JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 333-340
0021-9193/81/010333-08$02.00/0

Vol. 145, No. 1

Evidence for Involvement of Penicillin-Binding Protein 3 in Murein Synthesis During Septation but Not During Cell Elongation

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Furazlocillin (1 μg/ml) and piperacillin (5 μg/ml) bound specifically to penicillin-binding protein 3 (PBP-3) and not to the other major PBPs in intact *Escherichia coli* cells. The effect of this specific binding to PBP-3 on murein synthesis of elongating and synchronously septating cells was investigated in two thermosensitive division mutants, *E. coli* BUG6 and *E. coli* JE10730, the latter possessing a thermolabile PBP-3. Synchronous cell division was induced by shifting the cultures from the nonpermissive temperature (42°C) to 30°C. Both [^{14}C]diaminopimelic acid incorporation into murein of intact cells and [^{14}C]*N*-acetylglucosamine incorporation into murein of cells permeabilized with ether was inhibited by an average of 42% in septating cells. In filaments growing at the nonpermissive temperature, we detected no inhibition and, frequently, a 10 to 15% stimulation of murein synthesis. The two drugs, at concentrations used in the above experiments, bound exclusively to PBP-3 both in elongating and septating intact cells and in ether-treated cells. These results support the hypothesis that PBP-3 activity is exclusively required for septal murein synthesis.

The terminal stages of murein synthesis and its regulation, which distinguish the cell elongation process from the process of septation, the role of murein hydrolases in these processes, and the overall regulation of these synthetic and degradative activities with respect to each other, to chromosome replication, and to the stationary-phase condition are all important, little-understood aspects of the bacterial cell cycle. One approach to studying the relationship between murein biosynthesis and its regulation during the division cycle of *Escherichia coli* focuses on the role of the seven or more proteins present in the inner membrane to which penicillin binds covalently (3, 14, 16). This approach is based on the assumption that any protein that binds beta-lactam antibiotics is involved in murein metabolism.

Penicillin-binding protein 3 (PBP-3), the subject of this paper, is required for cell division in *E. coli*, as suggested by the following two lines of evidence: (i) benzylpenicillin and cephalexin at concentrations which cause *E. coli* to grow as filaments have been shown to bind somewhat selectively to PBP-3 in cell envelope preparations (14); and (ii) mutants of *E. coli* have been isolated in which thermolabile PBP-3 results in a thermosensitive defect of septation (15, 16). The function of PBP-3 in cell division is unknown. However, PBP-3 appears to be essential for septal murein synthesis. If PBP-3 has a direct role, the study of its function should reveal a biochemical basis for distinguishing between the murein synthesis required for septation and that which accompanies cell elongation.

In this paper we present evidence that during synchronous septation, murein synthesis is inhibited by the selective binding of the β-lactam antibiotics piperacillin and furazlocillin to PBP-3 in whole cells. These two beta-lactam antibiotics have little or no effect on the murein synthesis that occurs during formation of nonseptate filaments. Thus, PBP-3 is needed for murein synthesis during septation but not during elongation.

MATERIALS AND METHODS

Bacterial strains. Two temperature-sensitive filament-forming mutants of *E. coli*, JE10730 (16) (*ftsI*, with a thermolabile PBP-3, kindly supplied by Y. Hirota, National Institute of Genetics, Mishima, Japan) and BUG6 (1, 2, 13), were used throughout this study. Bacteria were routinely grown at 30°C in L broth without NaCl or glucose.

Measurement of murein synthesis in intact cells by incorporation of *meso*-[^{14}C]diaminopimelic acid (*meso*-Dpm). Cultures (600 ml) of *E. coli* BUG6 and *E. coli* JE10730 were grown with aeration by shaking at 30°C (dividing condition) to 0.35 U of absorbance at 585 nm. Fifty-milliliter portions were then incubated without shaking at 42°C (nondividing condition). After 60 min of growth at the nonpermis-

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sive temperature, a beta-lactam antibiotic was added as required, and the cultures were shifted back to 30°C and grown further with aeration. To ensure no delay in the attainment of septation, the temperature of the broth culture was rapidly cooled to 30°C by swirling the flasks in an ice bath (2). The cells were filtered (Millipore Corp., Bedford, Mass.; type HA; pore size, 0.45 nm) at 0, 10, 20, and 30 min after the shift and incubated for 10 min at 30°C in 10 ml of prewarmed incorporation medium (50 mM Tris-hydrochloride, pH 7.6; 0.2% glucose; 10 mM $MgCl_2$; 5 mM K_2HPO_4; 0.4 mM glutamic acid; 2 mM L-lysine; 1 mM L-alanine; 200 μg of chloramphenicol per ml) which contained 0.35 μCi of *meso*-[*U*-^{14}C]Dpm (specific activity, 315 mCi/mmol; Research Products International, Elk Grove Village, Ill.) as well as the required amount of the selected drug. Control samples with or without drug grown at 30 or at 42°C were filtered 20 min after time zero and similarly processed.

