REGULATION OF POLAR CAP FORMATION IN THE LIFE CYCLE OF *ESCHERICHIA COLI*

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Polar cap formation has been studied in synchronized *Escherichia coli* cells. It is dependent on a signal given after completion of a round of DNA replication. A 20 min time interval between the release of this signal and physical cell separation is probably the time required for the completion of polar caps. During this time murein is synthesized at an increased rate and cells are especially sensitive to penicillin.

1. INTRODUCTION

Bacterial cell division is normally coupled to DNA replication so that a chromosomal replication cycle has to be completed to allow subsequent cell division (1-2). A signal for division triggers a series of steps, some of which have been studied with synchronized cultures (2) or with mutants (3-4). However, no division step is clearly understood in molecular terms.

When a rod shaped *E. coli* cell divides, the new polar caps of the daughter cells are formed at the division site. During this process the morphology of the sacculus (5), which is the shape maintaining element of the bacterial wall, necessarily has to be modified. This occurs in a sequence of two reactions (6). First, covalent bonds are cleaved in the polymer murein-network (5) of which the sacculus consists. This step, which is accomplished by a set of murein hydrolases, provides both space and acceptor sites for the insertion of new material as a second step. By their localized and timed action, the murein hydrolases determine where, when and to what extent the sacculus undergoes morphological modifications in the cellular life cycle.

The action of murein hydrolases becomes evident when the correct insertion of murein precursors into the growing sacculus is prevented. Due to continued murein hydrolysis the mechanical stability of the wall is lost, and the cells lyse. This happens when the condensation of new material into the growing sacculus is blocked in the presence of penicillin (7-9). The antibiotic is thus a tool to measure murein hydrolase activity *in vivo*.

Depending on its concentration, penicillin can exert different morphological effects on *E. coli*. Polar cap formation is selectively inhibited by low doses, whereas cell elongation is blocked only at high concentrations of the drug (10-11). The selective effect of low penicillin concentration on polar cap formation was used to analyze the regulation of murein hydrolase

activity and its dependence on DNA replication in synchronized cultures of *E. coli*. In addition, the correlation between the activity of murein hydrolases and the rate of polar cap specific murein synthesis was studied. We believe that a study of polar cap specific murein synthesis should lead to an understanding of at least one of the many steps of bacterial cell division in molecular terms.

2. MATERIALS AND METHODS

(a) *Bacterial strains and media*

E. coli B/r-301 (F^-, leu^-, pro^-, lac^-, gal^-, trp^-, his^-, arg^-, thy^-, str^r, met^-, hsp^{K12}) is derived from strain HB 50 from H. Boyer. *E. coli* B/r/1 was obtained from D.J. Clark. Bacteria were grown in minimal-glucose medium, supplemented as necessary.

(b) *Synchronization of cultures*

Strain *E. coli* B/r-301 was synchronized in minimal glucose medium (generation time 42 min, Helmstetter and Cooper (12)) by a modified membrane selection technique, originally described by Helmstetter and Cummings (13). *E. coli* B/r/1, growing in M9 medium (14) with a generation time of 45 min, was synchronized according to the centrifugation technique of Mitchison and Vincent (15). Synchronous growth of the cultures was followed with a Coulter Counter combined with a multichannel analyzer.

(c) *Determination of the proportion of cells surviving penicillin pulses*

Penicillin G (1000 IU per ml) was added to synchronously growing cultures at different cell age. After a pulse incubation of 4 min, samples were diluted 100-fold into 0.9% NaCl, kept at 0° for 1 to 2 hr and finally plated on nutrient agar (Difco).

(d) *Determination of the rate of murein synthesis*

Originally we had tried to isolate a DAP dependent mutant from *E. coli* B/r-301 with which we could determine the rate of murein specific incorporation of DAP as a measure of the rate of murein synthesis. However, we found that *E. coli* B/r is not able to utilize exogenous DAP, probably because of the lack of a transport system; therefore we were unable to isolate a DAP-dependent mutant. Then, a mutant of *E. coli* B/r-301 was isolated which required D-glu for growth; however there was little difference between the amount of D-glu incorporated into the mutant and that incorporated into the parent strain. About 80% of the incorporated [^{14}C]D-glu could be liberated from the murein with lysozyme. This is the amount to be expected if D-glu is exclusively incorporated into murein (16). Finally the experiments were performed with *E. coli* B/r-301.

