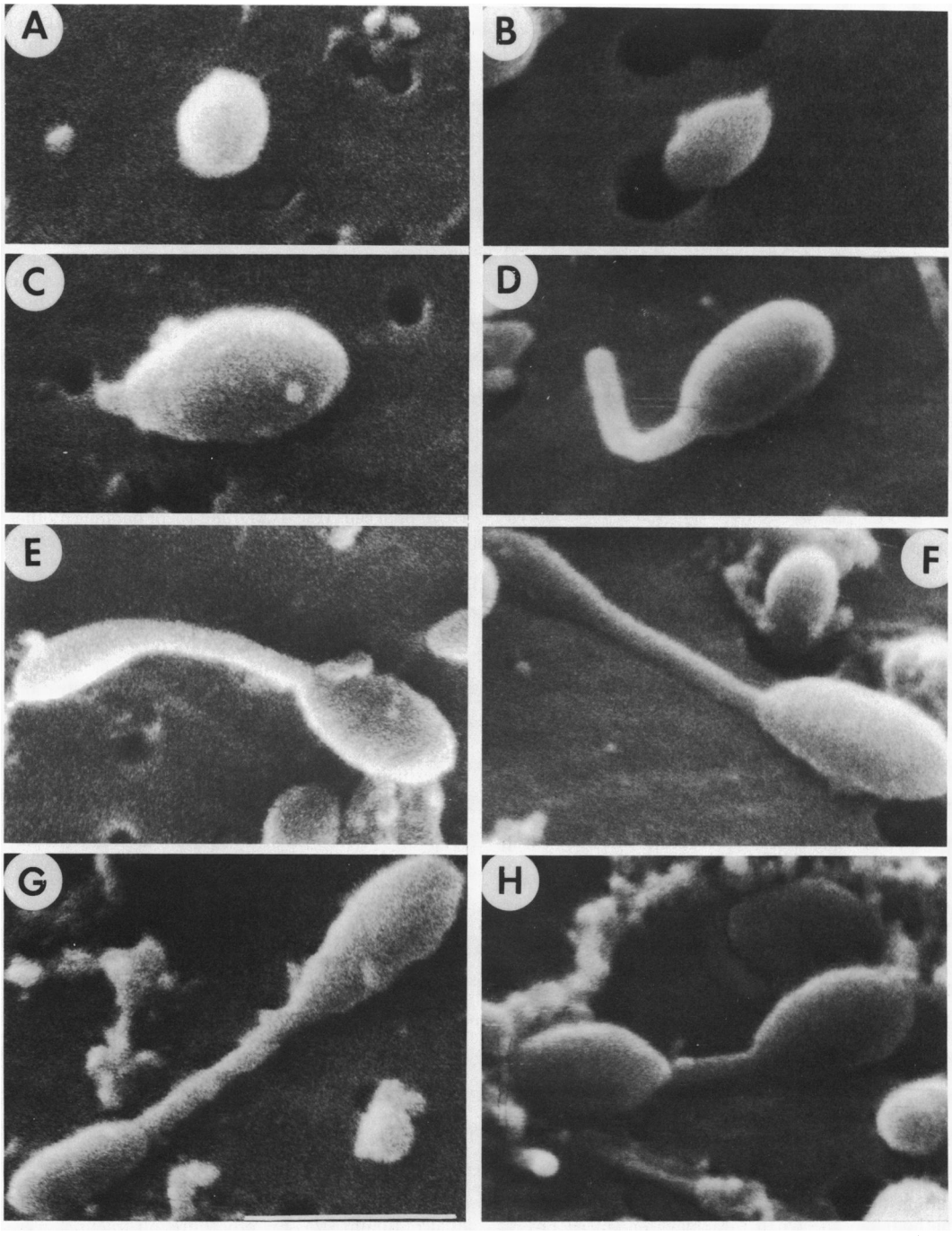

FIG. 1. *Scanning electron micrographs of the H. neptunium cell cycle. At intervals cells were removed from synchronous culture and fixed, stained, and scanned as described in text. Cells typifying each process or stage of morphological development are presented: (A) young swarmer cell, (B) swarmer maturation, (C) hypha outgrowth, (D) hypha elongation, (E) fully developed hypha (prebud), (F) bud initiation, (G) bud maturation, (H) mature bud (predetachment). Bar = 1 μm.*