At the end of the incubation, cells were harvested, washed twice in cold saline containing 10 mM $MgCl_2$, heated for 10 min at 100°C in 0.5 ml of water, and finally resuspended in 0.5 ml of 0.05 M ammonium acetate containing 500 μg of lysozyme (egg white lysozyme; Sigma Chemical Co., St. Louis, Mo.). After overnight incubation at 37°C, portions of the material solubilized by lysozyme digestion (i.e., not sedimented in 5 min at 5,000 × *g*) were mixed with 3 ml of scintillation fluid [2 liters of toluene, 1 liter of Triton X-100, 8 g of 2,5-diphenyloxazole (PPO), and 200 mg of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP)] and counted in a liquid scintillation spectrometer. The number of counts per minute was adjusted to the protein content determined on the pellet after lysozyme digestion (Bio-Rad protein assay).

Determination of the extent of peptide cross-linkage in murein synthesized under various conditions. The extent of cross-linkage was determined by the method described by Mirelman et al. (10), which measures the amounts of cross-linked and uncross-linked fragments in lysozyme digests.

Measurement of murein synthesis in ether-treated cells by incorporation of [^{14}C]UDP-*N*-acetylglucosamine. Cells were grown under dividing and nondividing conditions with or without antibiotic as described above. After harvesting, cells from 100 ml of culture were resuspended in 1 ml of basic medium (10) and stirred vigorously with an equal volume of ether for 60 s in order to make them permeable to nucleotides (17). Incubation mixtures (200 μl, final volume) were prepared as described elsewhere (10, 11). After 60 min of incubation at 30 or 42°C, the reaction was stopped by the addition of 5 ml of 10% cold trichloroacetic acid. The acid-precipitable material was collected on filters (Whatman GF/A). The material was washed twice with 10% trichloroacetic acid, ethanol, and acetone and dried. The dry filters were then immersed in toluene containing, per liter, 5.43 g of PPO and 100 mg of POPOP, and the radioactivity was measured in a scintillation counter.

Measurement of the rate of binding of beta-lactam antibiotics to the PBPs of intact and ether-treated cells. The method for treatment of intact and ether-treated cells with beta-lactam antibiotics is described in detail in the figure legends. After treatment, cell envelopes were prepared from both intact and ether-treated cells essentially as described by Spratt (14), suspended in 20 μl of 0.05 M phosphate buffer (pH 7), and incubated for 15 min at 30°C with either 2 μl of [^{14}C]benzylpenicillin (51 μCi/μmol; 50 μCi/ml; Amersham Corp., Arlington Heights, Ill.) or 2 μl of [^{125}I]furazlocillin (4,000 μCi/μmol; ~400 μCi/ml). The preparation and properties of [^{125}I]furazlocillin will be published elsewhere.

When [^{14}C]benzylpenicillin was used, the reaction was terminated by addition of 2 μl of 20% Sarkosyl (5) containing 1 mg of cold benzylpenicillin per ml. If [^{125}I]furazlocillin was used as radioactive probe, the reaction was stopped by addition of 1 ml of 50 mM phosphate buffer containing 1 mg of benzylpenicillin. Envelopes were centrifuged (100,000 × *g* for 15 min), rinsed, and suspended in 20 μl of 2% Sarkosyl. After 20 min at room temperature, the residue was removed by centrifugation, and 20 μl of supernatant was heated at 100°C for 3 min in 10 μl of 3× sample buffer (14) (0.15 M Tris, pH 6.8, 3% sodium dodecyl sulfate [SDS], 37% glycerol, 0.15 mM EDTA, 0.03% pyronin Y). PBPs were separated by SDS-polyacrylamide gel electrophoresis (7). Running gels contained 7.5% acrylamide and 0.15% methylenebisacrylamide (Bio-Rad Laboratories, Richmond, Calif.). Proteins labeled with [^{14}C]benzylpenicillin were located by fluorography (4, 8) essentially as described by Spratt (15) with the dried gels exposed to Kodak X-Omat RP5 X-ray film at −70°C for 19 days. When iodinated furazlocillin was used, films were exposed at −70°C for 12 to 24 h in the presence of an intensifying screen (DuPont Cronex Lightning Plus).

