Rapid β-lactam-induced lysis requires successful assembly of the cell division machinery

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β-lactam antibiotics inhibit penicillin binding proteins (PBPs) involved in peptidoglycan synthesis. Although inhibition of peptidoglycan biosynthesis is generally thought to induce cell lysis, the pattern and mechanism of cell lysis can vary substantially. β-lactams that inhibit FtsI, the only division specific PBP, block cell division and result in growth as filaments. These filaments ultimately lyse through a poorly understood mechanism. Here we find that one such β-lactam, cephalexin, can, under certain conditions, lead instead to rapid lysis at nascent division sites through a process that requires the complete and ordered assembly of the divisome, the essential machinery involved in cell division. We propose that this assembly process (in which the localization of cell wall hydrolases depends on properly targeted FtsN, which in turn depends on the presence of FtsI) ensures that the biosynthetic machinery to form new septa is in place before the machinery to degrade septated daughter cells is enabled. β-lactams that target FtsI subvert this mechanism by inhibiting FtsI without perturbing the normal assembly of the cell division machinery and the consequent activation of cell wall hydrolases. One seemingly paradoxical implication of our results is that β-lactam therapy may be improved by promoting active cell division.

amidase | cell wall hydrolase | *ftsN* | penicillin-binding proteins | peptidoglycan

Penicillin and other β-lactams have been used for over 60 years to treat bacterial infections. They covalently modify the active site of penicillin binding proteins, enzymes that synthesize and modify bacterial peptidoglycan (PG) (1, 2) and have been proposed to kill bacterial cells by changing the balance between PG synthesis and degradation (3, 4). Here, we study the mechanism of cell death by the clinically important class of β-lactams that target cell division.

Bacterial cell division proceeds through the ordered assembly of a protein complex called the divisome (5), which includes a target of the β-lactams, FtsI (PBP3) (6–8). FtsI (PBP3) is the only essential penicillin-binding protein (PBP) required for division. β-lactams that target FtsI, such as cephalexin (Keflex) and piperacillin (the active β-lactam in Zosyn), are among the world's largest selling β-lactams. Although the target of these β-lactams is known (8), the molecular mechanisms that lead to cell death are not understood. Cephalexin typically induces filamentation due to its ability to block the activity of FtsI. Ultimately, the long filaments die through an unknown mechanism.

Here we show that under certain conditions, treatment with cephalexin (and other FtsI specific β-lactams) can induce rapidly growing *Escherichia coli* to undergo rapid lysis at the division site. We find that rapid lysis by cephalexin at the division site requires the complete assembly of the divisome complex. This includes the FtsI-dependent recruitment of FtsN, the last essential component of the divisome (9), and, in turn, the FtsN-dependent recruitment of downstream hydrolases. Our studies provide a simple explanation for the ordered assembly of the divisome: it is a mechanism to ensure that the cell wall degradative machinery is not recruited until the biosynthetic machinery is in place with FtsN acting as critical link between the biosynthetic enzymes that synthesize the peptidoglycan during division and the degradative enzymes that facilitate daughter cell separation (10). This suggests that rapid lysis by cephalexin is due to its ability to block the activity of FtsI without disrupting normal divisome assembly and the accompanying activation of cell wall hydrolases.

Results

Cephalexin Induces Filamentation Followed by Lysis at Low Cell Density. Although FtsI inhibitors are often used to induce *E. coli* to filament (11), scattered reports indicate that they can also induce rapid cell lysis (12, 13). We found that midcell lysis is reproducible and requires cells to be growing exponentially in rich media at low cell densities [$<10^8$ cells/ml, supporting information (SI) Fig. S1] (13). Lysis also required concentrations of cephalexin above 10 μg/ml, levels at which it is specific for PBP3.

