

Beta-Lactam Antibiotics Induce a Lethal Malfunctioning of the Bacterial Cell Wall Synthesis Machinery

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SUMMARY

Penicillin and related beta-lactams comprise one of our oldest and most widely used antibiotic therapies. These drugs have long been known to target enzymes called penicillin-binding proteins (PBPs) that build the bacterial cell wall. Investigating the downstream consequences of target inhibition and how they contribute to the lethal action of these important drugs, we demonstrate that beta-lactams do more than just inhibit the PBPs as is commonly believed. Rather, they induce a toxic malfunctioning of their target biosynthetic machinery involving a futile cycle of cell wall synthesis and degradation, thereby depleting cellular resources and bolstering their killing activity. Characterization of this mode of action additionally revealed a quality control function for enzymes that cleave bonds in the cell wall matrix. The results thus provide insight into the mechanism of cell wall assembly and suggest how best to interfere with the process for future antibiotic development.

INTRODUCTION

Penicillin and related beta-lactam drugs are one of our oldest and most widely used antibiotic classes. They have long been known to interfere with bacterial cell wall assembly as part of their mode of action (Park and Strominger, 1957). The cell wall is an essential polysaccharide structure that surrounds most bacterial cells and protects their cytoplasmic membrane from osmotic rupture. It is built from the polymer peptidoglycan (PG), which consists of glycan chains with attached peptides used to crosslink adjacent glycans to form a matrix structure (Figure 1A).

Beta-lactams disrupt PG biogenesis by inactivating enzymes called penicillin-binding proteins (PBPs) (Tipper and Strominger, 1965). Bacteria encode a variety of PBPs that participate in PG assembly (Sauvage et al., 2008). The high-molecular-weight PBPs are the major PG synthases. They are subdivided into class A (aPBPs) and class B (bPBPs) enzymes (Figure 1B). aPBPs are bifunctional and possess both glycosyltransferase (GT) activity for polymerizing the glycan strands and transpepti-

dase (TP) activity for crosslinking them. bPBPs, on the other hand, are only known to possess TP activity. The primary target of beta-lactams is the TP active site of the synthetic PBPs, which is covalently modified by the drug. In addition to the PG synthases, beta-lactams also inhibit the low-molecular-weight PBPs. These factors belong to a large and diverse family of enzymes that cleave bonds in the PG matrix. Such enzymes, often referred to as PG hydrolases, are typically nonessential, but have been found to play important roles in morphogenesis (Uehara and Bernhardt, 2011).

The lethal activity of beta-lactams is thought to stem principally from the loss of wall integrity accompanied by cell lysis (Park and Strominger, 1957). According to the most widely accepted model, cell wall damage following beta-lactam treatment results from a drug-induced imbalance between the activities of cell wall synthases and hydrolases (Schwarz et al., 1969; Tomasz and Waks, 1975; Tomasz et al., 1970). This view is supported by the observation that PG hydrolase inactivation can prevent or delay beta-lactam-induced cell lysis (Chung et al., 2009; Heidrich et al., 2002; Tomasz, 1979; Tomasz and Waks, 1975; Tomasz et al., 1970; Uehara et al., 2009). However, surprisingly little mechanistic insight underlies this general framework for drug action. It remains largely unclear which PG hydrolases disrupt the wall following drug treatment and whether these autolysins are “induced” to damage the wall or are simply carrying out their normal physiological function in the absence of TP activity. Clues suggesting a more complex mode of action for beta-lactams than simple TP inhibition have also been reported. Surprisingly, in *Streptococcus pneumoniae* mutants blocked for cell lysis, beta-lactam treatment still promoted cell death with kinetics similar to lysing cells (Moreillon et al., 1990). Additionally, in *Escherichia coli*, beta-lactams were unexpectedly shown to cause a depletion of lipid-linked cell wall precursors prior to cell lysis (Kohlrausch and Höltje, 1991).

The lysis-independent effects of beta-lactams indicate that much remains to be learned about the events following TP inhibition and how they contribute to the killing activity of this vital class of antibiotics. These events have been difficult to elucidate because the effect of drug treatment on cell growth and morphology varies depending on the organism studied and on the number of different PBPs inhibited by the beta-lactam derivative used (Spratt, 1975). We overcame this challenge by studying the activity of mecillinam, a beta-lactam specific for a single essential PBP in the model gram-negative bacterium *Escherichia coli*

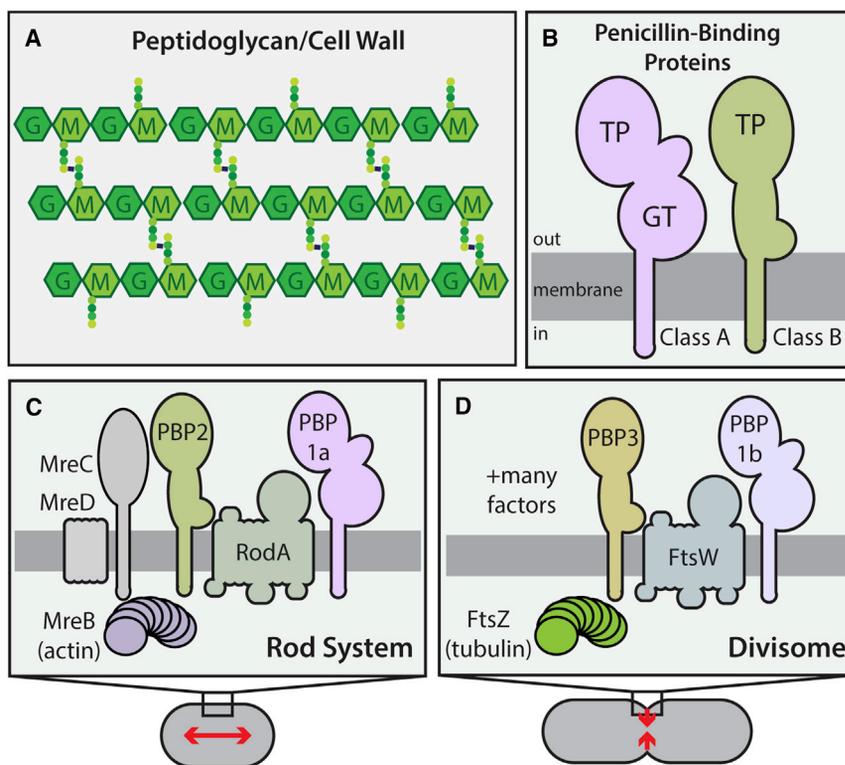


Figure 1. Peptidoglycan Structure and the Machines that Synthesize It

(A) The PG matrix consists of glycan chains with the repeating unit of N-acetylmuramic acid (MurNAc, M) and N-acetylglucosamine (GlcNAc, G). Attached to the MurNAc sugars are peptides (colored circles) used to form crosslinks between adjacent glycans.

