Probing the Catalytic Activity of a Cell Division-Specific Transpeptidase In Vivo with β-Lactams

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Penicillin-binding protein 3 (PBP3; also called FtsI) is a transpeptidase that catalyzes cross-linking of the peptidoglycan cell wall in the division septum of Escherichia coli. To determine whether the catalytic activity of PBP3 is activated during division, we assayed acylation of PBP3 with three β-lactams (cephalexin, aztreonam, and piperacillin) in growing cells. Acylation of PBP3 with cephalaxin, but not aztreonam or piperacillin, appeared to be stimulated by cell division. Specifically, cephalaxin acylated PBP3 about 50% faster in a population of dividing cells than in a population of filamentous cells in which division was inhibited by inactivation or depletion of FtsZ, FtsA, FtsQ, FtsW, or FtsN. However, in a simpler in vitro system using isolated membranes, acylation with cephalaxin was not impaired by depletion of FtsW or FtsN. A conflicting previous report that the ftsA3(Ts) allele interferes with acylation of PBP3 was found to be due to the presence of a thermolabile PBP3 in the strain used in that study. The new findings presented here are discussed in light of the hypothesis that the catalytic activity of PBP3 is stimulated by interaction(s) with other division proteins. We suggest that there might be allosteric activation of substrate binding.

Cell division in Escherichia coli requires approximately 10 proteins that localize to a ring structure at the division site. Most of these proteins are named Fts for filamentation temperature sensitive, because appropriate mutants form long, aseptate filaments under nonpermissive conditions. The Fts proteins localize to the division site in a defined sequence (Fig. 1) (8, 17, 23, 28, 30). The process starts with assembly of the tubulin-like protein FtsZ into a contractile ring at the middle of the cell. Subsequent recruitment of the other division proteins is thought to result in assembly of a large complex that is directly responsible for synthesis of the division septum. Protein-protein interactions in this hypothetical complex presumably serve to recruit proteins to the division site and to regulate their activities once they are there. One of the last proteins to localize to the septal ring is penicillin-binding protein 3 (PBP3; also called FtsI) (41, 42), a transpeptidase that catalyzes cross-linking of the peptidoglycan cell wall during division. Here we report on the use of a penicillin-binding assay in growing cells to test the idea that division proteins regulate the catalytic activity of PBP3.

β-Lactam antibiotics are often used as substrates to measure the catalytic activity of PBP3, because the physiological substrates used by the enzyme are not yet known (1). Some background will help clarify the principle of this assay. Transpeptidation is a two-step reaction. First, a serine residue (Ser307 of PBP3 [20, 27]) attacks the acyl D-alanyl—D-alanine bond of the donor peptide, resulting in the formation of an acyl enzyme intermediate and release of the terminal D-alanine. Subsequently, a primary amine in the acceptor peptide serves as the attacking nucleophile, with concomitant formation of a new peptide bond and release of the enzyme for further rounds of catalysis.

Many transpeptidases also react with β-lactam antibiotics, which mimic the acyl D-alanyl—D-alanine bond (35), but in this case the covalent acyl enzyme intermediate is essentially irreversible. Therefore, penicillin-binding proteins can be detected in polyacrylamide gels after treatment of whole cells or isolated membranes with a labeled β-lactam (33). The name PBP3 reflects the observation that it is 1 of about 12 (depending on the conditions) E. coli proteins observed in such assays (12). Because penicillin “binding” requires that the enzyme perform catalysis on the β-lactam, the assay gives some information on the catalytic state of the protein. For example, penicillin binding would not be observed if the catalytic serine were not activated to serve as a nucleophile or if the active site were occluded.

Evidence that the catalytic activity of PBP3 is indeed regulated in vivo comes from labeling studies using precursors of peptidoglycan (5, 44, 45). These studies indicate that the protein only engages in peptidoglycan synthesis in the developing septum. This finding can be explained in part by timed localization of PBP3 to the division site (42). However, while localization studies appear to account for the contribution of PBP3 towards septum assembly, they do not readily explain the apparent lack of a contribution to elongation of the peptidoglycan sacculus. This is an issue because PBP3 is present and distributed around the membrane in nondividing cells (43, 46).