Lack of Division Prevents Rapid Division Site Lysis by Cephalexin. Since cephalexin induces division site lysis, we asked whether lysis required proper assembly of the essential components of the divisome. We first treated cells in tubes with 50 μg/ml cephalexin. To test cephalexin at more concentrations, we proceeded to do detailed experiments by using 96-well plates since they provided a much larger number of assay points. Rapid lysis was also observed on plates at low cell density despite a difference in doubling time (approximately 22 min in tubes and approximately 35 min on plates). The results that follow include those that show a concordance between findings obtained with specific experiments done in tubes and plates (see *Materials and Methods*). SulA is a potent inhibitor of FtsZ ring formation, the first step in cell division (Fig. 1*A*) (14–16). Overexpression of SulA abolishes recruitment of divisome components to potential division sites, resulting in cells that increase in mass at wild-type rates but do not divide, thus growing as smooth filaments. We found that, after addition of cephalexin, wild-type cells lysed specifically at midcell-positioned bulges (Fig. 1*B*), often coincident with FtsZ rings (Fig. 1*D*). In contrast, SulA-expressing cells showed resistance to lysis (Fig. 1 *B* and *D*) at concentrations well above the minimum inhibitory concentration of cephalexin (Fig. 1*C*). Thus, the assembly of the cell division machinery is essential for rapid cephalexin-induced cell lysis and suggests the presence of an activation step in the assembly of the divisome that enables lysis.

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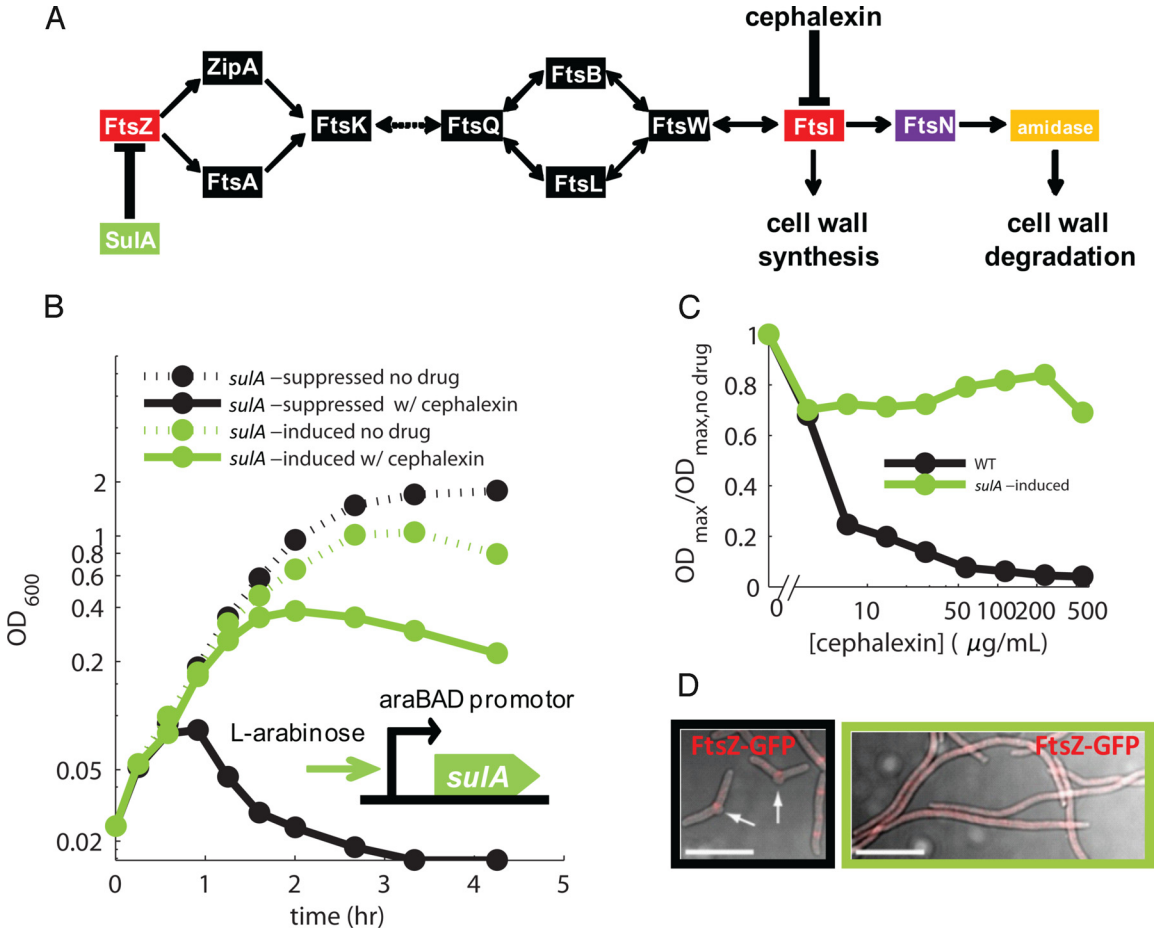

