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Interaction of FtsA and PBP3 Proteins in the *Escherichia coli* Septum†

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Mutations in the *ftsA* gene of *Escherichia coli* conferred a higher resistance to lysis induced by penicillin or by a combination of cefsulodin and furazlocillin. The *ftsA2* allele codes for an FtsA protein which is inactive at 42°C but is able to regain its activity once it is transferred back to 30°C; *ftsA2* filaments formed at 42°C in the presence of penicillin divided once the penicillin was removed and the temperature was lowered to 30°C. Potential septation sites in the filaments of wild-type cells treated in the same way remained inactive. The binding of a radioactively labeled derivative of ampicillin to penicillin-binding protein 3 (PBP3) was significantly decreased in strain D-3, containing the mutant allele *ftsA3*, when the binding assay was performed at the restrictive temperature. A molecular species able to cross-react with an anti-PBP3 serum was nevertheless found to be present in the envelope of D-3 cells. These observations suggested that the FtsA protein, a protein with a structural and regulatory role in septation, and PBP3, a protein enzymatically active in the synthesis of murein for septation, interact with each other.

The last visible stages of *Escherichia coli* division result in the physical separation of the two daughter cells. This process has been called septation. Septation involves a change in the overall direction of cell wall synthesis from longitudinal to transverse growth. The final result of septation is the formation of two new polar caps that comprise the three layers of the gram-negative wall. The intermediate stages of septation differ from one species to another (18).

Penicillin-binding proteins (PBPs) are a set of 8 to 10 membrane proteins which are involved in the final steps in biosynthesis of the murein layer of the cell wall; they are characterized by their ability to covalently interact with β-lactam antibiotics. Among them, PBP3 (coded for by the gene *pbpB* [*ftsI* or *sep*] [9]) plays an essential role in septation. Inactivation of PBP3, either by mutation or by selective inhibition with specific β-lactams, leads to filamentous growth of the cell (9, 25, 26).

Filamentation also occurs when the products of other genes are either missing or impaired. Some of these genes (*ftsQ*, *ftsA*, and *sulB* [*ftsZ*]) (3, 7, 13, 29) are located in a cluster adjacent to the *pbpB* gene. The product of the *sulB* gene has been postulated to be one of the targets of the SOS-induced inhibition of division (11). The product of the *ftsA* gene has a regulatory role during the last stages of the cell division cycle (7, 28, 29). In addition, the FtsA protein, identified as a 50-kilodalton polypeptide (12), has recently been shown to participate in the structure of the septum (30).

The fact that *fts* mutants are less susceptible than wild-type strains are to the bacteriolytic action of penicillin (19) suggests the existence of a relationship between the gene product of *fts* mutants and β-lactam-induced lysis. We investigated whether this relationship changes the binding of one β-lactam, [^{125}I]ampicillin, to PBP3 in *ftsA* mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the strains which were used are listed in Table 1. The mutant alleles *ftsA2* and *ftsA3* differ in that the thermal inactivation of the FtsA2 protein is reversible upon a shift back to permissive conditions (30°C), whereas the FtsA3 protein remains inactive after the thermal shock (42°C) even if it is returned to the permissive conditions (29, 30). The conditions of growth have been described (14).

Cell parameter measurements. Particle numbers (measured in a Coulter Counter [Coulter Electronics, Inc., Hialeah, Fla.]), optical density (OD) at 540 nm, cell length, and

TABLE 1. Bacterial strains, phages, and plasmids

Strain	Relevant genetic characteristics	Source or reference
OV-2	F$^-$ *ilv his leu trp*(Am) *thyA* (*deo*) *ara*(Am) *lac-125*(Am) *galU42*(Am) *galE tsx*(Am) *tyrT* [*supFA81*(Ts)]	8
D-2	OV-2 *leu*$^+$ *ftsA2*(Ts)	29
D-3	OV-2 *leu*$^+$ *ftsA3*(Ts)	28
D-3A	D-3 *ftsA*$^+$ (spontaneous revertant of D-3)	This work
D-3(λ16-2)	D-3 lysogenic for λ16-2 [*ftsA*$^+$]	This work
D-3(pNS28)	D-3 transformed with pNS28	This work
λ16-2	*ftsA*$^+$ *att*$^+$ *imm*^{21}	12
pNS28	*ftsA*$^+$ Ap^r	20

