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Caulobacter and *Asticcacaulis* Stalk Bands as Indicators of Stalk Age

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The prosthecae (stalks) of dimorphic caulobacters of the genera *Caulobacter* and *Asticcacaulis* are distinguished among such appendages by the presence of disk-like components known as stalk bands. Whether bands are added to a cell's stalk(s) as a regular event coordinated with the cell's reproductive cycle has not been settled by previous studies. Analysis of the frequency of stalks with *i*, *i* + 1, *i* + 2, etc. bands among more than 7,000 stalks of *Caulobacter crescentus* revealed that in finite (batch) cultures (in which all offspring accumulate), the proportion of stalks with *i* + 1 bands was regularly 50% of the proportion of stalks with *i* bands. This implied that the number of bands correlated with the number of reproductive cycles completed by a stalked cell. In chemostat-maintained perpetual cultures, the proportion was greater than 50% because stalked cells, with their shorter reproductive cycle times, contributed a larger proportion of offspring to the steady-state population than did their swarmer siblings. In *Asticcacaulis biprosthecum* cells, which bear twin prosthecae, the twins on a typical cell possessed the same number of bands. For both genera, stalk bands provide a unique morphological feature that could be employed in an assessment of age distribution and reproductive dynamics within natural populations of these caulobacters.

Stalked bacteria that divide asymmetrically and produce one motile and one stalked cell at each reproductive event were first described by Henrici and Johnson (8) on the basis of observations of cells attached to glass slides that had been submerged in a freshwater lake. Leifson (12) later detected a single flagellum at the pole of the motile cell by light microscopy of stained cells, and Houwink (9) provided electron micrographs of dividing cells, each with two distinctly different appendages, the stalk at one pole and the flagellum at the other; Houwink also detected the presence of bands in *Caulobacter* stalks. Bands are visible within ultrathin sections of both *Caulobacter* and *Asticcacaulis* stalks (15, 17, 23, 25); they are structurally integrated into the concentric layers of membranes and peptidoglycan of the stalk and are disorganized by lysozyme. Isolated bands are seen as disks composed of concentric rings (5, 10, 27). A similar disk of rings has been described by Coulton and Murray (1, 2) as the structure embedded in the outer membrane of *Aquaspirillum* cells through which the flagellum emerges from the cell surface.

The initial stages of stalk development can be studied in synchronously developing swarmer populations, although synchrony deteriorates after the first generation (4, 6, 29, 33). On the basis of studies of developing swarmer populations and of stalked cells growing attached to a solid substrate through 19 generations, Staley and Jordan (32) proposed that the stalk bands were added at regular intervals during stalk outgrowth, with one band appearing during each reproductive cycle completed by the cell. The bands would then serve as indicators of cell age by reflecting the number of cell cycles that had been completed by a cell since the generation in which the cell was a swarmer. This proposal was also made by Jones and Schmidt (10, 11), whose experimental evidence was not described. Swoboda and Dow (34) reached a contrary conclusion. They monitored initially synchronous populations through two to three rounds of reproduction and found that bands were either rare (0.03% of stalks) or occurred throughout the stalked population. They suggested that the addition of bands was a response to environmental stimuli and were not able to relate the sudden appearance of bands to the completion of synchronous cell fissions; they were also unable to identify an environmental stimulus of band formation.

In the present study, the question of the possible relationship between the number of stalk bands and the number of reproductive cycles completed by a cell was investigated statistically by the enumeration of bands in more than 7,000 stalks in populations of *Caulobacter crescentus* grown in finite and perpetual cultures and of *Asticcacaulis biprosthecum* prosthecae (see reference 30 for the definition of prostheca) grown in finite cultures. The results strongly support the inference that one band per stalk is added toward the end of each reproductive cycle, probably accompanying the process of cell division. Statistical analysis also allowed the calculation of the respective cycle times of stalked and swarmer siblings during steady-state perpetual cultivation in different nutrient fluxes.

(A preliminary report of this study has previously been presented [22].)

MATERIALS AND METHODS

Over a period of several years, electron micrographs of caulobacters that had accumulated in our respective laboratories had been examined for several aspects of caulobacter morphology. The records of stalk band numbers in cells grown under various conditions provided a sample sufficiently large for statistical analysis. The procedures presented here describe the various histories of the micrographs used as the data source for statistical analysis.

Bacteria and cultivation. *C. crescentus* CB2 and CB15 (17) and *A. biprosthecum* (16) were cultivated as suspended cells in aerated liquid media at 30°C. Cells were not in any case detached from solid substrata for examination by electron microscopy because mechanical detachment often breaks stalks, preferentially at the location of a band (Fig. 1). Cells were kept in suspension during cultivation by mechanical agitation; finite cultures were incubated on gyratory shakers operated at 150 to 180 rpm, and perpetual cultures were stirred at 240 rpm by magnetically rotated baffles and aerated by a flow of filter-sterilized air at 1 liter/min.