Five min pulses with 0.5 µC per ml of [^{14}C]D-glutamic acid (4.8 mCi per mmole; Calbiochem, Los Angeles) were administered in triplicate to a synchronized culture (see above) of *E. coli* B/r-301 at different cell ages. Incorporation of the label was stopped with trichloroacetic acid at a final concentration of 5%. Labeled cells were centrifuged together with carrier cells through a step gradient of 40% and 15% sucrose in 5% trichloroacetic acid for removal of excess label. Sedimented cells were transferred onto glass filter papers (Schleicher and Schüll, Dassel, Germany, No. 8), washed 4 times with 5% trichloroacetic acid, twice with ethanol and counted in a liquid scintillation counter.

3. RESULTS

(a) *A penicillin-sensitive step precedes cell division*

As shown in Fig. 1 the number of cells surviving penicillin pulses administered to a synchronized culture depends on cell age. For cells growing with a 42 min generation time there is a time interval of 15 min (average of 19 experiments, extremes 7 and 23 min) between the time when cells are most sensitive to penicillin and the occurrence of physical cell division. The period of maximum sensitivity to penicillin is therefore 5 to 10 min after the end of a round of DNA replication (12). A similar time interval between the completion of DNA replication and the penicillin sensitive step is found in cells growing with a generation time of 64 min. Under both conditions, min-

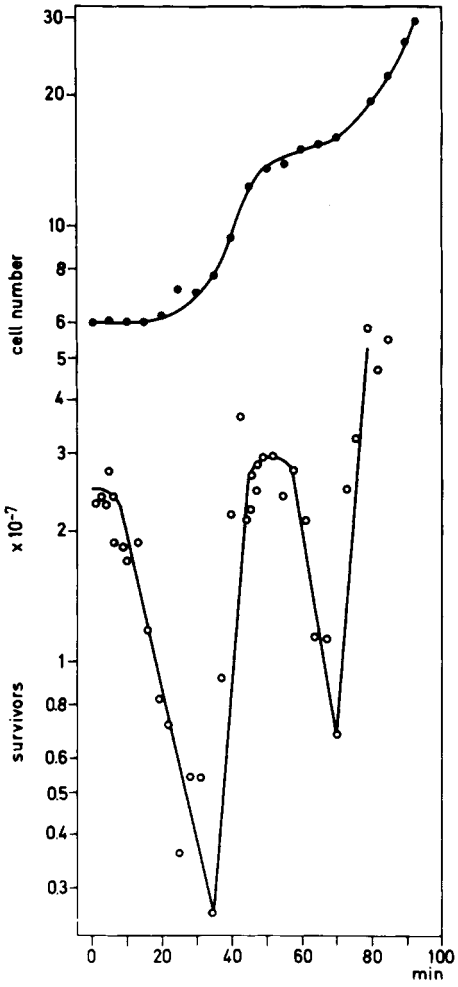

Fig. 1. *Penicillin sensitivity as a function of cell age.* Penicillin G was added to a synchronously growing culture of *E. coli* B/r-301 (●) at different cell ages. The number of survivors (○) after 4 min of penicillin treatment was determined by plating (Materials and Methods).

