Proc. Nat. Acad. Sci. USA
Vol. 72, No. 8, pp. 2999–3003, August 1975
Biochemistry

Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12

(β-lactam antibiotics/slab gel electrophoresis/binding protein mutants)

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Communicated by Arthur B. Pardee, May 20, 1975

ABSTRACT **The varied effects of β-lactam antibiotics on cell division, cell elongation, and cell shape in *E. coli* are shown to be due to the presence of three essential penicillin binding proteins with distinct roles in these three processes. (*A*) Cell shape: β-Lactams that specifically result in the production of ovoid cells bind to penicillin binding protein 2 (molecular weight 66,000). A mutant has been isolated that fails to bind β-lactams to protein 2, and that grows as round cells. (*B*) Cell division: β-Lactams that specifically inhibit cell division bind preferentially to penicillin binding protein 3 (molecular weight 60,000). A temperature-sensitive cell division mutant has been shown to have a thermolabile protein 3. (*C*) Cell elongation: One β-lactam that preferentially inhibits cell elongation and causes cell lysis binds preferentially to binding protein 1 (molecular weight 91,000). Evidence is presented that penicillin bulge formation is due to the inhibition of proteins 2 and 3 in the absence of inhibition of protein 1.**

The β-lactam group of antibiotics (penicillins and cephalosporins) exert a variety of effects on *Escherichia coli*. Most typical β-lactams inhibit cell division at low concentrations and produce cell lysis at high concentrations (1, 2). In addition, some of these compounds produce bulges in cells at intermediate concentrations (2). Another penicillin derivative (the amidino-penicillanic acid, mecillinam—previously called FL1060) produces ovoid-shaped cells at low concentrations without specifically inhibiting cell division or causing typical penicillin lysis (3–5).

Although it has been suggested (2, 6–8) that the effects of β-lactam antibiotics on cell division, elongation, and shape are due to the presence of distinct penicillin-sensitive enzymes specifically involved in peptidoglycan metabolism for these three processes, little evidence has been produced to substantiate this view. Multiple penicillin-sensitive enzymes (7, 9, 10) and penicillin binding proteins (8, 9, 11) with different sensitivities to β-lactam antibiotics are present in *E. coli*, and recently we have shown that one of the six penicillin binding proteins (protein 2) is specifically involved in the maintenance of the rod shape of *E. coli* (8).

Three of the *E. coli* penicillin binding proteins (proteins 4, 5, and 6) are not thought to be involved in the effects of typical β-lactams on cell elongation and division since several antibiotics fail to bind to these proteins at concentrations far above those that affect the growth of the cells (Spratt, in preparation).

The remaining two binding proteins (proteins 1 and 3) are the likely sites at which typical β-lactams act to inhibit cell division and cell elongation.

In this paper, I report that one of these proteins is specifically required for cell division and that the other is required for cell elongation; I also suggest a model to explain the varied effects of β-lactam antibiotics by their relative affinities for three proteins involved in cell division, elongation, and the maintenance of cell shape.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

METHODS

The organism used in these studies was *E. coli* K12 (strain KN126). It was grown in Difco Pennassay broth at 37° with vigorous aeration and harvested at late logarithmic phase. Mutants B6 and 6-30 were grown in the same medium at 30°.

Assay of Penicillin Binding Proteins. [^{14}C]Benzylpenicillin or [^{14}C]mecillinam (FL1060) were bound to purified cell envelopes [prepared by sonication and differential centrifugation (8, 12)], the inner membranes selectively solubilized with Sarkosyl NL-97 (13), and the binding proteins separated on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide slab gels and detected by fluorography (14) as described (8).

In experiments in which the residual penicillin binding proteins are measured after growth of cells in the presence of unlabeled β-lactam antibiotics, the membranes were prepared without the use of 2-mercaptoethanol to avoid the loss of bound penicillin.

Penicillin binding proteins were quantitated by densitometry of the x-ray films and measurement of the areas under each of the peaks.