Perpetual cultures employed a nonadhesive mutant of CB2, 2NY66R (24), to

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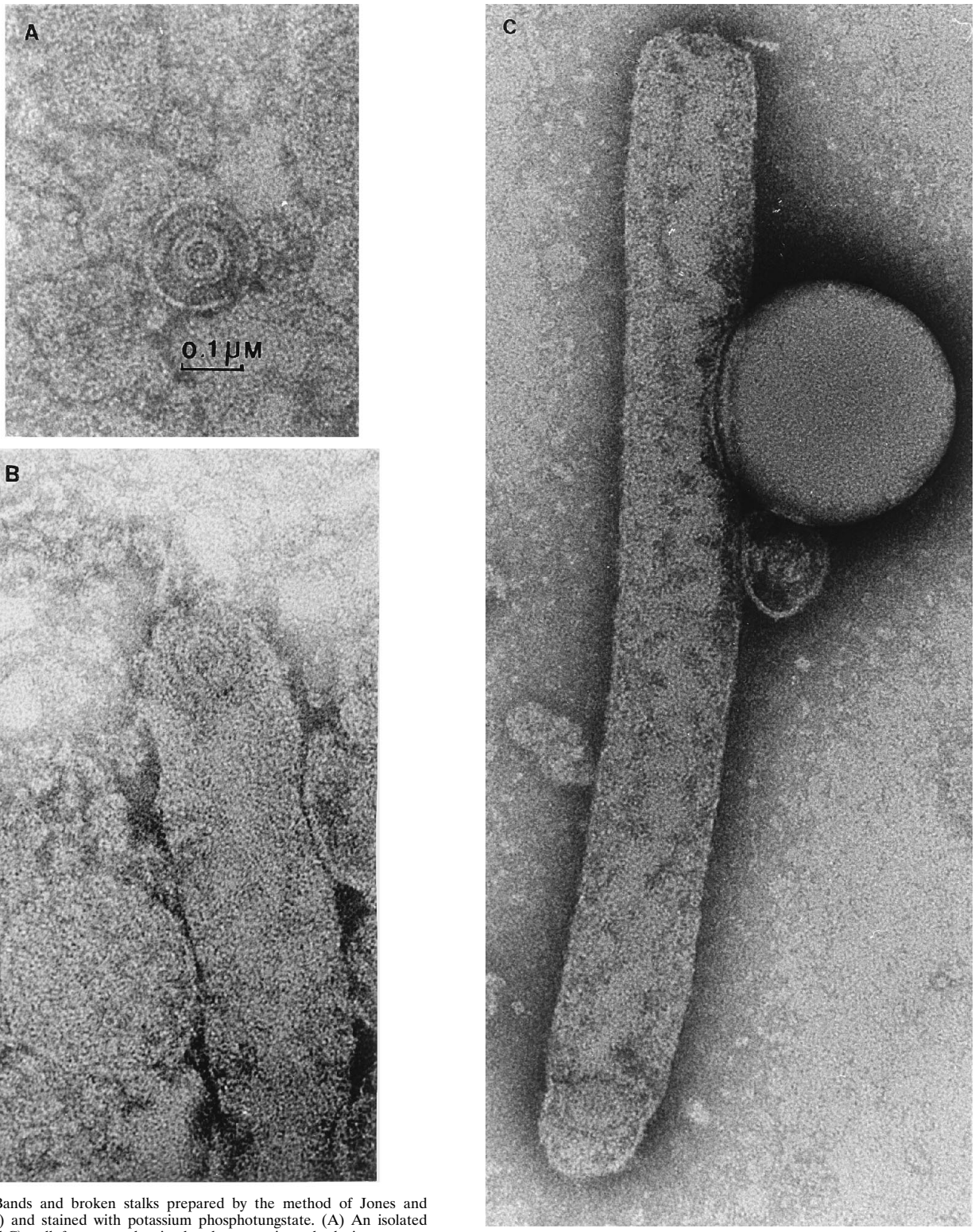

FIG. 1. Bands and broken stalks prepared by the method of Jones and Schmidt (10) and stained with potassium phosphotungstate. (A) An isolated band; (B and C) stalk fragments showing bands at one site or both sites, respectively, of stalk breakage.

avoid adhesion to the vessel wall and inserts. Cultures were maintained in New Brunswick BioFlo chemostats as described by Poindexter and Eley (24). Most cultures were operated with 350-ml culture volumes and flow rates from 14.6 to 87.5 ml/h. Three of the 27 cultures were operated with 1,400-ml culture volumes at flow rates of 58.4, 116.6, and 233.3 ml/h. The retention times for cultures ranged from 4 to 24 h.

Media. Peptone yeast extract (PYE) medium (17) was employed for some finite cultures. Hutner's imidazole glucose glutamate (HiGg) medium (18), sup-

plemented with 1 mM $CaCl_2$, was employed for defined media. The concentrations of glucose, glutamate, NH_4Cl, and Na- and K-phosphate were varied in order to impose growth yield limitation by C, N, P, or all three element sources simultaneously. In HiGg medium containing a gravimetric C/N/P ratio of 100:10:1, the three element sources are exhausted simultaneously; for example, the medium is balanced with respect to C, N, and P when prepared with 0.25% glucose–0.08% Na_1-glutamate · H_2O–0.025% NH_4Cl–0.405 mM phosphate. In finite cultures, the concentrations of glucose and Na_1-glutamate · H_2O were 0.1% (wt/vol) each and the concentration of phosphate varied from 0.025 to 4 mM. The concentrations used in the media delivered to perpetual cultures ranged from 0.0125 to 0.25% glucose, 0.0125 to 0.08% glutamate, 0.013 to 0.025% NH_4Cl, and 0.01875 to 0.48 mM phosphate.

Microscopy. Cells were prepared for microscopy either by collecting cells by centrifugation at 17,000 × *g* for 12 min and resuspending in distilled water or by diluting the culture directly into distilled water. Cells were allowed to settle onto nitrocellulose-coated copper grids, the fluid was gently drained away into a tissue, and the grids were allowed to air dry. The dried grids were shadowed at <10° with Pt-Pd (80:20; Fig. 2). A few specimens were alternatively stained with phosphotungstic acid during drying on grids. Cells grown in finite cultures were prepared from exponential phase, and cells grown in perpetual cultures were prepared from steady states.

Enumeration. Electron microscopical images of cells were recorded photographically, printed at a magnification of ×10,000 or greater, and examined with a hand magnifier for the enumeration of stalk bands. Every cell whose entire cell length and entire stalk length (if stalked) were visible was included in this enumeration. The presence or absence of a stalk and (for specimens from perpetual cultures) constriction was recorded, as was the number of bands in each stalk, exclusive of the band that typically occupies the distal tip of the stalk in *C. crescentus*. In all probability, that band serves the flagellum and is present prior to stalk initiation; it could not indicate stalk age. The morphologies of 10,325 cells, including 7,228 stalked cells, were recorded.

Stalks with very large numbers (12 or greater) of bands were encountered only infrequently. Since one such stalk would disproportionately influence analysis, such stalks were usually excluded from this analysis. The least statistically regular class of cells were P-limited stalked cells, particularly in chemostat populations. As usual, the stalks on these cells were considerably longer than those on cells that were not P limited (7, 21, 26), and the longer the stalk, the more susceptible it was to falling on or under another cell or across the bar of a grid. Such stalks were necessarily lost to analysis because only those stalked cells whose entire stalks could be viewed were enumerated. One consequence of this technical problem was a lower correlation coefficient for the enumeration of bands in the especially long stalks of P-limited populations, particularly those maintained in perpetual cultures (see Table 2).

