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Lytic Response of *Escherichia coli* Cells to Inhibitors of Penicillin-Binding Proteins 1a and 1b as a Timed Event Related to Cell Division

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In growing cultures of *Escherichia coli*, simultaneous inhibition of penicillin-binding proteins 1a and 1b (PBPs 1) by a β-lactam efficiently induces cell lysis. However, the lytic behavior of cultures initiating growth in the presence of β-lactams specifically inhibiting PBPs 1 suggested that the triggering of cell lysis was a cell division-related event, at least in the first cell cycle after the resumption of growth (F. Garcia del Portillo, A. G. Pisabarro, E. J. de la Rosa, and M. A. de Pedro, J. Bacteriol. 169:2410–2416, 1987). To investigate whether this apparent correlation would hold true in actively growing cells, we studied the lytic behavior of cultures of *E. coli* aligned for cell division which were challenged with β-lactams at different times after alignment. Cell division was aligned either by nutritional shift up or by chromosome replication alignment. Specific inhibition of PBPs 1 with the β-lactam cefsulodin resulted in a delayed onset of lysis which was coincident in time with the resumption of cell division. The apparent correlation between the initiation of lysis and cell division was abolished when cefsulodin was used in combination with the PBP 2-specific inhibitor mecillinam, leading to the onset of lysis at a constant time after the addition of the β-lactams. The results presented clearly argue in favor of the hypothesis that the triggering of cell lysis after inhibition of PBPs 1 is a cell division-correlated event dependent on the activity of PBP 2.

The final steps in the synthesis of the eubacterial murein sacculus are mediated by the penicillin-binding proteins (PBPs), a set of membrane-bound proteins characterized by their ability to interact with β-lactam antibiotics specifically at the active center, forming covalent, enzymatically inactive complexes (11, 24).

In *Escherichia coli* seven genetically independent PBPs have been identified (19). In exponentially growing cells it has been assumed that PBPs 1a and 1b (PBPs 1) catalyze the insertion of new precursors necessary for cell wall elongation; PBP 2 is needed for the maintenance of correct cell shape and seems to be involved in the initiation of cell wall growth; PBP 3 mediates septum formation at cell division; PBP 4 participates in postinsertional modification of murein; and PBPs 5 and 6 regulate the proportion of pentapeptide side chains in murein (11, 20, 21). However, with the exception of PBP 3, the physiological role of the individual PBPs is still under discussion (7).

The simultaneous inhibition of PBPs 1 by specific β-lactams triggers lysis in actively growing cells (19). However, it has been observed that bacteriolysis can be further stimulated by the concurrent inhibition of PBP 2, whose inactivation is not in itself bacteriolytic, suggesting that blockage of PBP 2 facilitates the lytic action of murein hydrolases when PBPs 1 are inhibited (3, 9).

Inactivation of PBPs 1 in cells initiating growth after the stationary phase leads to delayed lysis that takes place at the time of the first cell division, irrespective of the time of addition of the inhibitor, in a way apparently dependent on the termination of chromosome replication. However, simultaneous inhibition of all PBPs leads to a rapid lytic response without apparent connection to any particular period in the cell cycle (7).

In this communication we investigate the possibility that the triggering of cell lysis by inhibitors of PBPs 1 may also be linked to cell division in actively growing cells.

If the induction of lysis by inhibitors of PBPs 1 were coordinately timed with cell division, then any treatment of a culture which aligns division of the greater part of the bacterial population would lead to lysis only at the moment of division, irrespective of when the PBP 1 inhibitor was added. In particular, we have used treatments generating a temporary inhibition of cell division, nutritional shift up and chromosome replication alignment (14–16). The results obtained by this approach clearly favor our hypothesis.

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains *E. coli* AB1157 (K-12 F⁻ *thr-1 leu-6 proA2 hisG4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-33 rpsL31 sup-37*) (15) and its *thy* derivative, AB11570, obtained by trimethoprim selection of spontaneous *thy* mutants, were used throughout this work. Cells were routinely grown at 37°C with vigorous aeration, either in MOPS (4-morpholinepropane sulfonate) mineral medium (16) or in MC (mineral-citrate) medium (23) supplemented with glucose (4 g/liter), thiamine (1 mg/liter), thymine (50 mg/liter), 1 mM isopropyl-β-D-thiogalactopyranoside, and required amino acids (0.2 g/liter). In nutritional shift-up experiments LB broth (17), supplemented with thymine (50 mg/liter), was used as rich medium.