Assay of D-alanine carboxypeptidase 1A and 1B. The method for fractionation and assay was similar to previous methods (9, 12). The D-alanine carboxypeptidases were extracted from the envelope fraction of the cells from 600 ml of culture (absorbancy at 580 nm = 1.0) with 0.3 ml of 0.05 M Tris buffer, pH 8, containing 1% Triton X-100 and 1 mM 2-mercaptoethanol (TTM buffer) for 30 min at 0°C. After clarification by centrifugation at 100,000 × *g* for 15 min, the extract was placed on a column (3 cm by 0.7 cm in diameter) of DEAE-cellulose that had been equilibrated with TTM buffer. D-Alanine carboxypeptidase 1A was washed through the column with four 1-ml portions of TTM buffer. D-Alanine carboxypeptidase 1B was subsequently eluted from the column with four 1-ml portions of 0.2 M NaCl in TTM buffer.

To assay enzyme activity, 20 μl of each portion was mixed with 5 μl of a substrate mixture such that the final concentrations were 1.1 × 10^{-4} M UDP-Mur-NAc-L-ala-D-glu-*meso*-Dpm-D-ala-D-[^{14}C]ala (18,800 cpm), 5 mM $MgCl_2$, and 0.12 M Tris, pH 9. After incubation for 60 min at 37°C, the mixture was heated at 100°C for 2 min. After centrifugation, 20 μl of the supernatant was placed n a 0.2-ml bed of Dowex AG1 × 2H resin and washed onto the resin with 0.1 ml of water; after 2 min, [^{14}C]alanine was washed through the column with three 1-ml portions of water, dried, dissolved in 0.3 ml of water, mixed with 3 ml of the Triton X-100-based scintillation fluid, and counted.

Reagents. Cold UDP-Mur-NAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala was prepared by enzymatic addition of *meso*-Dpm and D-Ala-D-Ala to UDP-Mur-

NAc-L-Ala-D-Glu in a single-step procedure, and D-[^{14}C]alanine terminally labeled substrate was prepared by enzymatic addition of D-Ala-D-[^{14}C]Ala to UDP-Mur-NAc-L-Ala-D-Glu-*meso*-Dpm (1). [*U*-^{14}C]UDP-*N*-acetylglucosamine was purchased from International Chemical and Nuclear Co., Irvine, Calif. (specific activity, 32 mCi/mmol).

Furazlocillin (BAY K4999) was a gift from Delbay Pharmaceutical, Inc., Florham, N.J. Piperacillin was from Lederle Laboratories, Pearl River, N.Y. Chloramphenicol was purchased from Calbiochem, San Diego, Calif. Sodium lauryl sarcosinate was obtained from Chemical Additives Co., Farmingville, N.Y.

RESULTS

Effects of furazlocillin and piperacillin on cell septation. Of several new penicillins which we tested for their effects on cell shape, furazlocillin (BAY K4999) (18) and piperacillin (6) were found to cause filament formation without affecting the increase in cell mass. Comparison of the affinity of furazlocillin and piperacillin for the individual PBPs with cephalexin, another filament-inducing beta-lactam antibiotic, showed that both furazlocillin and piperacillin had much greater specificity for PBP-3 than did cephalexin (Table 1). Indeed, 50% saturation of PBP-3 occurred in 10 min at 30°C when cell envelopes were incubated with 0.05 μg of furazlocillin or 0.1 μg of piperacillin per ml. The other PBPs were 50% saturated only at much higher concentrations.

E. coli thermosensitive division mutants BUG6 and JE10730 (the latter possessing a thermolabile PBP-3) form filaments during growth at 42°C. If, after 60 min of incubation at 42°C, the cultures are shifted to 30°C by rapid, controlled cooling in an ice bath, cells septate in an almost synchronous way. In the experimental conditions adopted, a different timing of this process was observed with the two mutants. In BUG6, maximal septation occurred consistently 20 min after the temperature shiftdown, whereas septation occurred 35 min after the shiftdown in strain JE10730. Except for this difference in time of septation, the behavior of the two mutants was identical.

