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Cell-Cycle-Specific Fluctuation in Cytoplasmic Membrane Composition in Aerobically Grown *Rhodospirillum rubrum*

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Aerobic growth with synchronous cell division was induced in *Rhodospirillum rubrum* by starvation methods. Cells were harvested at different points in the cell cycle. Analysis of the composition of the cell envelope prepared by differential centrifugation or density gradient-purified cytoplasmic membrane obtained from cells at different times indicated that the protein/phospholipid ratio fluctuated with the cell cycle. The protein/phospholipid ratio of cell envelope from selection-synchronized cells also fluctuated with the cell cycle. These studies indicate that the phenomenon of cell-cycle-dependent fluctuation in membrane composition is not restricted to the intracytoplasmic chromatophore membrane of phototrophic cells.

Rhodospirillum rubrum is a purple nonsulfur facultatively phototrophic bacterium of the family *Rhodospirillaceae*. Members of this group, including *R. rubrum* and *Rhodobacter* (formerly *Rhodopseudomonas* [28]) *sphaeroides*, are well suited to studies of membrane structure and biogenesis (15, 17, 30, 42) because the formation of the intracytoplasmic chromatophore membrane (ICM) may be induced by alteration of growth conditions. When grown aerobically, members of this family are morphologically similar to most other gram-negative bacteria (18, 48). Upon removal of oxygen, formation of the ICM is induced. Previous studies have shown that the ICM is physically continuous with the cytoplasmic membrane (CM) (11, 13, 26, 46) and that the ICM is formed by invagination of the CM (17, 23).

Kaplan and co-workers have evaluated ICM formation in synchronously growing cell cultures of *Rhodobacter sphaeroides* (20–22, 37, 50, 51). These investigators have shown that ICM membrane components are inserted in a noncoordinate manner. While proteins are inserted continuously throughout the cell cycle (20), phospholipids are inserted at a specific point in the cell cycle (37). The combination of these phenomena results in a fluctuation in the ratio of protein to phospholipids (21, 37) in the isolated chromatophores and in their buoyant density (21). The fluctuation in ICM composition is also reflected in structural variation (22, 51). We have demonstrated that a similar cell-cycle-specific oscillation in the ratio of protein to phospholipids and buoyant density of ICM occurs in *R. rubrum* (41).

The relationship between cell cycle and membrane composition and structure has not been evaluated in either *R. rubrum* or *Rhodobacter sphaeroides* grown under aerobic conditions. This study of membranes obtained from synchronized *R. rubrum* demonstrates cyclic fluctuation in CM composition.

MATERIALS AND METHODS

Growth of organisms and induction of synchrony. *R. rubrum* S1 was grown in the medium of Ormerod et al. (43) modified by the substitution of 9.5 mM $(NH_4)_2SO_4$ for glutamate and the addition of 0.1% yeast extract (Difco Laboratories, Detroit, Mich.). Cultures of 500 ml in 2,800-ml Fernbach flasks were incubated at 30°C on a New Brunswick Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 300 rpm. Alternatively, cultures were incubated in a VirTis model 43-100 fermentor (VirTis Co., Inc., Gardiner, N.Y.) equipped with a 5-liter culture vessel; agitation was accomplished with dual impellers rotating at 400 to 450 rpm; aeration was provided by pumping compressed air at a rate of 2.5 liters/min through a stainless steel sparger located at the bottom of the fermentor vessel. Culture growth was monitored by optical density at 680 nm (OD_{680}) with a Beckman DU spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.; modified by Update Instruments, Madison, Wis.).

Synchronous cell division was induced by two methods. Phosphate starvation-induced synchrony was achieved by inoculating 1 volume of an asynchronous logarithmic culture (OD_{680}, 0.5 to 0.6) into 50 volumes of medium containing 1/10 (0.91 mM) the normal phosphate level (the amount of phosphate contributed by the yeast extract but not the inoculum was included in this calculation). Despite the low level of phosphate, the medium was adequately buffered, as the pH during growth was the same as that of standard medium. Incubation was continued until the increase in cell mass, while continuing, was no longer exponential (approximately 36 h, during which time the OD_{680} increased to approximately 0.4), at which point phosphate was added to bring the level to that of normal medium (9.1 mM). This induced division synchrony through two division cycles (Fig. 1a).