(B) Domain structure of the PG synthases. Both classes of PBPs have a single transmembrane domain with a large catalytic domain in the periplasm.

(C and D) Schematics highlighting the components of the two main PG synthetic systems in rod-shaped bacteria. Both systems require a dedicated bPBP (PBP2 or PBP3) and a SEDS (shape/elongation/division/sporulation) family protein (RodA or FtsW) for proper function. See text for details.

(Spratt, 1975). Our analysis revealed that, beyond simply inhibiting the TP activity of PBPs, mecillinam and other beta-lactams stimulate a deleterious futile cycle of cell wall synthesis and degradation by their target machineries that contributes to their lethal activity. Additional genetic analysis identified the enzyme responsible for beta-lactam-stimulated degradation of nascent PG. Characterization of the *in vivo* activity of this factor suggests a novel “quality control” function for cell wall cleaving enzymes in PG biogenesis. Our findings thus provide new insight into the cell wall assembly process in addition to uncovering an important mechanism by which beta-lactam antibiotics induce cell death.

RESULTS

Rationale

Like many rod-shaped bacteria, *E. coli* grows using two different PG biogenesis systems (Typas et al., 2012) (Figures 1C and 1D). The actin-like MreB protein and its partners constitute the Rod system, which catalyzes the insertion of new PG material along the cell body to promote cell elongation (Typas et al., 2012) (Figure 1C). The tubulin-like FtsZ protein, on the other hand, organizes the divisome to synthesize PG for the new daughter cell poles (de Boer, 2010) (Figure 1D). Each of these machineries requires an essential bPBP for their activity: PBP2 for the Rod system and PBP3 for the divisome (Typas et al., 2012) (Figures 1B–D). Proper PG biogenesis by these systems in *E. coli* is also thought to require the aPBPs, PBP1a and PBP1b, to promote glycan chain polymerization and crosslinking (Typas et al., 2012) (Figures 1B–1D).

with the Rod[−] FtsZ^{UP} cells growing and dividing as small spheres (Bendezú and de Boer, 2008; Kruse et al., 2005; Vinella et al., 1992). However, the mechanism by which increased FtsZ concentration suppresses the lethality of Rod system inactivation is not clear. In addition to genetic inactivation, Rod system function can also be blocked with the small molecules A22 (Gitai et al., 2005) or mecillinam (Spratt, 1975) (Figure 2A). A22 inactivates MreB (Gitai et al., 2005), and mecillinam is a beta-lactam that is specific for the TP active site of PBP2 (Spratt, 1975). Because PBP2 can be rendered nonessential by the overproduction of FtsZ (Vinella et al., 1993) (Figure 2B), we reasoned that studies of mecillinam presented a unique opportunity to study the consequences of PBP inhibition by beta-lactams under conditions where the target is not required for growth.

Mecillinam Induces a Lethal Malfunctioning of the Rod System

The lethal effects of inactivating MreB with A22 are suppressed by the low-copy vector pTB63, from which the *ftsQAZ* operon is expressed via its native promoters (a condition we will henceforth designate as FtsZ^{UP}) (Bendezú and de Boer, 2008) (Figure 2C). Surprisingly, however, FtsZ^{UP} cells remained sensitive to mecillinam (Figure 2C). Thus, blocking PBP2 function with mecillinam is lethal even under conditions where the protein itself is not essential (Figure 2B). This observation suggested that, beyond inactivation of the TP activity of PBP2, mecillinam is also conferring a dominant-negative function to the inactivated protein. We reasoned that this toxic activity of inhibited PBP2 may stem from the malfunctioning of the Rod system. To test this hypothesis, we treated cells with both mecillinam and A22

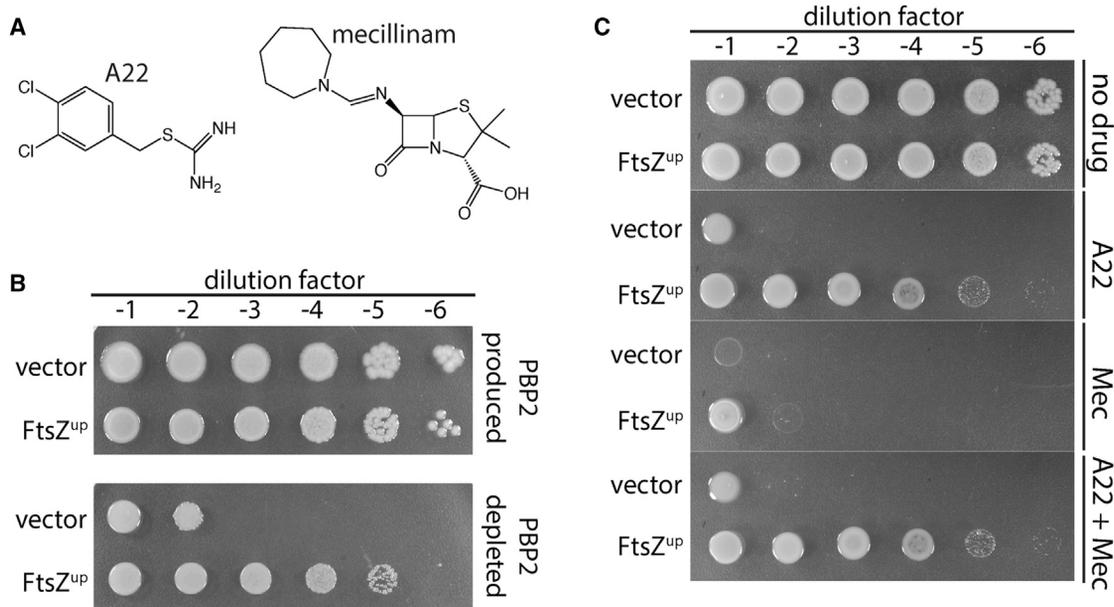


Figure 2. Mecillinam Induces a Lethal Malfunctioning of the Rod System

(A) Shown are the chemical structures of A22 and mecillinam.