Furazlocillin (1 μg/ml) when added at time zero completely inhibited cell division in both strains (Fig. 1). A higher concentration of piperacillin (5 μg/ml) was required to obtain a similar effect on both mutants. With lower concentrations (0.5 and 2 μg/ml, respectively), some septating cells were observed, whereas if higher concentrations were used (10 and 50 μg/ml), vacuoli appeared within the cytoplasm after a 30-min treatment (Fig. 1D).

Effect of delayed addition of the beta-lactam antibiotics and chloramphenicol on septation. Furazlocillin (1 μg/ml) added 5 min after the temperature shiftdown still completely inhibited cell septation, whereas if addition of the drug was delayed until 5 min before the expected burst in cell division, the number of septating cells was almost identical to that observed in the control culture. A similar timing of action was observed with piperacillin (5 μg/ml). Since both drugs, at the concentrations used, saturated their primary target (PBP-3) in less than 2 min (as shown in Fig. 2 and 3), the failure

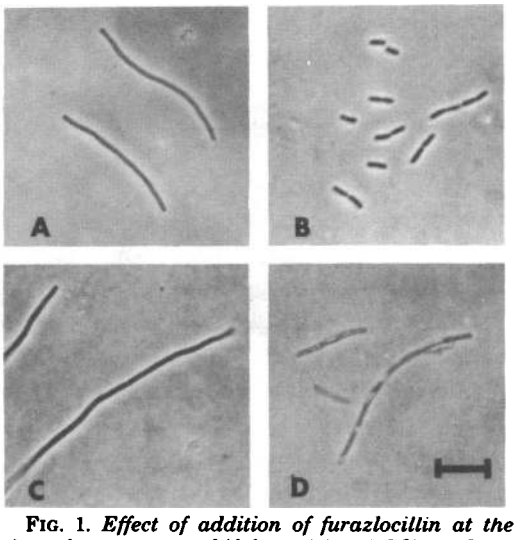

FIG. 1. *Effect of addition of furazlocillin at the time of temperature shiftdown (42 to 30°C) on shape of E. coli BUG6. (A) Untreated cells grown at 42°C for 60 min; (B) 30 min after the temperature shiftdown. Cells treated with furazlocillin at (C) 1 μg/ml and (D) 10 μg/ml for 30 min after the temperature shiftdown. Bar equals 3 μm.*

TABLE 1. *Concentrations of beta-lactam antibiotics required for 50% saturation of individual PBPs*[a]

Antibiotic	Concn required for given PBP								
	1A	1B	2	3	4	5	6	7	8
Cephalexin	4	>100	>100	25	25	>100	>100	>100	>100
Furazlocillin	3	6	0.5	0.05	6	6	12	0.8	0.8
Piperacillin	12	12	0.8	0.3	25	12	12	3	3

[a] Cell envelopes were incubated for 10 min at 30°C with twofold serial dilutions of the antibiotic to be tested. Then 2 μl of [^{125}I]furazlocillin was added to the suspension and incubation was continued for a further 15 min. See Materials and Methods for further details.

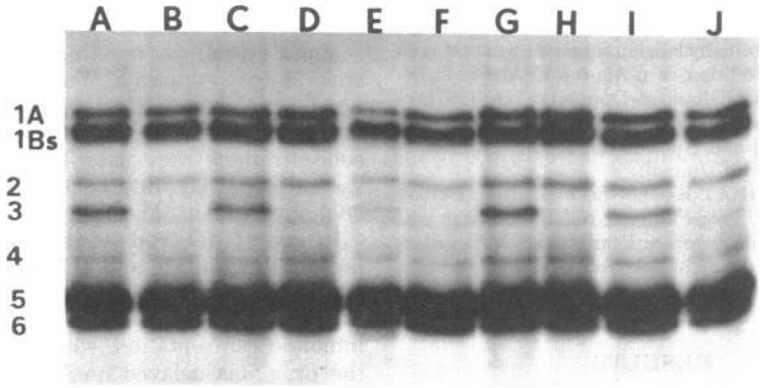

FIG. 2. *Binding of furazlocillin (1 μg/ml) to PBPs in intact cells of E. coli BUG6. Cells (80 ml for each sample) were grown at 30°C for two generations and then incubated at 42°C for 60 min. After this time, cultures were shifted down to 30°C and furazlocillin was added. Control and treated cells were harvested at 0, 10, 20, and 30 min after the shift. Control cultures were incubated at 42°C in the absence (A) and in the presence (B) of furazlocillin for 20 min. (C) 0-min control; (D) 0-min furazlocillin; (E) 10-min control; (F) 10-min furazlocillin; (G) 20-min control; (H) 20-min furazlocillin; (I) 30-min control; (J) 30-min furazlocillin. The amount of unreacted PBPs present in the cells was determined by using [^{125}I]furazlocillin as described in Materials and Methods.*