Might PBP3’s catalytic activity also be subject to allosteric regulation, such that the enzyme is turned on when it engages other division proteins at the middle of the cell? Several observations reported in the literature seem to support this possibility. One study reported that some fts mutations that block cell division render E. coli partially resistant to penicillin-induced lysis (29). Another study found that the binding of 125I-labeled ampicillin to PBP3 in membranes derived from an
FIG. 1. Recruitment of proteins to the septal ring of E. coli. The first event is polymerization of FtsZ into the FtsZ ring. Then FtsA and ZipA, which bind directly to the FtsZ ring, localize independently of each other. The remaining proteins localize subsequently in the order FtsK, FtsQ, FtsL, and YgbQ (codependently), FtsW, PBP3 (FtsI), and FtsN.

ftsA3(Ts) mutant was eliminated when those membranes were incubated at the nonpermissive temperature, as might be expected if an FtsA-PBP3 interaction modulates PBP3’s transpeptidase activity (36). Finally, a double mutation in a putative regulatory domain of PBP3 abolishes penicillin-binding activity (36). Finally, a double mutation in a putative regulatory domain of PBP3 abolishes penicillin-binding activity (36). Inactivation of PBP3 with an antibiotic would exacerbate the mild FtsZ ring defect observed when an fts mutant is grown at 42°C (by simultaneously inactivating two Fts proteins). It did not. Curiously, control experiments indicated that inactivation of ftsA or ftsQ slightly impaired cephalaxin binding to PBP3. This suggested the interesting possibility that assembly of PBP3 into the hypothetical protein complex that mediates division stimulates the transpeptidase catalytic activity and thus reactivity towards cephalaxin.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and reagents. Cells were grown in Luria-Bertani (LB) medium. L-Arabinose or D-glucose was added to a concentration of 0.2% to modulate the expression of genes controlled by the pBAD promoter. Chloramphenicol (30 μg/ml) and kanamycin (40 μg/ml) were used. Bocillin FL, a fluorescent penicillin derivative, was from Molecular Probes (Eugene, Ore.). Azytreonam was obtained from the University of Iowa Hospitals and Clinics (Iowa City, Iowa). All other antibiotics were from Sigma (St. Louis, Mo.).

Molecular biological and genetic procedures. Standard protocols for transduction, cloning and analysis of DNA, PCR, and electroporation were used (24, 31). The enzymes used to manipulate DNA were from New England Biolabs (Beverly, Mass.). PCR was performed with Herculease from Stratagene (La Jolla, Calif.). Oligonucleotides were from Integrated DNA Technologies (Coralville, Iowa). DNA sequencing was performed using dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase FS enzyme (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Oligonucleotides were from Integrated DNA Technologies (Coralville, Iowa). DNA was sequenced at the DNA Core Facility of the University of Iowa. Reactions were performed using dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase FS enzyme (Perkin-Elmer Applied Biosystems, Foster City, Calif.). The reactions were run and analyzed with a fluorescence automated sequencer (model 373 or 377; Perkin-Elmer Applied Biosystems).

Cloning of the ftsI allele from E. coli strain D-3. The ftsI gene from E. coli strain D-3 was amplified with primers P465 (CGGAATTCGGGTGGAAAGGATCGCCATC) and P469 (CATCTAGAGCAGTGCTCGCGAAGGTGCGT). The P465 and P469 primers anneal 70 bases upstream and downstream of ftsI. The resulting product was cloned into the same sites of pBAD18-Kan (15).

Plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genetic markers or features</th>
<th>Source, reference, or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD18-Kan</td>
<td>Arabinose regulation, Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD33</td>
<td>Arabinose regulation, Cam'</td>
<td>This study</td>
</tr>
<tr>
<td>pDSW478</td>
<td>pBAD18-Kan-fsl</td>
<td>This study</td>
</tr>
<tr>
<td>pDSW514</td>
<td>pBAD18-Kan-ftsI321(Ts)</td>
<td>This study</td>
</tr>
<tr>
<td>pBS38</td>
<td>Low-copy-number ftsQ42</td>
<td>This study</td>
</tr>
</tbody>
</table>

* TS, temperature sensitivity.
to create pDSW520. Plasmids derived from two independent amplifications were used for DNA sequencing. To facilitate penicillin-binding assays, the mutant ftsL allele was subcloned on a 1.6-kb SacI-XhoI fragment into pDSW478, an ftsL expression vector based on pBAD18-Kan. Plasmid pDSW478 carries “wild-type” ftsL with a strong ribosome-binding site and five additional amino acids at the N terminus (M EFPNKNK, where K is the second residue of native PBP3). Plasmid pDSW514 is the equivalent expression vector for the mutant ftsL allele from strain D-3. (The plasmid number [pDSW514 versus pDSW520] reflects the order in which the plasmids were entered into our collection, rather than the order in which they were constructed.)

