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Stalk Formation and Its Inhibition in *Caulobacter crescentus*

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Estimates of average rates of stalk formation over several generations of growth in *Caulobacter crescentus* showed that long-stalked Skl mutant and phosphate-starved wild-type cultures produce stalk material at about twice the rate of wild-type *C. crescentus* grown with adequate nutrients. Thus, the long stalks of Skl or phosphate-starved caulobacters are not merely a function of their longer doubling times. Inhibition of cell division of Skl 418 with mitomycin C (MC) caused production of cellular filaments and resulted in inhibition of stalk formation. There was no appreciable decrease in total cell mass or in rates of ribonucleic acid and protein synthesis in the MC-treated cultures as compared with controls, but stalk formation, which is normally dependent on these processes, was severely retarded. Average stalk lengths in MC-treated Skl cultures were 30% of those found in control cultures. MC-produced cellular filaments were also subjected to deoxyribonucleic acid analysis and ultrastructural examination. The deoxyribonucleic acid content of MC-treated bacteria was about 50 to 60% that of untreated bacteria. Hydroxyurea also was found to produce some cellular filaments and shorter stalks, but with accompanying decreases in growth rate and yield.

Caulobacter crescentus, a vibrioid bacterium, produces an extension of the cell known as a stalk or prostheca (10, 13). As shown by Stove and Stanier (15) and Poindexter (7), a vegetative population is a mixture of nonmotile stalked cells and motile nonstalked swarmers. Division occurs by transverse binary fission of a stalked cell, giving rise to a basal daughter bacterium which retains the stalk of the parent and an apical swarmer which is motile by means of a single polar flagellum. These events take place prior to the next division.

In nature and in very dilute media, a caulobacter may possess a stalk with a length of 20 μm or more (7). However, in pure batch cultures with adequate nutrients, the stalks are predominantly short, usually not exceeding 3 μm in length. The stalk of *C. crescentus* can attain various lengths, depending on the nutritional conditions, the number of generations a particular bacterium has possessed its stalk, and the state of the regulatory mechanism of stalk formation in a given strain. Phosphate starvation causes extreme stalk elongation to occur in wild-type *C. crescentus* (12). A caulobacter that has formed its stalk early in the course of growth in a batch culture will have a somewhat longer stalk than those stalked cells originating later in the culture because some stalk formation continues with each generation of that bacterium (14). However, due to the geometric increase in the numbers of bacteria, stalked cells with "older" stalks are relatively rare. Mutants (termed Skl strains) have been found which produce long stalks in complete nutrient medium, comparable in length to those of the phosphate-starved wild type (9). The change in regulation of stalk formation in the Skl mutants and in phosphate-limited wild type is related to changes in the intracellular pool levels of nucleoside triphosphates (11) and cyclic guanosine 3',5'-monophosphate. In morphogenesis of the *Caulobacter* swarmer cell, initiation of stalk formation appears to be an obligatory event which must occur before cell division can take place. We have become interested in the mechanism of control of stalk formation and its relationship to cell division. This problem was approached by inhibiting cell division with metabolic inhibitors and then determining the effect on continued stalk formation in the long-stalked mutant, Skl 418. Treatment of Skl 418 with mitomycin C (MC) demonstrated that continued stalk synthesis was limited by inhibition of cell division. We have also determined that the enhanced stalk formation observed in the Skl mutant is not merely a function of its longer generation time, but that the Skl mutant also has a more rapid rate of stalk formation.

MATERIALS AND METHODS

Organisms. *C. crescentus* strain CB15 (ATCC 19089) and Sk1 mutant strain 418 were used.

Media and growth conditions. Cultivation was performed in either a complex medium (PYE) or a synthetic medium (G-I) as described earlier (9). Liquid cultures were incubated at 30 C on a rotary shaker bath (New Brunswick Scientific, model G76) at 130 rpm. Growth was measured turbidimetrically using a Klett-Summerson photoelectric colorimeter with a no. 66 filter.