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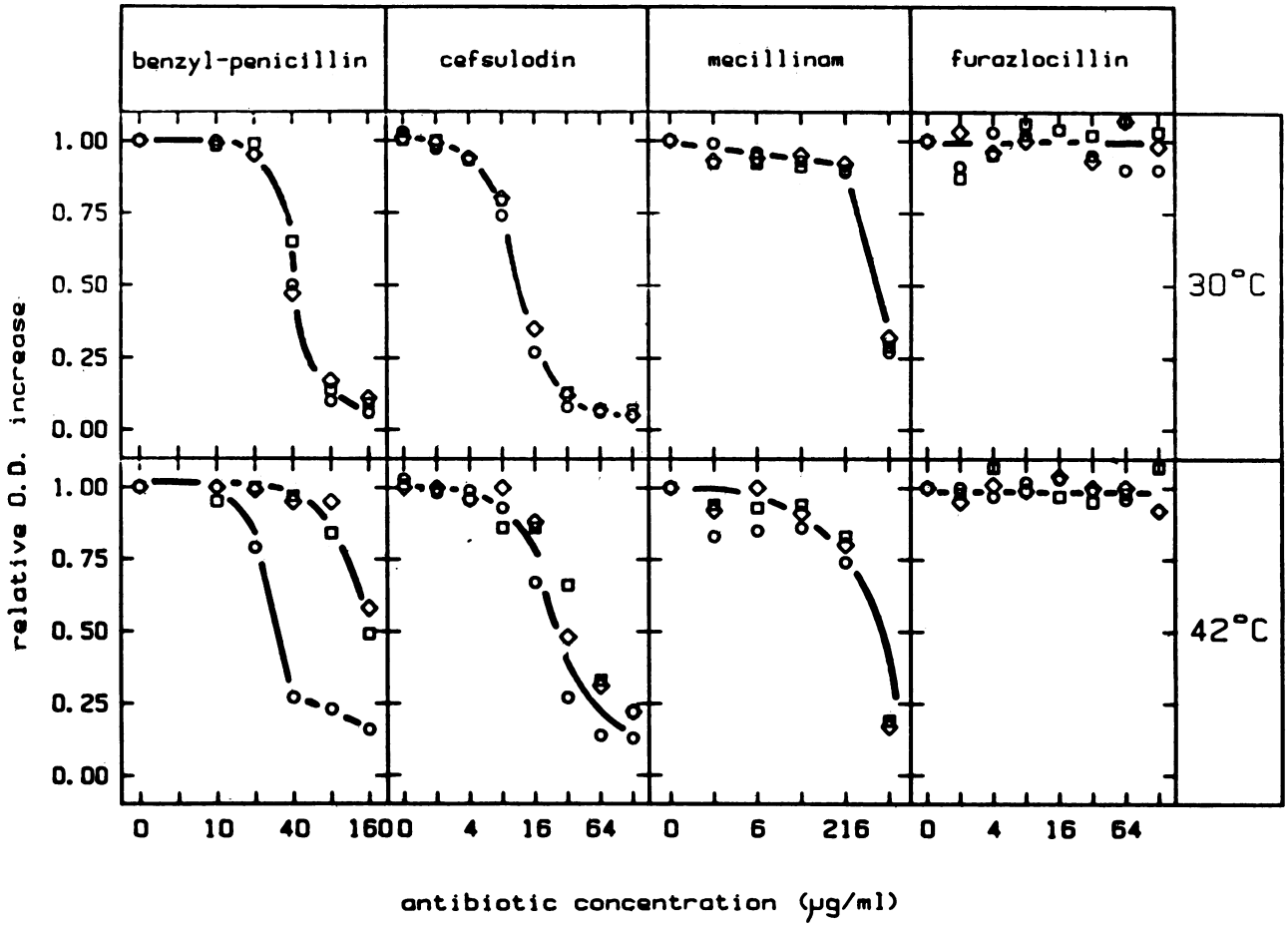

FIG. 1. Sensitivity to β-lactams of strains containing temperature-sensitive *ftsA* alleles. Cultures of strains D-2 (□), D-3 (◇), and OV-2 (○) growing exponentially at 30°C, with an OD near 0.08 were split in portions to which the indicated antibiotics were added at the final concentration indicated on the abscissa. Each portion was then split in two, and one half was incubated at 30°C, and the other half was incubated at 42°C. After two doubling times (as calculated for the portions receiving no antibiotic), the OD of each portion was measured. The plotted values for each concentration of the antibiotic were calculated as the final OD relative to the final OD measured when no antibiotic was present. In such a plot, a value of unity means that the growth of the culture was not inhibited, whereas values lower than unity indicate inhibition of growth and values below 0.25 indicate cell lysis.

septum-to-pole distance were all measured as previously described (30).