Assays of β-lactam binding in growing cells. For strains depleted of Fts proteins, a 5-ml culture was grown overnight at 37°C in LB medium containing kanamycin, chloramphenicol, and 0.2% arabinose. The next morning, this culture was resuspended in 20 ml of buffer 2 (20 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 0.5 mM EDTA) and centrifuged as described above. The washed membranes were resuspended in 800 μl of buffer 2 and stored at 4°C. The protein concentration was typically 5 mg/ml, as determined by a Bradford assay with bovine serum albumin as the standard.

The rate of acylation of PBP3 with Bocillin FL was determined in buffer 2 at a membrane protein concentration of 1.25 mg/ml. Tubes containing membranes in buffer were warmed to 37°C, and then reactions were initiated by addition of 1 μl of buffer 2 and storing site and five at −70°C. The protein concentration was typically 5 mg/ml, as determined by a Bradford assay with bovine serum albumin as the standard.

The rate of acylation with cephalixin was determined by competition with Bocillin FL. Membranes (membrane protein concentration of 1.25 mg/ml in buffer 2) were incubated for 30 min with 20 μM Bocillin FL and various concentrations of cephalixin, which was unlabeled. Reactions were stopped with an equal volume of 1× Laemmli sample buffer and analyzed as described above.

Determining the temperature stability of PBP3. Plasmids pDSW478 and pDSW514 express wild-type and mutant ftsL, respectively, under control of an arabinose-inducible promoter. Transformants were grown at 30°C to an OD600 of ~0.1, and then arabinose was added. After incubation, aliquots of 100 μl of culture per OD600 of 1.0 were resuspended in buffer 1 (5 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.5 mM EDTA) and centrifuged as described above. The washed membranes were resuspended in 800 μl of buffer 2 and stored at 4°C.

Detection and quantification of Bocillin FL bound to PBP3. Labeled proteins were separated on SDS–15% polyacrylamide gels and detected with a Typhoon 8600 imager (Amersham Pharmacia Biotech, Piscataway, N.J.). The instrument settings for gel scanning were as follows: excitation laser, 532-nm-wavelength light; short-pass emission filter, 526-nm-wavelength light; photomultiplier, 800 V; and pixel size, 100 μm. ImageQuant software was used to quantify the fluorescent signal. In some cases, such as Fig. 2B and Table 2, the fluorescent of the PBP2 band was used to normalize the signal from PBP3. Because PBP2 does not bind cephalixin, lane-to-lane differences in PBP2 fluorescent signal reflected sample handling errors. None of the conclusions reached in this study would be different if normalization was not done, but the standard deviations reported would be somewhat larger.

Western blotting. Samples were transferred to nitrocellulose membranes, and PBP3 was detected with a polyclonal antibody essentially as described previously (42).

RESULTS
Penicillin-binding assays in growing cells. We sought to test the idea that the catalytic activity of PBP3 is low in nondividing cells, where the protein is distributed around the membrane, but high in dividing cells, where the protein is localized to the division site and presumably makes contact with other division proteins. To test this hypothesis, we employed three β-lactams that bind selectively to PBP3, aztreonam, piperacillin, and cephalixin, because they could be added to growing cells without inducing rapid lysis. Previous studies have shown that piperacillin and aztreonam readily acylate PBP3 even at low concentrations, while cephalixin is a very weak acylating agent (1). It is important to realize that although the assay is traditionally called a “binding” assay, formation of an acyl enzyme complex requires that the protein perform catalysis on the β-lactam.