Separation of swarmers from stalked bacteria. In determining stalk formation rates and in testing the effects of inhibitors on stalk formation, relatively homogeneous populations of swarmers were needed to initiate the experiments. The centrifugal separation method of Stove and Stanier (15) was used. Swarmer bacteria were obtained by centrifugation at 1,517 × *g* using a Sorval XL centrifuge or at 2,590 × *g* in a Sorval SS-1 for 7 min at room temperature. Four washings with distilled water were used to obtain populations of swarmers that were at least 90% free of stalked bacteria.

Estimation of stalk elongation rates. For batch cultures, swarmer cell inocula, of 93 to 95% homogeneity, were used. When a known number of doublings (generations) had occurred, samples were taken and inactivated with 30% Formalin (0.1 ml/ml of sample), washed with distilled water, and sedimented at 20,000 × *g* in a Sorval RC2-B refrigerated centrifuge. The bacteria were then prepared for electron microscopy by a negative staining method, using 1% potassium phosphotungstate, pH 7.0 (1). Care was taken to prepare samples so that long-stalked bacteria would not be eliminated from the preparations. The reproducibility of the data depended on observing enough bacteria, consistent with the number of generations that had occurred in the culture, so that bacteria with the longest stalks, presumably originating from the first generation, could be evaluated. Generally, several thousand bacterial cells were scanned with a Philips 100B electron microscope and micrographs of several hundred, including ones with notably longer stalks, were taken at a magnification of ×1,000, suitable for measuring stalk length. Stalk lengths of those bacteria with the longest stalks (occurring at a frequency consistent with the number of doublings of the culture) were divided by the number of doublings or by the number of hours incubated in culture for that number of doublings to have occurred. For each set of conditions, repeated experiments and electron microscopy determinations were done to establish the reproducibility of the method. The frequency of residual stalked bacteria and their stalk lengths, occurring in the swarmer inoculum, were also determined and used as correction factors in the calculations.

In continuous culture experiments, a New Brunswick Bioflo (model C30) was used. Inocula were swarmers of *C. crescentus* strains. Medium feed rates were adjusted to equal 50% of the critical dilution rate values, since, at dilution rates greater than a critical value, steady-state conditions would not be maintained (16). Samples were usually taken before the cultures reached equilibrium, because only a limited number of doublings could be reasonably evaluated for estimations of stalk formation rates. For example, in an experiment involving eight doublings, one would be seeking long-stalked cells occurring at a frequency of one in 256. The continuous culture apparatus allowed evaluations of samples from longer-term exponential-phase cultures (i.e., more doublings) than the batch culture method. Stalk lengths and average rates of stalk formation were determined as for the batch cultures.

Chemicals. A stock solution of nalidixic acid (NAL) (Calbiochem) containing 5 mg/ml was prepared as described by Kantor and Deering (5). Stock solutions of hydroxyurea (HU) (Sigma) containing 14 mg/ml were prepared fresh daily and sterilized by filtration through a membrane filter (type HA, 0.45 μm, Millipore Corp.). MC was obtained from Calbiochem and Schwartz/Mann, and stock solutions were prepared by dissolving a quantity of inhibitor in sterile glass-distilled water.

Determination of filament-induction concentration in Sk1 418. The Sk1 418 strain was exposed to several concentrations of each specific inhibitor in G-I or PYE media to determine the concentration necessary to induce cellular filament formation. The concentrations of the antimetabolites which resulted in cellular filament formation as determined by direct microscope examination were used in subsequent experiments on stalk elongation. Filaments are here defined as those bacterial cells, excluding stalks, exceeding 5 μm in length.

The inhibitors MC, NAL, and HU were used to produce cellular filament formation and examine the effect of inhibition of cell division on stalk formation. The length of stalks of Sk1 418 after treatment with inhibitors and in control populations was determined using a Zeiss WL phase-contrast microscope equipped with a calibrated ocular micrometer. The stalks of 100 treated bacteria and those of 50 filamentous cells were measured at each concentration of inhibitor after 24 and 48 h of exposure. The lengths of the filamentous cells were also determined.

