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Effects of Furazlocillin, a β-Lactam Antibiotic Which Binds Selectively to Penicillin-Binding Protein 3, on *Escherichia coli* Mutants Deficient in Other Penicillin-Binding Proteins

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Furazlocillin binds selectively to penicillin-binding protein 3 (PBP-3), prevents septation of *Escherichia coli*, and allows the cells to form long filaments without lysis. The effect of furazlocillin on the morphology, autolysis, and murein synthesis of *E. coli* mutants deficient in either PBP-1A, PBP-1Bs, or PBP-2 was studied. The results reveal that PBP-1A and PBP-1Bs functions are not equivalent since furazlocillin affects the morphology, autolysis, and murein synthesis of PBP1A$^-$ mutants quite differently from that of PBP-1Bs mutants. Different "PBP-2$^-$" mutants were found to respond to furazlocillin in dramatically different ways: strain LS-1 cells formed elongated rods with a central bulge which eventually lysed, whereas SP6 cells formed stable "barbells" in which the two daughter cells were well separated but remained connnected by a thick central region.

At least nine penicillin-binding proteins (PBPs), located in the cytoplasmic membrane of *Escherichia coli*, can be detected with radiolabeled benzylpenicillin by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method (4). Mutants deficient in each of these proteins except for PBP-6, -7, -8 have been described (3, 8, 14, 15, 17, 18).

As suggested by Spratt (14), PBP-1, -2, and -3 seem to play essential roles in the physiology of *E. coli* cells. PBP-1 has since been resolved into two components, PBP-1A and PBP-1Bs, the latter consisting of three components determined by a single gene (17, 18). PBP-1Bs-deficient mutants have a severely reduced level of murein synthesis and show a decrease in the degree of cross-linkage of the murein peptide side chains (18). Round-shaped mutants lacking the ability to bind penicillin to PBP-2 have been described (3, 14, 17; L. Schmidt and J. T. Park, manuscript in preparation). PBP-2 can be considered essential because mecillinam, a highly active bactericidal β-lactam antibiotic which converts *E. coli* cells to osmotically stable spheres (6, 9, 12), binds specifically to PBP-2 (14) without affecting the activity of known penicillin-sensitive enzymes (9, 12). PBP-3 is clearly essential and is involved in cell septation. Temperature-sensitive mutants containing a thermolabile PBP-3 have been described which form filaments and do not septate when grown at the nonpermissive temperature (14, 17). Recently, biochemical evidence demonstrating a PBP-3 requirement for septal murein synthesis has been obtained in our laboratory (1).

Some of the studies cited above suggest that loss of one of the high-molecular-weight binding proteins may be compensated for by an increase in another PBP. Spratt et al. (15) reported that the loss of PBP-1A and -2 was accompanied by an increase in PBP-1Bs and -3. Tamaki et al. (18) reported that a partially temperature-resistant revertant of a PBP-1Bs$^-$ strain contained increased levels of PBP-1A and a two-step revertant contained increased levels of PBPs-2 and -4 as well.

To investigate the possible relationship of one PBP to another as suggested by these results, we have studied the effect of multiple inactivation of PBPs by combining mutational loss of one PBP with the inhibition of a second binding protein, PBP-3, by a β-lactam antibiotic which selectively binds to PBP-3. To find a suitable inhibitor, a number of β-lactam antibiotics were examined by a method designed to evaluate the affinity of β-lactam antibiotics for PBPs in growing cells. The antibiotic found which binds most selectively to PBP-3 was furazlocillin (19). The effect of furazlocillin on the morphology and biochemical characteristics of PBP-1A-, -1Bs-, and -2-deficient mutants is described.