Arithmetic for the analysis of perpetual cultures. The hallmarks of a perpetual steady state in constant-volume perpetual cultures operated as chemostats are constant population size and density in the vessel as cells are generated by binary fission and simultaneously removed by the linear flow of liquid from the reservoir through the vessel to the effluent. Population size and density remain constant as long as the production of cells by binary fission and the removal of cells by outflow to the effluent occur at equal rates.

In such cultures, the constancy of population size and density is dependent on the asynchrony of cell cycles occurring in the population. Waves of fission occur within the population, with each wave establishing an age cohort, a subpopulation of cells that pass through their cell cycles in synchrony with each other. Because cells of a given newborn cohort do not divide again until they are mature, the hydraulic flow through the vessel affects a cohort between waves of fission in the same manner in which it would affect inert particles in suspension, viz., it causes an exponential decline in their number. The dynamical interaction between successive waves of binary fission and exponential decline in cell number between waves of fission is illustrated in Fig. 3 for three populations: (i) a perfectly synchronous population comprising one cohort, (ii) an asynchronous population comprising 10 cohorts, and (iii) an asynchronous population comprising 10,000 cohorts (as in a typical chemostat culture). It is clear that the greater the number of age cohorts, the more stable the population size and density.

In perpetual steady state, the size of each cohort declines between waves of fission according to the relationship for exponential decay of nonreproducing particles:

$$N_t = N_0 \cdot 2^{-t/t_h} \quad (1)$$

where N_t is the number of cells of the cohort in the vessel at any time (t), N_0 is the number of cells initially comprising the cohort (i.e., immediately after they arose by a wave of fission), and t_h is the hydraulic half time of the system (the time required for the flow to remove one-half of suspended, nonreproducing particles; $t_h = R \cdot \ln 2$, where R is the hydraulic retention time such that $f \cdot R = V$, where f is the rate of liquid flow and V is the culture volume).

In a steady-state perpetual culture, the total population will sustain a constant size as long as two conditions are met: (i) the number of cohorts is large, and (ii) the average cell cycle duration (t_c) is the same for each cohort and equals the hydraulic half time (t_h) of the system. In such a situation, the cells of the cohort divide just as removal reduces the cohort to one-half the number of cells it contained immediately after its previous wave of fission. In this way, the size of each cohort fluctuates between 2N newborn cells and N mature cells and the number of cells in the cohort that divide in the vessel equals one-half the number of cells initially comprising the cohort; i.e., when $t = t_c = t_h$,

$$N_{t_c} = N_0 \cdot 2^{-t_c/t_h} = N_0/2 \quad (2)$$

When $t = t_c = t_h$, the cohort is represented at each wave of fission by the same number of dividing cells as were present at the onset of the previous wave of fission through the cohort.

In order to apply this concept to an analysis of *Caulobacter* populations in perpetual cultures, it was necessary to accommodate the two cell cycle times of *Caulobacter* cells as well as the effects of perpetual removal. Because fission of each mature cell yields one flagellated, nonstalked cell (an F sib; prospective cycle time = t_f) and one stalked cell (an S sib; prospective cycle time = t_s), the number of cells that divide equals the number of each type of cell that arises from each fission: $N_d = N_f = N_s$. In steady state, N_d is constant for each wave of fission, but because $t_f > t_s$, F sibs are not mature by the time their own stalked siblings are ready to divide. Consequently, in any wave of fission in a *Caulobacter* population, mature cells are derived from two different cohorts, F sibs from an older cohort and S sibs from a more recent cohort. The respective proportions of the two types of mature cells at the onset of a wave of fission are determined by their respective cycle times and the hydraulic half time of the system as follows:

$$N_d = N_f \cdot 2^{-t_f/t_h} + N_s \cdot 2^{-t_s/t_h} \quad (3)$$

where N_f is the number of cells that arose as F sibs in the older cohort, $2^{-t_f/t_h}$ is the proportion of those cells still present in the vessel at maturity, N_s is the number of cells that arose as S sibs in the more recent cohort, and $2^{-t_s/t_h}$ is the proportion of those cells still present in the vessel as the wave of fission begins. Because $N_d = N_f = N_s$, equation 3 can be simplified as

$$1 = 2^{-t_f/t_h} + 2^{-t_s/t_h} \quad (4)$$

Equation 4 predicts the proportion of each type of cell that will survive removal from the vessel long enough to mature and divide in the vessel. Because $t_f > t_s$, equation 4 (like the physical situation on which it is based) predicts that the proportion of S sibs that will mature in the perpetual-culture vessel will, in contrast to finite cultures, be greater than 0.500. If t_s is constant from the second generation onward so that successive divisions of the cells bearing stalks that began development in the first generation occur at regular intervals, then $2^{-t_s/t_h}$ of the S sibs of the first generation should produce offspring in the vessel, $2^{-t_s/t_h}$ of the S sibs of the second generation should produce offspring in the vessel, and so on for each successive generation. A semilogarithmic plot of descendant S sibs as a function of the number of generations should have a slope whose antilogarithm is equal to $2^{-t_s/t_h}$. The value of $2^{-t_f/t_h}$ can then be calculated from equation 4.

RESULTS

Rationale and interpretation of finite cultures of *C. crescentus*. In the first generation from the swarmer stage, there is a period prior to the initiation of stalk development during which the site at which the stalk will develop is occupied by the flagellum. In populations growing exponentially, this period accounts for approximately 25% of cell cycle time of the swarmer sibling and results in a swarmer cycle time that is about 1.33 times the cycle time of its stalked sibling (17). Once stalk development is initiated, the time from stalk initiation to completion of cell fission is approximately the same as the time between cell divisions for a cell that possesses a stalk at its inception. Consequently, 75% of one-half (i.e., 37.5%) of the stalks in a population should be new stalks (in the first generation of their existence) and the remainder should be at least one generation old (mature stalks). Among mature stalks, one-half (2^{-1}) should be in their second generation, one-quarter (2^{-2}) should be in their third generation, one-eighth (2^{-3}) should be in their fourth generation, etc.

Our hypothesis is that one stalk band is added to the stalk during each cell cycle after the generation in which the cell arose as a swarmer. Our prediction is that if one band is added with each cell cycle, including the first, then among the stalks in an exponentially growing population, 37.5% should have zero or one band; of the remainder, one-half (2^{-1}) should have two bands, one-quarter (2^{-2}) three bands, one-eighth (2^{-3}) four bands, etc.