Measurement of macromolecular syntheses. The incorporation of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) (New England Nuclear), [2-^{14}C]uridine (51.1 mCi/mmol) (New England Nuclear), L[^{14}C]leucine (240 mCi/mmol [Tracerlab]), or L[2,3-^{3}H]proline (45.7 Ci/mmol) (New England Nuclear) into cold trichloroacetic acid-insoluble material was used for measurement of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis, respectively, in Sk1 418. After two serial 24-h preincubations with unlabeled precursor (0.24 mg/ml), the bacteria were washed two times with distilled water, resuspended in G-I medium, and incubated with 0.25 or 0.5 μg of MC per ml and an appropriate concentration of the labeled precursor. A control, lacking MC, was included in each experiment. The initial cell densities of the MC-exposed and control cultures were the same; the concentrations of MC used in these experiments did not appreciably affect the rate of increase in turbidity or the final dry weight yields. Samples (1 ml) of the labeled cultures were removed at specified

intervals over a 24-h incubation period, placed in an equal volume of ice-cold 10% trichloroacetic acid with 100 μg of unlabeled precursor and allowed to stand in an ice bath for at least 30 min. The cold trichloroacetic acid-insoluble fraction was collected on membrane filters (type HA, 0.45 μm, Millipore Corp.) which had been gently boiled for 10 min in 0.1 M unlabeled precursor. The collected fraction was washed twice with 5 ml of ice cold 5% trichloroacetic acid. The filters were dried and counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3320, using the scintillation fluid Aquasol (New England Nuclear).

DNA determination. DNA was estimated using the diphenylamine reaction (2). Deoxyadenosine (Calbiochem) was used as a standard.

Determination of adenosine triphosphate pool level. Adenosine 5′-triphosphate (ATP) intracellular pool levels in MC-treated and untreated Skl 418 using the luciferin-luciferase reaction were determined by Gay Samuelson as described previously (11).

Electron microscopy. Thin sections of the MC-treated and untreated Skl 418 bacteria were observed on a Philips 300 electron microscope operated at 60 kV; cells were fixed using the method of Ryter and Kellenberger (8).

RESULTS

Estimation of stalk formation rates. Measurements of the longest stalks, in culture samples in which a known number of generations had occurred, showed that Skl 418 and phosphate-starved CB15 carry out stalk formation at a faster average rate than does CB15 grown with adequate nutrients. In Skl 418 and phosphate-starved CB15 the average lengths of stalk synthesized per hour were approximately twice that in PYE-grown CB15 (Table 1). The doubling times of Skl 418 and phosphate-starved CB15, which are longer than that of CB15 with adequate nutrients, also contributed to the stalk length, since more time is presumably available during each longer generation period for stalk synthesis.

In a second series of experiments done in continuous cultures, the effect of limiting organic nutrients (PYE medium diluted 1:10 which would then contain 0.02% peptone and 0.01% yeast extract) with and without added inorganic orthophosphate was examined. Adequate trace elements and combined nitrogen were supplied in all experiments. PYE medium diluted 1:10 contains 0.03 mM total phosphate, and this limiting amount in a synthetic medium is known to stimulate stalk formation (12). The rate of stalk formation of CB15 in the dilute PYE medium without added phosphate, and with a doubling time of 11 h, was 0.14 μm/h. In a dilute PYE medium with added orthophosphate (1 mM) and a doubling time of 10 h, the average rate of stalk formation was 0.065 μm/h. This suggests that a limitation of organic nutrients slows down stalk formation, but even while organic nutrients needed for synthetic processes are in limited supply phosphate starvation continues to have a stimulatory effect on stalk formation.