We examined the following *E. coli* K-12 mutant and parent strains: JE10528 (PBP-1A$^-$), JE10704 (PBP-1Bs$^-$), and their parent, PA3092 (17); AB47-18 (PBP-1Bs$^-$ PBP-5$^-$) (11); SP6 (PBP-1A$^-$ PBP-2$^-$) and its parent, KN126 (14); LS1 (PBP-2$^-$) and its parent, M7LD (Schmidt

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and Park, manuscript in preparation); LS90 (PBP-2$^-$ Lip$^+$ transductant of AT1325 *lip-9* (Schmidt and Park, manuscript in preparation); and JE7406 (PBP-1A$^-$ PBP-2$^+$ transductant of SP6) (11). All bacterial strains were grown in L broth (5) containing 0.2% (wt/vol) glucose as the carbon source and supplemented, when necessary, with the required amino acids.

Binding of β-lactam antibiotics to their target proteins in whole cells is affected by a number of parameters not relevant in binding experiments with sonicated cell envelopes, e.g., antibiotic permeability through the outer membrane, the presence of penicillinase, and de novo protein synthesis during growth (10, 20). Since we wanted to use furazlocillin to selectively bind PBP-3 in growing cells, we determined the concentration range in which furazlocillin binds selectively to PBP-3 under conditions of growth in L broth.

A representative SDS-PAGE autoradiogram obtained from furazlocillin binding experiments with parent strain PA3092 is shown in Fig. 1. At concentrations from 0.04 to 5 μg/ml, furazlocillin bound to PBP-3 and not to any of the other major PBPs. Similar data (not shown) were obtained for parent strains M7LD and KN126 and for mutants JE10528 (PBP-1A$^-$), LS1 (PBP2$^-$), SP6 (PBP1A$^-$ PBP2$^-$), and JE10704 (PBP-1Bs$^-$). Although antibiotic treatment was routinely carried out for 20 min, no significant difference in the binding pattern was obtained when cells were grown in the presence of antibiotic for 1 h. The concentration of furazlocillin required to saturate PBP-3 was reduced approximately one-half when the binding experiments were conducted in buffer rather than during growth in L broth, reflecting the contribution of de novo-synthesized PBPs to the binding pattern. The concentrations required for 50% saturation of PBP-3 were very similar when comparing related parent and mutant strains (Table 1). However, the minimal inhibitory concentrations (MICs) of the parent strains were significantly higher than those of the mutant strains. The MIC values for mutant strains LS90 (PBP-2$^-$), SP6 (PBP-1A$^-$ PBP-2$^-$), JE10704 (PBP-1Bs$^-$), and JE10528 (PBP-1A$^-$) were approximately 0.05 μg/ml, which corresponds roughly to the concentration required to half-saturate PBP-3 in 20 min (Table 1). On the other hand, the MICs for mutant LS1 (PBP-2$^-$) and the parental strains (0.5 to 0.6 μg/ml) were about 10-fold higher than the concentration needed to half-saturate PBP-3. These results exclude the possibility that the difference in sensitivity among the parent and mutant strains is due to an altered affinity of PBP-3 for β-lactams and support the hypothesis that the hypersensitivity is related to the mutational loss of a second PBP.

Growth of the parent strains in the presence of concentrations of furazlocillin which result in the antibiotic binding only to PBP-3 caused inhibition of cell division and formation of long filaments (Fig. 2B). Similar morphological effects were also evident when concentrations as