Induction of filaments by penicillin and recovery of septation. Cultures of the different strains each containing 10^7 cells per ml growing exponentially at 30°C were transferred to 42°C, and penicillin was added (final concentration, 30 μg/ml). After incubation at 42°C for 60 min for strains OV-2 and D-2 and also for 45 min in one experiment with strain OV-2, the cultures were passed through membrane filters (average pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), washed three times with an equal volume of prewarmed medium without antibiotic, and suspended in the same volume of fresh medium without penicillin at 30°C. When division resumed (30 min for strain D-2 and 15 min for strain OV-2), samples were withdrawn and fixed in an equal volume of saline (1.8% NaCl) containing formaldehyde (0.8%).

Binding of radioactive ampicillin to PBPs in cell envelopes and intact cells. Purification of cell envelopes and binding assays for the detection of PBPs were performed as described by Spratt (25). Binding experiments with intact cells were done as described by Berenguer et al. (4). PBPs were detected by using as a probe the radioactive derivative of ampicillin, *N*-(3-(4-hydroxy-5-[^{125}I]iodophenyl)propionyl) ampicillin ([^{125}I]ampicillin), prepared at a specific activity of 74 TBq/mmol by reacting ^{125}I-labeled Bolton and Hunter reagent (74 TBq/mmol; Radiochemical Centre, Amersham, England) with ampicillin by a modification of the method of Schwarz et al. (23). The binding assay was performed under conditions in which more than 90% saturation of the binding capacity of PBP3 was achieved (21, 23). The binding assays were performed in the presence of 200 μg of clavulanic acid per ml to inhibit the β-lactamase activity encoded by the plasmid marker when cells of strain D-3(pNS28) were used.

Labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis by using the method of Laemmli and Favre (10) and identified by autoradiography with Kodak X-Omat X-ray film (exposure for 1 to 3 days). The autoradiograms were scanned, and the resulting peaks were integrated to calculate the relative amount of binding to PBP3.

Detection of PBP3 by anti-PBP3 serum. Anti-PBP3 serum

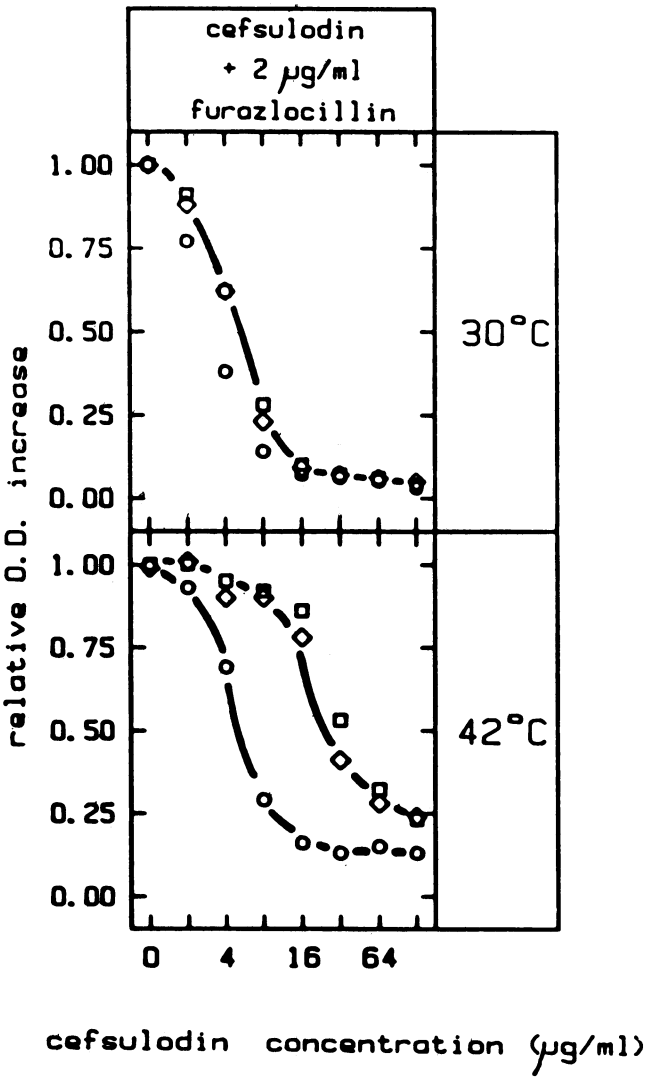

FIG. 2. Sensitivity to furazlocillin and cefsulodin of strains containing temperature-sensitive *ftsA* alleles. The procedure, calculations, and symbols are those described in the legend to Fig. 1, except that the furazlocillin concentration was kept constant (2 μg/ml) in all the cultures, whereas cefsulodin was added to each one at the concentration indicated on the abscissa.

was raised by using an antigen obtained from a *lacZ-pbpB* gene fusion which is able to overexpress the fusion product (details on the construction of the fusion and antibody production will be published elsewhere: J. A. Ayala, J. Plá, M. de Pedro, and D. Vázquez). Antibodies were obtained by the method of Tjian et al. (27). Western blots of electrophoretic separations of cell envelopes were performed by the method of Towbin et al. (31). Detection of the bound antibody was performed by coupling the antibody to ^{125}I-labeled protein A by the method of McConahey and Dixon (15).

