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Dependence of Cell Division on the Completion of Chromosome Replication in *Caulobacter crescentus*

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The relationship between chromosome replication and cell division in the stalked bacterium *Caulobacter crescentus* has been investigated. Two compounds, hydroxyurea and mitomycin C, were found to inhibit completely deoxyribonucleic acid (DNA) synthesis while allowing continued cell growth and elongation. When these inhibitors were added to exponentially growing cultures, cell division stopped after 38 min when hydroxyurea was used and after 33 min when mitomycin C was used. The period of continued cell division corresponds closely to the period previously determined for the postsynthetic gap (G2) in the DNA cycle of this organism. These results indicate that cell division is coupled to the completion of chromosome replication in *C. crescentus*.

Caulobacter crescentus is a gram-negative bacterium which displays a sequence of well defined developmental stages during growth (6, 8, 10). The three readily identifiable morphological forms are (A) the motile swarmer cell which carries a single flagellum at one pole, (B) the nonmotile stalked cell which has a polar outgrowth of cellular material known as the stalk, and (C) the dividing cell which possesses a stalk at one pole and a flagellum at the other (Fig. 1). Cell division is asymmetric and gives rise to a swarmer cell and a stalked cell. The stalked cell proceeds directly to division, whereas the swarmer cell must form a stalk before going on to divide (8).

As one approach to understanding the regulation of growth and morphogenesis in *C. crescentus*, an investigation was undertaken to determine whether cell division in this organism is dependent upon the completion of chromosome replication. The presence of this restriction in the cell cycle would be indicated if the period of continued cell division after inhibition of deoxyribonucleic acid (DNA) synthesis were limited to the length of the postsynthetic gap (G2) in DNA cycle (6; Degnen and Newton, *submitted for publication*). This type of coupling has been demonstrated in *Escherichia coli* strain B/r (1, 4) and in animal cells (7, 9). Recent results of Donachie, Martin, and Begg (2) with *Bacillus subtilis*, however, indicate that cell division in this bacterium is not dependent upon completion of replication.

We report here the identification of compounds specific for the inhibition of DNA synthesis in *C. crescentus* and the effects of these inhibitors on cell growth and division. The results indicate that cell division is coupled to the completion of chromosome replication in this organism.

MATERIALS AND METHODS

Bacteria and growth conditions. *C. crescentus* strain CB15 was obtained from the American Type Culture Collection (ATCC 19089). Cells were grown at 30 C in a water bath with gyrating shaking.

The defined minimal medium (M3), containing 0.2% carbon source, was prepared by the method of Poindexter (8).

Metabolic inhibitors. Solutions of hydroxyurea and mitomycin C (Sigma Chemical Co.) were prepared in distilled water. Hydroxyurea solutions were kept frozen until use; mitomycin C was prepared immediately before each experiment. Nalidixic acid (kindly supplied by S. Archer of the Sterling-Winthrop Research Institute, Rensselaer, N.Y.) was prepared in 0.2 N NaOH and frozen until use. When high concentrations of nalidixic acid were being tested, the desired amount was added directly to the culture as a solid. Neither method of addition altered the *p*H of the medium significantly. Phenethyl alcohol was obtained commercially (Eastman Organic Chemicals). Phleomycin (kindly supplied by W. T. Bradner of Bristol Laboratories, Syracuse, N.Y.) solutions were prepared in distilled water and kept at 4 C until use.