Fig. 1. Lack of division prevents cephalexin-induced cell lysis. (*A*) Bacterial cell division proteins localize according to a defined pathway. SulA inhibits the first step in cell division, FtsZ polymerization. (*B*) OD curves for strain (NWG593) expressing (green) and suppressing (black) *sulA* from an arabinose-induced promoter. 50 μg/ml cephalexin was added at t = 20 min. (*C*) Dose–response curves of the *sulA* induced and wild-type control showing their maximal OD, normalized to its no drug value, as a function of drug dosage. (*D*) Microscopy images of NWG593 show lysis under cephalexin treatment (50 μg/ml) occurred at mid-cell of *sulA*-suppressed cells (*Left*) and no lysis observed for *sulA*-induced cells (*Right*). FtsZ-GFP fluorescence images (red) show the disappearance of FtsZ ring in the *sulA*-induced strain. (Scale bar, 10 μm.) Cells were fixed at 30 min (−SulA) and at 80 min (+SulA) after the addition of cephalexin. For time-lapse movies of wild-type (JOE650) under different cephalexin concentrations, see Movies S1–S4. Note that cephalexin induces filaments, followed by mid cell lysis as in *D*.

To determine at which point of divisome assembly this activation step occurs, we examined strains carrying temperature sensitive mutations in *ftsI*, and 2 other divisome components, both of which are required for localization of FtsI to division site, *ftsA* and *ftsQ*. At the permissive temperature, *ftsA12*(Ts), *ftsQ1*(Ts), and *ftsI23*(Ts) strains behaved similarly to an isogenic wild-type strain, JOE309, with or without drugs. However, at the restrictive temperature (42 °C), all 3 temperature sensitive strains showed resistance to cephalexin-induced lysis (Fig. S2). These results are consistent with a variety of studies showing that *E. coli* strains with defects in cell division are resistant to lysis by certain β-lactams (17, 18).

FtsN Depletion Protects Against Cephalexin Killing Through a Mechanism Independent of the Drug Target Localization. That sensitivity to cephalexin requires the localization of FtsI supports the hypothesis that FtsI inhibition is the primary trigger of cephalexin-induced lysis. However, when we examined the role of FtsN, which is recruited subsequent to FtsI (Fig. 1*A*), we were surprised to find that the cells depleted of FtsN also failed to lyse rapidly (Fig. 2), a result similar to that found with mutations in *ftsI*, *ftsA*, and *ftsQ* (Fig. S2).

To confirm that FtsI was still localized under the FtsN depletion conditions (19) and that failure to lyse was not due to an unexpected defect in FtsI localization, we examined GFP-FtsI localization in FtsN-depleted cells treated with cephalexin. FtsI clearly localizes to nascent division sites along the filamentous cell with no evidence of lysis (Fig. 2*C*). In contrast, FtsN$^+$ cells lysed at midcell-localized bulges that contained FtsI (Fig. 2*C*). Thus the recruitment of FtsI to the FtsZ ring is not sufficient to enable fast cephalexin-induced lysis to occur. FtsN is apparently required for rapid lysis as well. Consistent with our findings, FtsN is properly localized to the divisome site in the presence of piperacillin, another β-lactam that specifically inhibits FtsI (20). We conclude that full assembly of all essential cell division proteins to the divisome is required for fast lysis.