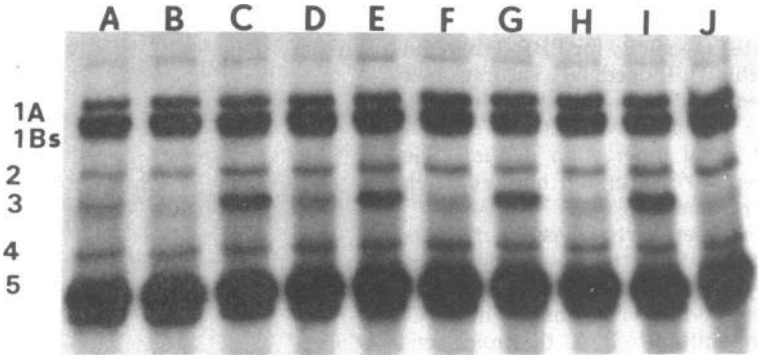

FIG. 3. *Binding of piperacillin (5 μg/ml) to PBPs in intact cells of E. coli BUG6. Legend as described for Fig. 2.*

to prevent cell division by very late addition suggests that a high level of PBP-3 activity is no longer required during the final 3 min preceding cell septation.

In contrast to BUG6, which could divide after the temperature shiftdown even in the presence of chloramphenicol, the mutant with thermolabile PBP-3 was not able to septate if chloramphenicol was present from time zero. This sensitivity indicates that sufficient PBP-3 is irreversibly inactivated during incubation at the nonpermissive temperature to require new synthesis of PBP-3 before septation can occur. However, if addition of chloramphenicol was delayed for 15 min after temperature shiftdown, at least 80% of the filaments divided.

Effect of furazlocillin and piperacillin on murein biosynthesis in intact and ether-treated cells. BUG6 cultured under nondividing conditions (i.e., at 42°C) incorporated 20% less [^{14}C]*meso*-Dpm into murein than cells grown at 30°C (Table 2). The degree of crosslinkage of the murein from dividing and nondividing cells was essentially the same (data not shown).

The effect of furazlocillin and piperacillin, at concentrations which inhibited cell division, on the amount of murein synthesized under different growth conditions is shown in Table 2. As can be seen, synthesis of murein by dividing cells (30°C), treated for 20 min with 1 μg of furazlocillin per ml, was inhibited by 24%. The inhibition actually varied from 17 to 30% in different experiments. A similar degree of inhibition was obtained with strain JE10730 and with wild-type strains of *E. coli* (KN126, PA3092, and M7LD) (data not shown). Murein synthesis was only slightly inhibited by piperacillin in cells grown

at 30°C. With higher concentrations of both drugs (40 μg/ml), a more marked inhibition was detected at 42°C (40%) than at 30°C (24%). This difference might be due to measurable binding of the drugs to PBP-2 that occurred only at the elevated temperature (data not shown). In contrast to the inhibition observed in dividing cells (30°C), the antibiotics actually caused a 10 to 15% stimulation of murein synthesis in nondividing cells (42°C) relative to the control (Table 2). There was no difference in the extent of cross-linkage in the insoluble murein formed by beta-lactam-treated filaments and untreated cells.

When murein synthesis was examined after the temperature shift, a rapid change in pattern was observed. Immediately after the shiftdown the effect of furazlocillin on murein synthesis was stimulatory, just as in the filaments growing at 42°C. Inhibition was evident in cells tested 10 min after the temperature shiftdown and increased to a maximum (30 to 55% in different experiments) at the time of maximal septation (Table 2). The percentage of inhibition (42%) was significantly higher than that observed in cells growing at 30°C and similarly treated. In cells labeled 45 min after the shift, the inhibition of murein synthesis by the antibiotics had dropped and was comparable to that seen in cells always cultured at the permissive temperature. All of the results obtained during shiftdown of BUG6 were confirmed in strain JE10730, except that the antibiotics did not stimulate murein synthesis by this strain at 42°C. This general pattern of inhibition (i.e., greatest inhibition 10 to 20 min after shiftdown and stimulation before shiftdown) was consistently observed in eight separate experiments.

