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Penicillin binding proteins: Role in initiation of murein synthesis in *Escherichia coli*

(cell growth/β-lactam antibiotics/peptidoglycan/crosslinking)

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ABSTRACT **The consequences of the specific inhibition of penicillin binding proteins (Pbps) by β-lactam antibiotics immediately before resumption of active growth in *Escherichia coli* suggest that inhibition of murein biosynthesis does not prevent the earlier steps of the initiation of cell growth in mass. The activity of Pbp 2 is apparently critical for the initiation of murein biosynthesis. Provided that Pbp 2 remains active, the other Pbps (1a, 1b, 3, 4, 5, and 6) can be inhibited without any noticeable effect on the initial rate of incorporation of new precursors into macromolecular peptidoglycan. These precursors are, in addition, inserted with a high degree of crosslinkage.**

When, after a long period in stationary phase, a bacterial cell starts active growth, one of the first biochemical events that must occur is the initiation of murein biosynthesis to permit the expansion of the rigid sacculus, whose elongation in bacillar bacteria like *Escherichia coli* conditions the growth of the cell. However, the lack of information on how murein biosynthesis begins constitutes, in our opinion, a serious gap in the present knowledge of the metabolism of the bacterial sacculus.

The crucial involvement of penicillin binding proteins (Pbps) in inserting new precursors into the murein and in shaping and dividing the growing bacterial cell (1–3) suggests that the study of their function in the early steps of murein and cell growth would be an appropriate way to begin research into this problem.

The characteristics of the Pbps of *E. coli* are fairly well known and have been reviewed recently (4, 5). It is generally accepted that Pbps 1a and 1b intervene in cell wall elongation, Pbp 2 is required for correct shaping of the cell, Pbp 3 participates in the formation of the septum, Pbp 4 is involved in postinsertional modifications of the peptidoglycan, and Pbps 5 and 6 regulate the number of pentapeptide side chains in the growing peptidoglycan.

In this paper, we study the way the beginning of cell growth and murein synthesis is affected by selective inhibition of those Pbps more probably involved in cell wall elongation and shaping.

MATERIALS AND METHODS

Bacterial Strains and Conditions of Growth. *Escherichia coli* W7 (*dapA*, *lysA*) (6) was used throughout this work. Bacteria were grown aerobically at 37°C in MC medium (7) supplemented with 10 mM glucose, 10 μg of *meso*/DD/LL-2,6-diaminopimelic acid (A_2pm) per ml, and 80 μg of L-lysine per ml. Under these conditions, cells enter stationary phase at an OD_{550} of 2.0.

In experiments with cells starting active growth, cultures kept in stationary phase with aeration for 24 hr at 37°C were diluted 1:5 with prewarmed medium of identical composition to initiate growth. The viability of the cultures in stationary phase remained constant (1.6×10^9 viable cells per ml) for more than 36 hr.

After the cells were fixed with 0.2% formaline/0.9% NaCl, particle concentration and mean cell volume were measured in a Coulter Counter ZBI particle analyzer coupled to a Coulter Chanalizer.

Antibiotics. Mecillinam and cefsulodin were generous gifts from Leo, Helsingborg, Sweden and Takeda, Osaka, Japan, respectively. Ampicillin and cefmetazole were obtained from Antibioticos, León, Spain.

Incorporation of Radioactive Precursors. *meso*/DD/LL-Diamino[3,4,5-^{3}H]pimelic acid ([^{3}H]A_2pm) (30 Ci/mmol; Comisariat a l'Energie Atomique, Service des Molécules Marquées, France; 1 Ci = 37 GBq) and L-[4,5-^{3}H]lysine (75 Ci/mmol; Radiochemical Centre) were used as precursors for the synthesis of peptidoglycan and proteins, respectively. The radioactive precursors were added to the cultures at the time of dilution, and double samples of 1 ml were taken at regular time intervals. Samples labeled with [^{3}H]A_2pm were mixed with 1 ml of 8% (wt/vol) $NaDodSO_4$, incubated in a boiling-water bath for 30 min, filtered through Millipore HAWP filters soaked in a solution of A_2pm (2 mg/ml), and washed with water at 90°C. Samples labeled with [^{3}H]lysine were mixed with 1 ml of ice-cold 10% (wt/vol) trichloroacetic acid, incubated for 1 hr in an ice-water bath, filtered through Whatman GF/C filters soaked in a solution of L-lysine (2 mg/ml), and washed twice with 10 ml of 5% trichloroacetic acid and then with 10 ml of cold ethanol. Once the filters had dried, radioactivity was measured by liquid scintillation counting.