Figure 2 presents results obtained when cells were depleted of FtsW, a membrane protein suspected of interacting directly with PBP3 (23). To do this assay, a strain engineered to express
to ftsW under control of an arabinose-dependent promoter was
grown in LB medium containing arabinose or glucose until the
glucose-grown cells became visibly filamentous; the average
lengths were 3.3 and 10.2 μm for cells grown in LB medium
containing arabinose and glucose, respectively. Then cepha-
lexin was added to both cultures, and growth continued for 30
min; the final lengths were 5.6 and 19.3 μm, respectively (the
arabinose-grown cells became filamentous because cephalaxin
inactivates PBP3). Cells were harvested, and the residual un-
acylated PBPs were detected with Bocillin FL.

Cephalexin appeared to acylate PBP1b, PBP3, and PBP4
under these conditions (Fig. 2A). Visual inspection of the gels
suggested somewhat more Bocillin FL binding to PBP3, and
therefore less cephalaxin binding, in the filaments grown in
various conditions (arabinose-grown versus glucose-grown
filaments, respectively). Then cephalaxin, or aztreonam. Antibiotic concentrations are in micrograms per
milliliter. The percentages of PBP3 bound are the means
of two (arabinose) or three (glucose) experiments, each done in triplicate.

Loss of PBP1b signal was surprising, as this protein is not
reported to have a high affinity for cephalaxin. Curiously, PBP1b
appeared to bind cephalaxin only when Bocillin FL counterla-
beling was done with detergent-extracted membranes. Similar
assays performed with spheroplasts did not imply acylation of
PBP1b with cephalaxin, nor did we see cephalaxin binding to
PBP1b in membranes isolated from sonicated cells (data not
shown). Therefore, we suspect that PBP1b was lost or inacti-
vated during sample work-up rather than acylated. There is
evidence that sometimes PBP1b forms a complex with several
other proteins involved in peptidoglycan synthesis, including
PBP3 (39). Perhaps binding of cephalaxin to PBP3 alters this
complex in a way that renders PBP1b more labile. Regardless
of the underlying mechanism, in those assays in which we did
see loss of PBP1b signal, it was not correlated with the cell
division status of the culture, so we did not study it further.

Binding of cephalaxin to PBP4 was consistently observed
and has been reported previously (10). Cephalexin acylated
PBP4 equally well in dividing and nondividing populations,
consistent with previous evidence that this protein is not re-
quired for cell division (12). (In Fig. 2A, it appears that slightly
less PBP4 bound cephalaxin in the glucose-grown filaments,
but this is an artifact of loading differences. When normalized
to PBP2, the fluorescence signals for a given cephalaxin con-
centration agree to within 5% in all cases [data not shown].)

Similar experiments were performed with aztreonam and
piperacillin. Each of these antibiotics was highly specific for
PBP3 under the conditions used. The extents of binding to

FIG. 2. Effect of FtsW depletion on acylation of PBP3 with β-lac-
tams. (A) Fluorescence image of a representative gel. Cells were grown
in the presence of cephalaxin for 30 min (cephalexin concentrations [in
micrograms per milliliter] are shown above the lanes), and then resid-
ual unacylated PBPs were detected by counterlabeling with Bocillin
FL, a fluorescent penicillin derivative. The cells were grown in the
presence of arabinose or glucose. Arabinose represses
ftsW; these cells exhibited normal morphology. Glucose represses
ftsW; these cells were filamentous. (B) Fraction of PBP3 acylated with cephalaxin, piperacil-
lin, or aztreonam. Antibiotic concentrations are in micrograms per
milliliter. The percentages of PBP3 bound are the means ± standard
deviations (error bars) of two (arabinose) or three (glucose) experiments, each done in triplicate.
PBP3 were quite similar in dividing and nondividing cells (Fig. 2B). If anything, there was slightly more binding in the nondividing cells. These results are consistent with a previous report that PBP3 readily reacts with pipercillin and furazlocillin in a temperature-sensitive mutant growing at the nonpermissive temperature (5).

We also investigated cephalxin binding to PBP3 when cell division was blocked by several other methods: inhibition of FtsZ ring assembly by induction of a sulA gene under arabinose control, shift of an ftsA(Ts) or ftsQ(Ts) mutant to 42°C, and depletion of FtsQ or FtsN (Table 2). In all cases, PBP3 was less reactive towards cephalxin in filamentous cells than in dividing cells. As with depletion of FtsW, the difference was apparent only at cephalxin concentrations that resulted in roughly 50% saturation of PBP3.