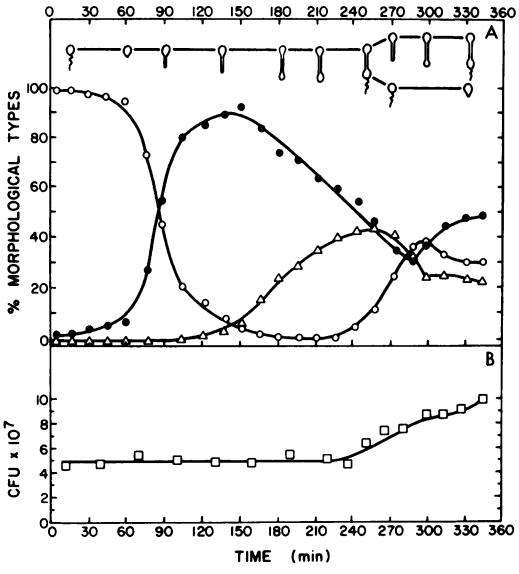

FIG. 2. *Proportions of morphological cell types during synchronous growth of H. neptunium swarmer populations. Cells were scored either as swarmer cells (○; see Fig. 1A and B); hyphal cells (●; see Fig. 1C to E); or budding cells (△; see Fig. 1F to H) as described in the text. Bottom: viable plate counts (□).*

the Roodyn and Mandel (15) filtration assay. Cellular proteins were labeled with L-[*methyl*-^{14}C]methionine (New England Nuclear Corp., Boston, Mass.; 100 mCi/mg) and assayed as previously described (5).

The efficiency of counting (Unilux II scintillation counter, Nuclear-Chicago Corp.) for both [^{14}C]adenine and [^{14}C]methionine ranged from 74 to 90%. Counts per minute were corrected to 100% efficiency (disintegrations per minute) by using a quench curve prepared by the alternate channel method. The data reported are results of at least three replicate experiments.

Scanning electron microscopy. Cells were centrifuged at 12,000 × *g* for 10 min at 4°C and fixed in 2.5% glutaraldehyde for 30 min. Fixed cells were washed six times in physiological saline and graded ethanol series and critical point dried. Specimens were shadowed with gold-platinum alloy, and micrographs were taken (Cambridge Stereoscan scanning electron microscope) with Polaroid type 52 film.

Synchronous growth. A size selection synchrony method (10) based on procedures developed by Moore and Hirsch (12) for *Hyphomicrobium* sp. strain B-522 was used. A relatively homogeneous swarmer cell population (80%) was obtained by significantly modified methodology, designed to achieve enhanced physiological synchrony and to diminish the attendant lag period of incipient cultures. A 0.5-ml amount of log-phase culture (5 × 10^8 bacteria per ml) was spread on marine agar in four to seven 130-mm-diameter petri plates. The plates were incubated at 36°C for 48 to 55 h and then refrigerated (4°C) for 12 to 14 h. Each plate was flooded with 7 ml of cold phosphate-buffered saline (PBS; 1.36 g of KH_2PO_4, 2.13 g of K_2HPO_4, and 8.5 g of NaCl per liter of water), and placed in a gyratory shaker bath (New Brunswick Scientific Co., model G76) at 200 rpm for 15 min. The wash was recovered and centrifuged at 3,000 × *g* for 15 min at 4°C (International Refrigerated Centrifuge, International Equipment Co.). The supernatants were discarded, and the pellets were resuspended in cold PBS at 1/10th their original volume by vigorous agitation on a Vortex mixer (45 s). The suspension was passed once through sterile 1.2-μm membrane filters (≅25 ml/47-mm filter; Millipore Corp.). The filtrate was agitated again for 45 s on a Vortex mixer and added to an appropriate volume of prewarmed (36°C), modified marine broth. A volume of 2× modified marine broth (22.4 g/liter) equal to the PBS inoculum was also added to the culture.

To monitor synchronous growth, plate counts, optical density measurements, morphological characterizations, and determinations of tracer incorporation were made as described above. Morphologically, cells viewed by phase-contrast microscopy were roughly classified as being either swarmer types (Fig. 1A and B), hyphal cells (Fig. 1C, D, and E), or budding mother cells (Fig. 1F, G, and H). A morphological diversity index [(number of cell types/number of total cells) × 100%] was calculated for each cell type in each sample after scoring at least 100 cells.