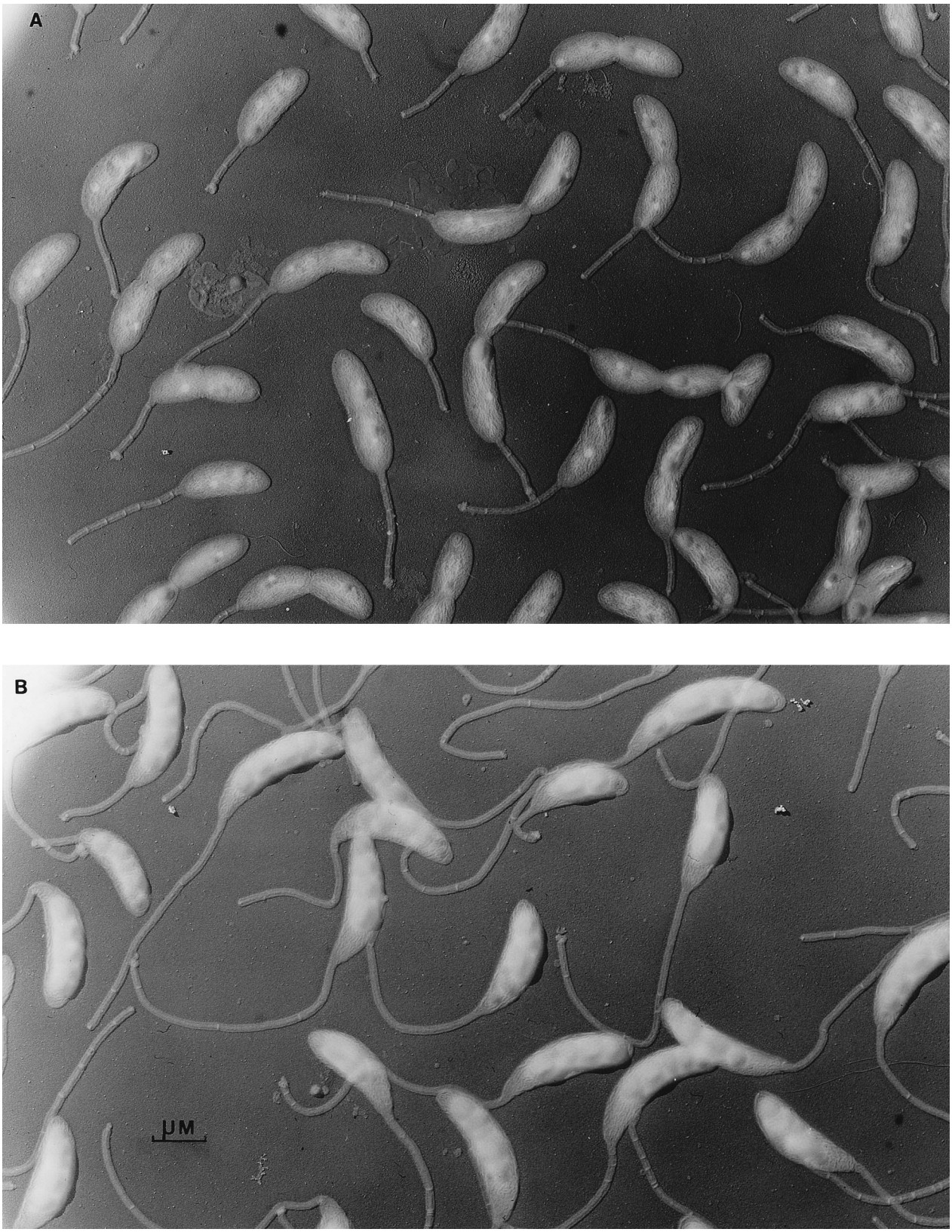

FIG. 2. *C. crescentus* cells from finite cultures in HiGg media containing adequate (A) and low-level (B) phosphate. In these negative images of shadowed preparations, the bands appear as bright stripes perpendicular to the long axis of the stalk.

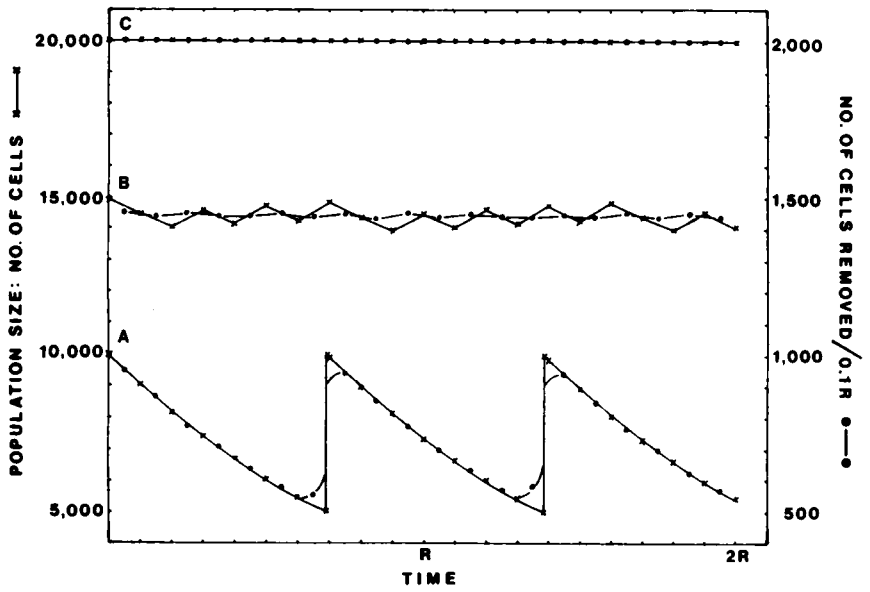

FIG. 3. Dynamic interaction between reproduction by binary fission and exponential decline of cells between fissions. ×, total population size in the growth vessel; ●, number of cells removed by liquid flow through the growth vessel during the interval $0.1R$, where R is the hydraulic retention time of the culture vessel ($R = V/f$), V is the culture volume, and f is the flow rate. (A) Dynamics of a perfectly synchronous population comprising a single cohort of cells with cycle time, t_c, equal to the hydraulic half time, t_h, where $t_h = R \cdot \ln 2$. (B) Dynamics of a population comprising 10 cohorts, each with $t_c = t_h$. A wave of fission occurs in one of the cohorts every $0.1R$. (C) Dynamics of a population comprising 10,000 cohorts, each with $t_c = t_h$, corresponding to a culture of 500 ml in which a wave of fission results from the entry of each 50-μl drop of fresh medium.