Effect of metabolic inhibitors on C. crescentus Skl 418. In morphogenesis of caulobacter swarmers, initiation of stalk formation normally must occur before cell division can take place (7). Here a situation which approximates the reciprocal relationship was examined: the effect of failure to complete normal cell division (resulting in cellular filament formation) on the continued stalk formation of Skl 418. After exposure of a swarmer cell inoculum to concentrations of inhibitors found to produce cellular filaments for a 24-h incubation period, the average length of stalks and cellular filaments of Skl 418 treated with metabolic inhibitors and stalk lengths of control bacteria were determined (Table 2). The average stalk length of bacteria treated with 1.0 μg of MC per ml was 27% of that for stalks in the untreated population; with exposure to 0.5 μg/ml, stalk lengths

TABLE 1. *Average stalk formation rates in Caulobacter crescentus*

Strain	Cultural condition	Medium	Doubling time (h)	No. of doublings	Maximum stalk lengths observed (μm)	Average stalk formation rates	
						μm/doubling	μm/h
SK1 418	Batch	PYE	6.5	3.7	7.3	1.96	0.30
SK1 418	Continuous	PYE	6.5	5.5	11.5	2.09	0.32
CB15	Batch	PYE	1.94	4.9	2.0	0.40	0.20
CB15	Continuous	PYE	1.94	8.0	2.3	0.28	0.14
CB15	Batch	G-I[a]	3.0	3.6	1.4	0.41	0.14
CB15	Continuous	G-I[a]	3.0	5.0	2.0	0.40	0.13
CB15	Continuous	G-I[b]	10.5	6.8	23.5	3.46	0.33

[a] G-I: glucose-imidazole synthetic medium with 1 mM inorganic orthophosphate.
[b] Synthetic medium with 0.01 mM inorganic orthophosphate.

that were 30% of those observed in control bacteria resulted.

The exposure of Sk1 418 to HU resulted in formation of filaments in 60 to 77% of the bacteria; stalk formation also was severely inhibited. In the population treated with 1.4 mg of HU per ml, the average stalk length was 6.4% of the controls, and with 0.7 mg/ml the stalks were 13% of the control population length.

The antibiotic nalidixic acid formed aberrant morphological cells, and few filaments were observed after exposing swarmer bacteria of Sk1 418 to various concentrations and times of exposure.

Growth yields of populations of Sk1 418 exposed to inhibitor and controls were compared (Table 3). Although HU caused notable cellular filament production and inhibition of stalk formation, the total cell mass was drastically reduced, and no inferences on the specific mechanism of inhibition of stalk formation in HU-treated cells could be made. With MC, the 0.5 μg/ml concentration gave growth yields comparable to the control.

Determination of macromolecular syntheses. Several antibiotics known to inhibit protein or RNA synthesis have been found to inhibit the initiation of stalk formation in *C. crescentus*: rifampin (6), actinomycin D, puromycin, streptomycin, and chloramphenicol (J. Schmidt, Ph.D. thesis, University of California, Berkeley, 1965; E. Haars, Ph.D. thesis, Arizona State University, Tempe, 1972). It is likely that RNA and protein synthesis are required for stalk formation. We measured the rates of macromolecular syntheses in MC-inhibited and control cultures to determine if differences that could be related to the inhibition of stalk formation occurred. The final turbidities of the populations were essentially the same in all experiments, which measured the uptake of ^{14}C- and ^{3}H-labeled precursors into cold trichloroacetic acid-insoluble cell constituents. De novo RNA synthesis was measured by the rate of incorporation of [^{14}C]uridine in MC-treated and control cultures of Sk1 418 (Fig. 1). After 24 h of exposure to

TABLE 2. *Effect of metabolic inhibitors on stalk length of Sk1 418*[a]

Metabolic inhibitor	Concn	Avg Stalk length (μm)	Avg filament length (μm)	Filaments in cell population (%)
Hydroxyurea	1.4 mg/ml	0.49	6.91	77
	0.7 mg/ml	1.09	6.41	60
Mitomycin C	1.0 μg/ml	2.01	11.61	55
	0.5 μg/ml	2.26	10.92	46
Control		7.56		

[a] Grown in G-I or PYE medium at 30 C for 24 h.