Synchronously dividing cells were obtained in a similar manner by starving the cells for malate. Malate starvation was achieved by growing the cells in 1/30 the normal malate level (approximately 41 h). After logarithmic growth ceased, malate was added to make the medium 0.57% malate. After an initial drop in cell number and turbidity, growth resumed with one synchronous division (see Fig. 2c).

Synchronously dividing cells were also obtained by a centrifugation selection procedure. An asynchronous logarithmic culture was centrifuged at 3,000 rpm (1,100 × *g*) for 10 min in a Beckman JA-10 rotor. A portion of the resulting supernatant fraction, which contained between 2.5 and 10% of the cells of the original population in repeat trials, was mixed with an equal volume of fresh medium, and incubation was continued. This selected population possessed division synchrony for two subsequent cycles. When an inoculum

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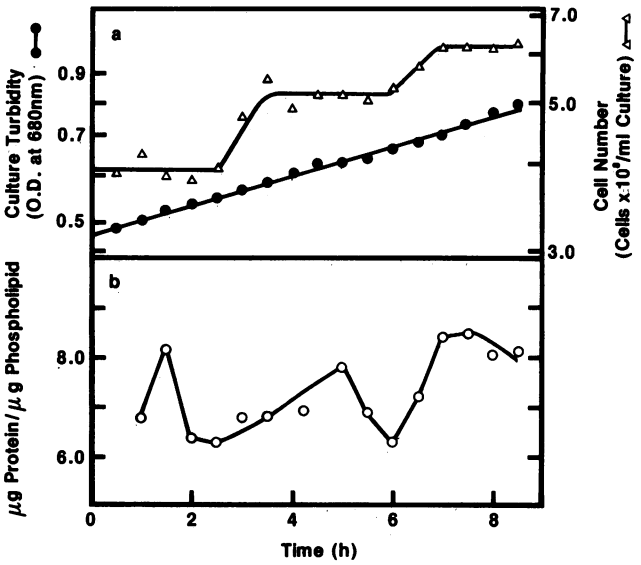

FIG. 1. Changes during phosphate starvation-induced synchronous aerobic growth of *R. rubrum*. (a) increase in cell mass and number. (b) Protein/phospholipid ratio of cell envelope.

consisting of a larger fraction of the population was used, the resulting culture did not exhibit synchronous divisions.

Synchronization of phototrophic cells was accomplished by stationary-phase cycling or high-light/low-light transition as described previously (41).

Cell enumeration and membrane purification. Culture samples were removed at 30-min intervals. Cell counts with a Neubauer hemacytometer and cell length measurements were done as described previously (41). In some experiments, the number of viable cells was determined by serial dilution and plate count. Cell mass was assessed by measurement of OD, as this was shown to be directly related to total cellular protein (41).

Cytoplasmic and outer membranes were purified by a protocol adapted from the method of Collins and Niederman (14). Cells were harvested and washed in 10 mM Tris hydrochloride buffer, pH 8.1. The washed cells were suspended in 25% (wt/wt) sucrose prepared in 10 mM Tris hydrochloride (pH 8.1) in the proportion of 5 g of cells (wet weight) per 24 ml of 25% sucrose. To lyse the cells, the following were added at 15-min intervals: 1/10 volume lysozyme (6.4 mg/ml) (Sigma Chemical Co., St. Louis, Mo.); 1/10 volume disodium EDTA (20 mg/ml); 5.0% (wt/vol) Brij 58 (polyoxyethylene cetyl ether) (Sigma Chemical Co.) to a final concentration of approximately 0.29%; 0.1 M $MgCl_2$ to a final concentration of 12.8 mM; and a few crystals of DNase I (Sigma Chemical Co.). Cell debris and remaining whole cells were removed by sedimentation at 750 × *g* for 10 min. The lysis procedure was performed at room temperature; harvesting and washing of cells and all procedures following lysis were performed at 0 to 4°C.