Chemicals. [^{14}C]Mecillinam (53 mCi/mmol) and unlabeled mecillinam were generous gifts from Dr. F. Lund of Leo Laboratories, Ballerup, Denmark. [^{14}C]Benzylpenicillin (54 mCi/mmol) was obtained from Amersham/Searle. Ampicillin was a gift of Bristol Laboratories. Cephalexin and cephaloridine were gifts of Eli Lilly and Co. Benzylpenicillin was a gift of E. R. Squibb and Sons.

RESULTS

Identification of β-lactam antibiotics with preferential effects on cell division, elongation, and cell shape

To identify penicillin binding proteins with distinct roles in cell division, elongation, and shape, we screened 13 antibiotics* for those which showed preferential effects on one of these three processes. Each antibiotic was added at a range of concentrations to cells of *E. coli* KN126 growing exponentially in Penassay broth at 37°, and the cells were examined at 15-min intervals under the phase contrast microscope.

* 6-Aminopenicillanic acid, ampicillin, benzylpenicillin, cephalexin, cephaloglycin, cephaloridine, cephalothin, cephalosporin G, cephamycin C, cefoxitin, dihydroampicillin, mecillinam, and penicillin V.

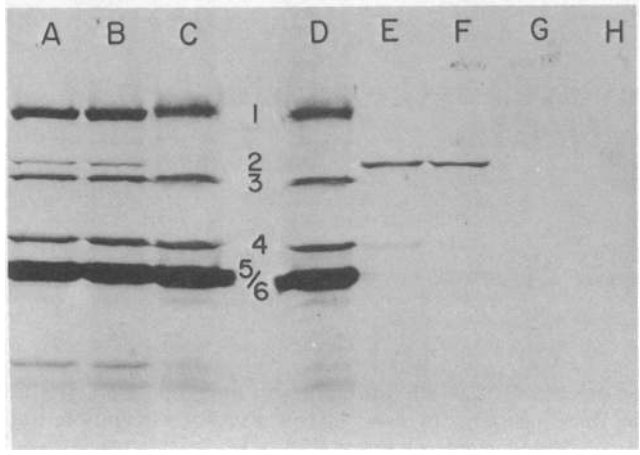

FIG. 1. Binding of [^{14}C]benzylpenicillin and [^{14}C]mecillinam to envelopes prepared from strains KN126 and B6. [^{14}C]Benzylpenicillin (31 μg/ml) or [^{14}C]mecillinam (2 μg/ml) were bound to purified cell envelopes prepared from strains KN126 or B6 grown in Penassay broth at 30° as described (8). The reaction was terminated, and the inner membrane selectively solubilized with Sarkosyl NL-97 (13), and separated on NaDodSO$_4$/polyacrylamide slab gels as described (8). The binding proteins were detected by fluorography (14) on Kodak RPRoyal x-ray film for 48 days at −70°. [^{14}C]Benzylpenicillin was bound for 10 min at 30° (A) or 42° (B) to envelopes prepared from strain KN126. [^{14}C]Benzylpenicillin was bound for 10 min at 30° (C) or 42° (D) to envelopes prepared from strain B6. [^{14}C]Mecillinam was bound for 10 min at 30° (E) or 42° (F) to envelopes prepared from strain KN126. [^{14}C]Mecillinam was bound for 10 min at 30° (G) or 42° (H) to envelopes prepared from strain B6. Proteins 5 and 6 are not fully resolved on this gel. In addition to the six binding proteins we consistently detect, two further minor binding proteins are also detected on this gel. Electrophoresis was from top to bottom.

Cell Shape. A binding protein involved in the maintenance of cell shape can be readily identified using the amidinopenicillanic acid, mecillinam (3, 8). Mecillinam is an ideal probe for cell shape, since it is specific for shape and does not initially inhibit cell division or cause typical penicillin lysis.

Cell Division. Most other β-lactam antibiotics inhibited division (without inhibiting cell elongation) at low concentrations and thereby caused filamentation; cell lysis occurred at higher concentrations. To probe for a penicillin binding protein involved in cell division we selected cephalexin, ampicillin, and benzylpenicillin, as these antibiotics showed the largest concentration range over which cell division was specifically inhibited without cell lysis occurring.

Cell Elongation. A binding protein involved in cell elongation was probed for with cephaloridine, since this β-lactam caused cell lysis (presumably by inhibiting cell elongation) at the lowest effective concentrations.