Characterization of the efficacy of the procedures used to obtain synchrony. The *H. neptunium* synchronization procedure was derived after three factors (low temperature, centrifugation, and filtration) that enriched the swarmer population were studied. Based on an observation that, at 4°C, hyphal cells released at least one generation of swarmers that did not exhibit further morphogenesis, the contribution of 4°C toward enhancing the swarmer population was examined. With other factors constant (see previous section), various synchrony starter cultures were prepared by eliminating the refrigeration step or by keeping lawns at 4°C for 12, 22, 48, or 65 h before centrifugation.

Second, centrifugation times and gravities were varied in combinations of 5, 10, 15, 20 min and 1,000, 2,000, 3,000, 4,000, and 5,000 × *g*. Lastly, Nucleopore filters (VWR Sci.) with pore diameters 0.6, 0.8, 1.0, and 3.0 μm and Millipore filters with pore diameters 0.65, 0.8, 1.2, and 3.0 μm were tested for their ability to retain all but swarmer forms. Procedures incorporating none, one, and two centrifugation and/or filtration steps were tested.

To monitor each variation, the final inoculum was morphologically characterized under phase microscopy and in photomicrographs, and the synchronous cultures were indexed (10) as described above. For representative procedural variations and also for the final optimum size sort procedure, all fractions were pooled (e.g., pellets and supernatants, filtrates and filter catches), and growth in marine broth was monitored by viable plate count to determine whether the manipulations required to induce synchrony also caused a lag in *H. neptunium* growth.

***H. neptunium* plasmid DNA.** A modification of a

technique used to rapidly isolate plasmids (3, 9) was employed. DNA samples were subjected to agarose gel electrophoresis and exposed to ethidium bromide (Sigma Chemical Co.), and the bands were visualized under long-wavelength UV light.

RESULTS

Synchronizing *H. neptunium*. An optimum procedure to induce synchrony was developed and characterized. Interestingly, when lawns of *H. neptunium* were maintained at 4°C for 12 to 65 h, the percentage of swarmer cells in the population (Fig. 1A) was increased two- to three-fold. However, as the duration of refrigeration was prolonged, the time to the initial cell division was lengthened so that 12, 22, 48, and 65 h of refrigeration yielded synchrony inocula that divided at 265, 280, 310, and 360 min, respectively. Thus, 12 h of cold induction, an optimal compromise that enhanced the swarmer population at least 2× without inducing a prolonged technique-induced lag period, was employed.

Centrifugation was then used to sort out swarmer cells. The best results were obtained at 3,000 × *g* for 15 min, whereby 30% of the cells, consisting almost entirely of maturer forms (Fig. 1D to H), remain in the supernatant. Finally, 1.2-μm-diameter (pore) filters retained most of the hyphal forms (Fig. 1C to H) and did not trap swarmer cells (Fig. 1A and B). Double filtration further reduced the heterogeneity, but ultimately was not adopted because this advantage was more than balanced by a longer lag phase with consequent dispersion of cell cycle events over longer intervals.

When *H. neptunium* was inoculated from marine agar lawns into marine broth, no lag period was detected. However, when it was subjected to the optimum synchronizing procedure, the trauma of the size sorting techniques induced a 15-min lag period. As evidenced by round morphological appearance (Fig. 1A) and high incidence of motility, 80% of the final synchrony inoculum was comprised of the youngest cell types, swarmer cells. The remainder was comprised of slightly maturer ovoid cells (19%, Fig. 1B) and hyphal forms (1%, Fig. 1C to H).

Timing of swarmer cycle morphogenesis. Three major cell types were microscopically scored: swarmer cells, hyphal cells, and budding cells (Fig. 2). The interval before hyphal outgrowth was roughly equated to the period of swarmer maturation, that between hyphal outgrowth and bud formation was taken as the period of hyphal growth, and that between bud formation and bud detachment was termed bud maturation (Table 1), although it is recognized that both bud maturation and septum formation (not monitored) occurred. In assessing the data, the appearance of each morphogenic marker was scored when first observed, when last observed, and when 50% of the population had progressed to the transition (e.g., bud formation), taken as the "transitional milestone."