Active Cell Wall Degradation Is Required for Rapid Lysis by Cephalexin. Why is FtsN required for rapid lysis in response to β-lactams? A specific function for FtsN is not known. However, it is required to recruit to the site of cell division the *N*-acetylmuramyl-L-alanine amidases (10) and the Tol-Pal complex (21), which is assumed to be involved in coordination of the separation of the outer membrane. Thus, recruitment of one of these could be essential for cell death upon FtsI inhibition. Amidases are involved in the degradation of peptidoglycan, which allows the daughter cells to separate, and it is plausible that recruiting an active amidase to the site of cell division when FtsI is inhibited

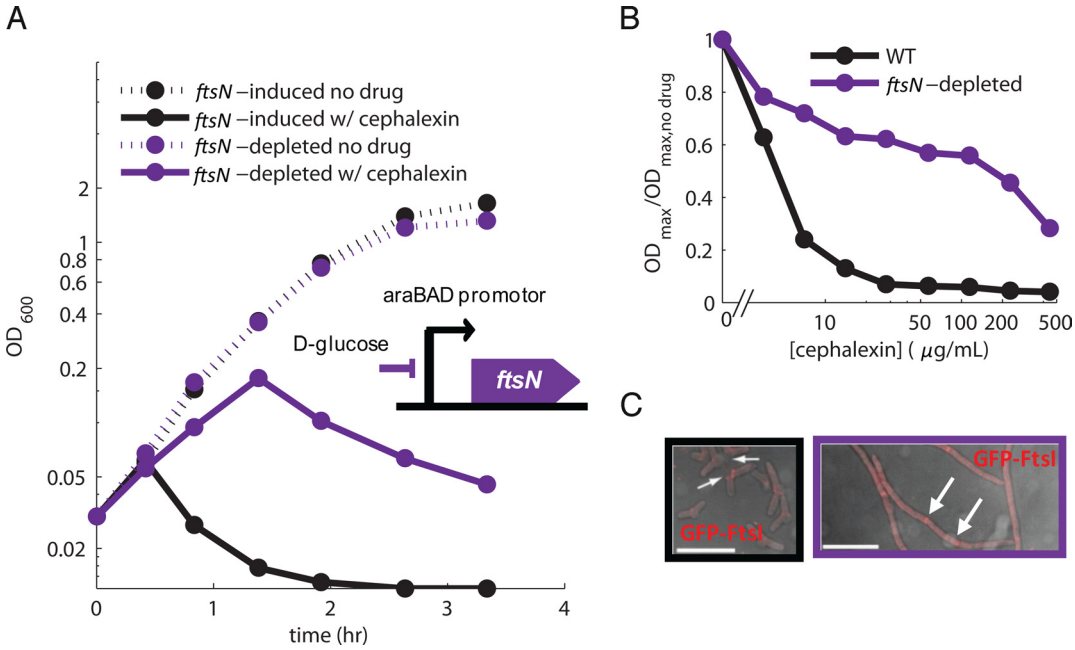

Fig. 2. FtsN depletion protects against cephalexin killing through a mechanism independent of the drug target localization. (*A*) OD curves for strain (HSC070) suppressing (purple) and expressing (black) *ftsN* from an arabinose-induced promoter; 50 μg/ml cephalexin was added at t = 0. (*B*) The protection of FtsN depletion against cell lysis operates along a wide range of drug dosage, as indicated by its higher normalized OD. (*C*) Microscopy images of HSC070 show lysis under cephalexin treatment (50 μg/ml) occurred at mid-cell of *ftsN*-induced (*Left*) and no lysis observed for *ftsN*-depleted cells (*Right*). (Scale bar, 10 μm.) GFP-FtsI fluorescence images (red) show that protection from lysis induced by cephalexin operates without affecting divisome localization of the drug target (FtsI) in the FtsN depletion strains. Cells were fixed at 30 min (+FtsN) and at 60 min (−FtsN) after the addition of cephalexin.

could provide a mechanism for fast lysis (22). Three N-acetylmuramyl-l-alanine amidases are found in *E. coli*: AmiA, AmiB, and AmiC (23). When all 3 of the genes encoding these proteins are deleted simultaneously, *E. coli* forms chained cells in which the cytoplasmic membrane and septal murein are synthesized normally but the septal murein is not able to separate into 2 daughter cells (23). AmiC is the only amidase that has so far been shown to localize selectively to the division site (10).