Bulge Formation. This was observed with only two of the β-lactams examined. One of these, ampicillin, was used as a probe of bulge formation. At low concentrations ampicillin inhibited cell division, resulting in the production of filaments. At slightly higher concentrations bulges were produced in the middle of the filaments, while lysis occurred at still higher concentrations.

Involvement of penicillin binding protein 2 in cell shape

Mecillinam competes for the binding of [^{14}C]benzylpenicillin to only one of the six penicillin binding proteins (protein 2), and we have suggested that protein 2 is involved in the maintenance of cell shape (8). Since [^{14}C]mecillinam is now available to us, we have studied the binding of this compound to *E. coli* directly. [^{14}C]Mecillinam, at physiologically effective concentrations (0.01–1.0 μg/ml), binds exclusively to a protein in the *E. coli* inner membrane that has the same mobility on NaDodSO$_4$/polyacrylamide slab gels as penicillin binding protein 2. No binding of mecillinam to the outer membrane was detected. Binding of [^{14}C]mecillinam was 50% saturated at 0.02 μg/ml for 10 min at 30°, which compares excellently with the value obtained previously (8). At higher concentrations of [^{14}C]mecillinam very slight binding occurred to some of the other penicillin binding proteins. Fig. 1E and F shows the binding of [^{14}C]mecillinam (2 μg/ml) to the *E. coli* inner membrane proteins.

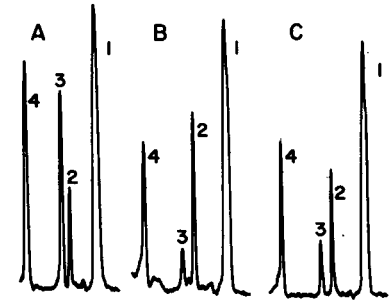

FIG. 2. Penicillin binding proteins in cells grown in the presence of cephalexin and benzylpenicillin. Cells of *E. coli* KN126 were grown at 37° in Penassay broth for 15 min (A) with no additions, (B) with 10 μg/ml of cephalexin, and (C) with 12 μg/ml of benzylpenicillin. Cells were then cooled on ice and purified cell envelopes were prepared without the use of mercaptoethanol (see *Methods*). [^{14}C]Benzylpenicillin (31 μg/ml) was added for 10 min at 30°, and the binding proteins were fractionated and detected as in Fig. 1. Densitometer tracings were made from the x-ray film. Binding proteins 5 and 6 are not shown. Aliquots of cells from (B) and (C) were also grown further at 37° to ensure that cell division was inhibited under these conditions.

Characterization of a mutant with an altered penicillin binding protein 2

Mutants resistant to mecillinam (10 μg/ml) were isolated at 30° on Penassay broth plates after nitrosoguanidine mutagenesis, and their penicillin binding proteins were examined. One of the five mutants examined (strain B6) failed to bind either [^{14}C]mecillinam or [^{14}C]benzylpenicillin to binding protein 2 (Fig. 1). Strain B6 grows slowly as round cells at 30°, in the absence of mecillinam, and fails to form colonies at 42°. It seems likely that strain B6 possesses a binding protein 2 that not only fails to bind mecillinam and benzylpenicillin, but also functions poorly and results in the growth of the strain as round cells. It is also possible that protein 2 is totally absent in this strain.

Involvement of penicillin binding protein 3 in cell division

When cells of strain KN126 are grown for 15 min at 37° in Penassay broth in the presence of a β-lactam that is a good specific inhibitor of cell division, the β-lactam binds mainly to protein 3. Fig. 2 shows the binding proteins still accessible to [^{14}C]benzylpenicillin after growth in the presence of unlabeled cephalexin or benzylpenicillin (at concentrations that just inhibit cell division). In both cases, the binding of [^{14}C]benzylpenicillin to protein 3 is greatly reduced compared to untreated control cells, while the binding to pro-