By approximately 85 min, 50% of swarmer stage *H. neptunium* formed hyphae (Fig. 2). Bud formation first became apparent about 135 min into the cycle and was more prevalent at 180 min. Although 1,000 cells periodically were scored during this time, it was difficult to discern new bud formation precisely, possibly resulting in a uniformly small percentage of budding cells being categorized as hyphal cells. Increases in the percentage of budding cells after 230 min reflected second generation bud formation, since mother cells began to form new buds almost as

TABLE 1. *Timing of morphogenesis of swarmer cell cycle of H. neptunium in synchronous culture in nonsynthetic medium*

Cell cycle stage		Time into cell cycle (min)			Approximate time (min) required for cell cycle event		% Cell cycle time required for event		
Observed morphological transition	Developmental process	Early population	Late population	T_{50}[a]	Occurrence interval	Duration of process	*H. neptunium*	*Hyphomicrobium* sp. B-522[b]	*R. vannielli*[c]
	Swarm maturation				0–85	85	32	34, 28	39
Hyphal outgrowth		45	150	85					
	Hyphal growth				85–180	95	36	28, 39	13
Bud formation		135	255	180					
	Bud maturation				180–265	85	32	38, 19	48
Bud detachment		235	295	265				14	

[a] Time in synchronous growth at which approximately 50% of the *H. neptunium* population had progressed to the transitional milestone.

[b] Data taken from Moore and Hirsch (12). The first set of numbers was derived from synchronous population, and the second set was from agar slide cultures of *Hyphomicrobium* sp. strain B-522.

[c] Data constructed from Whittenbury and Dow (17, 18), who further distinguish "landmark" events as motile period, flagellum shedding period, terminal maturation period, filament formation phase, bud synthesis, and daughter cell completion.

soon as the previous one was released (1). Bud detachment first occurred at 235 min, and about 50% of the population had divided by 265 min as determined by viable plate counts and by increase in the swarmer population. Based on these results, which have been frequently reproduced (±10 min), it was estimated that swarmer maturation required 85 min, hyphal growth required 95 min, and bud maturation required 85 min.

Viable plate counts increased from 5×10^7 cells per ml at 235 min to 8.5×10^7/ml (not 10^8/ml) at 295 min, because only 85% of the synchronous population initiated morphogenesis during the 6-h observation period. The remainder, swarmer cells having a granular cytological appearance, were in an extended lag period and consequently were ignored in the morphological data presented in Fig. 2. After 295 min, viable counts did not completely stabilize because the early segment of the population (e.g., mature swarmer cells comprising 19% of the inoculum) began to release a second generation of progeny. Based on a 265-min swarmer cycle and the occurrence of bud detachment over a 60-min interval, the division index of synchrony was 77%.

Modulation of macromolecular synthesis. Pulses of [^{14}C]adenine and [^{14}C]methionine, reproducibly incorporated into respective macromolecular fractions, provided an estimate of vacillations of total DNA, RNA, and protein synthesis during the swarmer cycle of *H. neptunium* (Fig. 3). Background counts, averaging 200 cpm in the NaOH-treated fraction, were attributed to an asynchronous population or repair activity or both.

Four discrete peaks of DNA biosynthetic activity were clearly evident. The first, occurring at 75 min, was thought to reflect actual DNA replication in an early maturing cell fraction, since 19% of the "synchrony start population" was comprised of mature swarmers (Fig. 1B). Nevertheless, we checked the possibility that it could have been due to an independently replicating plasmid and repeatedly failed to isolate any band of DNA that was distinct from chromosomal DNA.

A second and much more pronounced burst of DNA replication occurred from 150 to 210 min into the swarmer cycle, and its major component spanned 165 to 195 min. This coincided with the period when hyphal elongation was ceasing and bud formation was initiating. A third peak, at 240 to 300 min, was prominent at the time of bud release and second generation bud formation (mother cycle). The last round of activity, although shown, occurred when morphogenesis of the population was becoming less synchronized.