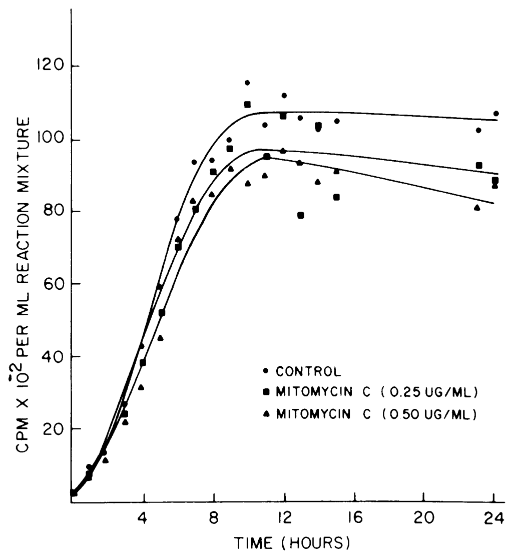

FIG. 1. *Incorporation of [^{14}C]uridine by mitomycin C-treated and control C. crescentus Sk1 418 cultures.*

TABLE 3. *Comparison of growth yield in inhibited and control Sk1 418 cultures*

Determination	Mitomycin C				Hydroxyurea			
	1.0 μg/ml		0.5 μg/ml		1.4 mg/ml		0.7 mg/ml	
	Actual reading	% of control	Actual reading	% of control	Actual reading	% of control	Actual reading	% of control
Turbidity[a]:								
treated	73	75	100	95	30	23	46	37
control	105		105		125		125	
Dry wt[b]:								
treated	1.91	65	2.64	90	0.45	20	0.72	32
control	2.94		2.94		2.23		2.23	

[a] Actual reading of turbidity is given in Klett units.
[b] Dry weight is given in milligrams per milliliter.

MC, Skl 418 had incorporated about 82% as much uridine as found in the control. The rates of uridine incorporation during active RNA synthesis of the populations appeared to be similar. Protein synthesis was determined by measuring the rate of incorporation of [^{14}C]leucine (Fig. 2). The incorporation of leucine increased for the first 5 h and then leveled off in both control and MC-treated populations, indicating no further net protein synthesis. Protein synthesis in MC-treated and control cultures was essentially the same. Protein and RNA syntheses in MC-treated and control cultures were followed concomitantly with the incorporation of [^{14}C]uridine and [^{3}H]proline. Again in this experiment, the incorporation of labeled precursors into RNA and protein in MC-treated and control bacteria was essentially the same.

Figure 3 illustrates the rate of incorporation of [^{3}H]thymidine as a measure of DNA synthesis. While initial incorporation in MC-treated bacteria approached that of the control, the final amount of radioactivity in the cold trichloroacetic acid-insoluble cell constituents at 24 h in the MC-treated population was 80 to 88% of that observed in the control.

DNA determination. A comparison of MC-inhibited and control Skl 418 cultures was made with respect to their DNA content using the diphenylamine reaction (Fig. 4). The DNA content based on dry weight determination of MC-treated Skl 418 (0.25 μg of MC per ml) after 24 h was found to be 85% of that of the control cells, whereas cells treated with 0.5 μg of MC per ml contained 51% of the amount of DNA present in the control populations. When the DNA contents were compared on a basis of direct cell counts (Fig. 5), it was found that after 4 h the DNA content was essentially the

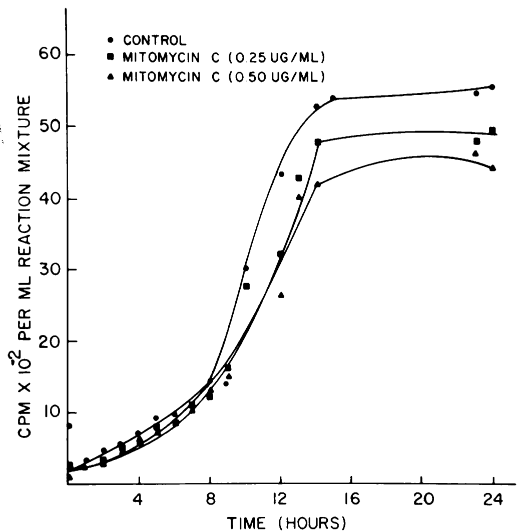

FIG. 3. *Incorporation of [^{3}H]thymidine by mitomycin C-treated and control Skl 418 cultures.*