Cell envelope was obtained from these cell extracts by centrifugation for 90 min at 50,000 rpm (184,000 × *g*) in a Beckman type 70Ti rotor. For purification of CM and outer membrane (OM), the resulting pellets were suspended in and dialyzed against 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5) and layered on linear 35 to 55% (wt/wt) sucrose gradients prepared in the same buffer. The gradients were centrifuged at 25,000 rpm (82,500 × *g*) for 17 h in a Beckman SW27 rotor. OM and crude CM fractions were collected from these gradients, and these membranes were recovered by centrifugation for 90 min at 184,000 × *g*. In some experiments a band of intermediate density was also formed. This band that was hybrid of CM and OM (C. R. Myers, Ph.D. thesis, University of Wisconsin, Milwaukee, 1987) has also been observed by investigators studying other organisms (3, 44). The CM fractions were resolved from contaminating ribosomes by centrifugation in 10 to 35% (wt/wt) sucrose gradients at 82,500 × *g* for 17 h in a Beckman SW27 or SW27.1 rotor. When no hybrid band was formed, 44% of the recovered membrane protein was in the CM fraction. Purified membranes were collected from the gradients and concentrated by centrifugation, followed by dialysis against 10 mM Tris hydrochloride, pH 7, prior to chemical analysis.

Analytical procedures. Membrane protein content was determined by the method of Lowry et al. (36) modified as described previously (12), with bovine serum albumin as the standard. Membrane phospholipids were extracted by the method of Bligh and Dyer (5) as described by Ames (2). Lipid phosphorus determinations were performed as described by Chen et al. (9) on lipid extracts that were wet-ashed by the method of Bartlett (4). Lipid phosphorus values were multiplied by a factor of 25 to calculate the quantity of phospholipid (37). RNA was determined by the method of Almog and Shirey (1) with yeast tRNA (Sigma Chemical Co.) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as adapted from Laemmli (35) as previously described (41).

RESULTS

Stationary-phase cycling and high-light/low-light transition procedures for inducing division synchrony in phototrophically growing *R. rubrum* have been reported (41). The use of high-light/low-light transition would be ineffective in inducing division synchrony in aerobic cells, as growth is chemotrophic. The use of stationary-phase cycling is also inappropriate, as it would not be possible in dense stationary-phase cultures to maintain the level of oxygen adequate to prevent induction (17) of the photosynthetic apparatus. Other synchronization techniques were therefore used for obtaining division synchrony in aerobic cells. The use of phosphate starvation for inducing synchrony in *Escherichia coli* has been reported (24, 31). This technique proved to be suitable for obtaining division synchrony in *R. rubrum*, as indicated by the observed stepwise increases in cell number (Fig. 1a and 2a). Increases in culture turbidity were continuous over the entire period during which cell number was monitored. Starvation for malate (Fig. 2c) also proved suitable for obtaining division synchrony in *R. rubrum*, but only one synchronous division was observed in cultures synchronized by this method. Also, cultures synchronized by malate starvation showed an initial drop in cell number and turbidity before growth began after supplementation with malate. Upon resumption of growth, continuous increases in turbidity and a discontinuous increase in cell number were observed.

Cultures induced to divide synchronously were used to examine cell-cycle-affected events. The protein and phospholipid contents of cell envelope obtained from cells at various stages of the cell cycle were examined. The protein/phospholipid ratio was shown to vary with the cell cycle (Fig. 1b). A decrease in this ratio occurred before each cell division. A 1.35-fold fluctuation was observed. The actual extent of fluctuation may be slightly greater because it

is possible that the minima and maxima did not coincide exactly with the points sampled. In additional trials, variations in the protein/phospholipid ratio of the cell envelope of 1.52 and 1.35 were measured.