Antibiotics. Cefsulodin, mecillinam, and furazlocillin were generous gifts from Laboratorios Abelló S.A. (Madrid, Spain), Leo Pharmaceutical Products (Ballerup, Denmark),

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and Bayer (Wuppertal, Federal Republic of Germany), respectively.

Nutritional shift up. Nutritional shift-up experiments were performed essentially as described previously (16) by dilution (1:4) of cultures growing in MOPS mineral medium with prewarmed LB broth.

Alignment of chromosome replication. Chromosome replication was synchronized in the *thy* strain AB11570 by successive periods of amino acid and thymine starvation, as previously described (14). An exponentially growing culture (doubling time, 60 min) was first deprived of amino acids for 90 min to permit the termination of ongoing rounds of chromosome replication while preventing the initiation of new ones. Afterwards, cells were deprived of thymine and amino acids were added back to the culture, which was further incubated for a time (120 min) long enough to allow the division of those cells ending replication during the period of amino acid starvation and to enable all cells in the culture to exceed the "initiation mass." The addition of thymine to cultures pretreated in this way triggers the initiation of a new round of DNA replication simultaneously in all of the cells.

Detection of cell lysis by the release of β-galactosidase activity. Cell lysis was detected by measuring the optical density (OD) of the cultures and β-galactosidase activity released into the growing medium. To increase sensitivity, cultures were grown in the presence of the β-galactosidase inducer isopropyl-β-D-thiogalactopyranoside (1 mM). Samples of 0.5 ml of culture were centrifuged (5,000 × *g*, 5 min) to sediment cells and the β-galactosidase activity in the supernatant was assayed as described previously (18).

Measurement of peptidoglycan synthesis. Peptidoglycan synthesis was analyzed by following the incorporation of the specific precursor *meso*-[3,4,5-^{3}H]diaminopimelic acid ([^{3}H]DAP; 35 Ci/mmol; Service des Molécules Marquées, Comisariat à l'Energie Atomique, Gif-sur-Yvette, France) into boiling 4% (wt/vol) sodium dodecyl sulfate-insoluble material, in the presence of lysine at 200 mg/liter to prevent metabolic conversion of the labeled precursor. Cultures were grown for a minimum of 10 generations in MOPS mineral medium in the presence of [^{3}H]DAP (0.05 μCi/μg, 10 μg/ml) before being diluted 1:4 in prewarmed LB medium supplemented with the radioactive precursor at the same concentration and specific activity. Repeated 0.5-ml samples were withdrawn throughout the experimental period, mixed with an equal volume of 8% (wt/vol) sodium dodecyl sulfate, and incubated at 100°C for 30 min. Samples were then filtered through HAWP membrane filters (Millipore Corp., Bedford, Mass.) and extensively washed with water at 60°C; after drying, radioactivity was measured by liquid scintillation.

RESULTS

Induction of delayed cell division by nutritional shift up and effect on the rate of murein biosynthesis. When *E. coli* AB1157 is subjected to a nutritional shift up, the growth rate in mass increases almost immediately, but cell division is transiently inhibited for approximately one (poor medium) generation time and then resumes at the new growth rate (16).

Figure 1 shows the behavior of a culture of *E. coli* AB1157 subjected to a nutritional shift up by growing the cells first in minimal MOPS-glucose medium (generation time, 70 min) and then transferring them to LB broth (generation time, 30 min). The change in the rate of growth in mass was almost immediate, whereas cell division stopped after the shift up and resumed 45 min later, as expected from previous work (15, 16).