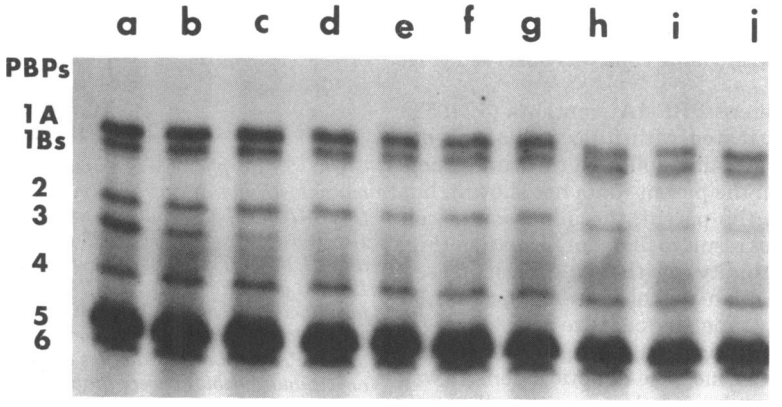

FIG. 1. *SDS-PAGE autoradiogram of PBPs of E. coli strain PA3092 treated in intact cells with increasing twofold concentrations of furazlocillin, from (b) 0.04 to (j) 12.5 μg/ml. Cells were grown in 800 ml of L broth to an absorbance of 0.5 U and then divided in 10 flasks (80 ml each). After addition of antibiotic, incubation was continued for an additional 20 min. Cells were immediately harvested and washed in phosphate buffer, and cell envelopes prepared as described (1). [^{125}I]furazlocillin (preparation to be described elsewhere) was then added to the washed envelopes (15 min at 30°C), and the reaction was terminated with excess cold benzylpenicillin. The radiolabeled PBPs were prepared for PAGE by standard procedures (1) and separated in a discontinuous buffer system (4) on a 7.5% acrylamide–0.15% bisacrylamide gel. PBPs were detected after overnight exposure of the gels to X-ray film at −70°C in the presence of intensifying screens (Cronex DuPont, Wilmington, Del.).*

TABLE 1. *Comparison of the MIC of furazlocillin for various strains and its binding affinity for PBP-3*

Strain	Furazlocillin concn required for 50% saturation of PBP-3[a] in intact cells	MIC[b] for furazlocillin (μg/ml)
M7LD (parent)	0.06	0.6
LS1 (*rodA*)	0.06	0.6
PA3092 (parent)	0.05	0.5
JE10528 (*ponA*)	0.05	0.1
JE10704 (*ponB*)	0.05	0.05
KN126 (parent)	0.10	0.6
SP6 (*rodA ponA*)	0.08	0.05
LS90 (*rodA*)	ND[c]	0.05

[a] Washed cells were incubated with different concentrations of unlabeled antibiotic for 20 min in L broth pH 7.0 before envelope preparation. SDS-PAGE of [^{125}I]furazlocillin-labeled envelopes and autoradiography were carried out as described in Fig. 1.

[b] Determined by dilution in broth inoculated with a 1:2,000 dilution of an overnight culture. The lowest antibiotic concentration at which no growth was evident after 24 h of incubation was reported as the MIC.

[c] Not done.

high as 50 μg/ml were used. Optical density of the culture increased at the same rate as that of the control for at least 3 h. No lysis occurred even after prolonged exposure to high concentrations of the antibiotic. In experiments performed using the MIC of the antibiotic, the number of survivors increased at a rate similar to that found for the untreated sample for the first 60 min of treatment, after which a plateau was reached. Upon prolonged incubation, a slight decrease in viable cells was observed (Fig. 3).

In two different PBP-1A$^-$ mutants (JE10528 and JE7406) treated with furazlocillin, the decrease in viable counts followed the same kinetics observed in the parent (data not shown), but in contrast to the parent, the inhibition of cell division did not result in filament formation (Fig. 2C). The turbidity of the culture increased slightly more than twofold during the first 90 min, at which time the cell size was heterogeneous and had an average length of four cell units. No further increase in optical density was observed over 3 h, and the average cell length was unchanged.

Furazlocillin produced completely different effects on the PBP-1Bs-deficient mutants JE10704 and AB47-18. The lack of PBP-1Bs, when combined with PBP-3 inhibition, caused formation of long filaments, but, contrary to that which happened in a normal strain, these filaments lysed abruptly after about 150 min of treatment (Fig. 3).