RESULTS

Effect of *ftsA* mutations on sensitivity to β-lactam antibiotics. The inhibition of growth caused by different β-lactams was examined with strains OV-2, D-2, and D-3 to determine whether the protection conferred by *fts* mutations against lysis by penicillin (19) was present in these strains, containing different alleles of the *ftsA* gene, and whether different β-lactams exerted different effects, depending on their particular mode of action.

The antibiotics chosen for this experiment included benzylpenicillin, which binds to all PBPs (25); cefsulodin, which binds preferentially to PBPs1 (17); mecillinam, a specific inhibitor of PBP2 (25); and furazlocillin, which acts preferentially on PBP3 (22).

The results (Fig. 1) indicated that when the product of the *ftsA* gene was active (strains D-2 and D-3 at 30°C and strain OV-2 at 30 and 42°C), cell growth was inhibited in a similar way by benzylpenicillin and cefsulodin; mecillinam, as expected, had little effect on cell mass increase except at very high concentrations. Furazlocillin had no effect on growth, when measured as increase in OD, but induced filamentation (data not shown). When the *ftsA* gene product was impaired (strains D-2 and D-3 at 42°C), inhibition of growth occurred to a similar extent to that observed in the presence of the active gene product, except in the presence of benzylpenicillin. In this case the strains containing mutant alleles of *ftsA* showed a higher resistance than did the wild-type strain.

One possibility was that the differential effect observed with benzylpenicillin on *ftsA* mutants was a result of the fact that among the β-lactams tested it was the only one able to simultaneously interact with PBPs1 and PBP3. Therefore, an experiment was done (Fig. 2) in which increasing concentrations of cefsulodin were added simultaneously with a constant concentration of furazlocillin (enough to cause filamentation by itself). The results showed that in this case the combined action of the two β-lactams inhibited growth and induced cell lysis in a fashion similar to that of benzylpenicillin alone when an active FtsA product was present. Inactivation of the FtsA protein at 42°C in strains D-2 and D-3 made these cells less susceptible to lysis even in the presence of both antibiotics.

These results led us to further examine the relationship between the *ftsA* gene and the proteins that covalently bind penicillin.

Effect of the presence of an impaired *ftsA* gene product on recovery of septation in penicillin-induced filaments. By observing the pattern of septation in filaments recovering from a block to division, investigators have recently shown that the *ftsA* gene product interacts with the septum (30). Low concentrations of benzylpenicillin induce the formation of filaments by inactivation of PBP3. This block to division is relieved once the antibiotic is removed from the medium (6). The pattern of septation was therefore studied in filaments of strains OV-2 and D-2 induced at 42°C by concentrations of benzylpenicillin that were sufficient to inhibit septation but cause no visible signs of lysis. Since the *ftsA2* gene product recovers its activity upon return of the strain to 30°C, cell division should occur at any position provided that the potential sites have not been damaged during filamentation. Division was inhibited by incubation with benzylpenicillin, or with benzylpenicillin accompanied by a temperature shift, for a period of time sufficient to allow the appearance of eight-unit-length filaments in both strains so that potential septation sites of several ages would be present. An additional experiment involving a shorter time of incubation was required to obtain filaments of four unit lengths in the wild-type strain. The method of representation of the results allowed the pooling of the two sets of data in one graph since the unit length for a given strain is invariant provided that the doubling time remains unchanged from one experiment to the next (8).