Binding of β-Lactam Antibiotics to Pbps. The interaction of unlabeled β-lactams with the Pbps was analyzed by means of competition experiments based on the method described by Spratt (1, 2). Samples containing 8×10^9 total cells were taken from cultures starting active growth in the presence of the selected β-lactams at 0, 1, and 2 hr after dilution of the stationary-phase culture. Cells were centrifuged (5 min at 10,000 × *g*), resuspended in 50 μl of 50 mM phosphate buffer (pH 6.8), mixed with 50 μl of *N*-[3-(4-hydroxy-5-[^{125}I]iodophenyl)propionyl]ampicillin (2000 Ci/mmol; 1 mCi/ml) (8), and incubated for 30 min at 37°C. To stop the reaction and solubilize the proteins, the samples were diluted in 0.5 ml of 2% $NaDodSO_4$/0.0625 M Tris·HCl/5% (vol/vol) 2-mercaptoethanol (pH 6.8), incubated for 20 min in a boiling-water bath, and centrifuged (30 min at 20,000 × *g*) to remove $NaDodSO_4$-insoluble material. After this, 75-μl aliquots of supernatant were fractionated by $NaDodSO_4$/PAGE as de-

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Abbreviations: Pbp, penicillin binding protein; A_2pm, DL-2,6-diaminopimelic acid.

scribed by Laemmli (9) in 8% (wt/vol) acrylamide gels. The Pbps were identified by autoradiography of the gels on Kodak X-Omat S x-ray films exposed for 10 days.

Analysis of Peptidoglycan by Paper Chromatography. After specific labeling of the peptidoglycan with [^{3}H]A_2pm, culture samples containing 6×10^{10} total cells were mixed 1:1 with 8% $NaDodSO_4$ and incubated for 45 min in a boiling water bath, after which peptidoglycan was extracted as described (10). Purified peptidoglycan was resuspended in 50 mM phosphate buffer (pH 4.9) at a concentration of 2 mg of murein per ml and was digested with *N,O*-acetyl-muramidase from *Chalaropsis sp.* (20 μg of enzyme per ml for 12 hr, 37°C), purified as described (11). The digests were subjected to descending paper chromatography on Schleicher & Schüll SS 2040b paper for 24 hr using the upper phase of a mixture of 1-butanol/acetic acid/water, 4:1:5 (vol/vol), as eluent (10). Muropeptides were identified by comparing their mobilities with those of purified bis(disaccharide) tetrapeptide (C3) and disaccharide tetrapeptide (C6) labeled with [^{3}H]A_2pm, which were run in parallel. Once dried, the chromatograms were cut into 0.5-cm-long pieces and soaked in 0.2 ml of water. Radioactivity was measured by liquid scintillation counting using Bray's fluid (12).

RESULTS

Fig. 1 shows the evolution of the main parameters of growth (cell mass, cell number, and mean cell volume) of a culture of *E. coli* W7 during resumption of active growth after a resting period of 24 hr. Under our experimental conditions, cell mass and mean cell volume began to increase 20–30 min after dilution of the resting culture, and cell division started 90 min later, when the cell mass had increased by 110%.

Selective inhibition of the Pbps was achieved by using β-lactam antibiotics with high specificity of binding. Mecillinam (10 μg/ml) was used to inhibit Pbp 2 (2), cefsulodin (150 μg/ml) was used to inhibit Pbps 1a and 1b (13), cefmetazole (formerly CS1170; 20 μg/ml) was selected to inhibit all the Pbps except 2 (14), and ampicillin (1 mg/ml) was chosen to inhibit all the Pbps simultaneously (2).

The ability of the selected antibiotics to interact with the Pbps of cells at the stationary phase and during resumption of growth was tested in competition experiments as described. The results (Fig. 2) confirm that, under our conditions, the antibiotics saturated their target Pbps. The lack of Pbp 3 in stationary-phase cells, as well as the reduced levels

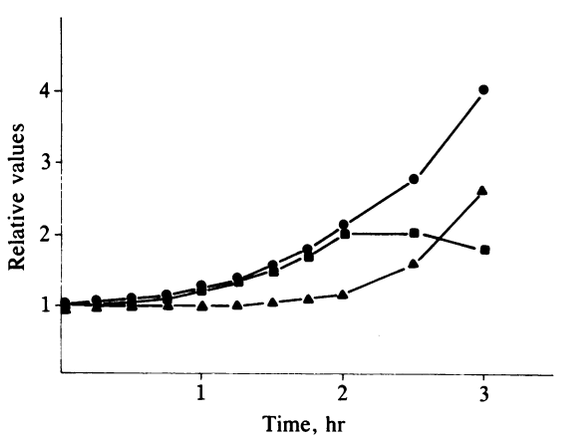

FIG. 1. Initiation of cell growth by *E. coli* W7. A 24-hr-old stationary-phase culture of *E. coli* W7 was diluted 1:5 (vol/vol) into new prewarmed medium. Initiation of growth was monitored by measuring OD_{550} (●), mean cell volume (■), and cell number (▲). Unit values correspond to 0.4 for OD_{550}, 0.95 μm^3 for cell volume, and 3.4×10^8 particles per ml for cell number.