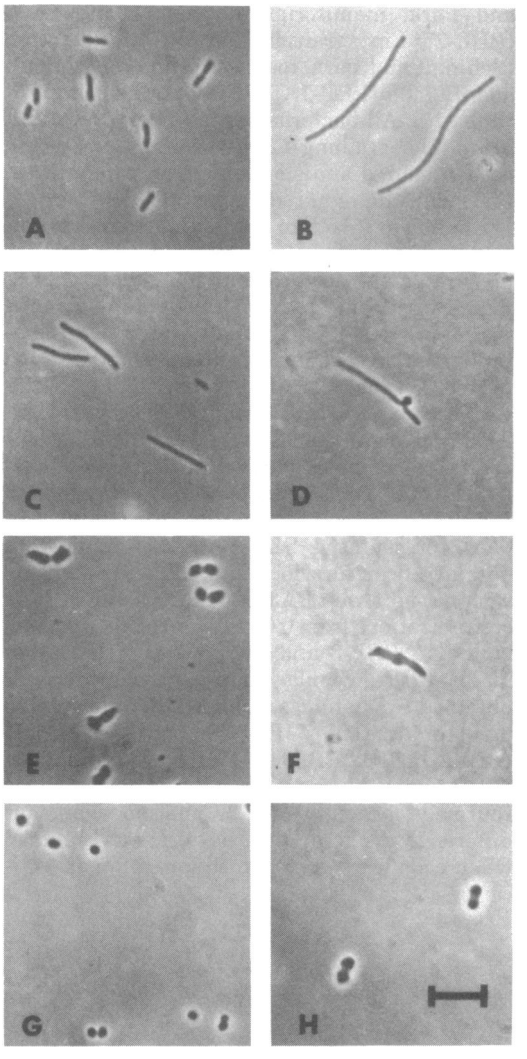

FIG. 2. *Morphology of parent and PBP-deficient mutant strains during treatment with furazlocillin (10× MIC). PA3092 (parent) untreated (A) and furazlocillin-treated (B); JE10528 (PBP-1A$^-$) treated (C) and JE10704 (PBP-1Bs$^-$) treated (D). PBP-2 mutant LS1 (E) and SP6 (PBP-1A$^-$ PBP-2$^-$) (G), treated with furazlocillin (F and H, respectively). Untreated PBP-1A and PBP-1Bs mutants were similar in morphology to PA3092 in (A). Samples of cultures grown with or without antibiotic were placed on a cover slip and pressed onto a microscope slide covered with a film of 0.5% Noble agar. Photomicrographs were taken after 120 min of antibiotic treatment except for LS1, which was taken after 45 min just before culture lysis. Bar equals 3 μm.*

Previous studies with PBPs-1A- and 1Bs-deficient mutants (15, 17, 18) have suggested that the functions of these two proteins are at least partially interchangeable. Our data indicate that

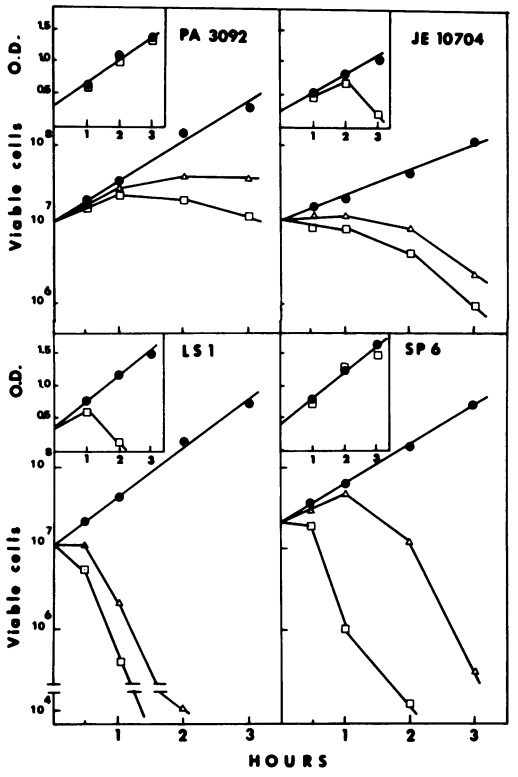

FIG. 3. *Effect of furazlocillin on the viability and culture turbidity (insert) of PA3092 (parent), JE10704 (PBP-1Bs⁻), LS1 (PBP-2⁻), and SP6 (PBP-1A⁻ PBP-2⁻). Drug was added to exponentially growing cultures at zero time. Symbols: (●, △) 1× MIC of furazlocillin; (○) 10× MIC of furazlocillin. Culture turbidity was measured at 585 nm. The number of viable cells was determined by removing samples of culture at regular time intervals and plating suitable dilutions on L agar plates. Colonies were counted after a 24-h incubation.*