(B) Conditional essentiality of PBP2. Cultures of strain HC439/pHC817 [Δ *pbpA* P_{lac}::*pbpA*] harboring either a vector control (pSC101, vector) or a derivative (pTB63, FtsZ^{up}) were serially diluted and spotted on LB agar with either 1 mM IPTG (PBP2 replete) or agar without inducer (PBP2 depletion). Plates were incubated overnight at 30°C and photographed. **C.** Cultures of strain MG1655 [WT] harboring either pSC101 or pTB63 were serially diluted and spotted on LB agar as in part B. Agar was supplemented with A22 (10 μ g/ml), mecillinam (1 μ g/ml), or both drugs as indicated.

to simultaneously inactivate the Rod system and the TP domain of PBP2. Indeed, inactivation of MreB with A22 in FtsZ^{up} cells completely suppressed the toxicity of mecillinam (Figure 2C). Genetic inactivation of the Rod system was also found to restore growth to FtsZ^{up} cells treated with mecillinam (Figure S1 available online). The contribution of Rod system malfunction to the lethal action of mecillinam was also apparent for cells lacking pTB63. Addition of mecillinam to a culture of wild-type cells led to a drop in culture OD₆₀₀ and a loss of viability that was reduced in severity by cotreatment with A22 (Figures S2A and S2B). The production of a PBP2 variant with a defective TP active site [PBP2(S330A)] also killed FtsZ^{up} cells (Figure S2C), and this toxicity could also be suppressed by A22 treatment (Figure S2C). Thus, the toxicity induced by mecillinam is specific for PBP2 inhibition, and not the result of an off-target activity. We conclude that blocking the TP activity of PBP2 causes the Rod system to malfunction in a manner that contributes to the lethal activity of mecillinam.

A Futile Cycle of PG Synthesis and Turnover Is Responsible for Mecillinam Toxicity

Results from Uehara and Park (2008) previously found that mecillinam treatment induced the degradation of newly synthesized PG while A22 blocks synthesis. We suspected that the observed turnover of nascent PG was key to the lethal mode of action of mecillinam. We therefore monitored PG synthesis and turnover using similar procedures to those of Uehara and Park (2008). Cells were metabolically labeled with [³H]-*meso*-diaminopimelic acid (mDAP), an amino acid unique to the peptide moiety of PG

(Figure 3A), following which, PG synthesis can be readily measured by monitoring the production of the major cytoplasmic precursor UDP-MurNAc-pentapeptide (UDP-MurNAc-pep₅) and the incorporation of label into the PG matrix. [³H]-mDAP labeling can also be used to quantify PG turnover. The major degradation products of PG are disaccharide fragments liberated by lytic transglycosylases (LTs) (Fisher and Mobashery, 2014) (Figure 3A). Rather than hydrolyzing the glycosidic bond, these enzymes promote the formation of an anhydro linkage between the C1 and C6 positions of MurNAc to generate GlcNAc-anhydro-MurNAc-peptides (GlcNAc-anhMurNAc-peptide). These degradation products are normally imported and processed by a number of enzymes in the cytoplasm for recycling (Fisher and Mobashery, 2014) (Figure 3A). We therefore used a strain lacking the amidase AmpD to trap PG turnover products and allow the quantification of PG degradation without complications of complete recycling (Uehara and Park, 2008) (Figure 3A).

To specifically monitor the effect of drug treatment on PG synthesis and degradation by the Rod system, divisome activity was blocked during the radiolabeling by expression of the FtsZ antagonist SulA prior to the addition of antibiotic(s). After 5 min of growth in the presence of drugs, [³H]-mDAP was added to the medium and growth was continued for 10 min (1/10th of a mass doubling). Cells were then harvested and extracted with hot (90°C) water. Soluble compounds (PG precursors and degradation products) were separated and quantified by HPLC and radiodetection. Material incorporated into the PG matrix was quantified as radioactive material released from the cell pellet by lysozyme treatment.