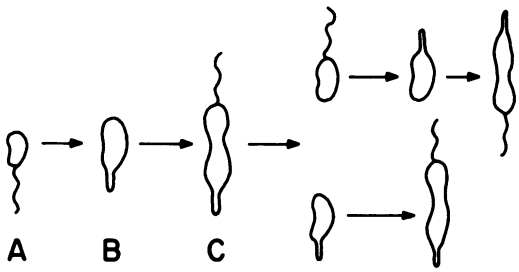

FIG. 1. *Life cycle of Caulobacter crescentus.*

RNA and DNA synthesis. Preliminary experiments indicated that neither thymine, thymidine, nor deoxyribose (*unpublished data*; J. Gerhardt, *personal communication*) are incorporated into the DNA of *C. crescentus* so that these labeled precursors cannot be used to follow nucleic acid synthesis in the organism. The most satisfactory method for following both ribonucleic acid (RNA) and DNA synthesis was found to be labeling with ^{3}H-adenine (New England Nuclear, Boston, Mass., 6 Ci/mmole). For determination of incorporation into the total macromolecular fraction, samples (0.1 ml) were pipetted onto Whatman GF/A glass filters (2.4 cm). The filters immediately were added to a solution of cold 5% trichloroacetic acid and allowed to stand on ice for 90 min. The trichloroacetic acid solution was changed twice during this period. The filters were then rinsed with cold 50% ethanol three times, dried, and counted in toluene scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (Packard Instruments). This measured the total incorporation of ^{3}H-adenine into DNA and RNA. Incorporation into DNA was determined as follows. Samples of 0.2 ml were removed and added to 2 ml of 1 N KOH containing 100 μg of bovine serum albumin per ml. The solutions were incubated overnight at 37 C, neutralized with 12 N HCl, precipitated with 2 ml of 20% trichloroacetic acid, and allowed to stand in the cold for 30 min. The samples were filtered onto GF/A filters, individually washed with 10% trichloroacetic acid containing 200 μg of cold adenine per ml, washed with 50% ethanol, dried, and counted in the toluene scintillation fluid. Incorporation into DNA averaged 9% of the total incorporation. The amount of radioactivity incorporated into material which was stable to both 1 N KOH and hot-acid degradation was less than 0.7% of the total incorporated counts and was considered to be negligible for purposes of calculation. RNA synthesis was calculated by subtracting the amount of radioactivity incorporated into DNA (*see above*) from the total radioactivity incorporated into the fraction precipitated by cold trichloroacetic acid.

RESULTS AND DISCUSSION

Metabolic inhibitors: effects on DNA and RNA synthesis. For the experiments to determine whether cell division in *C. crescentus* is dependent on chromosome replication, it was necessary to identify specific inhibitors of DNA synthesis. Five compounds known to inhibit DNA replication in *E. coli* were selected for testing. Three of these, hydroxyurea (11), mitomycin C (4), and nalidixic acid (3) had been shown to stop DNA synthesis immediately in vivo. Two, phenethyl alcohol (5) and phleomycin (12), had been found to inhibit initiation rather than continuation of DNA synthesis. The latter two compounds were tested since it was possible that their use would provide a tool for determination of the time required for chromosome replication (S) and G2; cell division in a nonsynchronous culture should continue for S + G2 minutes after addition of an inhibitor of chromosome initiation.

Only hydroxyurea and mitomycin C inhibited DNA synthesis without severely inhibiting RNA synthesis; the extent of inhibition by the two compounds is shown in Fig. 2. Nalidixic acid, phenethyl alcohol, and phleomycin were less specific in their action. At 108 μg/ml, nalidixic acid stopped RNA synthesis and only partially inhibited DNA synthesis. This effect contrasts with its specific inhibition of DNA synthesis in *E. coli* (3). Phenethyl alcohol and phleomycin were found to inhibit both DNA and RNA synthesis.

Metabolic inhibitors: effects on viability and growth. Figure 3 shows that the concentrations of hydroxyurea and mitomycin C needed to inhibit DNA synthesis completely (Fig. 2) were at least partially bacteriocidal. Growth continued in the presence of the two inhibitors, however, since hydroxyurea at concentrations of 0.3 to 6.0 mg/ml and mitomycin C at concentrations of 0.02 to 2.0 μg/ml induced filament formation. This observation is consistent with the failure of these inhibitors to block RNA synthesis. We concluded from these results that only hydroxyurea and mitomycin C were sufficiently specific for use in the experiments described below.

Cell division following inhibition of DNA synthesis in nonsynchronous cultures. Exponentially growing cultures of *C. crescentus* were treated with either hydroxyurea or mitomycin C. The effect of stopping DNA synthesis on cell division was determined by following the change in particle count before and after addition of the inhibitors (Fig. 4). Division continued for only 38 min after addition of hydroxyurea and for 33 min after addition of mitomycin C.

These results indicate that cell division in *C. crescentus* is dependent upon completion of chromosome replication. The average time of continued division after addition of the inhibitors (35 min) is in reasonably good agreement with the period of 30 min reported for the postsynthetic gap in the DNA cycle (6;