If, on the contrary, bands are added suddenly to all the stalks in a population in response to an environmental signal, as suggested by Swoboda and Dow (34), then during the course of incubation some number of generations should pass without bands appearing in the stalks; then when the putative environmental signal has been perceived, there should be bands in all the stalks and the number of bands per stalk should be either constant throughout the population or random.

The results of enumeration of the number of bands in the stalks of *C. crescentus* cells in samples of cultures from early, middle, and late exponential phase of seven finite cultures of strains CB2 and CB15 in PYE, eight cultures of strain CB2 in P-adequate HiGg, and five cultures of strain CB2 in low-P HiGg are presented in Table 1 and Fig. 4. Although medium composition appeared to influence band insertion in the first generation, neither the time of sampling during exponential phase nor the strain of *C. crescentus* influenced the distribution of bands among stalks; accordingly, like data were gathered, and the enumerations are reported as three classes of data, for PYE-grown populations, for P-adequate HiGg-grown populations, and for low-P HiGg-grown populations.

The first-generation stalks were interpreted as those with the fewest bands whose proportions rose as a function of the number of bands per stalk, presumably as stalks developed on cells

TABLE 1. Enumeration of bands in stalks of *C. crescentus* cells grown in finite cultures

No. of bands/stalk	PYE			P-adequate HiGg			Low-P HiGg		
	No.	Proportion		No.	Proportion		No.	Proportion	
		All	Non-first generation[a,d]		All	Non-first generation[b,d]		All	Non-first generation[c,d]
0	818	0.380		81	0.117		22	0.073	
1	635	0.295	0.483	195	0.282		66	0.219	
2	382	0.178	0.290	200	0.289	0.489	54	0.179	
3	184	0.086	0.140	105	0.152	0.257	74	0.245	0.463
4	68	0.032	0.052	65	0.094	0.159	41	0.136	0.256
5	47	0.022	0.036	25	0.036	0.061	23	0.076	0.144
6	15	0.007		14	0.020	0.034	15	0.050	0.094
7	2	0.001		3	0.004		4	0.013	0.025
8	0			4	0.006		3	0.010	0.019
Total stalks	2,151			692			302		

[a] The correlation coefficient for these cultures was −0.991, and the antilogarithm of the slope was 0.500.
[b] The correlation coefficient for these cultures was −0.995, and the antilogarithm of the slope was 0.509.
[c] The correlation coefficient for these cultures was −0.986, and the antilogarithm of the slope was 0.512.
[d] These proportions were calculated for stalks with the number of bands indicated; the numbers of such stalks in PYE, P-adequate HiGg, and low-P HiGg media were 1,316, 409, and 160, respectively.

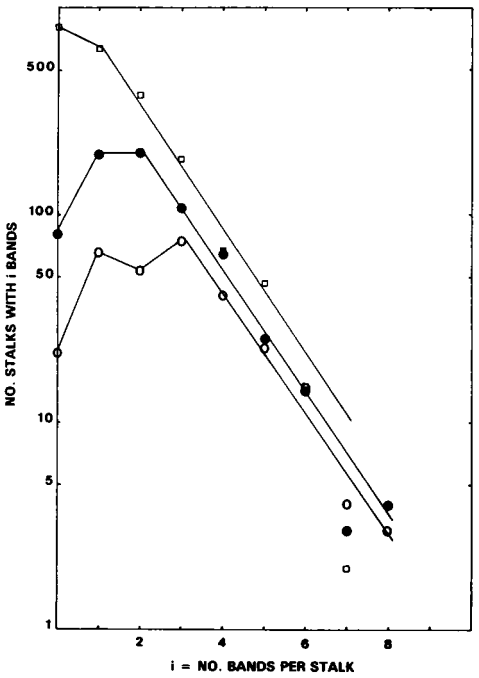

FIG. 4. Frequency of stalks with i bands in finite cultures of *C. crescentus* grown in PYE (□), P-adequate HiGg (●), and low-P HiGg (○). Lines represent the exponential regressions calculated as indicated in Table 1.

that arose from fission as swarmers (Fig. 4); mature stalks were interpreted as those whose proportions decreased exponentially as a function of the number of bands per stalk. Among mature stalks, stalks possessing i bands regularly accounted for twice the number of stalks possessing $i + 1$ bands, through at least five generations. Stalks with seven or more bands accounted for <1% of mature stalks and were not dependably encountered for enumeration in media with adequate levels of P. The antilogarithm of the slope of the exponential regression of number of stalks with i bands, as a function of i, is a direct expression of the relative proportion of each succeeding band number class within a population; according to the prediction, the slope should be 0.5 ($= 2^{-1}$). The slopes were 0.500, 0.509, and 0.512 for the PYE-grown, P-adequate HiGg-grown, and low-P HiGg-grown populations, respectively.

Rationale and interpretation of perpetual cultures of *C. crescentus*. The analysis of finite cultures was straightforward because all the products of cell division remained in the culture and could be accounted for and included in this analysis; stalks accumulated and aged (and added bands) as the cells continued to reproduce and the population sizes and densities increased exponentially. However, a population cannot expand indefinitely in an environment that provides only finite resources and the number of generations produced under constant conditions is limited. In contrast, constant-volume perpetual cultures operated as chemostats achieve a steady state that can be maintained for an indefinite number of generations in the same environment. Further, as long as the average cell cycle time (t_c) of the organism matches the hydraulic half time (t_h) of the system (see Materials and Methods), a single culture can be perpetuated through a succession of changes, achieving a steady state under each set of environmental conditions. Chemostat cultures, including some individual and some sequential populations, provided populations for our analysis of stalk band addition during stalk development under a variety of constant conditions.