As the ICM, which has been shown to oscillate in protein-phospholipid content (41), arises from invaginations of the CM (17, 23), it seemed likely that changes in the CM component of the cell envelope are responsible for the fluctuation in the cell envelope composition. This was tested by analyzing purified membrane fractions. The low yield of purified CM limited the number of points at which samples could be taken for analysis; for this reason, only one cell cycle was examined. Data for phosphate starvation-induced synchrony are shown in Fig. 2a and b. The protein/phospholipid ratio of the purified CM varied within the cell division cycle (Fig. 2b). This pattern of fluctuation was reproducible in repeat trials. CM was also purified from cultures synchronized by starvation for malate (Fig. 2c and d). A similar reproducible pattern of fluctuation in the composition of the CM was detected (Fig. 2d). For both techniques a variation of 1.7-fold was observed. Because it was not possible to sample two complete cell cycles for these experiments, this value must be considered a minimum of the extent of fluctuation. In any experiment such as this, the growth curve is not known until after the experiment is terminated.

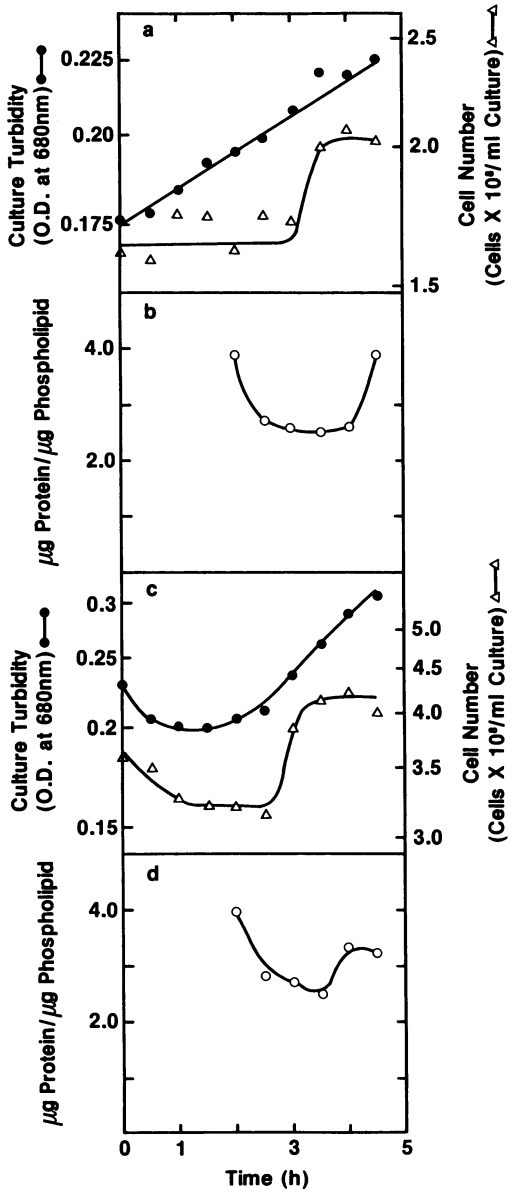

FIG. 2. Changes during phosphate starvation-induced (a and b) and malate starvation-induced (c and d) aerobic synchronous growth of *R. rubrum*. (a,c) Increase in cell mass and cell number. (b,d) Protein/phospholipid ratio of cytoplasmic membrane purified by density gradient centrifugation.

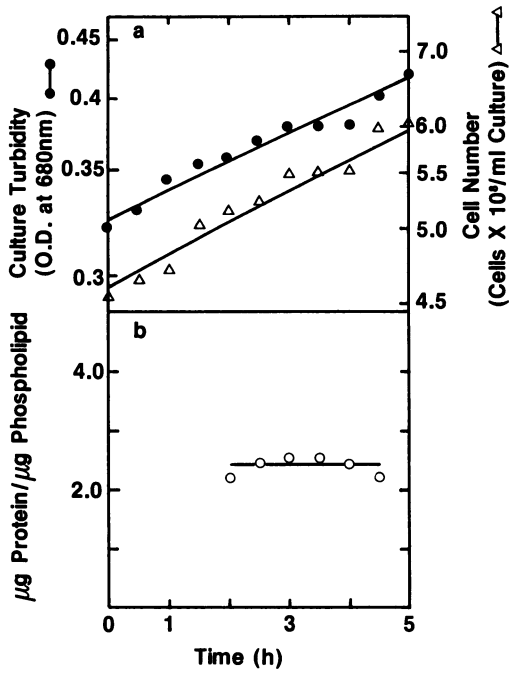

FIG. 3. Changes during asynchronous aerobic growth of *R. rubrum*. (a) Increases in cell mass and cell number; (b) protein/phospholipid ratio of cytoplasmic membrane (correlation coefficient, 0.93).