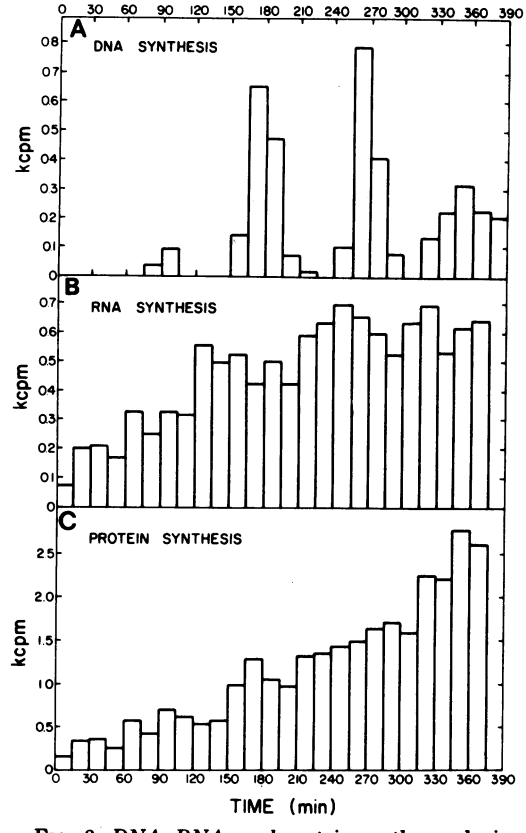

FIG. 3. *DNA, RNA, and protein syntheses during synchronous growth of H. neptunium swarmer populations. To measure DNA and RNA synthesis, 3-ml samples were removed from synchronously growing cultures (initial titer of 5×10^7 to 1×10^8 cells per ml) and labeled with 0.15 μCi of [^{14}C]adenine per ml for 15 min. To measure protein synthesis, 2.5-ml aliquots were added to a final concentration of 0.075 μCi of [^{14}C]methionine per ml for 15 min. During labeling, all samples were maintained at 36°C and aerated. Samples were prepared for counting, and radioactivity was determined as described in the text. Background counts of 0.2 kcpm were subtracted from the [^{14}C]adenine-treated samples, and 0.5 kcpm was subtracted from the [^{14}C]methionine-treated samples.*

As expected, periods of RNA and protein synthesis were not so discretely defined. However, on a per cell basis, both RNA and protein synthesis activities were lowest in the swarmer cell population and progressively increased as *H. neptunium* swarmer cells matured (Fig. 3). By setting levels of RNA and protein synthesis to 100% at the time of bud separation (265 min), relative values of activity were obtained (Table 2). Swarmer cell levels of RNA and protein synthesis were only about one-fourth those found in mother cells about to release daughter cells.

However, the data expressed on a per cell volume basis were perhaps more revealing (Table 2). Volumes were calculated roughly from measurements of the axis of various *H. neptunium* cell types, with the main cell body treated as a prolate spheroid, the hypha treated as a cylinder, and the swarmer cell or developing bud treated as a sphere (Table 2). Relative to this approximated cell mass, protein and RNA syntheses were demonstrated to have clearly ebbed during the period of hyphal outgrowth rather than during the period of swarmer maturation.

DISCUSSION

A number of selection and induction (with chloramphenicol) procedures were tested initially in our efforts to obtain maximum homogeneity of the zero time *H. neptunium* synchrony inoculum. Unfortunately, most of these procedures stressed the cells and led to a prolonged lag phase. Ultimately, the selection procedure used to synchronize *H. neptunium* was based on that developed for *Hyphomicrobium* sp. strain B-522 (12), extensively modified to accommodate the smaller size of our amino acid-requiring, marine bacterium. Beyond this, synchrony was significantly improved by the addition of an induction step (chilling) which enriched for *H. neptunium* swarmer cells. Chilling was not a useful adjunct for the synchronization of other budding bacteria (12, 17).

The optimum synchronization procedure was reproducible and provided good experimental resolution. Its main deficiency was the induction of a 15-min divisional lag. This is possibly similar to that observed by Moore and Hirsch (12), where swarmer maturation consumed 34% of a synchronous culture cycle but only 28% of the slide culture cycle. If this lag were to be translated into a developmental lag in the starting population, it is conceivable that swarmer maturation actually requires 70 min rather than 85 min and that the swarmer cycle requires 250 min rather than 265 min for completion. Considering the possibility that daughter cycle progeny may be formed and released more rapidly than mother cycle progeny, these estimates remain in general accord with those reported earlier with agar slide cultures in which mother cycle buds were formed and released at approximately 60-min intervals (1).