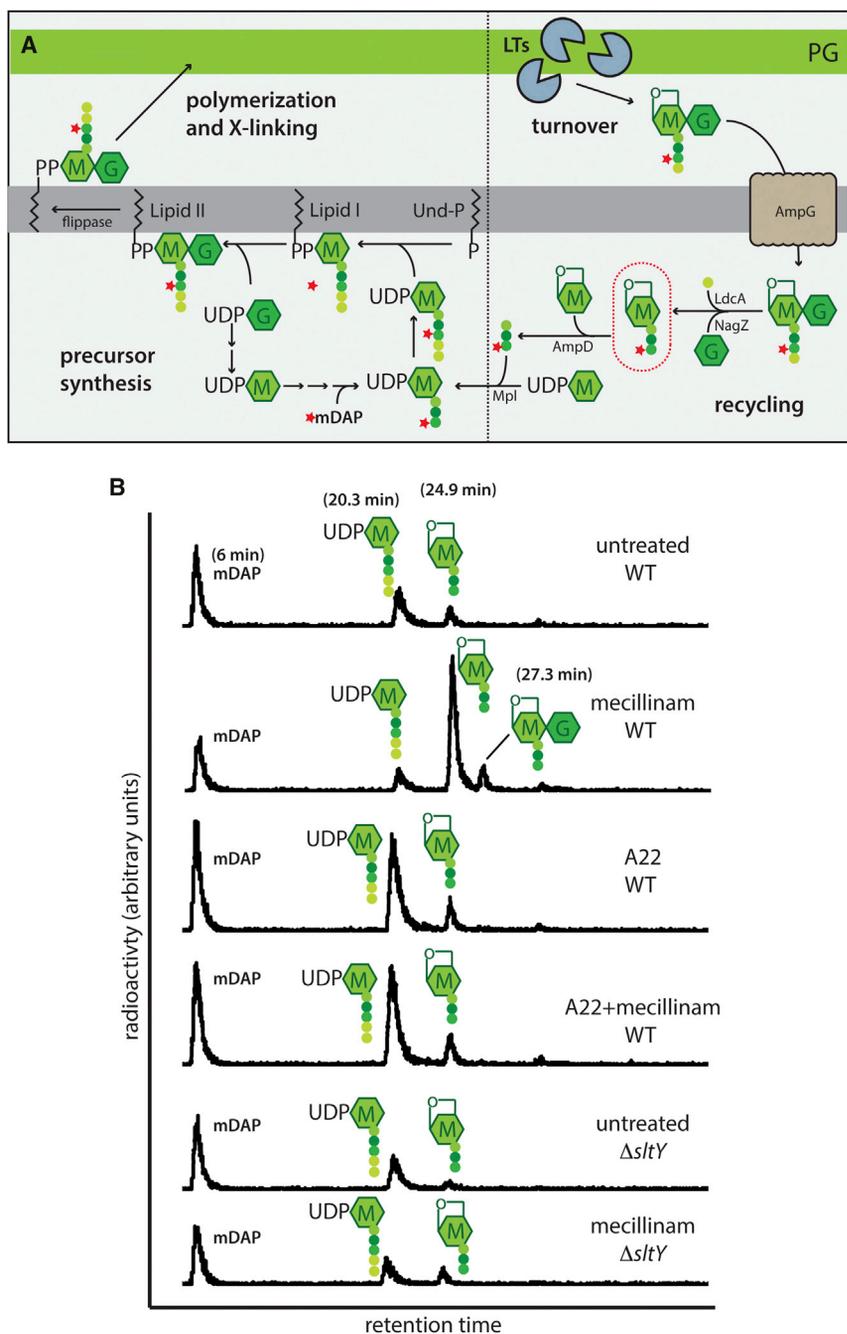


Figure 3. Measurement of PG Synthesis and Turnover following Mecillinam Treatment

(A) Schematic summarizes the PG synthesis and recycling pathways. Sugars and peptides are represented as in Figure 1A. The composition of the pentapeptide of the cytoplasmic precursors is L-Ala-gamma-D-Glu-meso-diaminopimelic acid (mDAP)-D-Ala-D-Ala. UDP-sugars are first made in the cytoplasm before being transferred to the lipid carrier undecaprenol-phosphate (Und-P). The final precursor lipid II is the substrate used by the PBP to form PG. As a result of the crosslinking mechanism and the activity of carboxypeptidase enzymes that remove the terminal D-Ala, the PG layer consists primarily of glycans with tetrapeptides. The turnover product circled in red will accumulate in a Δ ampD strain if nascent PG is degraded during a pulse labeling experiment. See text for details.

(B) Measurement of PG synthesis and turnover by the Rod system. Cells of TU278 [Δ lysA Δ ampD] or its Δ slt derivative (HC419) as indicated producing SulA to block cell division were pulse labeled with [3 H]-mDAP following treatment with the indicated drug(s) (10 μ g/ml each when added). Soluble metabolites were separated by HPLC and detected using an in-line radiodetector. The resulting chromatograms are shown. Peaks are labeled with schematics of the corresponding compounds. The identity of each peak is based on the results shown in Figure S3. See text for details.

In untreated cells, robust incorporation of label into the PG matrix was observed (Figure 4). Very little of the newly synthesized material was degraded as evidenced by the small amount of labeled anh MurNAc-peptides detected relative to the precursor pools (Figures 3B, 4, and S3). As reported by Uehara and Park (2008), we found the effects of A22 treatment differed dramatically from those induced by mecillinam. In cells treated with A22, incorporation of radiolabel into mature PG was significantly reduced as expected. This defect was accompanied by increased pools of the PG precursors mDAP and labeled UDP-MurNAc-pep₅ (Figures 3B, 4, and S3), suggestive of a late stage

block in the synthesis pathway. Only a modest increase in anh MurNAc-peptide accumulation was observed following A22 treatment, indicating that the drop in label incorporation into the PG matrix resulted primarily from the inhibition of PG synthesis. Similarly, in cells treated with mecillinam, incorporation of label into PG was also greatly reduced (Figures 3B and 4). However, as expected from the results of Uehara and Park (2008), a large pool of labeled turnover products was detected in mecillinam-treated samples (Figures 3B and 4). This observation confirms the conclusion that, rather than blocking PG synthesis by the Rod system like A22, mecillinam induced the degradation of nascent PG material produced by

the machine. Given that A22 suppressed the toxicity of mecillinam, we wondered if it would also block the apparent futile cycle of nascent PG synthesis and turnover induced by this beta-lactam. This indeed proved to be the case (Figures 3B and 4), thus linking the induction of nascent PG turnover with the lethal mode of action of mecillinam. PBP inhibition by beta-lactams was previously found to decrease cellular levels of the final PG precursor, lipid II (Kohlrusch and Hötje, 1991). Based on in vitro PBP assays, it was recently suggested that the decline in lipid II concentration may result from the stimulation of glycan strand synthesis