The results (Fig. 3) indicated that the pattern of septation

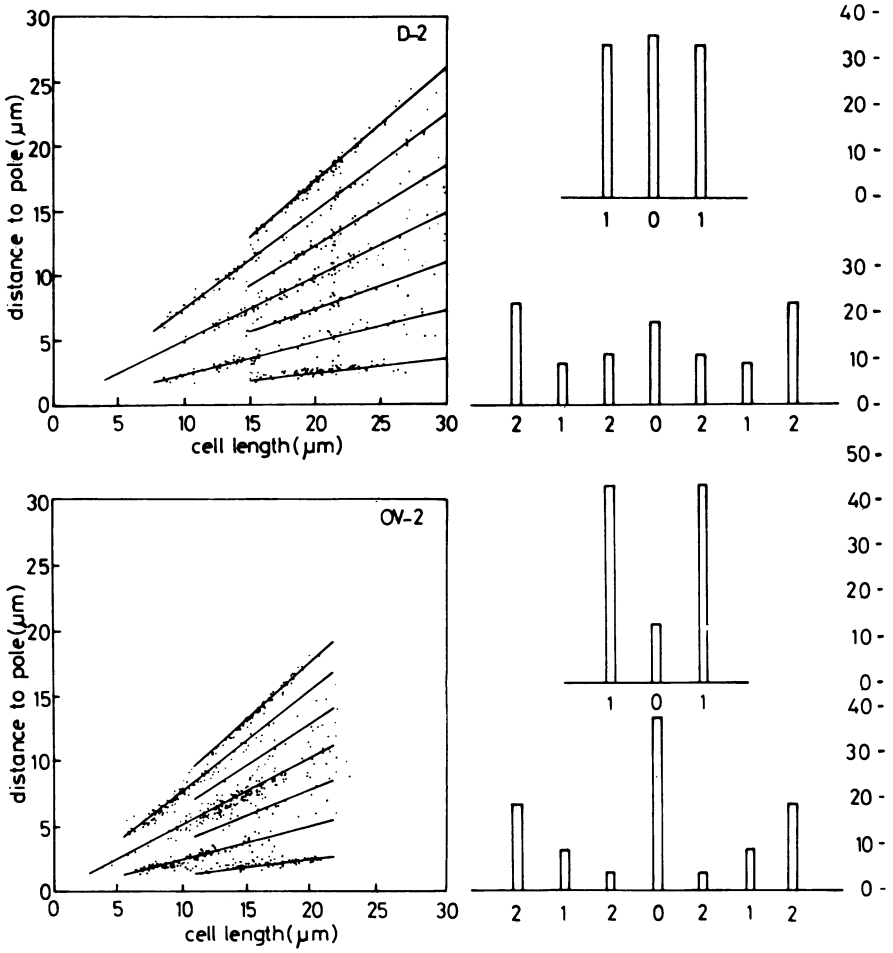

FIG. 3. Localization of septa in filaments induced by benzylpenicillin in the presence and absence of an active *ftsA* gene product. Filaments were induced at 42°C in the presence of 30 μg of benzylpenicillin per ml by the procedure described in the text. Measurements and plots were done as described by Tormo and Vicente (30). The number of septa and the total number of filaments measured (septa/filaments) were 286/152 in strain D-2 and 535/460 in strain OV-2. The OV-2 plot is a pool of the data from two experiments, as discussed in the text. The panels on the left show the length of each filament and the distance of each septum to each pole. The lines inside the panels are not regression lines but theoretical ones. For the histograms on the right, the data for four- and eight-unit-length filaments were pooled in the top and bottom histogram, respectively, of each pair. The border of the two sets was considered to be that length in which the potential division sites increased from three to seven. Septa were assigned to each potential site bisecting the surface between each pair of theoretical lines. The vertical scale indicates the percentage of total septa appearing at each position. Septa of age 0 in four-unit-length filaments were those formed during incubation at 42°C with benzylpenicillin; septa of age 1 in these filaments were formed during recovery at 30°C without benzylpenicillin. Similarly, septa of age 0 in eight-unit-length filaments were either initiated at 30°C and inactivated at 42°C with benzylpenicillin or formed early during incubation at 42°C with benzylpenicillin; septa of age 1 were formed during incubation at 42°C with benzylpenicillin, and septa of age 2 were mostly formed during recovery at 30°C without benzylpenicillin, although the possibility that some septa of age 2 had their origin in the latter part of incubation at 42°C cannot be totally excluded.

upon removal of the antibiotic and transfer to 30°C was not uniform for benzylpenicillin-induced filaments of the wild-type strain. Some positions, namely those corresponding to septa that were bypassed in the presence of benzylpenicillin, were inactivated for at least one generation after the removal of the inhibitor. This effect was particularly noticeable in the age 0 septa of filaments of four unit lengths. The pattern of recovery in filaments of eight unit lengths, although far from random, was harder to interpret. On the other hand, filaments of strain D-2 that had been induced by a similar treatment showed a quite different pattern of recovery. In this case septation, upon relief of the division blocks, occurred almost with the same probability at all potential septation sites, both in four- and eight-unit-length filaments. The higher proportion of septa found at the positions adjacent to the poles has been shown to be characteristic of the recovery of filaments (30). The results of these experiments suggested that the impaired product of the *ftsA2* gene protected the septa from inactivation caused by the addition of benzylpenicillin.