We changed several experimental parameters in an effort to increase the difference in the reactivity of PBP3 towards cephalxin in dividing cells compared to that in nondividing cells. These changes included prolonging the incubation time with cephalxin, letting the filamentous cells get longer before adding the cephalxin, and adding a protein synthesis inhibitor shortly before harvesting the cells. In each case, the difference in cephalxin binding observed was comparable to that reported above (data not shown). We also investigated the effects of expressing PBP3 from a plasmid, but the data could not be interpreted because overproduction of PBP3 resulted in the appearance of multiple bands that interfered with accurate bookkeeping. This problem prevented us from assaying mutant forms of PBP3 that are defective in localization and might be expected to bind cephalxin slightly less well than the wild type, at least when expressed at physiologically appropriate levels in growing cells.

Western blotting was used to investigate whether reduced acylation of PBP3 in filamentous cells might be due to changes in the amount of protein. Although there was some lane-to-lane variation in the PBP3 signal in these blots, it did not correlate with filamentation or cephalxin treatment (Fig. 3). We conclude that the variation seen reflects errors in sample handling and efficiency of protein transfer to membranes, not systematic differences in the amount of PBP3. In support of this interpretation, the strength of the PBP3 signal seen in Western blots was usually correlated with the strength of the PBP2 signal seen in Bocillin FL-binding assays (data not shown). Additional evidence that blocking division does not alter the amount of PBP3 was obtained when Bocillin FL binding was investigated in isolated membranes (see below).

Penicillin-binding assays in isolated membranes. We attempted to extend our observations to a simpler experimental system by assaying β-lactam binding to PBP3 in isolated membranes prepared from strains depleted of FtsW and FtsN. The FtsW depletion strain was grown in LB medium containing arabinose and LB medium containing glucose, and membranes were isolated from the resultant normal cells and filaments, respectively. At the time of harvest, the average length of arabinose-grown cells was 2.9 μm, while the average length of glucose-grown cells was 25.2 μm. Since we do not have an antibody against FtsW, we could not assess directly the levels of FtsW in these preparations.

The apparent rate of acylation of PBP3 with Bocillin FL was not affected by depletion of FtsW (Fig. 4A). It is worth noting that this assay was done with equal amounts of total membrane protein, so the fact that the binding curves are essentially superimposable indicates that depletion of FtsW did not cause a change in the amount of PBP3. To assay cephalxin binding, we used a competition assay in which membrane proteins were incubated for 30 min with 20 μM Bocillin FL and various concentrations of (unlabeled) cephalxin. Depletion of FtsW had no effect on the concentration of cephalxin needed to reduce Bocillin FL binding by 50% in this assay. The values obtained were 155 ± 29 and 166 ± 22 μM for FtsW-replete and -depleted membranes (mean ± standard deviation for six assays; 160 μM cephalxin = 55 μg/ml).

Next, we tested FtsN, another division protein considered likely to interact directly with PBP3 (2, 11). An FtsN depletion strain was grown on LB medium containing arabinose and LB medium containing glucose, and membranes were isolated from the resulting cells (average length, 2.6 μm) and filaments (average length, 21.4 μm). Since we have an antibody against FtsN, Western blotting was used to assess the level of this protein in the two membrane preparations. We also included a wild-type strain of E. coli in this comparison, because the depletion strain carries ftsN on a plasmid and is therefore expected to overproduce the protein. Western blotting of various dilutions of these membranes indicated that arabinose-

![FIG. 3. Amounts of PBP3 in FtsQ, FtsW, and FtsN depletion strains. Gels used for experiments reported in Table 2 were analyzed by Western blotting with a chemiluminescent substrate and exposure to film. Only the portion of the blot with PBP3 is shown.](http://jb.asm.org/)