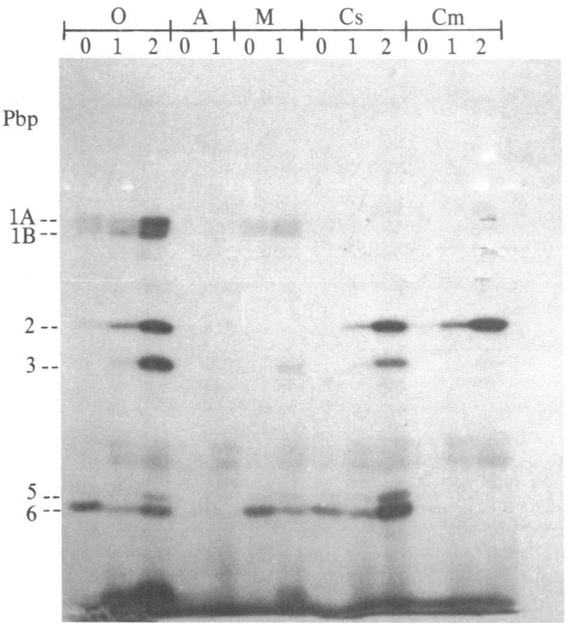

FIG. 2. Binding of β-lactams to the Pbps of *E. coli* W7. Ampicillin (1 mg/ml), mecillinam (10 μg/ml), cefsulodine (150 μg/ml), and cefmetazole (20 μg/ml) were added to parallel 24-hr-old stationary-phase cultures of *E. coli* W7 30 min before diluting 1:5 (vol/vol) with new prewarmed medium containing the same antibiotic at an equal final concentration. A fifth culture, untreated with antibiotics, was used as control. Samples (8×10^9 total cells) were taken at time zero and 1 and 2 hr after dilution and were incubated with *N*-[3-(4-hydroxy-5-[^{125}I]-iodophenyl)propionyl]ampicillin to identify the Pbps unaffected by the treatment with antibiotics. In the cultures treated with ampicillin and mecillinam, no 2-hr samples were taken because of the onset of cell lysis in the cultures. Lanes: O, Control culture; A, ampicillin at 1 mg/ml; M, mecillinam at 10 μg/ml; Cs, cefsulodin at 150 μg/ml; Cm, cefmetazole at 20 μg/ml. Numbers indicate the time in hours at which samples were taken.

of binding of the radioactive antibiotic to Pbps 1 and 2, is a physiological property of these proteins whose capacity of binding to β-lactam antibiotics is modified depending upon the state of growth of the cells (15).

To analyze the consequences of inhibiting the Pbps at the early stages of active growth, we added the selected antibiotics at the final concentrations indicated above to 24-hr-old stationary-phase cultures of *E. coli* W7, and the antibiotics were allowed to act on the cells for 30 min before the cultures were diluted in new medium containing the antibiotic at the same final concentration to permit initiation of growth. The action of the same β-lactams on exponentially growing cultures was followed for comparative purposes.

The results of this experiment (Fig. 3) indicated that none of the antibiotics prevented initiation of cell growth, even at concentrations producing severe damage to exponentially growing cells, although all of them blocked cell division effectively as indicated by the fact that the cell number remained constant at the initial value (3.4×10^8 particles per ml) until the onset of cell lysis. The biphasic character of the initiation of growth in the presence of mecillinam was somewhat variable when repeated. In most cases (three times out of five experiments) it was as shown in Fig. 3. However, in other experiments, a period of cell lysis, with a drop in OD_{550} of 30–40%, occurred between the two waves of growth. The differences observed in the action of ampicillin and cefmetazole, which does not bind to Pbp 2, suggest that Pbp2 might be involved in the initiation of cell wall synthesis.