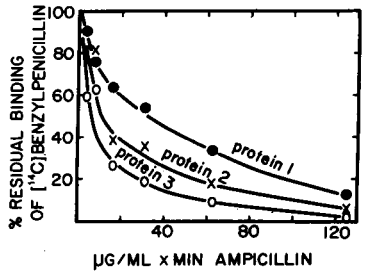

FIG. 3. Competition of ampicillin for the penicillin binding proteins. Washed cell envelopes of strain KN126 were preincubated with a range of ampicillin concentrations and the binding proteins remaining accessible to benzylpenicillin detected by the addition of a saturating concentration of [^{14}C]benzylpenicillin (31 μg/ml for 10 min at 30°). The binding proteins were fractionated and detected as described in Fig. 1, and the amount of each binding protein remaining accessible to [^{14}C]benzylpenicillin was measured by densitometry of the x-ray film. ●, Protein 1; ×, protein 2; ○, protein 3.

teins 1, 2, and 4 is little altered. Proteins 5 and 6 are also unaffected (data not shown).

We have also implicated protein 3 in cell division by studying the affinities of the penicillin binding proteins for β-lactams that are good inhibitors of cell division. Fig. 3 shows the binding of a saturating concentration of [^{14}C]benzylpenicillin to cell envelopes after prebinding a range of ampicillin concentrations. In common with all β-lactams that are good specific inhibitors of division, ampicillin competes more effectively for penicillin binding protein 3 than for proteins 1 or 2.

Characterization of a mutant with an altered penicillin binding protein 3

Since penicillin resistance can be acquired by a variety of means unrelated to changes in penicillin binding proteins (10), we adopted a selective technique (15) to isolate a mutant with a thermolabile binding protein 3.

Cells were mutagenized with nitrosoguanine, and colonies resistant to a low level of cephalexin (5 μg/ml) were isolated at 30° on Penassay broth plates. From these colonies temperature-sensitive cell division mutants were isolated. By using this selection procedure it was hoped to isolate mutants that had a protein 3 that was more resistant to cephalexin at 30° and was thermolabile at 42°. Inactivation of protein 3 at 42° would result in the inhibition of cell division. Membranes

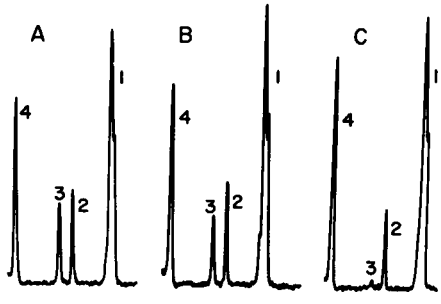

FIG. 4. Penicillin binding proteins in strain 6-30. Washed cell envelopes were prepared from cells of strain 6-30 grown in Penassay broth at 30°. (A) [^{14}C]Benzylpenicillin (31 μg/ml) was added for 10 min at 30°. (B) [^{14}C]Benzylpenicillin (31 μg/ml) was added for 2 min at 42°. (C) Cell envelopes were incubated at 42° for 11 min and then [^{14}C]benzylpenicillin (31 μg/ml) was added for a further 2 min at 42°. Penicillin binding proteins were fractionated and detected as described in Fig. 1. Densitometer tracings were made from the x-ray film. Binding proteins 5 and 6 are not shown.

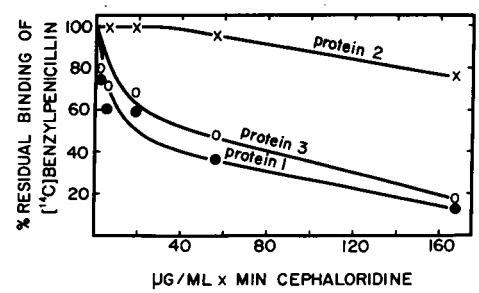

FIG. 5. Competition of cephaloridine for the penicillin binding proteins. Washed cell envelopes of strain KN126 were preincubated with a range of cephaloridine concentrations, and the binding proteins remaining accessible to benzylpenicillin were detected by the addition of a saturating concentration of [^{14}C]benzylpenicillin (31 μg/ml for 10 min at 30°). The binding proteins were fractionated, detected, and quantitated as described in Fig. 3. ●, Protein 1; ×, protein 2; ○, protein 3.

were prepared from mutants grown in Penassay broth at 30°, and the binding of [^{14}C]benzylpenicillin was assayed at 30° and 42°.