although both mutations result in hypersensitivity (i.e., reduced MIC values) to an antibiotic which specifically binds to PBP-3 and inhibits septation, the morphological and growth responses of these mutants are quite different. The reports that the PBP-1Bs⁻ mutation results in a greatly reduced rate of murein synthesis of a low-cross-linked product (17, 18) may help explain why furazlocillin eventually causes PBP-1Bs strains but not normal *E. coli* to lyse. However, the suggestion that PBP-1Bs is essential for cell elongation (15) is no longer tenable. In fact, our results suggest that PBP-1A, rather than PBP-1Bs, is needed for prolonged elongation, at least in the absence of a functional PBP-3.

The response of the PBP-2 mutant, LS1, to cell septation inhibition was unique. These cells, which are severely reduced but not completely deficient in PBP-2 binding activity, lysed abruptly after 60 to 90 min of growth in the presence of 5 μg of furazlocillin per ml (Fig. 3). The morphological change of LS1 treated with furazlocillin is shown in Fig. 2 (compare 2E with 2F). Cells were able to elongate, although to a limited extent and in a very peculiar way; nearly all of the cells in the culture had an enlarged central structure with two thick arms of approximately one cell length extending in opposite directions. This shape resembles the short filament with central bulge obtained after treatment of normal rod-shaped *E. coli* with penicillin G or ampicillin.

Mutant SP6 (PBP-1A⁻ PBP-2⁻) and a transductant, LS90, containing the PBP-2⁻ gene of SP6, reacted in a different manner than LS1 to the binding of PBP-3 by antibiotic treatment. Cultures of SP6 grown in the presence of furazlocillin at the MIC and 10 times the MIC increased in turbidity, with no apparent lysis for 3 h at a rate similar to that of the untreated control (Fig. 3). The number of viable cells increased only slightly, reached a plateau, and finally decreased 1.5 log units after 3 h. Unlike LS1, the PBP-2⁻ mutants SP6 and LS90 were unable to elongate in the presence of furazlocillin and, in general, the round cells enlarged in size commensurate with the increase in turbidity of the culture. About 30% of the SP6 cells formed a cylindrical connection of variable diameter between the two spherical daughter cells, resembling the shape of a barbell (Fig. 2H).

Interestingly, *Klebsiella pneumoniae* Mir M7, a pH conditional morphology mutant with a round shape at pH 7 (13), was observed to undergo morphogenic changes similar to those in LS1 when treated with cephalexin, a β-lactam antibiotic acting on PBP-3 (G. Botta et al., Abstr. XII, Int. Congr. Microbiol., 1978 p. 69). It is remarkable that in the *Klebsiella* strain PBP-2 was present in normal amounts (G. Botta, unpublished data).

The remarkable difference between the PBP-2-deficient mutants in terms of their sensitivity to furazlocillin and shape change during treatment with furazlocillin suggests that their mutational defects may be different. Apparent loss of PBP-2, as judged by inability to bind penicillin, results in round-shaped mutants which are altered in their ability to initiate elongation as a rod (3, 14; Schmidt and Park, manuscript in preparation). However, loss of PBP-2 function may not be complete in these mutants since the lethality of mecillinam, a β-lactam antibiotic that binds specifically to PBP-2 (14), indicates that PBP-2 is essential. The extent of PBP-2

TABLE 2. *Effect of furazlocillin on murein synthesis in PBP-deficient mutants and their parents*[a]

Strain	% Inhibition of murein synthesis by furazlocillin at given concn (μg/ml)		
	0.1	1	10
M7LD	13	27	34
LS1 (PBP-2⁻)	13	23	28
PA3092	15	21	24
JE10528 (PBP-1A⁻)	15	18	27
JE10704 (PBP-1Bs⁻)	25	36	62
KN126	15	16	34
SP6 (PBP-1A⁻ PBP-2⁻)	28	54	82