As with finite cultures, populations cultivated with phosphorus as the sole limiting nutrient presented a class of data different from those populations for which carbon, nitrogen, or all three nutrients simultaneously limited the growth yield (population density) of cultures. The imposed reproductive rate (t_h) varied from 2.7 to 16.6 h (R varied from 4 to 24 h), but variation in reproductive rate did not affect the proportions. Accordingly, enumerations were gathered into two sets of like data, one set from 16 P-adequate populations and one set from 11 P-limited populations. Each cell was also scored for the presence of a constriction indicating that it was in the process of fission, thus allowing the data for dividing and nondividing cells to be sorted and analyzed separately within each set of like data. Constricted cells were not encountered among the 1,498 nonstalked cells examined from these cultures; all 866 constricted cells were stalked. The results of enumeration are presented in Table 2 and Fig. 5.

The data in Fig. 5 are plotted on semilogarithmic coordinates, with the logarithm of the number of stalks with i bands plotted as a function of the number of bands per stalk (i). The antilogarithms of the slopes were 0.58 for P-adequate cells and 0.63 for P-limited cells. (The relatively low correlation coefficient [−0.972] for P-limited stalks becomes −0.994 when the number of stalks is calculated as a function of the number of pairs of bands per stalk, but the slope remains 0.63; the low correlation coefficient seemed to be attributable to the difficulty of enumerating bands in the very long multibanded stalks of P-limited cells [Fig. 2].)

The antilogarithm of each slope would equal the proportion of each succeeding class of stalks that was derived from the preceding class of stalks, i.e., the proportion of each succeeding generation of stalked cells derived from the preceding generation's S sibs. Accordingly, in populations whose growth was not P limited, about 58% of the S sibs reproduced in the growth vessel; the remainder of divisions, about 42%, would be accounted for by the divisions of F sibs that had not been removed by the time they completed their longer cell cycles. In P-limited populations, about 63% of the S sibs reproduced in the growth vessel and only about 37% of the F sibs did so.

By substituting these proportions in equation 4 (see Materials and Methods), the relative durations of the cycle times of the two types of siblings could be calculated as follows. For P-adequate cells, $1 = 0.42 + 0.58$, $2^{-t_f/t_h} = 0.42$, $t_f/t_h = 1.25$, $2^{-t_s/t_h} = 0.58$, $t_s/t_h = 0.79$, and $t_f/t_s = 1.58$. For P-limited cells, $1 = 0.37 + 0.63$, $2^{-t_f/t_h} = 0.37$, $t_f/t_h = 1.43$, $2^{-t_s/t_h} = 0.63$, $t_s/t_h = 0.67$, and $t_f/t_s = 2.13$. For comparison, if t_f/t_s was a constant 1.33, as in microcultures in PYE (17), the values of $2^{-t_f/t_h}$ and $2^{-t_s/t_h}$ would be 0.45 and 0.55, respectively. Therefore, the slopes imply that $t_f > 1.33 \cdot t_s$ in nutrient-flux-limited cultures and that the difference between t_f and t_s is considerably greater when the nutrient whose flux is limiting is phosphorus.

Analysis of finite cultures of *A. biprosthecum*. Pate and coworkers (15, 16) reported the isolation of an unusual kind of caulobacter which they called *A. biprosthecum*. Cell division is asymmetric, as in other caulobacters, producing a monoflagellate swarmer and a prosthecate sibling. In *A. biprosthecum*, the development of a prostheca is lateral and the typical prosthecate cell bears two prosthecae with their long axes perpendicular to the long axis of the rod-shaped cell. Even very early stages of outgrowth can be detected at two sites on a single cell (Fig. 6). Sometimes called pseudostalks (15, 31) because they lack the adhesiveness of *Caulobacter* stalks (17), *Asticcacaulis* prosthecae are otherwise identical in structure to *C. crescentus*

TABLE 2. Enumeration of bands in stalks of *C. crescentus* cells grown in perpetual cultures

No. of bands/stalk	P-adequate medium						P-limited medium					
	Nondividing cells		Dividing cells		All cells[a]		Nondividing cells		Dividing cells		All cells[b]	
	No.	Proportion	No.	Proportion	No.	Proportion[c]	No.	Proportion	No.	Proportion	No.	Proportion[c]
0	403	0.185	12	0.017	415		162	0.157	1	0.006	163	
1	469	0.215	94	0.133	563		221	0.214	13	0.081	234	
2	501	0.230	208	0.294	709		197	0.191	39	0.242	236	
3	344	0.158	179	0.253	523	0.439	155	0.150	29	0.180	184	0.339
4	188	0.086	91	0.129	279	0.234	120	0.116	31	0.193	151	0.279
5	122	0.056	53	0.075	175	0.147	63	0.061	13	0.081	76	0.140
6	66	0.030	36	0.051	102	0.086	54	0.052	16	0.099	70	0.129
7	40	0.018	19	0.027	59	0.050	23	0.022	7	0.043	30	0.055
8	26	0.012	7	0.010	33	0.028	11	0.011	3	0.019	14	0.026
9	15	0.007	5	0.007	20	0.017	14	0.014	3	0.019	17	0.031
10	6	0.003	2	0.003	8		7	0.007	3	0.019	10	
11	3	0.001	0		3		5	0.005	2	0.012	7	
12	0		0		0		0		1	0.006	1	
13	0		1	0.001	1		0		0		0	
Total stalks	2,183		707		2,890		1,032		161		1,193	

[a] The correlation coefficient for these cultures was −1.000, and the antilogarithm of the slope was 0.584.
[b] The correlation coefficient for these cultures was −0.972 (see text for discussion of this exceptionally low correlation coefficient), and the antilogarithm of the slope was 0.632.
[c] These proportions were calculated for stalks with three to nine bands; the numbers of such stalks in P-adequate and P-limited media were 1,191 and 542, respectively.

stalks, including the presence of bands with the same ultrastructure (27).

To examine the hypothesis that stalk band insertion is a regular feature of a caulobacter's life cycle, the bands in the stalks of *A. biprosthecum* cells growing exponentially in PYE medium were enumerated. Only a small sample (40 cells) was examined (Table 3). Among these, 35 were prosthecate cells. Of these 35 cells, 31 bore two prosthecae, with zero to nine bands per stalk. In 23 cells, the number of bands was the same in each stalk; in 7 cells, one stalk had one more band than the other stalk; and in the 31st cell, the difference was two. Thus, 30 of 31 (97%) prostheca pairs contained the same number of bands or the number differed by only one. It seems reasonable to conclude that band addition in *A. biprosthecum* is a regular feature of its cell cycle and may be a simultaneous event in its twin prosthecae.