Fig. 4 shows the binding to strain 6-30 at 30° and 42°. At 30° this strain binds less [^{14}C]benzylpenicillin to protein 3 than does the parent strain [compare the ratio of peak 2 to peak 3 in the mutant (Fig. 4A) with that of the parent (Figs. 1A and B and 2A)]. At 42° the ability to bind penicillin is lost within 11 min in the mutant but not in the parent strain. Since no other binding proteins are thermolabile in strain 6-30, this temperature-sensitive cell division mutant has a thermolabile protein 3. Since our other experiments have implicated protein 3 in cell division, the temperature-sensitive cell division phenotype appears to be caused by a thermolabile penicillin binding protein 3.

Involvement of penicillin binding protein 1 in cell elongation

The cell elongation probe, cephaloridine, is unusual in that it is the only β-lactam we found that causes cell lysis at the lowest effective concentrations.

Cephaloridine is also the only β-lactam that shows a higher affinity for binding protein 1 than for protein 2 or 3. Fig. 5 shows the binding of a saturating concentration of [^{14}C]benzylpenicillin to proteins 1, 2, and 3 after prebinding a range of cephaloridine concentrations. We believe that protein 1 is involved in cell elongation and that inhibition of this protein by β-lactams results in cell lysis.

Involvement of penicillin binding proteins 2 and 3 in the formation of penicillin bulges

Since bulge formation is an alteration of cell shape, we considered the possibility that it was due to the inhibition of protein 2. Most β-lactams bind with low affinity to protein 2 such that mecillinam-like cell shape changes are not observed. If a β-lactam showed higher affinities for proteins 2 and 3 than for protein 1, cell shape and division should be altered before lysis occurred.

Since inhibition of protein 2 (by mecillinam) results in continuing cell division and the maintenance of normal rod shape for about 50 min at 37°, the effect of additionally inhibiting cell division (blocking protein 3) should be to produce a normal rod-shaped filament during the first 50 min. After 50 min the blocking of protein 2 should result in the production of the cell shape changes (bulges) within these filaments. In support of this model, the appearance of bulges by ampicillin can be mimicked by the simultaneous addition

Table 1. Effects of β-lactam antibiotics on growth of *E. coli* predicted by their relative affinities for penicillin binding proteins

Relative affinities of binding proteins 1, 2, and 3 for a β-lactam antibiotic*	Morphological effects produced by three arbitrary concentrations of a β-lactam antibiotic			β-Lactam showing this behavior
	Low concentration	Medium concentration	High concentration	
1 > 2 or 3	Lysis	Lysis	Lysis	Cephaloridine
2 > 1 > 3	Ovoid cells	Lysis	Lysis	6-Aminopenicillanic acid
2 > 3 > 1	Ovoid cells	Filaments with bulges	Lysis	None known
2 ≫ 1 or 3	Ovoid cells	Ovoid cells	Ovoid cells	Mecillinam
3 > 1 > 2	Filaments	Lysis	Lysis	Benzylpenicillin
3 > 2 > 1	Filaments	Filaments with bulges	Lysis	Ampicillin

* 1 = cell elongation; 2 = cell shape; 3 = cell division.

of a β-lactam that binds to protein 2 (mecillinam) and one that binds preferentially to protein 3 (cephalexin).

As expected from this model, ampicillin had a high affinity for protein 3, slightly less affinity for protein 2, and even less affinity for protein 1. One other antibiotic (dihydroampicillin) also produces bulges and shows a similar order of affinities for binding proteins 1, 2, and 3.

DISCUSSION

Table 1 summarizes a model by which the particular effects of a β-lactam antibiotic on the division, elongation, and shape of *E. coli* are explained by the relative affinity of that antibiotic for three penicillin binding proteins with specific roles in these three processes.