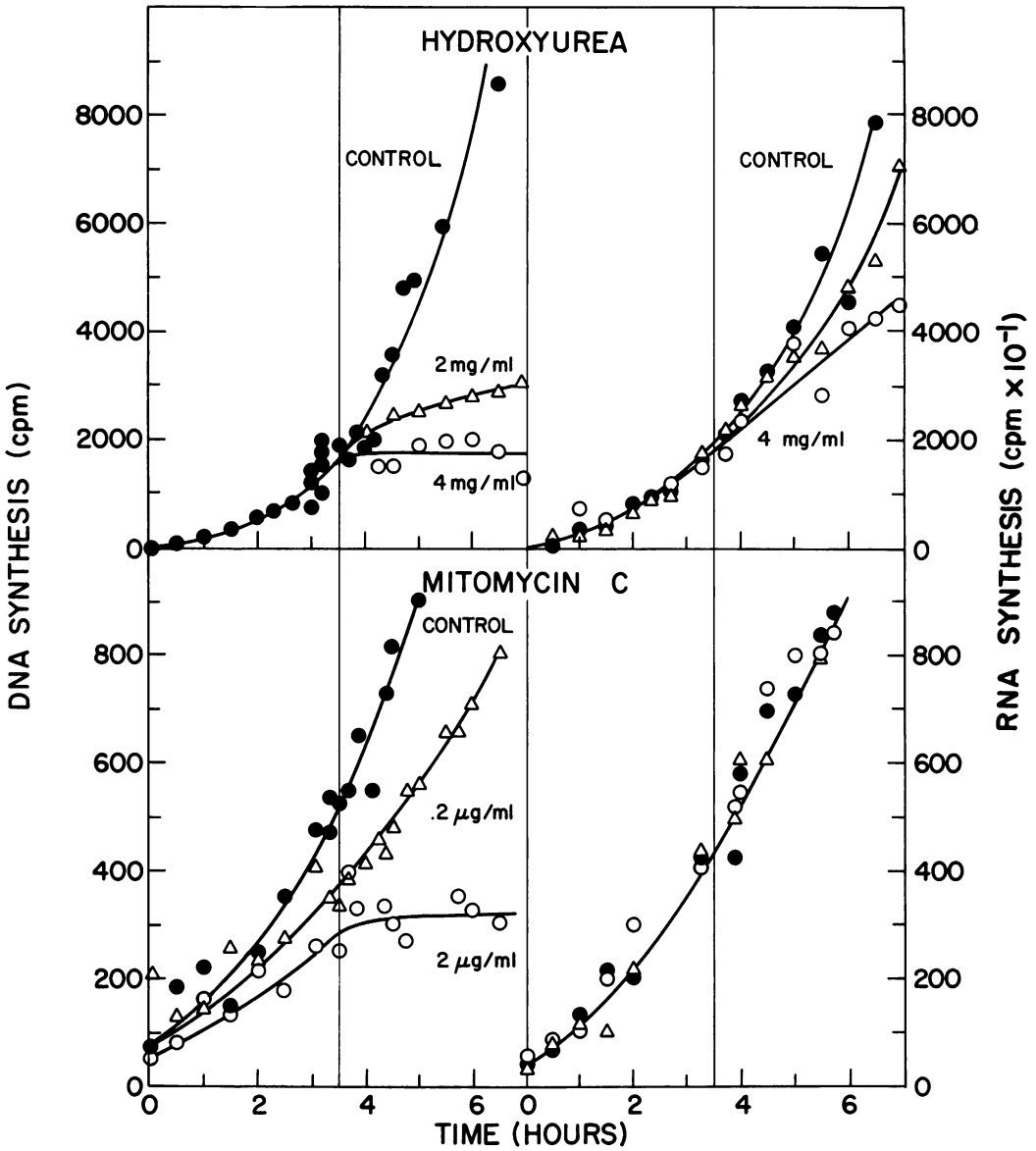

FIG. 2. *Effect of hydroxyurea and mitomycin C on RNA and DNA synthesis in C. crescentus. Exponential cultures of C. crescentus were grown in glucose minimal medium to a density of at least 10^8 cells/ml. Each culture was divided into several portions, one to serve as a control and the others to test various concentrations of inhibitors. These portions were added to flasks containing unlabeled adenine to a final concentration of 10 μg/ml and ^{3}H-adenine to a final specific activity of 0.1 Ci/mmole. The cultures were incubated and sampled for incorporation for at least one generation, the inhibitor was added, and the incubation continued. RNA and DNA were determined as described in the text. Addition of inhibitor is indicated by the vertical line. Incorporation is expressed as counts per minute per 0.1 ml of culture. Hydroxyurea: control (●); 2 mg/ml (△); 4 mg/ml (○). Mitomycin C: control (●); 0.2 μg/ml (△); 2 μg/ml (○). The two panels on the left represent DNA synthesis and two panels on the right represent RNA synthesis. The doubling time in the absence of inhibitors was 150 min.*

Degnen and Newton, *submitted for publication*). The length of G2 was determined from labeling experiments with both stalked and swarmer cells, growing in minimal salts-glucose medium. Thus, the number of cells which divide after addition of the inhibitors can be accounted for largely by those cells which have at least two completed chromosomes.