The CM preparations were also analyzed by electrophoresis; the profiles of CM were essentially identical at all stages in the cell cycle and were identical to those of CM purified from asynchronously growing cultures (data not shown). No bands corresponding to OM were detected. No evidence suggesting a cell-cycle-specific fluctuation of OM composition was detected by analysis of OM preparations obtained from starvation-synchronized cells.

In contrast to the results obtained with synchronous cultures, CM isolated from an asynchronous culture had a constant (correlation coefficient, 0.93) protein/phospholipid ratio (Fig. 3).

To confirm the finding of cell-cycle-specific fluctuation in membrane composition and to eliminate the possibility that perturbations caused by starvation were responsible for these observations, an experiment was done with an additional synchronization technique. Centrifugation selection has been described as a means for obtaining division synchrony in other bacteria (19, 25) and was successfully used to obtain division synchrony in *R. rubrum*. Because only a small percentage of the population is selected, cell densities are approximately 10-fold less than those for the other synchronization techniques. This limited the number of points at which samples could be taken for analysis. Also,

TABLE 1. Increases in cell number and mass in synchronously dividing cultures[a]

Mode of growth	Cell division	% increase in no. of cells during synchronous division[b] (mean ± SD)	% increase in culture mass during synchronous division[c] (mean ± SD)	No. of expt
Aerobic	Phosphate starvation induction	26.7 ± 5.2, 21.3 ± 1.0	25.0 ± 10.8, 15.6 ± 5.1	8
	Malate starvation induction	26.3 ± 3.2	41.7 ± 18.3	4
	Centrifugation selection	25.2 ± 4.4, 25.6 ± 5.3	Not determined	8
Phototrophic	Stationary-phase cycling induction	31.3 ± 3.2, 25.5 ± 6.2	28.7 ± 8.8, 22.3 ± 5.0	5
	High-light/low-light transition	27.5	31.8	1

[a] Values for both division 1 and division 2 are shown for some experiments. Estimation of percent increase in cell number and mass in the second division was based on three experiments for phosphate starvation and four experiments for centrifugation selection.
[b] Percent increase in cell number = $[(N-N_0)/N_0] \times 100$, where N is the cell number after division and N_0 is the cell number before division.
[c] Percent increase in culture mass = $[(A-A_0)/A_0] \times 100$, where A is the turbidity after division and A_0 is the initial turbidity, for the same time intervals during which cell number increases were calculated. For cultures synchronized by malate starvation, A_0 was equal to the lowest value.

cell envelope was analyzed because cell densities were too low to provide sufficient material for purification of CM. In this experiment, the protein/phospholipid ratio of the cell envelope fluctuated with the cell cycle. These findings are consistent with our findings for cells which were induced to divide synchronously.

In a previous report (41) we noted that in phototrophic cultures synchronized by two different techniques, only ca. 25% of the cells were dividing during the synchronous divisions. Nondividing cells were nongrowing but viable. It is now evident that the same situation applies to aerobic cultures synchronized by three different techniques (Table 1). Similar results were also obtained with cultures synchronized by starvation for either biotin or sulfate (unpublished data). With each synchronization technique, cell division resulted in a 26% increase (average for all divisions monitored in trials of all techniques) in cell number, accompanied by a 27% increase in cell mass as measured by turbidity (Table 1).

For all synchronization techniques, mass doubling times, as indicated by culture turbidity increases, of the synchronous populations were close to those of asynchronous cultures (Table 2). For the synchronous cultures, approximate 12-h mass-doubling times were accompanied by cell divisions at approximately 3-h intervals (Table 2).