Results obtained testing ether-treated cells were similar to those obtained in whole cells (Table 3). However, inhibition of murein synthesis in ether-treated cells at 30°C by piperacillin was higher than in intact cells and close to the values obtained with furazlocillin. Thus, it seems likely that the relative ineffectiveness of piperacillin in intact cells is due to the outer membrane barrier.

Binding of furazlocillin and piperacillin to PBPs in intact cells and ether-treated cells. Since the concentrations of the two antibiotics required to bind PBP-3 in cell envelope preparations were lower than those found effective in inhibiting cell septation and murein synthesis, we decided to investigate the binding pattern under the experimental conditions selected for the above-described studies. This was a necessary step in order to establish a relation between the observed inhibition of murein synthesis and the exclusive binding to a single PBP.

In intact cells, the antibiotics bound exclusively to PBP-3 of cells grown under all conditions when treated with either furazlocillin or piperacillin under the same experimental conditions used for the Dpm incorporation studies (Fig. 2 and 3). The binding occurred very rapidly since even in cells to which the antibiotic was added at 0 min and harvested almost immediately, no PBP-3 was available for reaction with [^{125}I]furazlocillin (Fig. 2 and 3). PBP-3 was still the only protein binding either antibiotic at concentrations up to 20 μg/ml (data not shown). At a 40-μg/ml concentration of either piperacillin or furazlocillin, some binding to PBP-2 was detectable (Fig. 4). It is interesting that PBP-2 appeared to be more accessible to the unlabeled beta-lactam antibiotics when intact cells were

TABLE 2. *Incorporation of [U-^{14}C]meso-Dpm into murein by intact cells of E. coli BUG6*

Growth conditions	Incorporation (cpm)[a]		
		Cultures treated with:	
	Control	Furazlocillin, 1 μg/ml	Piperacillin, 5 μg/ml
30°C (dividing cells)	100	76	95
42°C (nondividing cells)	80	93	87
Time (min) after shiftdown to 30°C			
0	80	93	90
10	80	65	68
20	100	57	59
30	100	80	77

[a] Expressed as percentage of counts incorporated per milligram of protein relative to the control at 30°C.

TABLE 3. *Incorporation of [^{14}C]N-acetylglucosamine into trichloroacetic acid-precipitable material from ether-treated cells of E. coli BUG6*

Growth conditions	Incorporation (cpm)[a]		
		Cultures treated with:	
	Control	Furazlocillin, 1 μg/ml	Piperacillin, 5 μg/ml
30°C (dividing cells)	100	82	77
42° (nondividing cells)	80	95	97
Time (min) after shiftdown to 30°C			
0	80	95	80
10	70	70	65
20	112	58	60
30	100	90	70

[a] Expressed as in Table 2.

treated at 42°C than when grown at 30°C or in late times after the temperature shiftdown. The complete disappearance of PBP-2 in furazlocillin- or piperacillin-treated filaments in comparison with the partial binding that occurred in dividing rods or septating filaments was noticed consistently in several experiments.

The binding of [^{125}I]furazlocillin and [^{14}C]benzylpenicillin was also examined in cells of BUG6 and strain JE10730, treated with ether and then with the unlabeled beta-lactam antibiotic. Both furazlocillin (1 μg/ml) and piperacillin (5 μg/ml) bound only to PBP-3, in complete agreement with the results obtained in intact cells. However, when a 40-μg/ml concentration of either drug was used, PBP-2 was also completely saturated with the drug, and PBP-1A and -5 were at least 50% saturated. Results obtained with furazlocillin on ether-treated BUG6 and strain JE10730 are shown in Fig. 5. In this experiment, ether-treated cells were incubated with the unlabeled drug for 15 min, but prolonged incubation (1 h, as in the experiments for murein synthesis by ether-treated cells) did not noticeably alter the results. It should be noted that strain JE10730, grown at 30°C, possessed a reduced level of PBP-3 in comparison with wild-type *E. coli*.