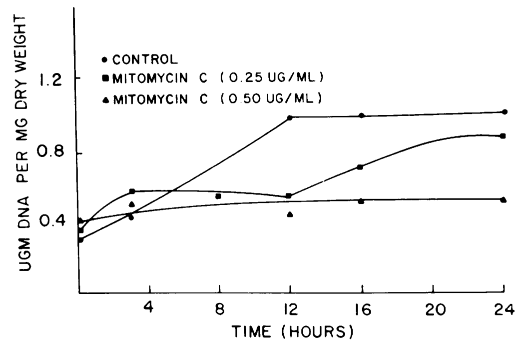

FIG. 4. *Deoxyribonucleic acid content of mitomycin C-treated and control Skl 418, compared on the basis of dry weight determinations.*

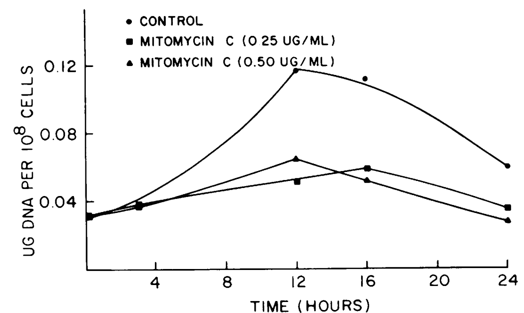

FIG. 5. *Deoxyribonucleic acid content of mitomycin C-treated and control Skl 418, compared on the basis of direct cell counts.*

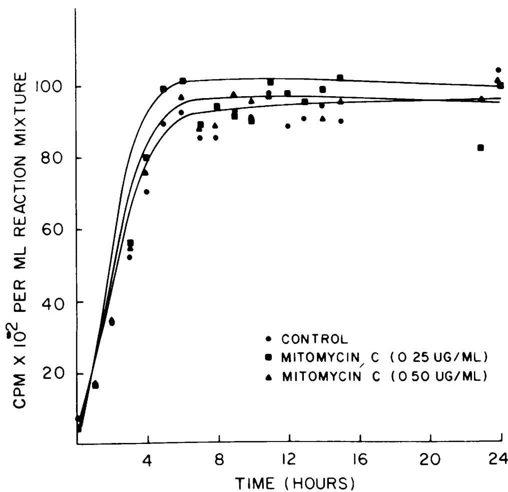

FIG. 2. *Incorporation of [^{14}C]leucine by mitomycin C-treated and control Skl 418 cultures.*

same in the MC-treated and untreated population, but after 24 h, the MC-treated filaments, which are about three to four times longer than a normal Sk1 bacterial cell, contained about 46 to 60% of the DNA in untreated bacteria. No lysis of the MC-treated cultures was observed.

Electron microscopy of MC-treated Sk1 418. In comparison with the untreated control (Fig. 6A), thin sections of MC-treated Sk1 418 showed more extensive development of mesosomes (Fig. 6 B and C). Mesosomes were often located at intervals along the filamentous cells