Effect of different *ftsA* alleles on binding of [^{125}I]ampicillin to PBPs. The results of the experiments described in the two preceding sections led us to formulate the hypothesis that the *ftsA* gene product could interact with some of the PBPs, because the impaired FtsA protein counteracted to some extent the effects of penicillin on growth, lysis, and recovery of septation. One likely candidate for a role in this interaction was PBP3 because it is a protein that is essential for septation (25, 26). The results of experiments designed to test the validity of this hypothesis are shown in Fig. 4, 5, and 6 and Table 2. Binding of [^{125}I]ampicillin was done in isolated cell envelopes (Fig. 4 and Table 2) and intact cells (Table 2)

of strains OV-2 (wild type), D-2, and D-3. The results were analyzed by the electrophoretic and autoradiographic procedures described in Materials and Methods. Binding of the labeled antibiotic to all PBPs was normal in the wild-type strain and in strain D-2 under all conditions of growth and binding. However, binding to PBP3 was preferentially reduced when either whole cells or cell envelopes of strain D-3 were incubated at 42°C during the binding assay, irrespective of the temperature used for growth of the culture. Binding to PBP3 in strain D-3 was also reduced when the culture had been grown at 42°C and the binding was carried out at 30°C, particularly when whole cells were used for the binding assay. Otherwise, binding to the other PBPs, or to PBP3 when both growth and binding were done at 30°C in strain D-3, showed a similar pattern, both in gels and densitograms (results not shown for the latter), to that found in the wild-type strain under the same conditions of growth and binding. Other changes observed in the binding at different temperatures to other PBPs were not specific to strain D-3 and were also observed in the wild-type strain. These results confirmed that inactivation of the *ftsA* gene product in strain D-3 rendered the cells, or membranes, unable to bind [^{125}I]ampicillin to PBP3, whereas it had no major effect on the binding ability of the rest of the PBPs.

To determine whether this was due to a decreased amount of PBP3 in the cell envelope, an experiment was performed in which an anti-PBP3 serum, obtained as described in Materials and Methods, was used to assay for PBP3 in the wild-type strain and the mutant D-3 strain. The results (Fig. 5) demonstrated the presence of a molecular species with a molecular weight similar to that of PBP3 and able to cross-react with the serum in the envelopes of strain D-3. The bands that migrated faster than PBP3 and were labeled by the anti-PBP3 serum were products related to PBP3 since these bands did not appear in preparations from *pbpB* mutants (data not shown). Their prominence in all the bindings at 42°C (Fig. 4) suggested that none of our strains contained additional mutations in the *pbpB* gene. Because strain D-3 was derived by phage P1-mediated cotransduction of the *ftsA3* allele into strain OV-2 and has been shown to be complemented by a lambda bacteriophage containing the *ftsA*$^+$ allele but not the *ftsA*(Ts) allele (29), it is unlikely that our present observations were due to a faulty PBP3, unless the PBP3 found in strain D-3 was temperature sensitive for binding to the antibiotic but not for its physiological functions. This latter possibility contradicts current ideas on the mode of action of β-lactams on the PBPs (5). Moreover, construction of strains derived from D-3, by either spontaneous reversion to temperature resistance or introduction of an *ftsA*$^+$ allele, allowed us to exclude this possibility because the PBP3 found in these strains was able to bind [^{125}I]ampicillin at 42°C (Fig. 6).

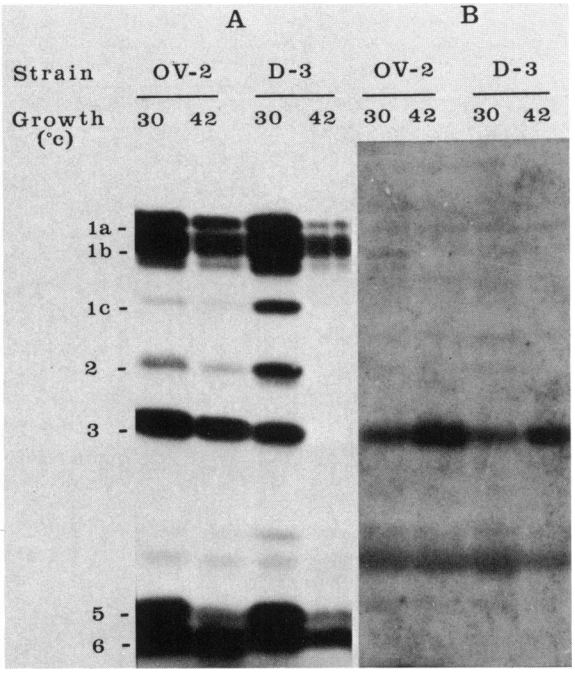

FIG. 5. Detection of PBP3 in the cell envelope of strain D-3. Cell envelopes of strains OV-2 and D-3 growing at the temperatures indicated were obtained by the procedure described in the text. One half of the samples were used for [^{125}I]ampicillin binding at 37°C; the other half were separated, without further treatment, on the same gel. A blot on nitrocellulose paper was obtained of the whole gel. (A) Autoradiogram of the half-blot containing [^{125}I]ampicillin-labeled PBPs. (B) Anti-PBP3 serum bound to the unlabeled half and detected by the methods described in the text. The numbers and letters on the left identify the PBPs.