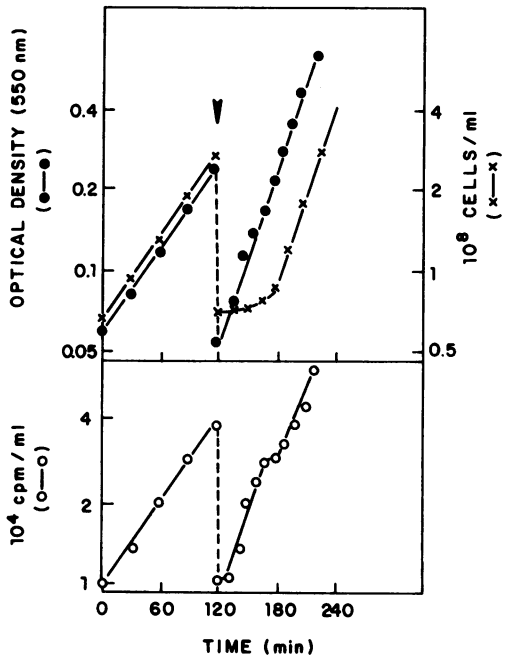

FIG. 1. Cell division and peptidoglycan synthesis in cultures of *E. coli* AB1157 subjected to nutritional shift up. A culture of *E. coli* AB1157 grown for 10 generations in minimal MOPS-glucose medium supplemented with [^{3}H]DAP (0.05 μCi/μg, 10 μg/ml) at 37°C was diluted 1:4 in prewarmed LB broth plus [^{3}H]DAP as above. Samples were periodically removed to measure the OD (●), particle number (×), and radioactivity incorporated as sodium dodecyl sulfate-insoluble material (○).

For our purpose it was important to make sure that the shift-up treatment did not induce a transient inhibition of peptidoglycan synthesis, as this could lead to erroneous conclusions. This point was checked by following the incorporation of the murein precursor [^{3}H]DAP as sodium dodecyl sulfate-insoluble material throughout the shift up. The result is also shown in Fig. 1 and indicates that there is no inhibition of murein synthesis. This point was further confirmed in experiments with the *dapA lysA* double mutant strain W7 (10) (data not shown).

Induction of cell lysis by β-lactam antibiotics in cultures subjected to nutritional shift up. The β-lactams used to specifically inhibit selected PBPs were cefsulodin (100 μg/ml), which binds to PBPs 1; mecillinam (10 μg/ml), which blocks PBP 2; and furazlocillin (1 μg/ml), which binds to PBP 3 (4, 9, 19).

The effect of the inhibition of PBPs 1 by cefsulodin was studied by adding the antibiotic, alone or in combination with mecillinam or furazlocillin, to a series of parallel cultures at different times after the nutritional shift up. The occurrence of lysis was monitored in two ways: following the variation in OD of the cultures and measuring the activity of β-galactosidase released into the medium by lysed cells. The lytic behavior of the cultures closely matched the expectations. In the cultures treated with cefsulodin, lysis started in all instances by the time that cell division was due to resume (40 min after the shift up) (Fig. 2). Furthermore, cultures were able to increase substantially in OD before the onset of lysis. Therefore, mass growth throughout the time