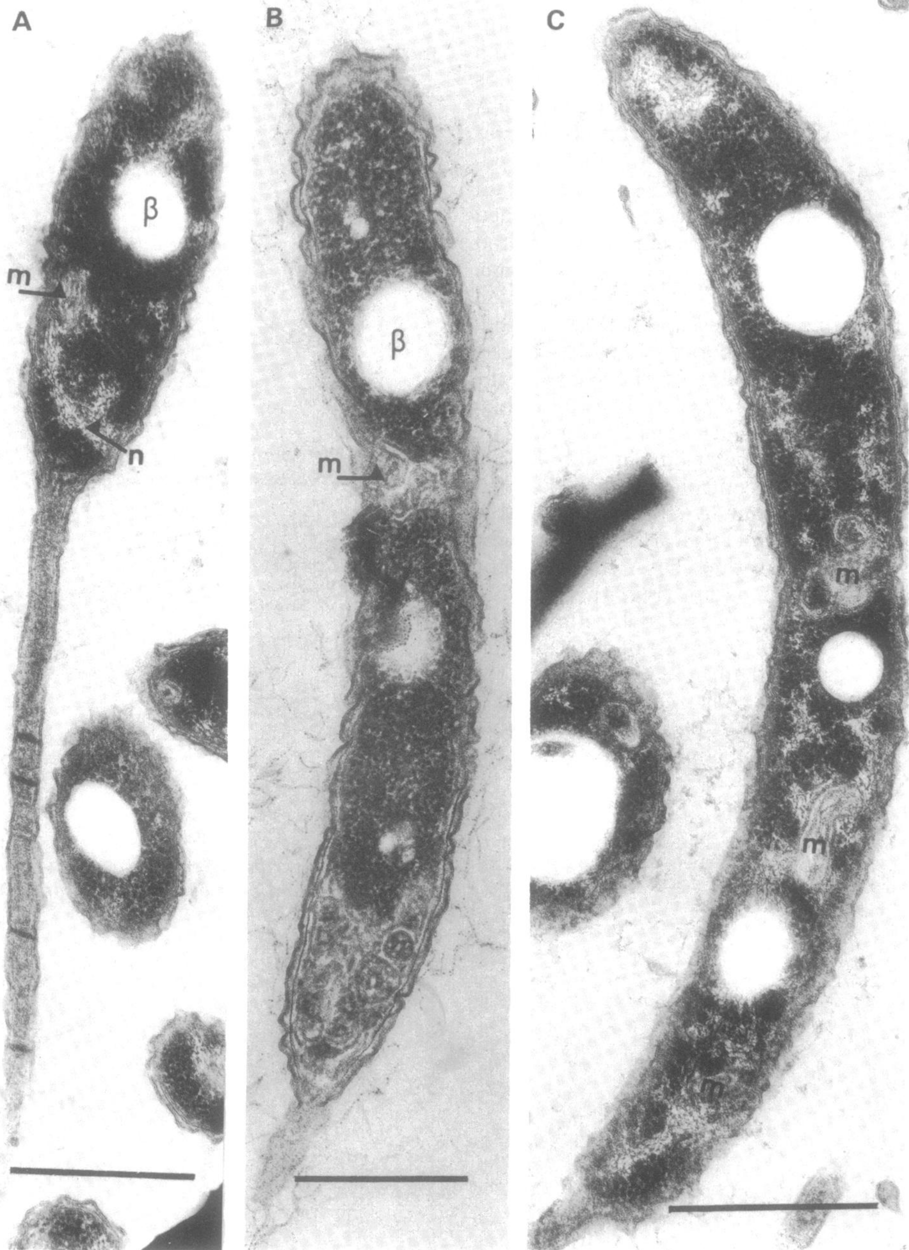

FIG. 6. *Ultrastructure of Sk1 418. Abbreviations: M, mesosome; β, poly-beta-hydroxybutyrate deposit; N, nucleoplasm. (A) Control. (B) Mitomycin C-induced filament. (C) Mitomycin C-induced filament with several mesosomes. The bars represent 0.5 μm.*

that would be consistent with normal Skl 418 cell lengths; however regions in the MC-treated bacteria where cell division events appeared to be in progress were not often observed. The dispersion of nucleoplasm in the randomly-sectioned MC-treated bacteria appeared to be more variable than in the control population. In many MC-treated filaments, nucleoplasm was not very evident (Fig. 6B). This could be due to lesser amounts of nucleoplasm per cell in the MC-treated bacteria (as suggested by the data in Fig. 5 and 6), or to an arrangement of nucleoplasm that often left it unobserved without the aid of serial sections. Since the Skl 418 MC-filaments usually had a corkscrew morphology and thus occurred in multiple planes, thin sections revealing a substantial length were uncommon.