We investigated the effects of cephalexin treatment on mutant strains in which the amidase genes were deleted, first individually and then in all pairwise combinations (Fig. 3 and Fig. S3). In the absence of antibiotic treatment, the single gene deletion strains grow normally, as previously reported (23). Two of the double mutants, ΔAΔB and ΔAΔC, show a chaining phenotype but, contrary to previous reports (23), ΔBΔC separates normally (see HSC087, HSC083, and HSC088 in Table S1). In the presence of cephalexin, fast lysis was observed for all of the single gene deletion strains, confirming the functional redundancy of the amidases in our system. However, while the ΔBΔC strain resembled the single mutants (Fig. S3 *B* and *D*), neither the ΔAΔB nor the ΔAΔC strains displayed fast lysis upon cephalexin treatment (Fig. 3 *A* and *B* and Fig. S3 *A* and *C*). We concluded, therefore, that action of these amidase pairs is critical for cephalexin-induced fast lysis. These results are consistent with findings of Bernhardt et al. (24) that depletion of lytic enzymes makes cells less sensitive to β-lactams.

Sensitivity to β-Lactams Depends on the State of Divisome Assembly. The ΔAΔB or ΔAΔC mutations protect the cells against killing by cephalexin (Fig. 3). If this protection results from the specific balance between synthesis and degradation of PG at the divisome, then it should be specific to β-lactams that target FtsI. We considered, therefore, the effects of 3 other β-lactams (Fig. S4): ampicillin, which inhibits multiple targets (PBP1a, PBP1b, and PBP3), cefsulodin, which is selective for PBP1a and PBP1b (25), and piperacillin, another selective inhibitor of PBP3. We first measured the protection of *sulA*-induced cells against these additional drugs and confirmed that inhibition of divisome assembly protects very powerfully against fast lysis promoted by cephalexin (Fig. 4) and piperacillin (Figs. S5 and S6) but less against ampicillin and even less so for cefsulodin (Fig. 4). We then tested the effect of these drugs in the FtsN depletion cells and the amidase double knockouts (Fig. 4). We found, again, that the profile of protection of FtsN depletion and the amidase double mutant strains match well their pattern of selectivity to the divisome. These results clearly demonstrate that the mechanism of cell death mediated by FtsN depletion and the amidase double deletion strains is not a result of some nonspecific tolerance of these mutants to general cell-wall stress but is specific to β-lactams that disrupt FtsI activity.

It is interesting to note that the protective effect of disrupting divisome assembly against each of the drugs varied depending on the component targeted. Blocking division by SulA confers greater protection than blocking FtsN recruitment, which is more protective than deleting amidases. This is not completely surprising since blocking FtsZ ring formation would be expected to rapidly and completely eliminate divisome assembly while depletion of FtsN is necessarily incomplete. Garcia and de Perdro also reported similar findings when treating *ftsZ(ts)* and *ftsI(ts)* mutants with cefsulodin (18). The observation that amidase deletion does not confer as much protection as blocking division at earlier stages is also noteworthy. The divisome assembly pathway is more complex than the simplified linear model depicted in Fig. 1*A* and may contain additional branches. In particular, there are many hydrolases involved in degrading PG. In particular, EnvC and the related LytM-like hydrolases appear to contribute to lysis and their presence may be responsible for the partial effects we see (24).

Discussion

For rapid division site lysis to occur upon inhibition by β-lactams, cell division must be "activated," meaning that divisome assem-