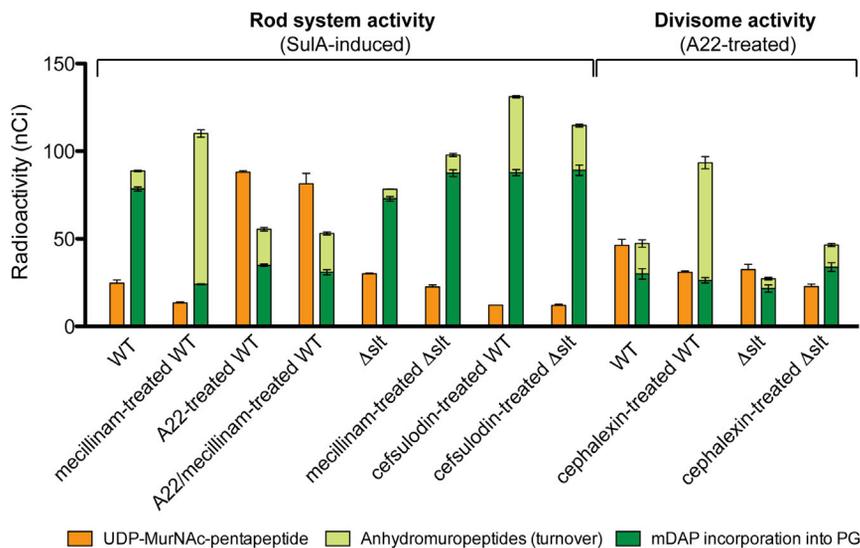


Figure 4. Quantification of PG Synthesis and Turnover following Beta-Lactam Treatment

Cells of TU278 [$\Delta lysA \Delta ampD$] or its Δsit derivative (HC419) were grown, radiolabeled, and analyzed as in Figure 3. The amount of UDP-MurNAc-pentapeptide and total anhydromuropeptides produced were quantified from the area under the peaks in HPLC chromatograms. Radiolabel incorporation into PG was determined by quantifying the amount of label released from cells by lysozyme. Antibiotic concentrations used were: mecillinam (10 $\mu\text{g/ml}$), A22 (10 $\mu\text{g/ml}$), cephalexin (10 $\mu\text{g/ml}$), and cefsulodin (100 $\mu\text{g/ml}$). Results are the average of three independent experiments with the error bars representing the standard deviation. The drop in precursor levels between untreated and mecillinam-treated WT cells is significant ($p < 0.005$). See text for details.

when TP activity is blocked (Banzhaf et al., 2012). A dual mode of action for beta-lactams was thus proposed where TP inhibition and substrate depletion act synergistically to promote efficient bacterial killing (Banzhaf et al., 2012). Our results indicate that the futile-cycle of PG synthesis and turnover induced by beta-lactams is likely to be the main driver of the PG precursor depletion observed here and by others (Kohlrusch and Höltje, 1991; Uehara and Park, 2008) (Figure 3B and 4). To investigate the contribution of precursor depletion to the lethal activity of mecillinam, we generated strains that overexpress *uppS* or *murA*, which encode enzymes required for the synthesis of the lipid-carrier Und-P or UDP-MurNAc, respectively (Figure 3A). Importantly, both strains gained partial resistance to mecillinam (Figure S2D). Additionally, studies by D'Ari and colleagues (Vinella et al., 1993) showed that high-level overproduction of FtsZ (a 7 \times increase from pZAQ [Begg et al., 1998], as opposed to a 2 \times increase from pTB63 [Bernhardt and de Boer, 2005]), a condition where the divisome may more effectively compete with the Rod system for PG precursors, can partially suppress the lethal action of mecillinam. We conclude that mecillinam not only blocks the TP active site of PBP2, but by doing so, induces a futile cycle of cell wall synthesis and turnover by the Rod system that contributes to its bactericidal activity, at least in part, by depleting PG precursor pools.

Futile Cycle Induction Is a Common Property of Beta-Lactams

We investigated whether inhibiting PBPs other than PBP2 also stimulates the degradation of nascent PG. Cephalexin is a beta-lactam that preferentially targets PBP3, the cell division-specific bPBP (Spratt, 1975). To monitor the effect of cephalexin, we used a modified version of the labeling method described above. Instead of blocking division with SulA, cell cultures were treated with A22 to block Rod system activity prior to [^3H]-mDAP addition. This procedure allowed us to focus the analysis on divisome-mediated PG synthesis. In the absence of cephalexin, robust incorporation of label into PG by the divisome was detected, but with a higher basal level of PG turnover

than when the activity of the Rod system was monitored (Figures 4 and S4A). This observation is consistent with previous results (Uehara and Park, 2008), and most likely reflects the activity of the cell separation PG hydrolases. Importantly, just as with mecillinam, cephalexin treatment led to a dramatic increase in the degradation of nascent PG (Figures 4 and S4A).

The effect of blocking the TP activity of aPBPs was analyzed using cefsulodin, a beta-lactam that is specific for PBP1a and PBP1b (Curtis et al., 1979). Using labeling conditions identical to those for the mecillinam studies, we also observed a significant increase in nascent PG turnover upon cefsulodin treatment (Figures 4 and S4B). However, this increase was not accompanied by a corresponding drop in label incorporation into the PG matrix, presumably because PBP2 and other components of the Rod system remain functional when the TP activity of aPBPs is blocked. We conclude that the induction of nascent PG degradation is a common property of beta-lactams regardless of the growth process or class of PBP they target.

Slt Is Responsible for Nascent PG Turnover following Beta-Lactam Treatment

We reasoned that a genetic selection for mutants resistant to mecillinam might reveal the cell-wall-degrading enzyme responsible for the nascent PG turnover induced by beta-lactams. Notably, selections for mecillinam-resistance were performed many years ago by several laboratories (Spratt, 1975; Vinella et al., 1992; Wachi et al., 1987). This genetic analysis was instrumental in the initial identification of the genes encoding the Rod system (*mreBCD*, *pbpA*, and *rodA*). In retrospect, however, these selections were more complicated than originally appreciated because they were carried out in genetic backgrounds with wild-type FtsZ levels. Thus, based on our current understanding, these early mecillinam-resistance selections were actually demanding mutants with two changes: (1) those that increase FtsZ levels to render the Rod system nonessential, and (2) those that generate defects in the Rod system or other factors in order to alleviate the toxicity of the futile cycle of PG synthesis and degradation. Consistent with this view, the classic mecillinam-resistant mutants *mre-129*

and *mre-678* with lesions in the *mreBCD* operon (Wachi et al., 1987) were shown to produce elevated levels of FtsZ (Bendezú and de Boer, 2008). Therefore, to simplify the genetic analysis, we revisited the selections for mecillinam resistance in an FtsZ^{up} genetic background.