As the *A. biprosthecum* cell elongates during growth and then divides across its long axis, it may eventually lose one or both prosthecae (16). Four of the 35 prosthecate cells recorded bore only one prostheca each; two of these prosthecae possessed 8 bands, and two of them 11 bands. This is consistent with older stalks (i) possessing larger numbers of bands and (ii) being more susceptible to loss during a previous cell fission.

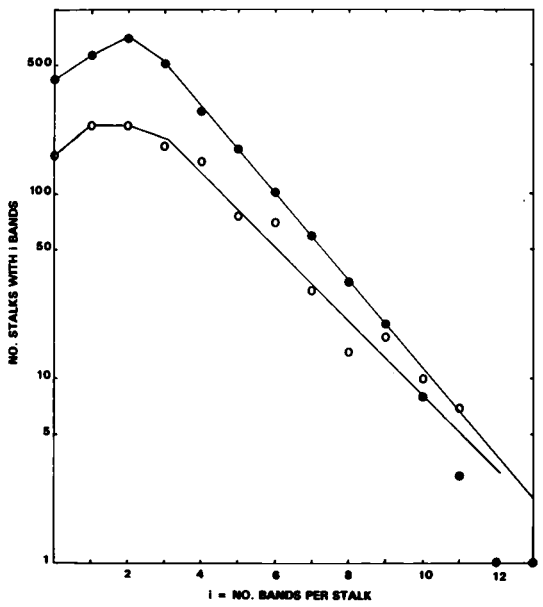

FIG. 5. Frequency of stalks with *i* bands in perpetual cultures of *C. crescentus* grown in HiGg media. ●, non-phosphate-limited cultures; ○, phosphate-limited cultures. Lines represent the exponential regressions calculated as indicated in Table 2.

DISCUSSION

A statistical analysis of 7,228 stalked cells has revealed that the number of bands in a *C. crescentus* cell's stalk reflects the number of reproductive cycles completed by that cell since its inception as a swarmer sibling. This is clearly evident among stalks possessing three or more bands, regardless of whether cells have been cultivated in complex or defined media, media containing adequate phosphorus relative to other nutrients, or media in which growth was P limited, or whether the growth system was closed (finite cultures) or open (perpetual cultures). Therefore, (i) there were half as many mature stalks with $i + 1$ bands as mature stalks with i bands in finite-culture populations (Fig. 4) and (ii) the proportion of nondividing cells with i bands was roughly equal to the proportion of dividing cells with $i + 1$ bands in perpetual cultures (Table 2).

Events in the first generation, during which a swarmer cell sheds its flagellum and initiates the development of its stalk, were less regular. On the assumptions that (i) swarmer cycle time in finite cultures during exponential phase would be 1.33 times the stalked-cell cycle time, as it is in microcultures and synchronous cultures, and (ii) stalks do not lose bands, first-generation stalks would account for 37.5% (= 0.50/1.33) of the stalks with the fewest bands. That proportion of stalks possessed zero bands in PYE-grown cells, zero or one band in

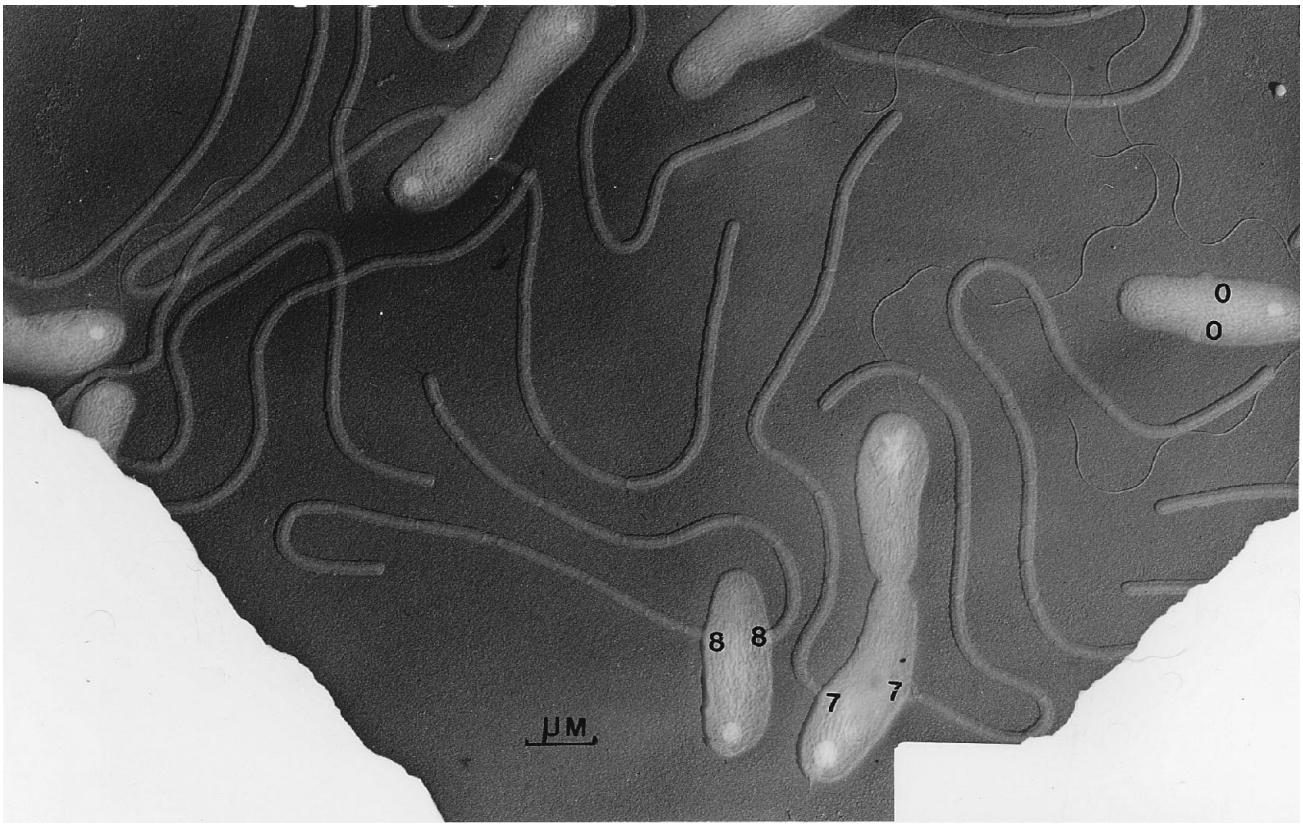

FIG. 6. *A. biprosthecum* cells from a finite culture in PYE medium. The number at the base of each prostheca indicates the number of bands in that prostheca.