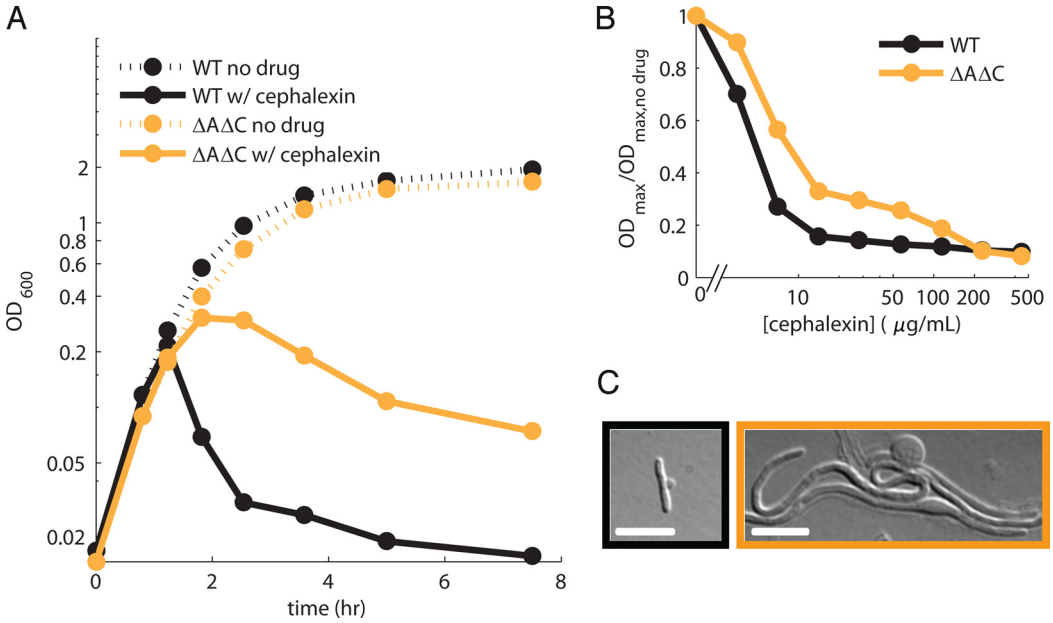

Fig. 3. Cell wall degradation enzymes are required for fast lysis by cephalexin. (*A*) OD as a function of time for wild type (JOE309; black) compared to amidase double knockouts (HSC087: Δ*amiA* Δ*amiC* (ΔAΔC); orange) in the presence (solid lines) and absence (dotted lines) of cephalexin; 50 μg/ml cephalexin was added at t = 48 min. (*B*) Dose–response curves showing maximal OD as a function of drug dosage for wild-type and Δ*amiA* Δ*amiC* strains. (*C*) Microscopy images show lysis for wild-type cells (JOE309; black) under cephalexin treatment (50 μg/ml) occurred at midcell and defects in cell separation leading to delayed lysis of Δ*amiA* Δ*amiC* strain (HSC087; orange). These images are snapshots taken from Movie S4. For the other 2 amidase double knockouts [HSC086: Δ*amiA* Δ*amiB* (ΔAΔB); HSC083: Δ*amiB* Δ*amiC* (ΔBΔC)], see Fig. S3.

bly must be complete and, specifically, amidases must be recruited to the division site. The ordered recruitment of FtsI, FtsN, and then the amidases apparently functions to coordinate the degradative activity of the amidases with the synthetic activity of FtsI. This ordinarily means that old peptidoglycan is not degraded unless the divisome is functioning properly. Consistent with previous studies suggesting a role for FtsN in activation of the divisome and initiation of septation and separation of daughter cells (5, 26–28), our results suggest that recruitment of FtsN functions to ensure that cell wall degradation does not take place unless new cell wall is being incorporated at the emergent poles. Also consistent with a regulatory rather than, for instance, an essential enzymatic role, mutants that are missing FtsN can be suppressed by mutations in another component of the divisome, FtsA (29).

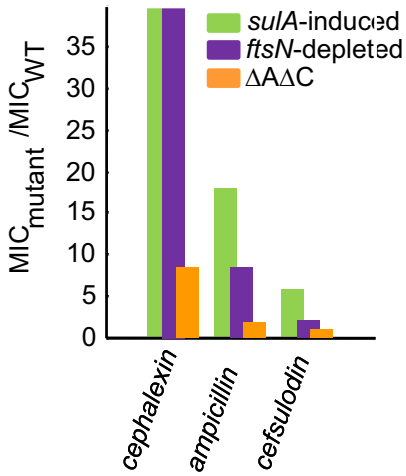

Fig. 4. Sensitivity to β-lactams depends on the state of divisome assembly. The bar graph shows the relative change in MIC for mutant versus wild-type strains under treatment with cephalexin (PBP3 selective), ampicillin (PBP1a, PBP1b, and PBP3), or cefsulodin (PBP1a and PBP1b selective). The ratio of MICs for both *sulA*-induced and *ftsN*-depleted cells under cephalexin treatment is greater than 35, but *sulA* induction confers better protection than *ftsN* depletion.