MG1655/pTB63 cells were mutagenized with a transposon and plated on LB agar supplemented with mecillinam. Similar to prior studies, two classes of resistant mutants were isolated: those that remained spherical in the absence of mecillinam and those that regained rod shape when the drug was removed. As expected, all of the permanently spherical mutants possessed transposon insertions that mapped to genes encoding components of the Rod system (data not shown). Mutants in the second class, however, retained rod-shaped without mecillinam and therefore must have a functional Rod system. We suspected that a subset of these mutants were likely to harbor insertions in genes important for the degradation of nascent PG following mecillinam treatment. One such mutant had an insertion in the *slt* gene encoding a periplasmic cell-wall-degrading enzyme called Soluble Lytic Transglycosylase (Slit).

Because LTs cleave glycan strands to generate the ^{anh}MurNac-peptide products that accumulate following beta-lactam treatment, we focused on the *slt* mutant. To test whether Slit was responsible for beta-lactam-induced turnover of nascent PG, we performed the radiolabeling assay in a Δ *slt* strain. Without treatment, the loss of Slit function did not affect net PG synthesis by either the Rod system or the divisome, but the basal rate of turnover in each case was reduced (Figure 3B and 4). Significantly, following mecillinam or cephalexin treatment, the Δ *slt* strain displayed a dramatically reduced level of nascent PG turnover relative to the WT strain. Moreover, inactivation of Slit restored wild-type levels of PG incorporation into the matrix of mecillinam-treated cells (Figure 4). Slit also appeared to be important for PG turnover in cefsulodin-treated cells, but unlike the situation with drugs that inactivate bPBPs, a significant proportion of the turnover following aPBP inhibition was found to be Slit independent (Figure 4). Notably, the turnover products produced upon cefsulodin treatment had a much greater ^{anh}MurNac-pentapeptide content (25%) than those observed following mecillinam treatment (4%) (Figures 3B and S4B). This observation suggests that uncrosslinked, nascent PG with a relatively high proportion of pentapeptides is formed by the cefsulodin-treated cells and that such material is subject to destruction by LTs other than Slit. Nevertheless, we conclude that Slit is likely to be the major enzyme responsible for nascent PG turnover following beta-lactam treatment.

Differential Effect of Slit Inactivation on Mecillinam Sensitivity Depending on FtsZ Levels

Curiously, in contrast to our results, Slit inactivation was previously found to result in mecillinam hypersensitivity rather than resistance (Templin et al., 1992). However, in a separate study, loss of Slit function was shown to increase mecillinam resistance in a *Salmonella typhimurium* mutant already partially resistant to the drug (Costa and Antón, 2006). Because the original observation of mecillinam hypersensitivity for a Δ *slt* mutant was made with otherwise wild-type cells, and our selection for resistance was performed with FtsZ^{up} cells, we suspected that the apparent

contradiction was related to cellular FtsZ concentration. Indeed, we confirmed that for cells lacking Slit and producing normal levels of FtsZ, the minimal inhibitory concentration (MIC) of mecillinam is almost ten times lower (0.04 μ g/ml) than that of wild-type cells (MIC = 0.3 μ g/ml). Conversely, as expected from our genetic analysis, FtsZ^{up} cells defective for Slit showed much higher resistance to mecillinam (MIC > 10 μ g/ml) than those with functional Slit (MIC = 0.3 μ g/ml). The change in mecillinam sensitivity of Δ *slt* cells relative to wild-type as a function of FtsZ levels was readily visualized on solid medium (Figure 5A). Importantly, Δ *slt* cells are not generally more sensitive to agents that target the Rod system; wild-type cells and a strain inactivated for Slit displayed the same A22 sensitivity (Figure S5A).

How can Slit inactivation result in both hypersensitivity and resistance to mecillinam? To investigate this conundrum, we compared the effect of mecillinam on the growth and morphology of wild-type and Δ *slt* cells with and without increased FtsZ production. Microscopic analysis following treatment with mecillinam for 3 hr revealed that cells lacking Slit undergo a morphological change at a much lower mecillinam concentration than wild-type cells. Moreover, the concentration of drug that leads to this shape defect was strongly correlated with the concentration that induces a growth defect in Δ *slt* cells (Figures 5 and S6). As with the growth phenotype, the morphology of Δ *slt* cells was only hypersensitive to mecillinam; wild-type and Δ *slt* cells were found to undergo shape changes at equivalent A22 concentrations (Figure S5). It has been well documented that the loss of rod shape results in cell division defects and poor growth unless FtsZ is overproduced (Bendezú and de Boer, 2008). We therefore conclude that the mecillinam hypersensitivity of Δ *slt* cells with normal FtsZ levels results from the morphological changes and consequent division inhibition induced by low doses of drug. On the contrary, when Δ *slt* cells have elevated FtsZ levels, they overcome the adverse effects of the shape alterations just as mutants with defects in the Rod system are suppressed by FtsZ overproduction.

Aberrant PG Crosslinking in Δ *slt* Cells Treated with Mecillinam

The shape change observed for Δ *slt* cells at low doses of mecillinam suggests that when uncrosslinked PG produced by beta-lactam-targeted synthetic complexes is not destroyed by Slit, it can be misincorporated into the PG matrix to alter morphology. To investigate this possibility, we analyzed the composition of purified cell walls (sacculi) isolated from wild-type or Δ *slt* cells following mecillinam treatment. In wild-type cells, the total amount of PG isolated following mecillinam treatment was reduced as expected, but the composition of the isolated material, including the relative degree of crosslinking, did not change dramatically (Figure 6A and Table S1). The muropeptide profile of sacculi from untreated Δ *slt* cells was similar to that observed for wild-type PG. However, following mecillinam treatment, major alterations were observed (Figure 6B and Table S1). Strikingly, muropeptides with PBP-generated crosslinks, those linked via the fourth residue of one peptide and the third residue of the other (a 4-3 crosslink), were almost completely depleted from mecillinam-treated Δ *slt* sacculi. This loss was accompanied by a corresponding increase in