The evidence for a penicillin binding protein involved in the maintenance of the rod shape of *E. coli* is very strong. Mecillinam is a powerful probe for this protein (protein 2) since it has a specific action on cell shape over a very wide concentration range (3). Studies with both [^{14}C]mecillinam and [^{14}C]benzylpenicillin show that the mecillinam binds exclusively to one inner membrane protein (molecular weight 66,000). A mecillinam-resistant mutant (strain B6) has been isolated that fails to bind [^{14}C]mecillinam or [^{14}C]benzylpenicillin to this protein. The enzymatic function of this protein seems to be seriously impaired since the mutant grows slowly as round cells at 30° and fails to form colonies at 42°. A study of revertants of strain B6 will be required to understand the relationship between temperature-sensitivity, mecillinam-resistance, cell shape, and the altered binding protein.†

The evidence for the involvement of distinct penicillin binding proteins in cell division and cell elongation is less strong than the evidence for a protein involved in cell shape, since no β-lactam with absolute specificity for the former proteins is known. A good correlation does exist between a β-lactam being a good inhibitor of cell division and having a high affinity for protein 3 (molecular weight 60,000). Furthermore, when cells were grown for 15 min in the presence of a concentration of benzylpenicillin or cephalexin that just inhibits division, the β-lactams were bound preferentially to protein 3. Strong additional evidence for the involvement of protein 3 in cell division comes from the isolation of a temperature-sensitive, cell division mutant (strain 6-30) with a thermolabile binding protein 3.

The mutants B6 and 6-30 are examples of mutants resistant to β-lactams in which the resistance has been correlated with an altered penicillin binding protein. Strain 6-30 is also an example of a cell division mutant in which the biochemical basis of the lesion is in some way understood.

Penicillin binding protein 1 (molecular weight 91,000) is considered to be involved in cell elongation. β-Lactams that cause inhibition of cell elongation and cell lysis at the lowest effective concentrations show a higher affinity for protein 1 than for protein 2 or 3.

A possible explanation for bulge formation by some penicillins is that it is due to the inhibition of protein 2 in addition to protein 3 (see Table 1). Most β-lactams do not produce bulges in our strain, since protein 1 has a higher affinity for most β-lactams than protein 2, such that lysis occurs before any effects on cell shape are observed. The only two β-lactams that give typical penicillin bulges in our strain (ampicillin and dihydroampicillin) are also the only two β-lactams that show higher affinities for proteins 2 and 3 than for protein 1.

The precise role of the three essential penicillin binding proteins is not clear. Inhibition of protein 1 stops cell elongation and results in cell lysis, presumably by the continued action of peptidoglycan hydrolases in the absence of peptidoglycan synthesis. Protein 1 is proposed to be required for all peptidoglycan synthesis. It possibly acts as a transpeptidase which introduces new precursors into the growing peptidoglycan chains (16).

Peptidoglycan synthesis continues when protein 2 or 3 (or both) are inhibited, and the peptidoglycan produced under these conditions is not seriously defective, since ovoid cells (produced by inhibiting protein 2), filaments (produced by inhibiting protein 3), and cells with bulges (produced by inhibiting proteins 2 and 3) are osmotically stable.

Protein 3 may be required for the synthesis of peptidoglycan for cross walls, but not for that for side walls. It is also possible, however, that this protein acts transiently at the end of a round of DNA replication to alter the direction of peptidoglycan growth at the potential division site. Synthesis of both cross walls and side walls would then be due to the action of protein 1.

Inhibition of protein 2 does not appear to affect pre-existing cell wall growth sites, since the resulting shape change

† Note Added in Proof. A revertant of strain B6 has been obtained which has a normal rod morphology, grows at 42°, and is sensitive to mecillinam. This revertant has regained the ability to bind [^{14}C]mecillinam and [^{14}C]benzylpenicillin to penicillin binding protein 2.

does not occur for about 50 minutes in an exponential culture (5), and never occurs if DNA synthesis is blocked (17). It is interesting to note that bulge formation also required continuing DNA synthesis (2, 18), and only occurs after a similar time lag (2). One possibility is that protein 2 acts transiently in the cell cycle to ensure that elongation at newly introduced growth sites occurs in the correct rod configuration (James, manuscript in preparation).

I would especially like to thank Dr. Frantz Lund for the generous gift of [^{14}C]mecillinam and Dr. Arthur B. Pardee for support and encouragement. I would also like to thank Drs. Jack L. Strominger and Austin Newton for reading the manuscript and Ms. Mary Cupples for providing clean glassware. Supported by U.S. Public Health Service Grant CA11595 to Dr. A. B. Pardee.

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