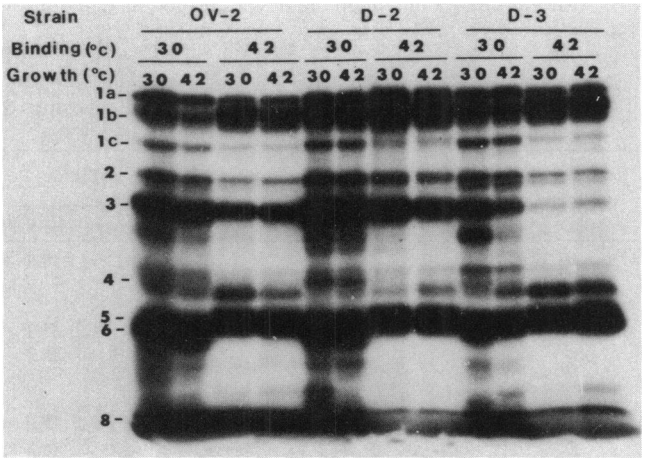

FIG. 4. Binding of [^{125}I]ampicillin to cell envelopes purified from strains OV-2, D-2, and D-3 grown at 30 or 42°C. Samples (50 μl) of purified cell envelopes (10 mg of protein per ml) prepared from strains OV-2, D-2, and D-3 grown at 30°C or after 60 min of incubation at the restrictive temperature (42°C) were preincubated for 10 min at 30 or 42°C before addition of [^{125}I]ampicillin to a final activity of 3.7 kBq/ml. After an additional incubation for 15 min, the samples were further processed as indicated in Materials and Methods. The samples are identified by the name of the strain, the temperature at which the binding assay was performed, and the temperature of growth. The numbers and letters on the left identify the PBPs.

DISCUSSION

Although bacterial cell division is a complex event and although many genetic lesions have a pleiotropic effect on cell division (24), several genes have been identified as specifically involved in the process of cell division in *E. coli* (for recent reviews, see references 16 and 32). We are nevertheless far from being able to describe bacterial growth and division at a molecular level. Recent results have implicated the product of *ftsZ*, a gene found in the cluster of division genes near min 2.5 of the *E. coli* standard map (1), with the SOS-induced inhibition of cell division because *ftsZ*

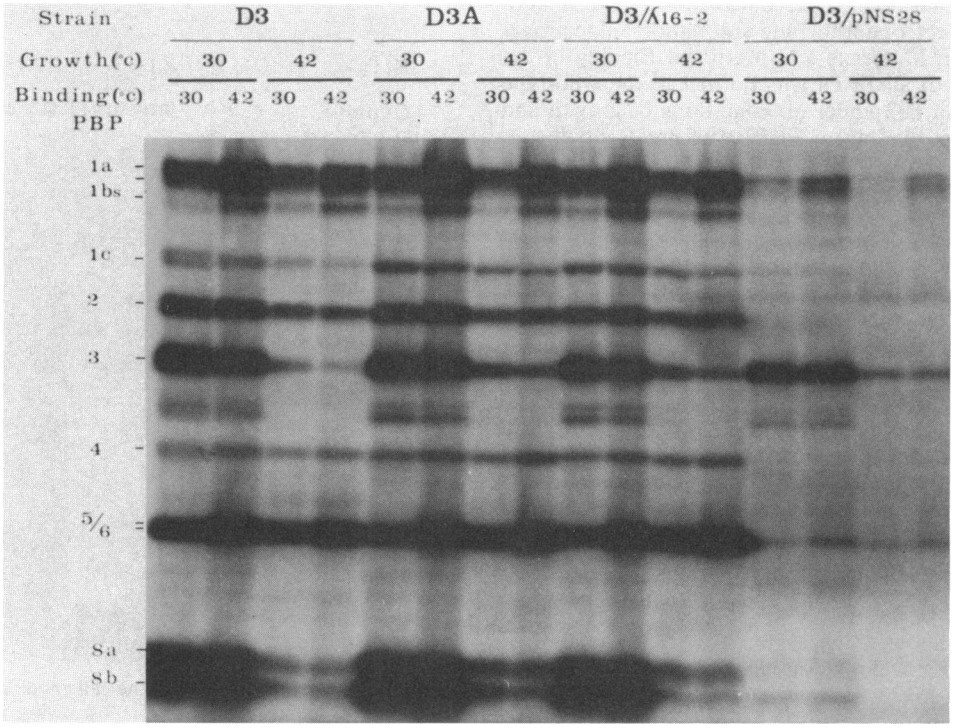

FIG. 6. Binding of [^{125}I]ampicillin to whole cells of strains D-3, D-3A, D-3(λ16-2), and D-3(pNS28). Samples (50 μl) of a concentrated suspension containing 2 × 10^9 cells of each strain were preincubated for 10 min at 30 or 42°C before the addition of [^{125}I]ampicillin to a final concentration of 20 kBq/ml. Binding was performed in the presence of clavulanic acid for samples of strain D-3(pNS28). The rest of the procedure was done as described in the legend to Fig. 4.