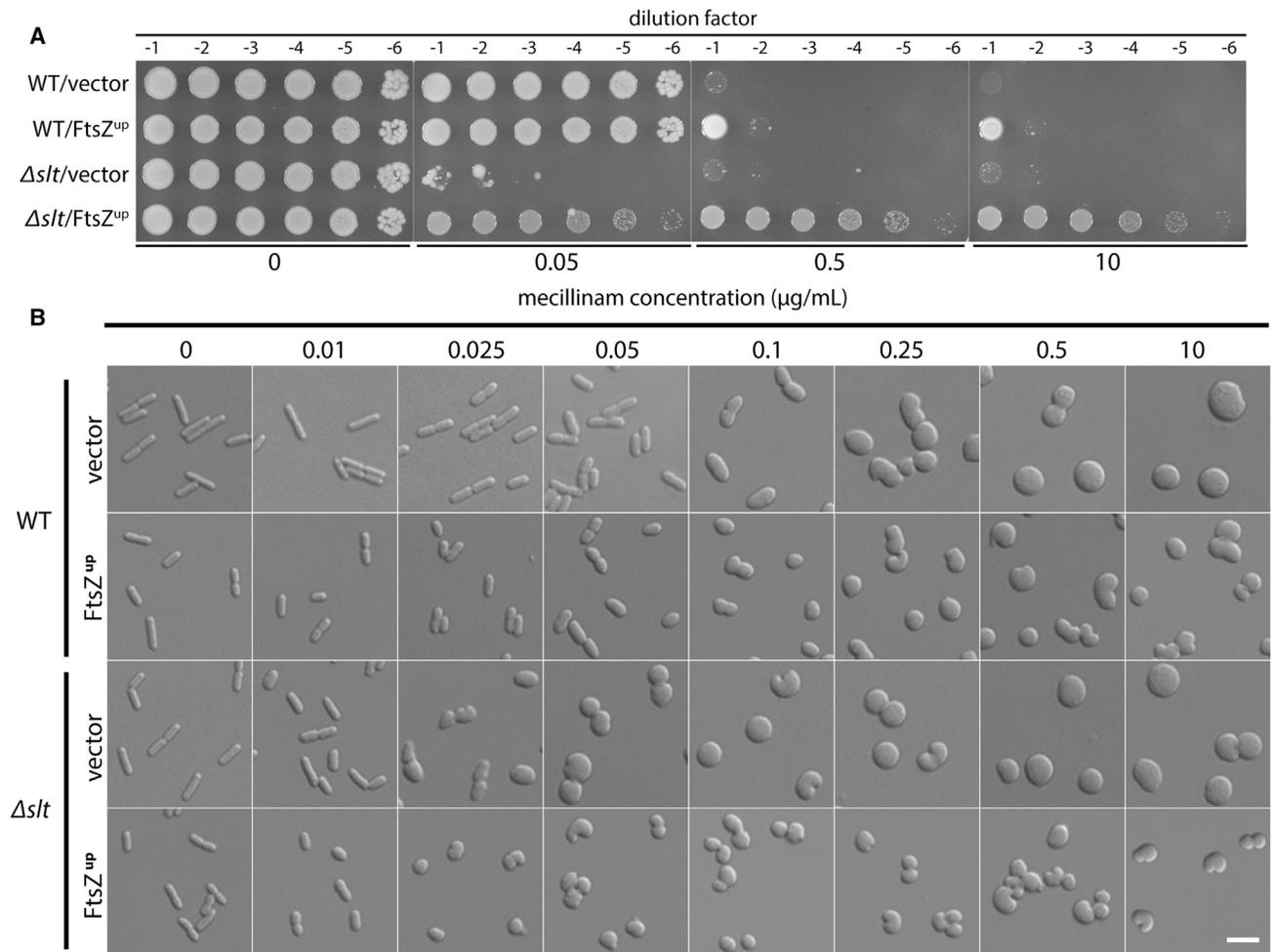


Figure 5. Effect of Mecillinam on the Growth and Morphology of Cells Defective for Slt

(A) Cells of MG1655 [WT] or HC408 [Δ slt] harboring either pSC101 (vector) or pTB63 (FtsZ^{up}) were grown overnight, diluted, and spotted on agar containing the indicated concentration of mecillinam as described for Figure 2B.

(B) The same cells were grown to exponential phase and diluted to an OD₆₀₀ of 0.025 into LB medium containing the indicated concentration of mecillinam. Growth was continued for an additional 3 hr and the cells were fixed. The fixed cells were then imaged on agarose pads using DIC optics. Scale bar, 4 microns.

muropeptides with an alternative 3-3 (mDAP-mDAP) crosslink formed by L,D-transpeptidases. Thus, the failure to degrade uncrosslinked PG produced by beta-lactam-targeted synthetic machines indeed results in the aberrant incorporation of PG into the matrix. We conclude that the observed misincorporation is most likely responsible for the shape defects induced in Δ slt cells treated with low levels of mecillinam. Furthermore, the accompanying drug hypersensitivity displayed by these mutants suggests that, in cells with wild-type levels of FtsZ, the shape changes induced by PG misincorporation are worse for viability than the consequences of the futile cycle, at least at low drug concentrations. Accordingly, the growth of Δ slt cells with the vector control is more adversely affected by low doses of drug than the corresponding WT cells (Figure S6). At higher drug concentrations, however, functional Slt becomes detrimental, presumably due to the increased burden of the futile cycle as a higher proportion of Rod complexes become targeted (Figure S6).