[a] Murein synthesis was determined as [^{14}C]Dpm incorporation into lysozyme-digestible murein. Cultures were grown to mid-log phase in L broth followed by continued growth in the presence of antibiotic for 20 min. A 50-ml culture sample was filtered and suspended in 10 ml of a medium especially designed for murein synthesis in the absence of protein synthesis (1), containing [*U*-^{14}C]Dpm (0.1 μCi/ml, 315 mCi/mmol) to which furazlocillin was added as required. After labeling, cells were harvested and digested with egg white lysozyme as described elsewhere (1). Portions of the lysozyme-solubilized radioactivity were counted in a toluene-based scintillation fluid (1).

function in the several "PBP-2⁻" mutants examined is suggested by their morphology and by their response to furazlocillin. Furazlocillin caused LS1, which is a thick, irregularly shaped ovoid cell when grown in L broth, to elongate for about one generation, whereas SP6 and LS90 increased in cell mass but maintain their spherical shape. From this observation one would predict that their mutations are at different loci within the *rodA* gene resulting in (i) lesser (SP6, LS90) or greater (LS1) amounts of synthesis of PBP-2 or (ii) synthesis of altered gene product with lesser (SP6, LS90) or greater (LS1) degrees of PBP-2 function.

Cell wall synthesis in the presence of various concentrations of furazlocillin was measured by incorporation of [^{14}C]diaminopimelic acid (Dpm) as described elsewhere (1). The inhibition of murein synthesis in the parent strains ranged from 13 to 27% in the presence of furazlocillin at 0.1 to 1 μg/ml (Table 2). At the highest concentration tested (10 μg/ml), only a small increase in inhibition was observed with the parental strains. Murein synthesis by JE10528 (PBP-1A⁻) and LS1 (PBP-2⁻) was inhibited to a degree similar to that observed in the parents. In JE10703 (PBP-1Bs⁻) and SP6 (PBP-1A⁻, PBP-2⁻), [^{14}C]Dpm incorporation was more severely affected by furazlocillin (Table 2). Furazlocillin had no significant effect on the degree of cross-linkage of murein even at the highest concentrations used (data not shown).

The small amount of low-cross-linked murein synthesized in PBP-1Bs mutants (17, 18) may, in the presence of furazlocillin binding to PBP-3, be subject to autolysis (i.e., culture lysis). Murein synthesized under conditions of PBP-3 inactivation and some limited PBP-2 function, as in the LS1 strain, is apparently more susceptible to hydrolysis by lytic enzymes since LS1 lyses when grown with furazlocillin and SP6 and LS90 do not. The greater sensitivity of SP6 (and LS90) to growth and murein synthesis inhibition by furazlocillin suggests that PBP-3 plays a more important role in these cells and that perhaps the greater loss of PBP-2 function directly or indirectly causes a loss of autolytic potential.

The contribution of PBP-3 function to total murein synthesis in exponentially growing cells is small (unpublished data), but the timing during the cell cycle of its function in septal murein synthesis must be critical. For example, furazlocillin inhibits murein synthesis of synchronously dividing BUG6 cells but not of BUG6 when grown as filaments (1). Therefore it is not surprising that inhibition of PBP-3 by furazlocillin does not result in greater inhibition of total murein synthesis. The inhibition of murein synthesis by furazlocillin in the PBP-1Bs⁻ strains, as well as the PBP-2⁻ mutants whose murein synthesis is more sensitive to inhibition by furazlocillin, may reflect the fact that these strains are already reduced in the amount of murein synthesized in the absence of antibiotic (30 and 50%, respectively; unpublished data) and that the remaining murein pathway is more dependent on PBP-3.

In conclusion, we have examined the effect of multiple inactivation of PBPs by combining the mutational loss of one PBP with specific binding of a β-lactam to a second binding protein, PBP-3. The data presented clearly indicate that simultaneous inactivation of PBP-1A and -3, PBP-1Bs and -3, or PBP-2 and -3 is severely inhibitory to cell growth and viability. These results provide additional insight into the functions of PBP-1A, -1Bs, and -2, especially with regard to the role they play in murein synthesis and cell integrity.

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