P-adequate HiGg-grown cells and included 92% of the one-banded stalks; and zero, one, or two bands in low-P HiGg-grown cells and included 46% of the two-banded stalks. As is evident in Fig. 4, the proportions of those stalks in each medium did not fall in the same class of data (regarded here as enumerations of mature stalks) that generated the exponential decline of proportion at the rate of 50% per added band.

TABLE 3. Enumeration of bands in prosthecae of *A. biprosthecum*

No. of bands/prostheca in twin prosthecae		No. of cells (n = 41)
First prostheca	Second prostheca	
None[a]	None	6
0	0	7
1	1	3
2	2	1
3	3	2
4	4	0
5	5	1
6	6	5
7	7	1
8	8	2
9	9	1
1	3	1
2	3	1
5	6	1
6	7	3
7	8	2
8	None	2
11	None	2

[a] Refers to the prostheca, not the number of bands.

In perpetually cultivated populations, the differences in band number distribution for nondividing and dividing cells implied that first-generation stalks possessed zero, one, or two bands. Among unconstricted cells, the proportion (35 to 40%; three-fourths of one-half of the stalks) of stalks that were first generation was accounted for by stalks with zero or one band. Among constricted cells, only 1.5% lacked bands and only 12% possessed only one band; the 35 to 40% with the fewest bands was accounted for almost entirely by two-banded stalks plus, in P-limited populations, a fraction of the three-banded stalks. Evidently, the greater the severity of phosphorus limitation relative to that of other nutrients, the greater the number of bands added in the first generation. This was expected, because swarmers provided with all growth nutrients except phosphate develop very long stalks with as many as four bands, although fission does not occur at all in such populations (19).

Staley and Jordan (32) concluded that the addition of a band occurred during or shortly after cell division. The statistical analysis reported here implies even more narrowly that in reproducing populations, the new band is added while the cell is constricted. Whether during the cell's first fission that band is the first band appears to vary with the nutritional environment.

From the conclusion that at least after the swarmer cycle, one band is added per cell cycle, band addition joins the cascade of gene expression related to motility (reviewed in references 14 and 28) as a regular, endogenously regulated event in the caulobacter cell cycle. The marked similarity of band ul-

trastructure to that of flagellar plates suggests further that bands may be a part of that same regulatory system. If the dividing caulobacter cell prepared a flagellar plate for each pole, one plate would be associated with the new flagellum, but the other would be assembled at the stalked pole and be carried out as a stalk band during subsequent stalk extension. One implication of this interpretation is that among flagellated but nonmotile mutants, there could be some whose motility fails because of the lack of a flagellar plate; such mutants should also lack stalk bands. It was not determined whether the *flbT* mutant that appeared to initiate stalk development more than once at a given pole was unusual in its placement of bands (5).

Our analysis of bands in the prosthecae of *A. biprosthecum* was cursory and involved only one population. Nevertheless, it is clear that the typical cell of this species bears two prosthecae with equal numbers of bands. This unusual organism, which has been isolated only once, produces flagellar-plate-like structures (i.e., stalk bands) for each of its twin prosthecae, possibly once each cell cycle, yet bears only a single, subpolar flagellum in its swarmer stage. If *C. crescentus* mistakes its stalked pole for a potentially flagellated pole, *A. biprosthecum* compounds the developmental error in number and in location on the cell surface but nevertheless probably reveals its age through its bands. In contrast, bands do not appear at all in stalks of *Prosthecobacter fusiformis*, whose cell cycle lacks a flagellated stage (3, 31).

In both finite and perpetual cultures, the marked influence of phosphorus availability on caulobacter morphogenesis was evident. Schmidt and coworkers (7, 26) showed that high levels of P_i (the P source used in HiGg media) reduced the average stalk length in both finite and perpetual cultures. This effect was later attributed to the dependence of stalk development on calcium availability (20), which is reduced in high-phosphate media, presumably because of the inability of cells to utilize calcium phosphate. Ca-limited perpetual cultures were not included in the analysis reported here because the cell morphology was so highly irregular, stalk development was so minimal, and reproduction was so erratic that steady states were difficult to sustain (22a).

Longer stalks are also associated with an increased capacity for phosphate acquisition (20, 21). A relatively restricted physiologic capacity for phosphate acquisition by the cell prior to development and maturation of its stalk could account for the greater difference in swarmer cycle time relative to stalked-cell cycle time in P-limited perpetual cultures than in P-adequate perpetual cultures.

The occurrence of reproduction by binary fission vis-à-vis the linear output of cells from perpetual cultures has seemed paradoxical because both fission products are viable and the population (like all populations of reproducing units) should be capable of exponential expansion. The seeming paradox is reconciled by the concept that a steady-state population comprises a large number of age cohorts, with each cohort experiencing periodically two opposing processes (Fig. 3) that change its size: waves of fission that suddenly double the cohort's size, and the constant removal of cells by hydraulic flow that reduces the cohort's size exponentially between waves of fission. In an adequately asynchronous population, waves of fission follow so regularly upon each other and successive cohorts are so close in age that the population appears to be constant in size while it simultaneously reproduces by binary fission and yields cells to the effluent at a constant, linear rate. Our analysis of stalk band distribution in caulobacters growing in perpetual cultures was based on this concept.

This concept could be applied to an analysis of natural populations of caulobacters. Determination of the stalk band number distribution could reveal whether selective loss of an age group occurred during a population decline. With modification, the concept and the equation could be employed in an analysis of a competition between species in perpetual cultures. It could also explain the dynamics of so-called quiescent (13) microbial populations, those naturally occurring populations that do not expand although they are demonstrably capable of transformations of environmental substances and of reproduction by binary fission. Such populations are probably subject to constant attrition by viruses, predators, starvation, and adverse abiotic factors, such as radiation, desiccation, oxygen damage, and fluctuations in temperature and pH, just as populations in perpetual cultures operated as chemostats or turbidostats are subject to the constant attrition of outflow from the growth vessel. Nevertheless, like perpetual cultures, active natural populations could persist at a fairly constant size by balancing asynchronous or intermittent binary fission against environmental attrition.

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