Cell length measurements were done in some of the experiments to determine whether the population of nondividing cells was growing or nongrowing. If the nondividing cells were growing, percent increases in average cell length and mass would be significantly greater than percent increases in cell number. For phosphate starvation and stationary-phase cycling-induced synchronous cultures (average for all divisions in which cell length was also monitored), percent changes in mean cell length of the population (31%) correlated well with percent increases in cell number (30%) and cell mass (25%), suggesting that the population of nondividing cells was also nongrowing. In some experiments for several of the synchronization techniques, viable-cell counts were done concurrently with the direct cell counts. In all instances examined, viable-cell counts demonstrated that approximately 90 to 100% of the cells measured by the direct cell counts were viable, which indicates that a large population of the cells in the synchronous cultures were viable but nondividing.

DISCUSSION

The starvation (Fig. 1 and 2) and centrifugation selection techniques are useful in achieving division synchrony in aerobic cultures of *R. rubrum*. However, all of these procedures resulted in a low percentage of dividing cells (Table 1). Despite the failure of these synchronization techniques to establish cultures in which all cells were dividing, these cultures were adequate for demonstration of cell-cycle-specific changes.

The extent of fluctuation (at least 1.7-fold) in the protein and phospholipid contents (Fig. 2) of CM from synchronized aerobic cells approximated the protein-phospholipid fluctuations (2.0-fold) observed in ICM from synchronous phototrophic cells (41), although the absolute values of protein and phospholipid were slightly higher in the latter. In view of the presence of nondividing cells, the real extent of the fluctuation in *R. rubrum* CM composition may be greater than was observed. Insufficient material was recovered to analyze both cell envelopes and CM in the same experiment. However, comparisons made between preparations from different experiments indicate that the results were consistent. As determined by recovery after fractionation of the cell

TABLE 2. Mass-doubling times and time between cell divisions

Mode of growth	Cell division (method)	Mean calculated mass doubling time[a] (h) ± SD	No. of expt	Mean time elapsed between synchronous divisions (h) ± SD	No. of expt
Aerobic	Synchronous (phosphate starvation induction)	13.2 ± 4.3	8	3.0 ± 0.5	3
	Synchronous (centrifugation selection)[b]			2.5 ± 0.4	4
	Asynchronous	13.1 ± 1.6	2	—[c]	—
Phototrophic	Synchronous (stationary-phase cycling induction)	11.6 ± 2.8	5	3.2 ± 0.3	5
	Asynchronous	11.0	1	—	—

[a] Extrapolated by logarithmic regression analysis to determine time required for initial cell mass to double.
[b] Data for selection-synchronized cells not determined due to very low culture turbidity.
[c] —, Not applicable.

envelope, the CM contributed at least 44% of the envelope membrane protein. This must be considered a minimum, because recovery of the CM was reduced by centrifugation through two successive density gradients; the OM was obtained by a single gradient step. If it is assumed that CM and OM contribute equal protein to the cell envelope fraction, a 1.7-fold variation in CM composition results in a 1.35-fold variation in cell envelope composition, provided that additional protein (approximately 23% of the total) contributed by ribosomes is present in the unfractionated cell envelope. The presence of ribosomes in the cell envelope fraction has been demonstrated previously (14), and RNA was detected in the envelope fractions used in this study. The level of RNA measured is consistent with ribosomal proteins composing 10 to 45% of the crude cell envelope fraction.