The effect of inhibition of Pbps by ampicillin on the initiation of protein synthesis was investigated in experiments in which [^{3}H]lysine (final specific activity, 0.2 μCi/μg)

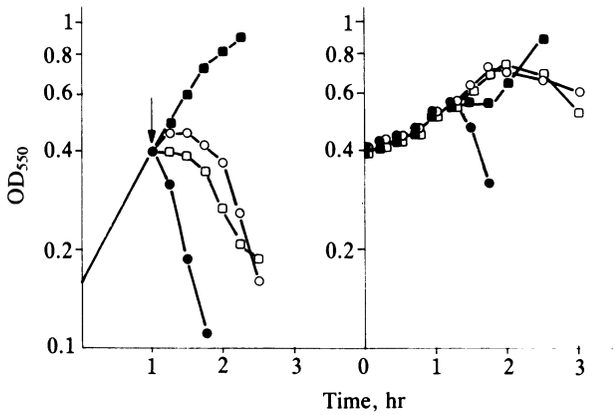

FIG. 3. Effect of β-lactam antibiotics on cultures of *E. coli* W7 growing exponentially and starting active growth. Ampicillin (1 mg/ml), mecillinam (10 μg/ml), cefsulodin (150 μg/ml), and cefmetazole (20 μg/ml) were added to parallel exponentially growing cultures of *E. coli* W7 at the time indicated by the arrow (*Left*) or to 24-hr-old stationary-phase cultures (*Right*) 30 min before 1:5 dilution with new prewarmed medium containing the same antibiotic at an equal final concentration. Time zero indicates the time at which the cultures were diluted. ●, Ampicillin at 1 mg/ml; ○, cefsulodin at 150 μg/ml; ■, mecillinam at 10 μg/ml; □, cefmetazole at 20 μg/ml.

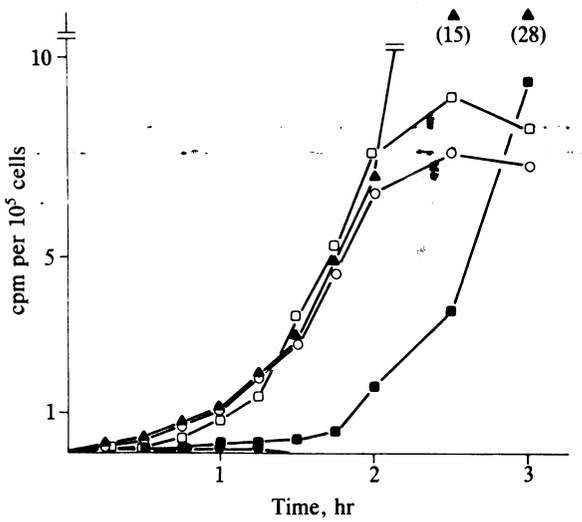

FIG. 4. Incorporation of [^{3}H]A_2pm by cells initiating growth in the presence of β-lactam antibiotics. The indicated β-lactams were added to 24-hr-old stationary-phase cultures 30 min before 1:5 dilution in new prewarmed medium containing the same antibiotic at an equal final concentration and [^{3}H]A_2pm at 1.25 μCi/μg. Samples were taken at the times indicated and processed as described. Time zero is the time at which cultures were diluted. ●, Ampicillin at 1 mg/ml; ○, cefsulodin at 150 μg/ml; ■, mecillinam at 10 μg/ml; □, cefmetazole at 20 μg/ml; ▲, untreated control. The ampicillin-treated culture was followed only for 75 min because of the onset of rapid cell lysis at this time.

was added at the time of dilution to cultures starting growth in the presence of ampicillin at 1 mg/ml. As a negative control, the incorporation of the radioactive precursor into a parallel culture treated with chloramphenicol (100 μg/ml) instead of ampicillin was also measured. The results indicate that the action of ampicillin did not affect protein synthesis during the initiation of growth. After 60 min, the ampicillin-treated cultures had accumulated 70.425 cpm of [^{3}H]lysine per ml, which compares with the 78.075 cpm/ml accumulated by an untreated control culture. Background incorporation in the presence of chloramphenicol was 8.637 cpm/ml.

The incidence of the inhibition of Pbps on the synthesis of macromolecular peptidoglycan during the initiation of growth was analyzed by following the incorporation of the specific precursor [^{3}H]A_2pm into boiling 4% $NaDodSO_4$-insoluble material. The experimental protocol was identical to that described above except that [^{3}H]A_2pm was added to a final specific activity of 1.25 μCi/μg to the cultures at the time of dilution.