This model suggests that FtsI-selective β-lactams induce rapid midcell lysis because they inhibit the activity of FtsI without disrupting its ability to localize to the Z-ring and to recruit FtsN and the amidases. This subverts the normal regulatory mechanisms of the cell: amidases begin to degrade the old cell wall despite the cell's inability to properly synthesize new septal peptidoglycan. At this time, it is not clear whether it is simply the localization of FtsN and/or the amidases that permits lysis or if an additional activation step must occur that is, itself, dependent on proper divisome assembly, such as initiation of constriction.

The notion that β-lactam sensitivity can be modulated by the division status of the cell is not new. For example, Garcia and de Perdro (18), in examining the effects of β-lactam antibiotics on cells depleted of PBP1b reported that inhibition of FtsI led to rapid lysis, but if cell division was blocked before recruitment of FtsI, no lysis occurred. This implied that recruitment of FtsI is necessary for this form of lysis. Our results that amidase recruitment is sensitive to the physical presence of FtsI in the divisome provide an explanation. At the same time, their observation that deleting PBP1b sensitizes cells to rapid lysis suggests that drug treatment may partially inhibit PBP1b as well as FtsI. Indeed, other PBP's, such as PBP1b, are known to play a role in division (18, 30). In fact, the weaker protection we observe on treatment of the double amidase knockouts suggests that other non-FtsN-regulated hydrolyases also play a role in the mechanism of lysis.

Non-FtsI selective β-lactams (cefsulodin) show less protection from division site lysis when divisome assembly is blocked, presumably because killing by these antibiotics also involves effects on elongation machinery. Nonetheless, it seems reasonable that misregulation of the active degradation machinery required for normal cell elongation could be involved in the ability of these β-lactams to lyse cells.

We note in closing that the recognition that cells must be in a state of division for rapid lysis by FtsI-selective β-lactams may have important clinical implications since FtsI selective β-lactams are widely used. Whether actively induced or a by-product of stress brought on by the host immune response, filamentation is a key survival strategy for a variety of bacterial pathogens, including *E. coli*, *Mycobacterium tuberculosis*, *Salmonella*, and *Legionella* (31, 32). While FtsN does not appear in all bacterial species, we presume that all species use some sensing mechanism to ensure that cell wall synthesis and degradation are coordinated. Coordinated regulation of cell wall degradation could be achieved by controlling either localization or activity (or both) of the amidases/hydrolyases. Therefore, although counterintuitive, it may be possible to develop antibiotics that induce bacterial cell division by prematurely activating the cell division amidases (33).

Materials and Methods

Bacterial Strains, Plasmids and Media. Bacterial strains, plasmids, and primers used in this work are listed in *SI Text* (Table S2 and Table S3). All experiments were performed in Miller's LB medium. β-lactam antibiotics were all freshly prepared in pure water before each experiment (stock solution loses activity over time).

Monitoring the Effects of Cephalexin on Different Strains by $OD_{600\ nm}$ in Tubes SulA-Inducible Strain (NWG593, Fig. 1*B*). Cells were grown with 0.2% glucose/LB media to suppress *sulA* expression from pBAD33 vector, at 37 °C until $OD_{600\ nm}$ ≈0.3 and then harvested and washed with LB media twice. Cells were diluted 1:10 into prewarmed LB media with 0.2% glucose to suppress or with 0.2% arabinose to induce *sulA* expression. In this and subsequent experiments, expression of the FtsZ-GFP with 10 μM IPTG had no effect of lysis as identical results were seen in the absence of IPTG. Cephalexin (50 μg/ml) was added at 20 min after *sulA*-induction and cell growth was monitored by OD at 600 nm wavelength ($OD_{600\ nm}$).