A cell-cycle-specific fluctuation in ICM composition of phototrophically grown *Rhodobacter sphaeroides* has been reported (21, 37). The basis of this phenomenon in *Rhodobacter sphaeroides* is cell-cycle-specific accumulation of phospholipid in the ICM, as increases in total cellular protein were determined to be continuous while increases in total lipid phosphorus were stepwise (37). A burst of phospholipid synthesis occurs just prior to cell division in synchronously dividing populations of phototrophic *Rhodobacter sphaeroides* (33). Phospholipid synthesis has been localized in the cytoplasmic membrane of *Rhodobacter sphaeroides* (7, 16, 47), and phospholipid transfer activity has been demonstrated (6, 10, 49). Some investigators have reported fluctuation in the rates of phospholipid synthesis (8, 29, 45) and stepwise increases in total phospholipid content (40) during the cell cycle of *E. coli*. The cell-cycle-specific fluctuation of the protein/phospholipid ratio in *R. rubrum* CM reported here is consistent with the possibility that phospholipid is synthesized at a specific point in the cell cycle. Moreover, these studies indicate that cell-cycle-specific changes in membrane composition are not restricted to the ICM of phototrophic cells and suggest that this phenomenon may also occur in obligately chemotrophic bacteria as well.

These studies with synchronously dividing aerobic cells extend and confirm our previous finding (41) that not all cells are dividing in synchronized *R. rubrum* cultures. Since all synchronization techniques resulted in approximately the same low percentage of dividing cells, it is doubtful that the phenomenon was procedurally induced. The correlation between measurements of increase in cell length, number, and mass suggest that the nondividing cells were also not growing. However, as viable-cell counts were essentially identical to direct cell counts, it seems that all cells remained viable even though the majority were nondividing or nongrowing over the intervals during which synchronous growth was monitored.

Absolute cell number increases were compared for successive divisions for the synchronization techniques that provided more than one synchronous division. If the daughter cells resulting from the first synchronous division were also those which divided during the subsequent division, then only 50 to 60% of the daughters from division 1 grew and divided in division 2. This was reflected in the lower percent increase in cell number and mass observed for division 2 in most cases (Table 1). As it was not possible to maintain synchronous cultures beyond two divisions, it cannot be determined whether this reduction in percent increase in cell number and mass is a continuing trend. The data obtained from two divisions suggest that after each division approximately one-half of the daughter cells, while remaining viable, exhibit a temporary inability to grow and divide. This could be attributable to the distribution of a factor to only one of the daughter cells.

R. rubrum S1 is known to harbor a plasmid of approximately 50 kilobases (34; J. Nesta and M. L. P. Collins, unpublished). We tested the possibility that loss of this plasmid by unequal partitioning during cell division occurs, but found no evidence to support this. Colonies formed on plates inoculated from synchronous cultures were uniformly found to contain plasmid DNA (unpublished data).

We are not aware of other studies on synchronous populations which demonstrate a low percentage of dividing cells. However, studies of *E. coli* have demonstrated the presence of an unstable inducible division inhibitor, SfiA (SulA) (38, 39). The product of the *sfiA* gene, induced after DNA damage as part of the SOS response (27), acts as an inhibitor of septation, leading to the formation of multinucleate filaments. Division but not growth is affected. The experimental evidence on synchronous populations of *R. rubrum* presented in this report suggests that both cell division and cell growth are temporarily halted in the nondividing cells. It is possible that an unstable factor is responsible for a reversible inhibition of growth and division in *R. rubrum*, resulting in the occurrence of nongrowing and nondividing cells observed in this study. It is also possible that the DNA molecules distributed to the two daughter cells are in some way different (32), so that one of the cells does not grow and divide under the culture conditions used. This is speculative, and we cannot rule out the alternative possibility that different subpopulations are involved in successive divisions. In view of the finding that the mass doubling times for synchronous and asynchronous cells were similar (Table 2), it is tempting to suggest that a similar pattern of growth occurs for both. Perhaps cells in asynchronous cultures have a nongrowing and nondividing stage in the cell cycle. The data in Tables 1 and 2 are approximately consistent with portions of the population dividing in successive divisions, resulting in division of all of the cells within one mass-doubling time. Distinction between the possibilities that division is restricted to a small population of cells (that is decreasing in proportion with respect to the total) and that different subpopulations divide in successive divisions is made difficult by the inability to determine the growth curve for one complete mass doubling in these synchronous cultures. The mass-doubling times were determined by extrapolation from the observed increases in cell mass by logarithmic regression analysis.

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