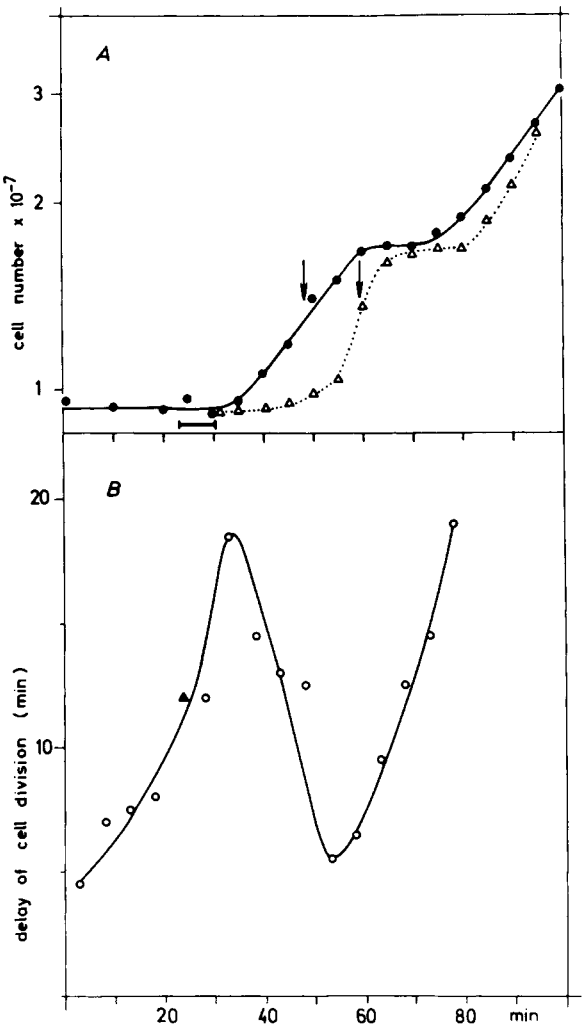

Fig. 2. *Penicillin induced delay of cell division.*

A. Determination of the delay time of cell division. *E. coli* B/r/1 was synchronized as described in Materials and Methods. In the experiment shown, penicillin G (40 IU per ml) was added at 23 min cell age. Seven min later an excess of penicillinase (3000 IU per ml) was added and cell division followed in the Coulter Counter. Arrows indicate times of division (i.e. the inflection points) of the experimental sample (△) and of an untreated control (●). Incubation time with penicillin is represented by a bar.

B. The delay of division as compared to the control. The delay of cell division was determined as described in A (▲) in a series of experiments in which penicillin pulses were given at different cell age. The delay times found are plotted versus the time at which the penicillin pulse was started (abscissa).

imum sensitivity to penicillin is reached at about 8 min after division. The oscillation of penicillin sensitivity during the life cycle is not due to a variation of cell permeability to penicillin. Survival curves determined at various cell ages show a constant lag of about 2 min followed by a decrease in the number of surviving cells at different rates. The variations of killing rate are the reason for the oscillation of penicillin sensitivity.

Not only high, but also low concentrations of penicillin, show an age dependent effect. Pulses utilizing 40 units per ml of the antibiotic lead to a delay in cell division. This delay probably reflects the time required to repair the lesions caused by the drug. As shown in Fig. 2, maximal delay is observed if the pulse is administered at the time when maximum penicillin sensitivity occurred as determined in the experiment shown in Fig. 1.

(b) *The penicillin sensitive step is coupled to the DNA replication cycle*

DNA synthesis was inhibited in synchronized cultures by thymine starvation or by the addition of nalidixic acid (17) at a cell age when chromosome replication was not yet completed. Under these conditions we found no age dependent oscillation in the percentage of cells surviving the penicillin treatment. This indicates that the signal which triggers the penicillin sensitive step was no longer generated (Fig. 3A, B). The only effect observed was unspecific killing (about 50% survivors; see Fig. 1) which might be due to penicillin induced cell lysis at the sites of cell elongation, as postulated by Donachie and Begg (18). Inhibition of protein synthesis (starvation for leucine, Fig. 3C, or addition of chloramphenicol) also prevented the age dependent oscillation of penicillin sensitivity. Obviously, the signal for division after completion of a round of DNA replication can only be expressed when protein synthesis is allowed.

(c) *Microscopic examination of penicillin treated cells*

In the presence of penicillin at low concentration, bulges are formed at the division site instead of normal polar caps (10). We asked at which cell age the potential for bulge formation arises. Penicillin was added to a synchronized culture at various cell ages and the proportion of cells with bulges was determined after a fixed period of further incubation. To prevent a release of signals for cell division when additional rounds of chromosome replication could be completed in the course of the experiment, nalidixic acid was added together with penicillin.

As represented in Fig. 4, penicillin induced bulge formation is maximal when the drugs are given at or shortly after the time when a round of replication has been completed. This is about the cell age at which the cells are most sensitive to penicillin.

(d) *Rate of murein synthesis during the life cycle*

The timing of the penicillin sensitive division step reflects the function of a mechanism which regulates localized murein synthesis during polar cap formation. Therefore we expected to find an age dependent variation in the overall rate of murein synthesis in the life cycle.

The rate of murein synthesis was determined at different cell ages in synchronized cultures with pulses of the murein specific amino acids D-glutamic acid (D-glu) and 2.6-diaminopimelic acid (DAP), respectively (5). The rate of incorporation of D-glu per cell or per cell mass ($OD_{550\ m\mu}$) into *E. coli* B/r-301 is maximal at the same cell age at which the cells are most sensitive to penicillin (Fig. 5 and Fig. 1). Similar results are obtained when murein synthesis is determined by following the incorporation of DAP into synchronized (19) *E. coli* K12-W7 (20).