FtsN Depletion Strain (HSC070, Fig. 2*A*). Cells were grown at 37 °C with 0.2% arabinose/LB media to $OD_{600\ nm}$ ≈0.3 with 2 μM IPTG at 37 °C and washed with fresh LB twice. Next, cells were resuspended in LB media and diluted 1:150 into LB media with 0.2% arabinose to induce or 0.2% glucose to suppress *ftsN* expression with 2 μM IPTG to express FtsI-GFP or 10 μM IPTG to express FtsZ-GFP at 37 °C. Cephalexin (50 μg/ml) was added at 80 min after *ftsN*-depletion and cell growth was monitored by OD at 600 nm wavelength ($OD_{600\ nm}$).

Amidase Double Deletion Strains (Fig. 3*A* and Fig. S3 *A* and *B*). Cells were grown in LB media from a single colony until OD_{600} ≈0.4 and diluted 1:10 into 2 LB cultures. Cephalexin was added to one culture but not the other. Cell growth and lysis were monitored by $OD_{600\ nm}$ with JOE309 serving as a control for drug responses.

Measuring Dose–Response Curves of Different Strains Under 3 β-Lactams by $OD_{500\ nm}$ on 96-Well Plates. All strains (NWG593, HSC070, amidase double knockouts, and their wild-type control) were grown and diluted in specific conditions as described above; 100 μL diluted cell culture was transferred into each well of a 96-well plate (Corning Costar 3596, flat bottomed with lid, polystyrene wells). Different concentrations of β-lactams were prepared by serial dilutions (1:2) and added to the plate with cell cultures. This plate was incubated at 37 °C with aeration by shaking. Aluminum foil was used to wrap up the plates to prevent media evaporation during incubation. OD at 500 nm was recorded at every 20 min after addition of drugs. A fitting was done to convert $OD_{500\ nm}$ on 96-well plate into standard $OD_{600\ nm}$. Cell cultures of different cell density were measured both by standard $OD_{600\ nm}$ using SmartSpec 3000 (Bio-Rad) and $OD_{500\ nm}$ on 96-well plate using a Victor III (PerkinElmer) multilabel counter; OD data presented in all figures were results after the following calibration, thus in standard $OD_{600\ nm}$.

$$OD_{600} = -8.74\ OD_{500}{}^2 + 8.29\ OD_{500}\ (R^2 = 0.9968)$$

Dose–response curves ($OD_{max}/OD_{max,\ no\ drug}$ against [β-lactam] (μg/ml), see Figs. 1*C*, 2*B*, 3*B*, S3 *C* and *D*, and S4) are all generated based the OD curves acquired on plates.

Microscopy. For all experiments involving various *E. coli* mutants, cells were harvested and chemically fixed at 30 min (−SulA or +FtsN), or at 60 min (−FtsN), or at 80 min (+SulA) after the addition of cephalexin and prepared for microscopy following the procedure of Chen et.al (34). Fixative [100 μL of 16% paraformaldehyde, 0.4 μL of 25% glutaraldehyde, and 20 μL of 1 M sodium phosphate (pH 7.4)] was added to a 500 μL aliquot of cell culture. The mixture was incubated for 15 min at room temperature and for 15 min on ice. Cells were then washed 3 times with PBS [10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 15 mM KCl] and resuspended with 50 μL of PBS per $OD_{600\ nm}$ unit of 0.1 of the original sample. Fixed cells were allowed to adhere to a 15-well multitest slide (ICN Biomedicals) pretreated with polyl-lysine (Sigma) by incubation at room temperature for 10 min. Slides were then washed 5 times by adding PBS and aspirating to remove free-floating cells. This was followed by one wash with PBS containing 50% glycerol. Finally, 50 μL of PBS containing 50% glycerol was added before sealing with a cover glass; differential interference contrast (DIC) and fluorescent images were taken using an inverted confocal microscope (Zeiss LSM 510 Meta system) with a 100 × plan-Apochromat oil immersion objective and filter sets to visualize EGFP. All images were binned 2 × 2. Grayscale GFP images were assigned to the red channel and overlayed on grayscale DIC images to create the resulting RGB images.

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