DISCUSSION

The close relationship among specific binding of furazlocillin or piperacillin to PBP-3, inhibition of cell septation, and a significant decrease in murein synthesis during septation is the main finding of this report. The observed inhibition of murein synthesis demonstrable only during septation strongly suggests that PBP-3 activity is specifically required, although in an as-yet-unknown way, for septal murein synthesis. Presumably the inhibition is greatest during the period of synchronous septation that occurs after the temperature shiftdown because septation-specific synthesis represents a major portion of the total murein synthesis under these conditions.

In BUG6 growing as filaments at the nonpermissive temperature, murein synthesis was not inhibited; in fact, it was stimulated by furazlocillin and piperacillin even though the antibiotics selectively bound to PBP-3. This result indicates that PBP-3 does not participate significantly in the murein synthesis that occurs during cell elongation. The stimulation can be attributed to the fact that furazlocillin and piperacillin inhibited the D-alanine carboxypeptidases (Table 4) and hence more of the nascent murein was present in a form that could be cross-linked to the existing wall, as has been suggested for the stimulatory effect observed with ampicillin (11). The inhibition of septal murein synthesis and the lack of inhibition of the murein synthesis required for cell elongation by furazlocillin and piperacillin are evidence that at least two pathways for murein synthesis exist and operate during the cell cycle. Whether the pathways differ in terms of substrates, final product, enzymes, or all of these is unknown at present, but the apparent lack of activity of PBP-3 during cell elongation suggests that different enzymes are involved and their activities are regulated during the cycle.

The inhibition of D-alanine carboxypeptidases by furazlocillin and piperacillin raises an important issue that must be considered in interpreting the role of PBPs in murein synthesis based on studies with antibiotics. As shown in Fig. 2 and 3 and Table 1, neither furazlocillin nor piperacillin bound to the principal D-alanine car-

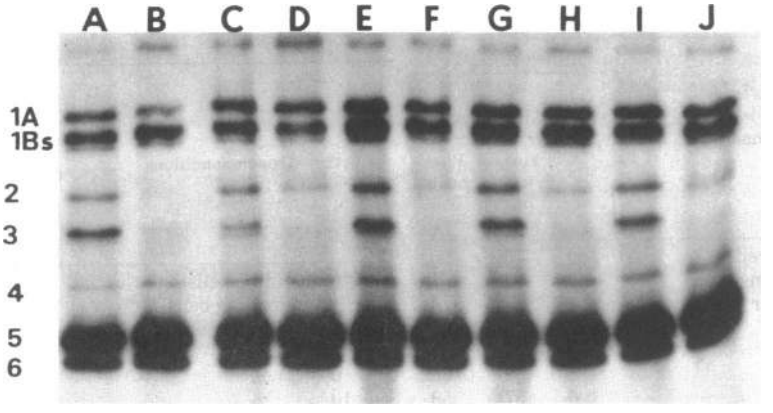

FIG. 4. *Binding of furazlocillin (40 μg/ml) to PBPs in intact cells of E. coli BUG6. Legend as described for Fig. 2.*