4. DISCUSSION

All our experiments support the conclusion that in the life cycle of *E. coli* a signal, emitted only after the end of a round of chromosome replication, triggers localized murein synthesis which is extremely sensitive to penicillin (10). This synthesis is essential for polar cap formation and eventually leads to cell division. In some division mutants the ordered sequence of these events may be altered (3, 21-23).

Our results are consistent with the results of previously reported experiments but not with their interpretation: "that maximum (penicillin) sensitivity occurs at the time of division" (24). This latter coincidence is fortuitous, since the previously reported experiments were performed with a medium in which the end of a round of DNA replication and cell division (25) occurred at about the same time.

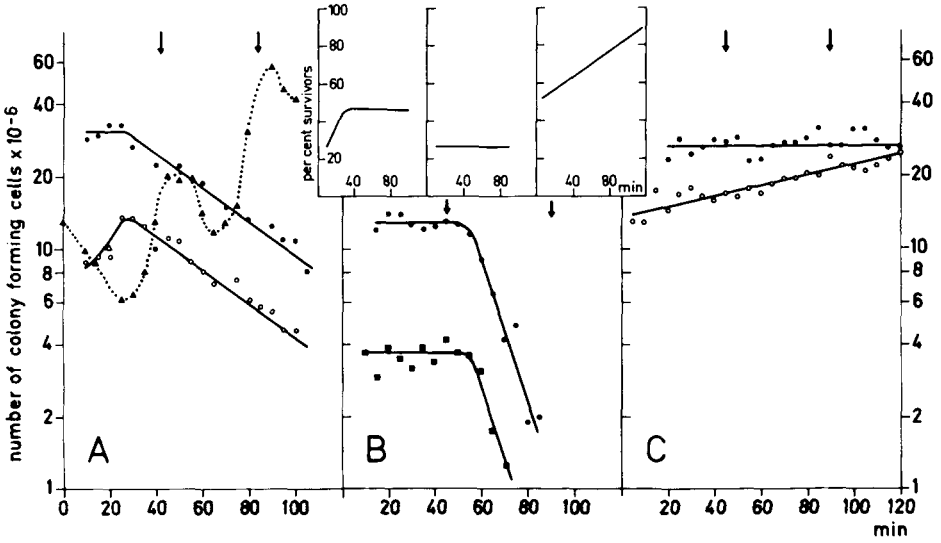

Fig. 3. *Effect of inhibition of DNA or protein synthesis on penicillin sensitivity of synchronized cultures.*

A. *E. coli* B/r-301 was synchronized as described in Materials and Methods. The number of colony formers was determined after 5 min pulses with 1000 IU per ml of penicillin G (··▲··▲··), after addition of 10 µg per ml of nalidixic acid at the cell age of 10 min (—●—●—), and after addition of 10 µg per ml of nalidixic acid at the cell age of 10 min and subsequent 5 min pulses of 1000 IU per ml of penicillin G at different cell ages (—○—○—).

B. *E. coli* B/r/1 *thy*⁻ was synchronized as described in Materials and Methods. Thymine was removed at cell age 0 min. Number of colony formers was determined after 6 min pulses of 900 IU per ml of penicillin G (■) and in a penicillin free control (●).

C. *E. coli* B/r/1 *leu*⁻ was treated as in B except that leucine instead of thymine was removed (○).

Arrows indicate time of synchronous division in the untreated control. Inserts show per cent viable cells after penicillin treatment as calculated from the curves (penicillin free control taken as 100 per cent).

The oscillation of the rate at which murein specific amino acids are incorporated need not necessarily reflect a variation of the rate of murein synthesis. It could be mimicked by an oscillation of the intracellular pool size of these amino acids. It is difficult to exclude this by experiments. However, because the oscillations of penicillin sensitivity are synchronous and since our experiments were done with two different strains and amino acids, we favour the hypothesis that it is the rate of murein synthesis which oscillates in the life cycle of *E. coli*.

Construction of new polar caps, after inhibition of cell division with low concentrations of penicillin, requires both repair of the lesions caused by the drug and the synthesis of new polar cap murein. The delay times found are remarkably long (Fig. 2). Under normal conditions, the capacity of the cell for fast polar cap formation also appears to be quite limited. The long time interval between the end of DNA replication and cell separation (25) may be the time required for polar cap formation.