Determination of ATP intracellular pool level. The ATP pool levels were determined in MC-treated and control populations (Table 4). The levels of intracellular ATP observed for the MC-treated and control cultures are within the usual range for Skl mutants (11). Much higher levels of intracellular ATP or exogeneously supplied ATP correlated with shorter stalks in *C. crescentus;* during exponential growth the wild-type strain CB15 with its slower rate of stalk formation contained about four times as much ATP as its SK1 418 mutant. Since the MC-treated cells did not contain a significantly elevated amount of intracellular ATP over the control population, it was unlikely that the inhibition of stalk formation in the presence of MC resulted from an accumulation of ATP in the filamentous bacteria.

DISCUSSION

Stalk formation proceeds at a faster average rate in Skl mutant 418 than in its parent strain of *C. crescentus* CB15. The exaggerated stalk formation of Skl 418 facilitated the examination of the effects of cell division inhibition on stalk formation.

The rates of stalk formation estimated here are average values taken over several generations of growth, and do not take into account the possibility that stalk synthesis may be more rapid during the first generation of the bacterium, when the swarmer differentiates into a stalked bacterium, than it is in stalked bacteria which have possessed their stalks for several generations. This is suggested by comparing our data with that of Staley and Jordan (14). Also it has been suggested that stalk formation is discontinuous during a generation period, proceeding for a time and then stopping, only to begin again for that stalked bacterium during its next generation (14). The average hourly rates as determined here may require further refinement to adequately describe the caulobacter's morphogenesis over one generation or through several generations of growth.

TABLE 4. *ATP intracellular pool levels in mitomycin-treated and control Skl 418 cultures*

Time of culture incubation (h)	ATP (pmol/mg dry wt)		
	Mitomycin C treated		Control
	0.25 μg/ml	0.50 μg/ml	
9	646	588	449
24	258	360	218

The stalk formation rates estimated here bring up an interesting question as to why stalk material is synthesized at a more rapid rate in Skl mutant and phosphate-starved wild-type cultures, while their overall growth rates are much slower than the normal wild type. Transcriptions (or translations) involved specifically in stalk formation may be stimulated in these aberrant situations. We know that nucleoside triphosphate pool levels and cyclic guanosine 3′,5′-monophosphate concentrations play a role in the regulation of stalk formation (11), but genetic regions involved in stalk formation have not been identified.

Thymidine has been reported to be an unsatisfactory DNA label in *C. crescentus* (3, 4), but mutant strain Skl 418 used in this study did appear to incorporate it into acid-insoluble cell material. The de novo synthesis of DNA, measured by [^{3}H]thymidine incorporation, showed the MC-treated and untreated bacteria to behave quite similarly. The total amount of DNA in MC-treated bacteria was lower than in control bacteria, however. This suggests that there is some disruption of DNA metabolism and unbalanced growth in the MC-treated cultures. Degnen and Newton (4), using MC at a concentration of 2 μg/ml, have shown that stoppage of chromosome replication in *C. crescentus* resulted in a cessation of cell division. At the lower concentration used by us (0.5 μg/ml), there was formation of cellular filaments and lesser amounts of DNA per cell, but DNA synthesis was only partially inhibited.

With mitomycin C a correlation between inhibition of cell division and inhibition of stalk formation in Skl 418 was found, whereas RNA and protein synthesis approached that of the control culture. The inhibition of stalk formation in the presence of MC may be due to an interrelationship between cell division and the control of stalk formation, or there may be a

similar site of action of MC in its inhibition of both cell division and stalk formation. We speculate that expression of the DNA regions responsible for stalk formation is interfered with in the MC-induced filaments, due to their aberrant DNA content, and as a result stalk formation is discontinued prematurely. In the control Sk1 bacteria, transcription is continued so that formation of the stalk proceeds, faster than in the wild type and as long as the bacterium is viable, resulting in the characteristic long stalk of the Sk1 mutant.

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