The results of the experiment are shown in Fig. 4. With the exception of ampicillin, which inhibited peptidoglycan synthesis completely as expected, the effect of the β-lactams used was quite surprising. In the presence of cefsulodin (inhibition of Pbps 1a and 1b) or cefmetazole (inhibition of all Pbps except Pbp 2), the cells retained the ability to synthetize macromolecular peptidoglycan at a rate similar to that in the untreated control cells for about 2 hr, when the incorporation of new material suddenly stopped. In the presence of mecillinam, [^{3}H]A_2pm was incorporated at a comparable rate. However, initiation of the incorporation was considerably delayed (90 min). The late initiation of murein synthesis in mecillinam-treated cultures cannot be attributed to degradation of the antibiotic. The amount of mecillinam left in the culture media after 3 hr of incubation was still high enough to induce formation of ovoid cells in an exponentially growing culture of the same bacteria, mixed 1:1 with a filtrate of the mecillinam-treated culture.

To find out whether the macromolecular peptidoglycan synthetized in the presence of cefsulodin and cefmetazole was normally crosslinked, its degree of crosslinkage was measured by paper chromatography of the degradation products obtained after digestion with the *N,O*-acetylmuramidase of *Chalaropsis sp.* The experimental method was similar to that indicated above. The cultures were incubated in the presence of [^{3}H]A_2pm (specific activity, 1.25 μCi/μg) for 2 hr, and peptidoglycan was purified and treated as described. The results (Table 1) clearly showed that the murein synthetized in the presence of the antibiotics was highly crosslinked, pointing, therefore, to the participation of murein-transpeptidase activities in the synthesis of this material.

Observation under the electron microscope of the cells treated with ampicillin, cefsulodin, and cefmetazole showed that they remained rod-shaped throughout the treatments, while in the presence of mecillinam they became ovoid (2) (data not shown).

DISCUSSION

The observation that resting cells are able to start growth in the presence of a high concentration of ampicillin, increasing their size and synthetizing proteins at rates similar to those of untreated cells for a considerable period of time (60–80 min), suggests that initiation of cell growth in mass is independent of the functional state of the Pbps and, therefore, of the capacity of the cells to start murein biosynthesis.

The capacity of cells starting growth to increase their cell mass significantly (40–50%) in the absence of murein synthesis, before the onset of lysis, suggests that the cell wall

Table 1. Crosslinkage of the murein synthetized by *E. coli* W7 cells starting active growth in the presence of cefsulodin or cefmetazole

Antibiotic added	[^{3}H]A_2pm in dimers, cpm	[^{3}H]A_2pm in monomers, cpm	Crosslinkage*
None	9,426	9,289	0.336
Cefsulodin	11,623	10,445	0.357
Cefmetazole	13,512	9,645	0.412

*Expressed as the molar fraction of dimers.

might be able to undergo certain structural rearrangements, allowing a moderate enlargement of the cell.

The differential action of ampicillin and cefmetazole or cefsulodin on the initiation of murein biosynthesis suggests that all Pbps except Pbp 2 are dispensable in the early steps of this process, as far as net incorporation of new material into the sacculus is concerned. Furthermore, the peptidoglycan synthetized in the presence of cefsulodin or cefmetazole acquired a degree of crosslinkage even higher than that of the murein synthetized by the control cells, indicating that Pbp 2 might have a transpeptidase activity *in vivo* able to produce a highly crosslinked peptidoglycan. The recent finding of a DD-transpeptidase activity associated to purified Pbp 2 in *in vitro* systems (16) strengthens our argument considerably.

Inhibition of Pbp 2 by mecillinam at the beginning of cell growth did not prevent initiation of peptidoglycan synthesis, although it was drastically delayed (>60 min). When it did take place, it was at the same moment in the process as that at which cell lysis was triggered after inhibition of Pbps 1 by cefsulodin or cefmetazole. These results suggest that, during initiation of cell growth, Pbps 1 became active in peptidoglycan biosynthesis and necessary for the maintenance of cell integrity at a relatively late stage, timed independently of the activity of Pbp 2.

By assuming that the action of the β-lactams used in this study is exclusively due to the inhibition of the Pbps to which they bind, our observations could be interpreted in the following way: When cell growth begins, Pbp 2 mediates the synthesis of peptidoglycan required for the initial elongation of the sacculus until the cells become close to the moment of division, when a second mechanism of synthesis (most probably mediated by Pbps 1 and 3) becomes essential for the first round of cell division to occur. This hypothesis agrees well with the observation of James *et al.* (17) that mecillinam inhibits an early event in the *E. coli* cell cycle. It is conceptually based on the model proposed by Satta *et al.* (18) to explain cell wall growth in *Klebsiella pneumoniae*.

Apparently our results question previous reports postulating that in *E. coli* Pbps 1, particularly 1b, are the main peptidoglycan-synthetizing enzymes involved in the elongation of the murein sacculus (1, 4, 16). However, most information on this subject has been obtained by using either *in vitro* systems or exponentially growing cells, whose functional requirements for peptidoglycan synthesis might differ substantially from those of cells starting growth.

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