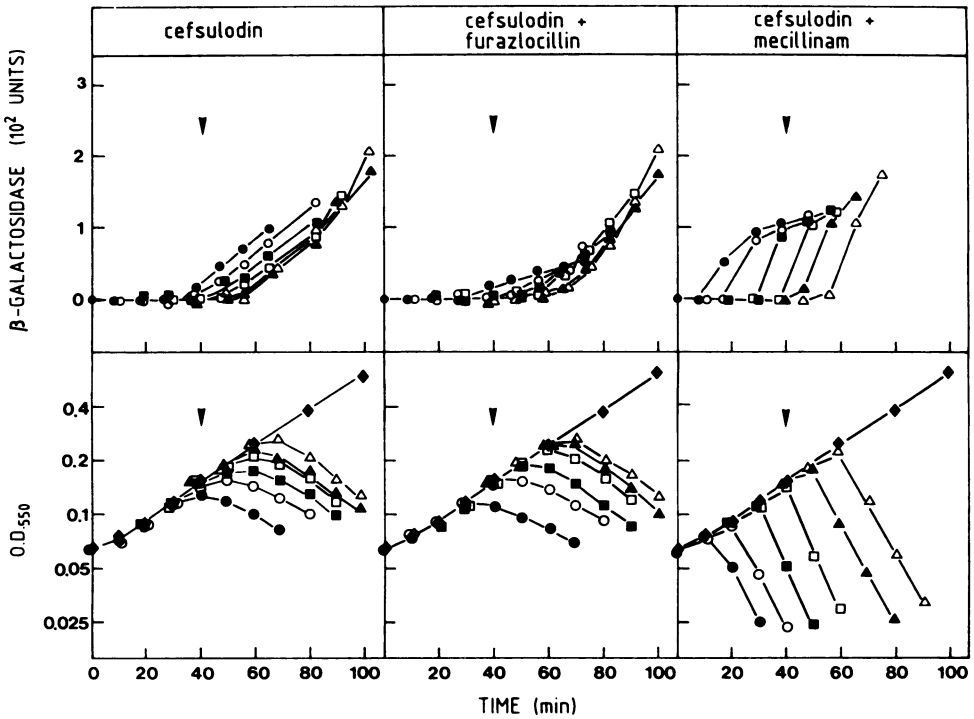

FIG. 2. Effect of β-lactam antibiotics in cultures subjected to nutritional shift up. A culture of *E. coli* AB1157 was subjected to nutritional shift up and divided into subcultures which were treated with cefsulodin (100 μg/ml) alone or in combination with furazlocillin (1 μg/ml) or mecillinam (10 μg/ml) at the following times: ●, 0 min; ○, 10 min; ■, 20 min; □, 30 min; ▲, 40 min; and △, 50 min. ◆, Growth of an untreated subculture. Induction of lysis was followed by measuring the OD of the cultures and the β-galactosidase activity released to the medium. The arrow indicates the time of initiation of cell division in the control culture.

between the addition of cefsulodin and the onset of lysis was apparently unaffected by the antibiotic. However, the simultaneous addition of mecillinam and cefsulodin evoked a drastically changed response of the cells, which now lysed at a constant time 10 min after the addition of the drugs. In fact, the increase in OD was in all cultures comparable to that in a similarly treated exponentially growing culture, between 15 and 20%. In contrast, the effect of cefsulodin was not modified by the simultaneous presence of furazlocillin, indicating that the inhibition of PBP 3 did not affect the lytic response of the cells after blockage of PBPs 1.

Effect of β-lactams on cultures subjected to alignment of chromosome replication. To confirm the above results, cultures of *E. coli* AB11570 were subjected to chromosome replication alignment as an alternative method (14). As septation depends on the termination of DNA replication (6), this kind of treatment results in a delayed and partially synchronized round of cell division.

To study the effect of β-lactams on cells treated in this way, the initiation of DNA replication was triggered by the addition of thymine to a series of pretreated aligned subcultures (see Materials and Methods) which were subsequently challenged with antibiotics at different times and then followed in OD, particle number, and β-galactosidase activity released into the medium.

Figure 3A shows the evolution in OD and particle number of a culture of *E. coli* AB11570 throughout the treatment. Cell division resumed about 40 min after the addition of thymine. Figures 3B and C show the response of the cultures to the addition of cefsulodin alone and of cefsulodin plus mecillinam, respectively. The results were comparable to those of the shift-up experiment. The inhibition of PBPs 1 at any time during the first 30 min resulted in the onset of cell lysis at the time that cell division was expected to occur. Furthermore, while the simultaneous inhibition of PBP 3 by furazlocillin had no effect on the action of cefsulodin (data not shown), blockage of PBP 2 by mecillinam again caused a change in the response of the cells, which under these conditions lysed at a fixed time (10 min) after the addition of the antibiotics. Measurements of released β-galactosidase throughout the experiment confirmed the turbidimetric results (data not shown).

DISCUSSION

Earlier studies on the effect of β-lactams in *E. coli* cells initiating growth indicated that cell lysis caused by the inhibition of PBPs 1 is a timed event in the first division cycle, in the sense that irrespective of the time of inhibition, lysis actually occurs only when the cells reach a particular stage, coincident with the initiation of cell division. This raised the question of whether the apparent timing of the lytic response to the inhibition of PBPs 1 would also be exhibited by actively growing cells. An affirmative answer would suggest that PBPs 1 act in a periodic fashion in the cell cycle, specifically during septum formation.

The experimental approach selected was based on the assumption that the triggering of cell lysis by the inhibition of PBPs 1 would be linked to some early event in septation. We therefore studied lysis of *E. coli* cells treated with β-lactams under conditions in which cell division was aligned either by nutritional shift up or by chromosome replication alignment. Neither of these methods yields synchronous cultures, but rather they induce a temporary inhibition of cell division