### TABLE 2. Effect of blocking cell division on binding of cephalxin to PBP3 in growing cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell division target or mutation</th>
<th>% of PBP3 acylated by cephalxin&lt;sup&gt;a&lt;/sup&gt; in:</th>
<th>No. of independent expts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dividing cells</td>
<td>Filaments</td>
</tr>
<tr>
<td>EC906</td>
<td>FtsZ (induces sulA)</td>
<td>62 ± 3</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>EC297</td>
<td>ftsA12(Ts)</td>
<td>76 ± 3</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>EC433</td>
<td>ftsQ(Ts)</td>
<td>77 ± 3</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>JOE170</td>
<td>FtsQ depletion</td>
<td>78</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>EC912</td>
<td>FtsW depletion</td>
<td>87</td>
<td>57 ± 1</td>
</tr>
<tr>
<td>JOE565</td>
<td>FtsN depletion</td>
<td>71 ± 12</td>
<td>34 ± 6 ± 3</td>
</tr>
<tr>
<td>Unweighted avg</td>
<td></td>
<td>75</td>
<td>44</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cephalxin was used at a concentration of 10 μg/ml, and Bocillin FL counterlabeling was done with spheroplasts rather than envelopes prepared by detergent extraction as in Fig. 2.

<sup>b</sup> Mean ± standard deviation given for strains EC297, EC433, and JOE565 for which more than one experiment was performed.
grown cells had about 50-fold more FtsN than normal, while glucose-grown cells had about 50-fold less FtsN than normal (data not shown).

The rate of acylation of PBP3 with Bocillin FL was not affected by depletion of FtsN (Fig. 4B). Likewise, competition assays failed to reveal any effect of FtsN depletion on acylation with cephalaxin. The concentrations of cephalaxin required to reduce acylation of PBP3 with Bocillin FL by 50% were 184 ± 22 and 185 ± 46 μM for membranes with and without FtsN, respectively (four experiments).

Analysis of the ftsA3(Ts) E. coli strain D-3. Our inability to find evidence that depletion of FtsW or FtsN prevents binding of β-lactams to PBP3 in isolated membranes was troubling in view of a previous report that inactivation of ftsA can abolish

the ability of PBP3 to acylate itself with ampicillin (36). FtsA is an early recruit to the division site and therefore is less likely than FtsW or FtsN to interact directly with PBP3, a late recruit (Fig. 1). We therefore decided to reinvestigate the ftsA3(Ts) mutant.

Strain D-3, which was used in the previous study, was obtained from the E. coli Genetic Stock Center at Yale University. We confirmed that this strain formed colonies on LB agar at 30°C, but not at 42°C, and that colony formation could be rescued by plasmid pBS58, which carries ftsQAZ (4). We also amplified the ftsA gene by PCR and confirmed the presence of the previously reported mutation, C719→T, which changes threonine 240 to isoleucine (32). Finally, we used Bocillin FL to confirm that acylation of PBP3 was significantly reduced when membranes from strain D-3 were preincubated at 42°C and that loss of activity could not be attributed to the loss of PBP3 protein as judged by Western blotting. Other PBPs from strain D-3 were not significantly affected by incubation at 42°C. Likewise, PBP3 from a wild-type strain was not much impaired by this treatment. Taken together, these results confirm the previously reported observations (36).

We then considered the possibility that this apparent ftsA3(Ts) effect might be due instead to a cryptic temperature-sensitive mutation in ftsI, which encodes PBP3. The ftsI gene from strain D-3 was amplified by PCR and cloned into a plasmid vector. Plasmids derived from two independent amplifications were used for DNA sequencing and revealed three ftsI mutations: C861→T, which is silent; C1114→T, which changes Pro372 to Ser; and C1675→T, which changes Arg559 to Cys. Both amino acid substitutions are in the penicillin-binding (transpeptidase) domain (26). We designated this allele ftsI321.

To determine whether the ftsI321 allele encodes a thermolabile PBP3, we subcloned the relevant portion of the mutant gene into an arabinose-based expression vector for wild-type ftsI. Both expression vectors were transferred into DHB4, a strain not known to have any mutations affecting cell division. Isolates were grown at 30°C and induced with arabinose to overproduce the plasmid-encoded PBP3. This results in roughly 50-fold overproduction of PBP3 (data not show), so the presence of a small amount of chromosomally encoded wild-type PBP3 from the host strain does not affect subsequent assays. Cells were harvested, and membranes were prepared by extraction of soluble proteins with a nonionic detergent. Membranes were preincubated at 42°C for various lengths of time, transferred to 30°C, and assayed for binding of Bocillin FL. The half-life of the mutant PBP3 at 42°C was about 6 min, whereas that of the wild-type PBP3 was about 50 min (Fig. 5). We infer that ftsI321 encodes a thermolabile PBP3 and that the ftsA3(Ts) allele has no effect on the penicillin-binding activity of PBP3 in isolated membranes.