As delineated by the transitions of hyphal outgrowth, bud formation, and bud detachment, the developmental processes of swarmer maturation, hyphal growth, and bud maturation (plus separation) consume 32, 36, and 32%, respectively, of the duration of the 265-min *H. neptunium* daughter cycle in synchronous culture in marine broth. These values are not markedly different from those reported for *Hyphomicrobium* sp. strain B-522, but differ from those reported for a photosynthetic, budding bacteria, *R. vannielii* (Table 1). Although *H. neptunium* may be only superficially related to *R. vannielii*, it is nonetheless interesting that *H. neptunium* hyphal growth requires 95 min (36% cycle time) whereas *R. vannielii* requires only 45 ± 5 min (13% cycle time) for filament formation despite the observation that the appendages of both bacteria are of similar size (17).

DNA synthesis during the swarmer cell cycle of *H. neptunium* appears to be discontinuous. The period of augmented [^{14}C]adenine incorporation from 75 to 105 min is not ascribed to chromosome replication since only one nucleoid is found in *H. neptunium* (R. Weiner, unpublished data) and in other budding bacteria (13, 17) during this period of swarmer development. It probably does not reflect plasmid replication either, since no extrachromosomal DNA could be isolated from *H. neptunium* just as none

TABLE 2. *Relative levels of RNA and protein synthesis during H. neptunium swarmer cell cycle*

Cell cycle stage	Avg cell volume[a] (μm^3)	% Cell volume[b]	% RNA and protein synthesis/cell no.[c]		% RNA and protein synthesis/cell volume[d]	
			% RNA	% Protein	% RNA	% Protein
Swarm maturation	0.19	27	28[e]	22[e]	103[e]	81[e]
Hyphal growth	0.49	70	40	46	57	65
Bud maturation	0.63	90	85	81	94	90
Bud detachment	0.70	100	100	100	100	100

[a] Calculated as reported by D. Danald (M.S. thesis, University of Maryland, College Park, 1977).

[b] Average cell volume at cell cycle stage/average cell volume at bud detachment stage (0.70 μm^3).

[c] Amount of tracer incorporation during cell cycle stage (from Fig. 3)/incorporation at bud detachment stage/relative cell number.

[d] Amount of tracer incorporation during cell cycle stage (from Fig. 3)/incorporation at bud detachment stage/percent cell volume.

[e] These figures theoretically could be larger, since they were calculated from measurements made during synchrony interval 0 to 85 min. The population may have been physiologically repressed during the first 15 min of outgrowth (see text).

could be isolated from *R. vannielii* (17). It seems likely that this peak, 1.5×10^2 versus 1.5×10^3 cpm in later peaks, represents DNA replication in an asynchronous element (19%) of the population.

The second peak of [^{14}C]adenine incorporation appears to reflect a period of chromosome replication that occurs between 150 and 225 min into the cycle. Because some elements of the synchronous population, no doubt, initiated and terminated replication ahead of others, the actual interval of cellular DNA replication is believed to be even more condensed. In any event, it coincides with the end of the period of hyphal growth and the period of bud maturation. The timing of DNA replication during the daughter cycle of *H. neptunium* is, therefore, analogous to that in *R. vannielii* (17) but probably not to that in *Hyphomicrobium* sp. strain B-522 (13).

The third period of [^{14}C]adenine incorporation into the DNA fraction, observed from 240 to 300 min, occurred during bud detachment and new bud formation (mother cycle). These data indicate that the *H. neptunium* swarmer cycle of 265 min has a G_1 period of approximately 180-min duration, whereas the mother cycle of 60 to 85 min has little or no G_1 period. This is generally consistent with a recently proposed unified model for the cell division cycle (2).

RNA and protein syntheses are also modulated during the *H. neptunium* swarmer cycle. On a per cell basis, the activity of both anabolic processes significantly increase as the swarmer cells mature. This age-dependent increase in biosynthetic activity has also been observed in *Hyphomicrobium* sp. strain B-522 (13). On the other hand, the decrease in protein synthesis/cell volume during hyphal growth coincides with observations in *R. vannielii* (17). Macromolecular synthetic activity, associated with bud formation through bud separation, should be more cautiously interpreted since synchrony becomes more random with time (10).

Although throughout this discussion comparisons have repeatedly been made with other budding bacteria, it should be emphasized that *H. neptunium* is a unique species (6), serving in its own right as a model for the study of procaryotic differentiation. Future investigations may determine phylogenetic relationships among budding bacteria and permit more enlightened generalizations of their biology.

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