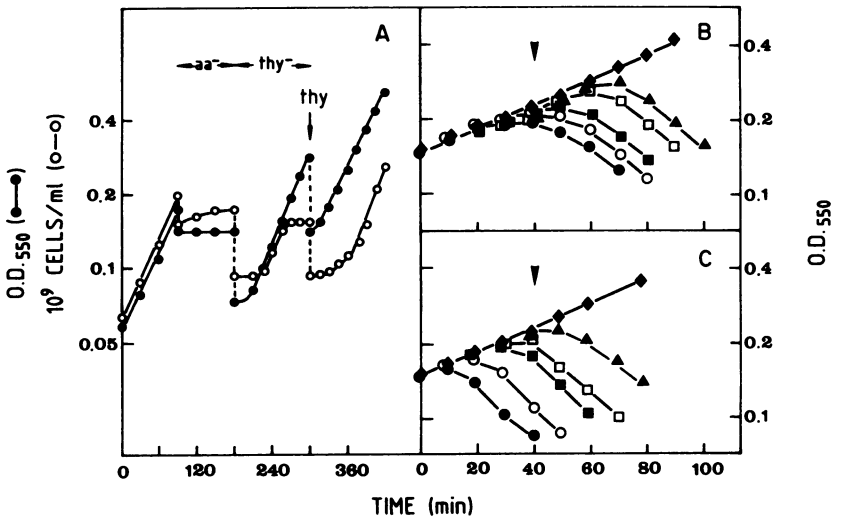

FIG. 3. Effect of β-lactam antibiotics in cultures subjected to chromosome replication alignment. A culture of *E. coli* AB11570 was aligned for chromosome replication as described in Materials and Methods and was divided into subcultures at the time when DNA replication was allowed to restart by the addition of thymine; these subcultures were treated at different times with cefsulodin (100 μg/ml) alone or in combination with mecillinam (10 μg/ml) at different times. (A) Evolution of OD (●) and cell number (○) throughout the treatment for chromosome alignment; (B) evolution of the subcultures treated with cefsulodin; (C) evolution of the subcultures treated with cefsulodin plus mecillinam. Symbols: ●, 0 min; ○, 10 min; ■, 20 min; □, 30 min; ▲, 40 min; ◆, untreated subculture. The arrow indicates the time of initiation of cell division in the control culture.

without simultaneous blockage of cell growth. Under these conditions potentially division-proficient cells accumulate and divide almost simultaneously when the temporary inhibition is released. Nevertheless, the confirmation of our hypothesis will have to wait for the development of an efficiently strain-independent synchronization method yielding balanced growth.

The results obtained by these two very different methods were highly consistent and clearly indicated that when PBPs 1 alone were inhibited at any time during the inhibition of cell division, the initiation of lysis always took place coincidentally with, or shortly before, septation, in a fashion resembling that described for cells at the initiation of growth (8). This suggests that the murein hydrolytic system of the cell is responsive to the inhibition of PBPs 1 only during a narrow time window during the cell cycle, coincident with the initiation of cell division. Our observations further suggest that PBPs 1 are involved in an early event in septum formation. This could explain why the inhibition of PBP 3, an essential but supposedly "late" protein in septum formation, had no effect on the lytic response to inhibitors of PBPs 1. However, the apparent connection between cell division and the change in the responsiveness of murein hydrolases to the inhibition of PBPs 1 seems to be modulated by PBP 2 activity, as indicated by the fact that the inhibition of this protein by mecillinam promotes an immediate response of the murein hydrolases when an inhibitor of PBPs 1 is present.

Our observations suggest that an active PBP 2 is by itself able to maintain murein synthesis throughout the elongation period, up to a point when a septation-related event occurs, making the activity of PBPs 1 necessary to avoid the otherwise fatal, uncontrolled action of the murein hydrolases. From a mechanistic point of view this could be explained in at least two ways; either PBP 2 is the main synthetic enzyme for cell wall elongation and PBPs 1 are required only during a reduced period at septation, or PBP 2 is able to take over the function of PBPs 1 during the cell elongation period but not during septation, when greater murein synthetic activity is required. A possible modulation of PBP 2 activity by a cell division-related event would not be surprising; in fact, it was suspected long ago (5, 13), and recent findings indicating that PBP 2, PBP 3, RodA, and FtsA may form a complex certainly support this view (1, 2, 12, 22). The present results suggest that PBPs 1 may also participate in the septation process.

Although the experimental systems that we used may still be far from reflecting the physiology of balanced growth, we consider that the results reported here favor the idea that the triggering of cell lysis in response to the inhibition of PBPs 1 is a timed event in the cell cycle.

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