DISCUSSION

Penicillin-binding assays as probes of transpeptidase catalytic activity. These studies were undertaken to test the idea that interactions between PBP3 and other division proteins stimulate PBP3’s transpeptidase activity during cell division. The underlying assumption in our approach is that transpeptidase activity will be reflected in reactivity towards β-lactams.
This assumption is probably valid only for some aspects of the transpeptidation reaction. For example, if the catalytic serine is occluded or not activated as a nucleophile or if the oxanyon hole that stabilizes the transition state is not formed in the enzyme, then the rate of acylation will probably be negligible with β-lactams or the authentic peptide substrates. However, because the peptide substrates are much larger than β-lactams, some conformations of the enzyme that do not bind the peptides might still bind the antibiotics, and different β-lactams might bind differently and therefore give different results. Finally, penicillin-binding assays as performed here give no information on the second step (deacylation) of the transpeptidase reaction.

The ftsA3(Ts) strain D-3 contains a thermolabile PBP3. When we initiated these studies, the most compelling evidence for regulation of the catalytic activity of PBP3 by another division protein was the report that inactivation of the ftsA3(Ts) allele abolished penicillin binding to PBP3 (36). We found this interpretation to be in error; the strain employed in those studies harbors a thermolabile PBP3 with two amino acid substitutions in the transpeptidase domain. The ftsA3(Ts) mutation was induced with nitrosoguanidine, which is known to cause frequent secondary mutations (13, 14).

Penicillin-binding assays in isolated membranes. Based on the results of localization studies, the two division proteins most likely to interact directly with PBP3 are FtsW and FtsN (2, 23). Accordingly, we isolated membranes from FtsW and FtsN depletion strains and assayed acylation of PBP3 with Bocillin FL and cephalaxin. Depletion of FtsW or FtsN did not appear to affect the rate of acylation with either antibiotic. Either FtsW and FtsN do not regulate the transpeptidase activity of PBP3, or if they do, regulation occurs only in the context of protein complexes that are lost during membrane isolation. The latter possibility must be taken seriously in view of the evidence from protein localization studies that recruitment of division proteins to the septal ring depends on the presence of an FtsZ ring (30). Our membrane preparations lack FtsZ.

Penicillin-binding assays in growing cells. Using β-lactams to probe the catalytic activity of PBP3 in growing cells makes it possible to assay the enzyme under conditions in which it is active in cell wall synthesis. These conditions cannot be replicated in vitro yet (1). We found that inhibition of cell division by a variety of methods had no detectable effect on the apparent rate of acylation of PBP3 with aztreonam or piperacillin but slightly reduced acylation with cephalaxin. The magnitude of the effect observed with cephalaxin depended upon the assay conditions, especially the concentration of antibiotic, but is likely to be in the range of 1.5- to 2-fold. The lower value is based on the observation of about 60 to 80% acylation of PBP3 with 5 to 10 μM cephalaxin in dividing cells, compared to about 40 to 60% in filaments (Fig. 2 and Table 2). The higher value takes into consideration that in an E. coli population growing with a doubling time of about 30 min, only about half of the cells exhibit septal localization of PBP3 (41–43). While an effect of twofold or less is small, we think it is likely to be an underestimate. If PBP3 is engaged in cell wall synthesis in the dividing population, then peptide substrates are probably competing with cephalaxin for access to the active site. Protection of the enzyme by peptide substrates has been invoked previously to explain why the MICs for some β-lactams are higher than would be expected on the basis of binding affinities determined in isolated membranes (7, 21).

We infer that the catalytic site of PBP3 is properly assembled even in nondividing cells, when PBP3 does not appear to be engaged in cell wall synthesis. If the catalytic site adopted a different (presumably more active) conformation when PBP3 engages other proteins at the division site, we would have expected to see much faster acylation with all three antibiotics in dividing populations compared to nondividing populations. However, the finding that acylation with cephalaxin is somewhat sensitive to the division status of the cells suggests that some aspect of PBP3 function is modulated by interaction of PBP3 with other division proteins. We suggest that this is substrate binding and that the small effect seen with cephalaxin might reflect a much larger effect on binding of the authentic peptide substrates used in cell wall synthesis. Several of our observations are consistent with this interpretation, including lack of a difference in acylation rate in isolated membranes (no complexes present under these conditions regardless of the presence or absence of FtsW or FtsN), lack of a difference in acylation rate at high cephalaxin concentrations (which overcome weak binding), and lack of a difference in acylation rate with aztreonam or piperacillin (these antibiotics bind differently to the enzyme than cephalaxin does).