is an allele of *sulB* (11). It has been shown, as well, that *ftsA*, another gene in this cluster, codes for a protein whose synthesis is dependent on DNA replication. This protein is required during the last stages of the cell cycle and is a structural component of the septum (29, 30). Genetic and biochemical studies of *pbpB*, the gene which codes for PBP3, indicate that this protein, which has transglycosilase and DD-transpeptidase activities, is active in the process of septation (22).

Our results indicated that at least two of these proteins, namely FtsA and PBP3, the genes for which map within the 2.5-min cluster, interact with each other. This was suggested by the observation that mutations in the *ftsA* gene rendered cells more resistant to lysis induced by either benzylpenicillin or a combination of cefsulodin and furazlocillin (Fig. 1 and 2). Also, the presence of an inactivated FtsA protein protected the potential septation sites from being inactivated by benzylpenicillin (Fig. 3). Finally, the presence of the *ftsA3* allele prevented the binding of [^{125}I]ampicillin to PBP3 at the restrictive temperature, whereas it showed no major effect on the binding of the antibiotic to other PBPs (Fig. 4 and Table 2). Moreover, our observation that this effect on the binding to PBP3 could be found in both intact cells and isolated membranes indicated that the product of *ftsA3* may be associated with the bacterial envelope.

The fact that the product of the *ftsA* gene has been postulated to be a termination protein (28, 29), together with our present observations, suggests that the bacterial septum is a place in which regulatory proteins may interact with structural proteins and enzymes. Broome-Smith et al. have speculated that, because of the inhibition of division that takes place in cells in which an altered form of PBP3 is overexpressed, PBP3 may form part of a division complex (5). Although PBP3 has been clearly identified as a septation

TABLE 2. Binding of [^{125}I]ampicillin to PBP3 in cell envelopes or intact cells of strains containing different *ftsA* alleles

Strain	Temperature (°C) for:		Binding (%) to[a]:	
	Binding	Growth	Cell envelopes	Intact cells
OV-2 *ftsA*$^+$	30	30	24.5	46.1
		42	31.7	47.7
	42	30	17.0	27.1
		42	20.5	25.3
D-2 *ftsA2*(Ts)	30	30	28.7	37.3
		42	32.9	34.8
	42	30	28.0	17.3
		42	22.0	26.4
D-3 *ftsA3*(Ts)	30	30	19.2	27.3
		42	10.7	9.2
	42	30	1.3	0.5
		42	1.7	<0.1

[a] Percentage of the total binding of [^{125}I]ampicillin to each sample, measured as described in the text.

protein (26), it is found in the cell at an almost constant concentration during the *E. coli* division cycle (33). On the other hand, synthesis of the FtsA protein just preceding septation is sufficient for septation (7). Our observation that both proteins may interact suggests a possible model to combine the enzymatic activity of PBP3 with the potential timing properties of FtsA to allow septation at a precise time during the division cycle.

Benzylpenicillin interacts with all of the PBPs, but it preferentially binds to PBP3 at low concentrations (25); the protective effects of some *ftsA* alleles against this antibiotic and against the combined action of cefsulodin and furazlocillin (Fig. 2) could then be attributed to the diminished interaction between the β-lactams and PBP3 at the restrictive temperature. Inactivation of the FtsA protein at the restrictive temperature could then prevent, to some extent, the damage caused by the antibiotics that bind to PBP3, even in strain D-2 in which binding of β-lactams to PBP3 would not be affected. However, morphologies of filaments derived from *ftsA* (2, 29) and *pbpB* (2) mutants do not support this alternative.

The binding of [^{125}I]ampicillin to PBP3 may therefore be affected by microenvironmental factors, such as some mutations in the *ftsA* gene (Fig. 4 and Table 2). Genetic tests, including complementation analysis, should be used, in addition to binding studies, to unambiguously identify mutations in genes coding for these types of proteins that may exert their action in the cellular locations, such as the bacterial septum, in which a highly ordered structure is likely to exist.

ACKNOWLEDGMENTS

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