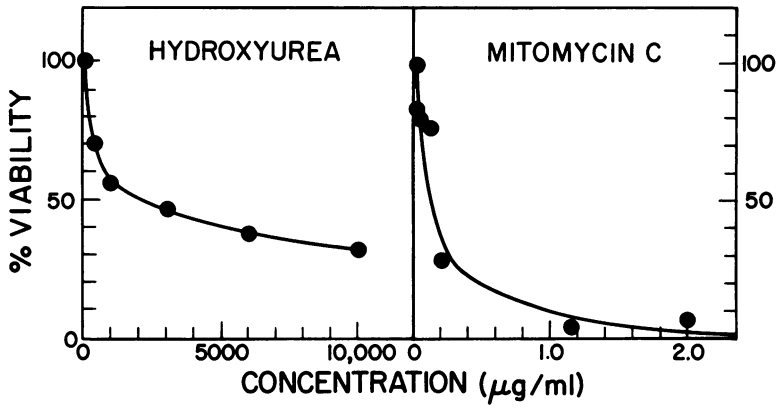

FIG. 3. *Effect of inhibitors on viability of C. crescentus. Exponential cultures of C. crescentus grown in glucose minimal medium to a density of at least 10^8 cells/ml were divided into equal parts, and inhibitors were added to the specified concentrations at zero time. One culture was not treated with inhibitor, and this served as a control. Viable counts were taken at intervals to compare the viable count of the inhibited culture with that of the control after one doubling of the latter. Viable counts in the inhibited culture are expressed as a percentage of those in the control after one doubling time and plotted versus concentration of inhibitor added.*

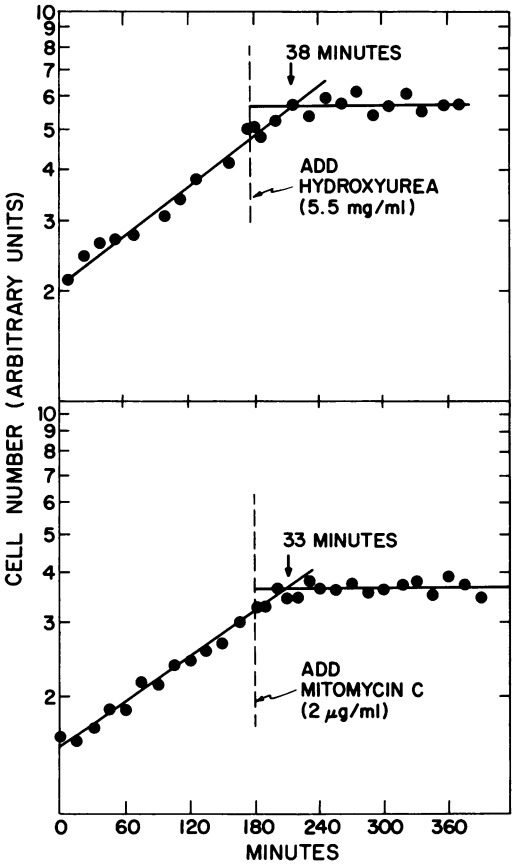

FIG. 4. *Cell division after inhibition of DNA synthesis in C. crescentus by hydroxyurea or mitomycin C. Exponential cultures of C. crescentus were grown in glucose minimal medium to a density of at least 10^8 cells/ml. The culture was monitored for approximately one doubling time, and then hydroxyurea or mitomycin C were added to final concentrations of 5.5 mg/ml or 2 μg/ml, respectively, and the incubation was continued. Cell number was determined by diluting samples into saline containing 10% Formalin and counting on a model B Coulter counter with a particle size distribution plotter (Coulter Electronics, Chicago, Ill.). Cell number is expressed in arbitrary units. Addition of inhibitor is indicated by the vertical line.*

Experiments with *E. coli* have shown that, when chromosome replication is blocked in these cells, division is also blocked after a short period of time (1, 4). The coupling between the completion of chromosome replication and cell division described here in *C. crescentus* and previously in *E. coli* is not characteristic of all bacteria, however. Donachie et al. (2) have recently reported that, in several strains of *B. subtilis*, cell division continued for an extended period in the absence of DNA synthesis, and that enucleated cells were eventually produced. It will be of interest to see whether the regulatory mechanism which couples cell division to the completion of chromosome synthesis in *C. crescentus* and *E. coli* is common to gram-negative bacteria but not to gram-positive bacteria such as *Bacillus subtilis*.

ACKNOWLEDGMENTS

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