Genetic studies support the notion that the binding site for cephalaxin overlaps but is not identical to those for peptide substrates and that the binding site for cephalaxin differs in important respects from those for aztreonam and piperacillin. Hedge and Spratt selected for E. coli mutants resistant to cephalaxin and obtained strains with lesions in the transpeptidase domain of PBP3 (18, 19). Despite extensive efforts, only a few mutations that resulted in substantial cephalaxin resistance were recovered, implying that very few amino acids in PBP3 can be changed to reduce the affinity for cephalaxin without
compromising transpeptidation. In other words, the binding sites for cephalexin and peptide substrates are similar but not identical. Perhaps more surprising was the finding that cephalexin-resistant mutants that produced an altered PBP3 with about 500-fold-lower affinity for cephalexin have no detectable cross-resistance against aztreonam or piperacillin.

A few less-interesting hypotheses might also account for faster acylation of PBP3 in dividing cells than in nondividing cells. One potential explanation is that the cephalexin concentration in the periplasm of filamentous bacteria might be lower. This would be the case if filamentation retarded diffusion of cephalexin into the periplasm, enhanced efflux, or stimulated a β-lactamase. However, if a lower cephalexin concentration in the periplasm were the basis of our observations, we would expect to see reduced acylation of PBP4, which we do not. PBP4 reportedly binds cephalexin less well than PBP3 (10), so it should be more sensitive to PBP3 to the antibiotic concentration. In addition, most mechanisms of excluding cephalexin would also be likely to exclude aztreonam or piperacillin to some extent, but no evidence for reduced binding of these antibiotics was obtained, even at sub saturating concentrations (Fig. 2B). Another potential explanation for slower acylation with cephalexin in filaments than in dividing cells would be differences in the amount of PBP3 in two cell types, but this hypothesis is hard to reconcile with the finding that acylation with aztreonam and piperacillin was not reduced in the filaments (Fig. 2B). Moreover, direct measurements of PBP3 levels by Western blotting (Fig. 3) and penicillin-binding assay (Fig. 2A and Fig. 4) did not reveal any consistent differences in PBP3 levels that could be correlated with filamentation or cephalexin treatment.

It is interesting that the reactivity of PBP3 towards cephalexin was reduced by inactivation or depletion of proteins needed for recruitment of PBP3 to the septal ring (FtsZ, FtsA, FtsQ, and FtsW) as well as depletion of a protein (FtsN) that localizes after PBP3. This finding implies that localization of PBP3 is not sufficient for activating the transpeptidase activity. Comparison to serine proteases. An analogy can be made to the serine proteases, which employ a catalytic mechanism similar to that of the PBPs and are subject to inactivation by small molecule inhibitors, such as diisopropylphosphofluoridate (DFP). DFP forms a relatively long-lived covalent adduct with the catalytic serine, but essentially it does so only if the enzyme is active as a protease. Thus,zymogens do not react readily with inhibitors that modify the catalytic serine. For example, chymotrypsin reacts about 4 orders of magnitude more rapidly with DFP than does chymotrypsinogen (25), because in the latter the oxazolone bond involved in transition state stabilization is not formed (40).

Other modes of regulation. Allosteric interactions that activate the catalytic function of PBP3 during division are an attractive, but by no means the only, way to spatially and temporally regulate the transpeptidase. There is evidence to suggest that PBP3 uses a special substrate for the transpeptidation reaction (3), although this has been questioned (12, 38). In addition, it is worth noting that the reaction catalyzed by PBP3 is unusual in that both the enzyme and its substrates are fixed in space, so conformation changes brought about by various Fts proteins during division might give PBP3 access to the transpeptidation substrates without affecting the structure of the active site per se. Either of these alternative modes of regulation could effectively restrict enzyme activity in time and space but would not be reflected in different reactivities towards freely diffusible artificial substrates such as β-lactams.

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