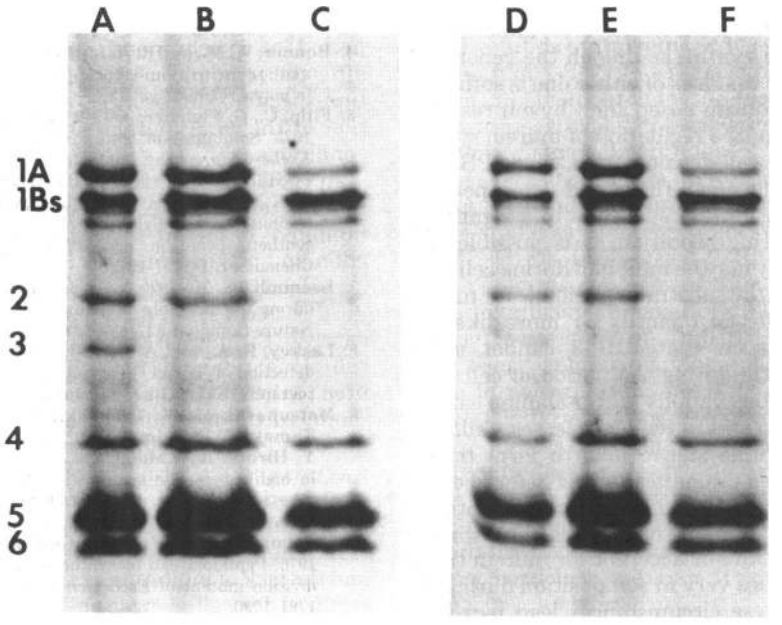

FIG. 5. *Binding of furazlocillin to PBPs in ether-treated cells of E. coli BUG6 (left) and E. coli JE10730 (right). Cells were grown at 30°C to mid-log phase, harvested after addition of cold basic medium, washed, and then treated with ether. After washing to remove ether, cells were resuspended in 200 μl of the same medium used for [^{14}C]N-acetylglucosamine incorporation, and furazlocillin was added at the final concentrations indicated. Incubation was carried out for 15 min at 30°C. After this time, the amount of unreacted PBPs present in the cells was determined by using [^{14}C]benzylpenicillin as described in Materials and Methods. (A) BUG6; (B) BUG6, 1 μg of furazlocillin per ml; (C) BUG6, 40 μg of furazlocillin per ml; (D) JE10730; (E) JE10730, 1 μg of furazlocillin per ml; (F) JE10730, 40 μg of furazlocillin per ml.*

boxypeptidases (PBP-4 and PBP-5) nor to any other PBP except PBP-3, and yet at the concentrations used in these experiments, furazlocillin and piperacillin inhibited Triton-solubilized D-alanine carboxypeptidase 1A and D-alanine carboxypeptidase 1B by over 95% (Table 4). When assayed in ether-treated cells, where the enzymes are embedded in the membrane, less but still significant inhibition was observed (Table 4).

The issue, then, is whether a given beta-lactam antibiotic is exerting an effect on a particular PBP without binding to it. For those PBPs of unknown function, this problem will cast a cloud of uncertainty. In the present experiments, we believe that we are justified in concluding that PBP-3 is involved specifically in septal murein synthesis because we compared septum-forming cells with filament-forming cells in the same antibiotic environment wherein only PBP-3 is bound and PBP-3 is known by genetic studies to be essential for septation (15, 16). It has recently been found that beta-lactam antibiotics bind to several minor inner membrane proteins (G. Botta and J. T. Park, manuscript in preparation) and that among these the one designated

TABLE 4. *Inhibition of D-alanine carboxypeptidase 1A and 1B activity by furazlocillin and piperacillin*

Expt no.	Concn (μg/ml)	% Inhibition of given carboxypeptidase		
		1A	1B	1A + 1B
1, Triton-solubilized enzymes				
Furazlocillin	0.1	50	51	
	1	90	86	
	10	98	98	
Piperacillin	0.5	67	63	
	5	97	94	
	50	97	99	
2, ether-treated cells				
Furazlocillin	1			37
Piperacillin	3			58

PBP-1C, because of its position between PBP-1Bs and PBP-2 on SDS-polyacrylamide gel electrophoresis, binds furazlocillin about as readily as does PBP-3. Y. Hirota (personal communication) has evidence that a minor PBP in this approximate location is required for cell division. Hence, we cannot rule out the possibility that simultaneous inhibition of PBP-1C and PBP-3

by furazlocillin is necessary for inhibition of septal murein synthesis, though the genetic evidence argues that loss of either one is sufficient.

Another problem posed anew by our results is the question of the regulation of murein synthesis during the cell division cycle. Since PBP-3 is present in cells at all times and yet appears to participate in murein synthesis in a significant way only during septation, it is possible that PBP-3 activity may be inhibited during cell elongation or that the substrates available for murein synthesis differ in quantity or more likely in composition such that PBP-3 cannot utilize them efficiently during the period of cell elongation. The observations that D-alanine carboxypeptidase II activity (which releases D-alanine from tetrapeptide side chains to form tripeptides) increases dramatically just before cell division (1) and that D-alanine carboxypeptidase I (10) is also more active at this time suggest that the substrate or acceptor for murein transpeptidation does vary in composition during the cell cycle. These circumstances lead us to the prediction that PBP-3 is a murein transpeptidase which prefers or requires tripeptide in the acceptor position. We believe that it should now be possible to test this hypothesis and to investigate the differences between septal and lateral murein synthesis by the use of appropriate model systems, beta-lactam probes, and mutants.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 05090 from the National Institute of Allergy and Infectious Diseases and by National Science Foundation grant PCM-7804326.

G.A.B. was supported, in part, by a NATO-CNR junior fellowship.

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