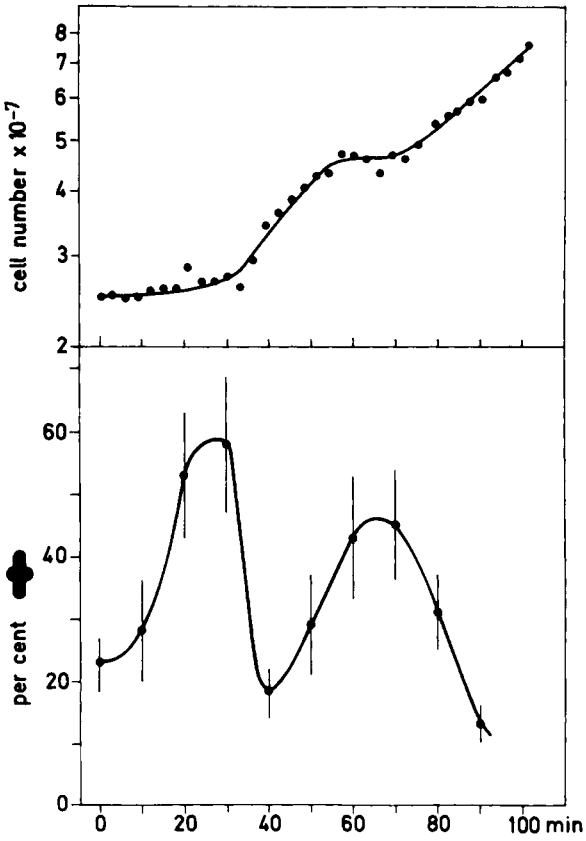

Fig. 4. *Penicillin induced bulge formation in synchronized cultures. E. coli* B/r-301 was synchronized as indicated in Materials and Methods. Samples were transferred at different cell age to medium containing 12% sucrose, 10 µg per ml of nalidixic acid, and 20 IU per ml of penicillin G, final concentrations, and incubated for 130 min to allow development of bulges. The proportion of cells with bulges was determined with a phase contrast microscope and plotted versus the time of transfer into the bulge forming medium. Bars represent standard errors.

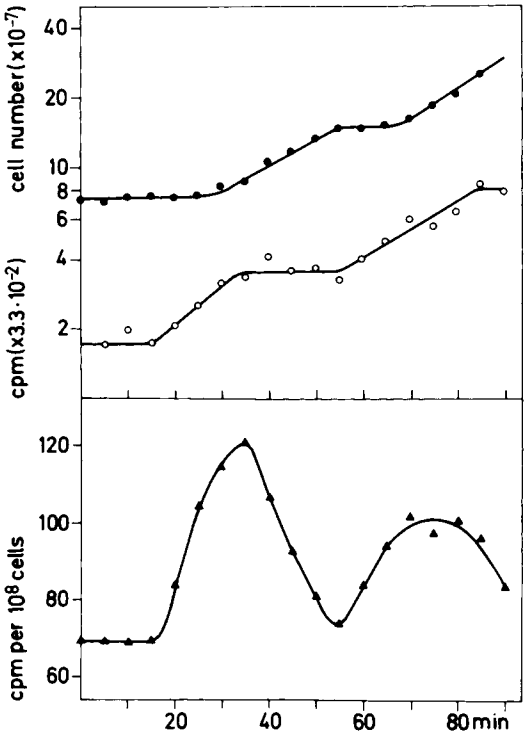

Fig. 5. *Rate of murein synthesis during the life cycle.* *E. coli* B/r-301 was synchronized and cell number (●) measured as indicated in Materials and Methods. The rate of murein synthesis as determined by incorporation of murein specific label (Materials and Methods) into cells from 0.5 ml of culture is plotted versus cell age (○). The amount of label found in 10^8 cells (▲) was calculated from curves ● and ○.

Polar cap formation, as measured by penicillin sensitivity, starts at about the same cell age at which the number of sites for cell elongation doubles (26) according to the unit cell hypothesis of Donachie and Begg (18). However, these two events are distinct from each other by a different sensitivity to penicillin and are subject to different regulatory processes, reflected in their relationship to DNA replication. Sites for cell elongation can double in the absence of DNA synthesis (26), whereas initiation of polar cap formation occurs as the result of a completed DNA replication cycle.

Recently, D.J. Clark informed us that David Groves in his laboratory found an oscillation of penicillin sensitivity during the life cycle of *E. coli* similar to that described in this article.

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