DISCUSSION

A Common Theme for the Mode of Action of Bactericidal Drugs

Bactericidal drugs promote cell death while bacteriostatic agents merely stop bacterial growth. Antibiotics belonging to both general categories interfere with essential cellular pathways or enzymes. However, studies of the mode of action of clinically important drugs in the aminoglycoside and fluoroquinolone classes provide examples of how bactericidal agents typically do more than just inhibit their target enzymes. Aminoglycosides disable the proofreading capacity of the ribosome, causing the production of mistranslated proteins that are ultimately thought to cause irreversible membrane damage and death (Davis, 1987). Similarly, fluoroquinolones bind to DNA gyrase and topoisomerase IV, leading to the formation of stable drug-enzyme-DNA complexes that block DNA replication and promote the formation of double-strand breaks (Hooper, 2001). Thus, rather

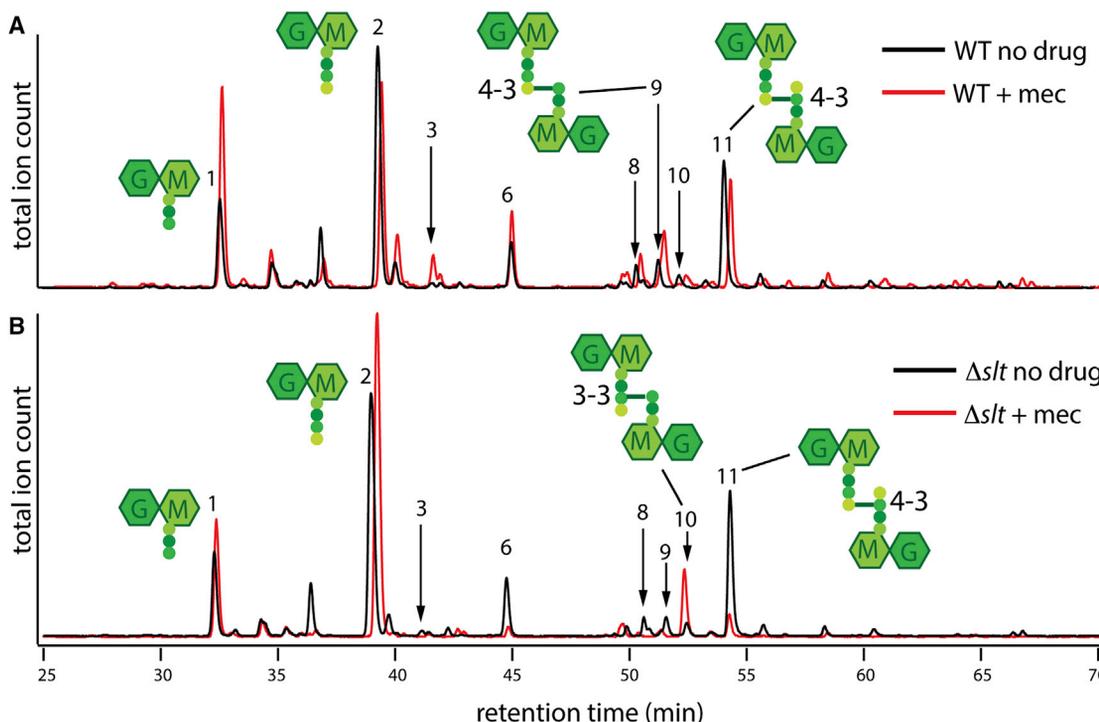


Figure 6. Muropeptide Composition of PG following Mecillinam Treatment

Cells of MG1655 [WT] (A) or HC408 [Δ slt] (B) were grown to an OD_{600} of approximately 0.5 and diluted 1:20 into fresh LB medium with or without mecillinam (10 μ g/ml) as indicated. Growth was continued for an additional 3 hr and PG sacculi were prepared from cells of each culture. The purified PG was then digested with the muramidase mutanolysin and the resulting muropeptides were reduced and analyzed by LC/MS. Total ion count chromatograms are shown with the chromatograms of the mecillinam-treated samples off-set for clarity. Note that the scales of each chromatogram are not identical. They were scaled to show the relative muropeptide composition rather than the total amount of material. For example, the total peak area in the chromatogram from mecillinam-treated WT cells is one third that of the corresponding untreated sample even though an equivalent of twice the volume was injected. Schematics of several major muropeptide products are shown near their corresponding peaks with the numbers referring to the type of crosslink (4-3 v.s. 3-3). The identities for all labeled peaks and the quantification of their relative amounts are listed in Table S1. The results were reproducible over two biological replicates of each sample and three technical replicates for each biological replicate.

than simply inhibiting an essential activity, these drugs disrupt key functions of their target such that the activity of the crippled enzyme or multicomponent machine becomes toxic and reduces viability. Whether beta-lactams elicit a similar toxic malfunctioning of their target has remained unclear.

The widely accepted view of the mode of action of beta-lactams is that by inhibiting the TP activity of the PBPs, these drugs simply disrupt the balance between PG synthases and the action of PG hydrolases working to expand the cell wall matrix (Tomasz, 1979). Here, we show that beta-lactams derange the process in a more direct and insidious manner than this general framework suggests. Our results indicate that TP inhibition induces the turnover of nascent PG material produced by the targeted complex. In the case of PBP2 targeting by mecillinam, we clearly show that this turnover contributes significantly to the lethal action of the drug by inducing the depletion of cellular PG precursor pools such that even nontargeted PG synthetic complexes are likely to be adversely affected by beta-lactam treatment. Our findings thus connect the killing mechanisms of three important and widely used antibiotics (aminoglycosides, fluoroquinolones, and beta-lactams); they all stimulate a dominant-negative activity in their target pathway to induce systemic toxicity. Further-

more, all three drug classes typically function against multiple targets: aminoglycosides target ribosomes made from rRNA encoded by several genetic loci per genome (Davis, 1987), fluoroquinolones target gyrase and topoisomerase IV (Hooper, 2001), and beta-lactams typically target several different PBPs in a given organism (Curtis et al., 1979). It has been discussed previously that interfering with the activity of multiple cellular targets is an important property of monotherapies needed to reduce the incidence of mutational resistance (Silver, 2011). However, the potential benefit of such therapies inducing a dominant-negative activity in the affected pathway in addition to hitting multiple targets has been under appreciated. The combination of these properties likely enhances antibiotic effectiveness because, even if one of the targets is mutationally altered to block drug binding, the sensitivity and lethal malfunctioning of the other targets will be dominant over the lone resistant allele. Thus, the principles learned from this and other studies of the mode of action of our classic antibacterial therapies indicate that, rather than searching for simple inhibitors of essential enzymes as has been common practice, antibiotic discovery efforts should ideally be seeking new molecules that induce a lethal malfunctioning of multiple cellular targets.

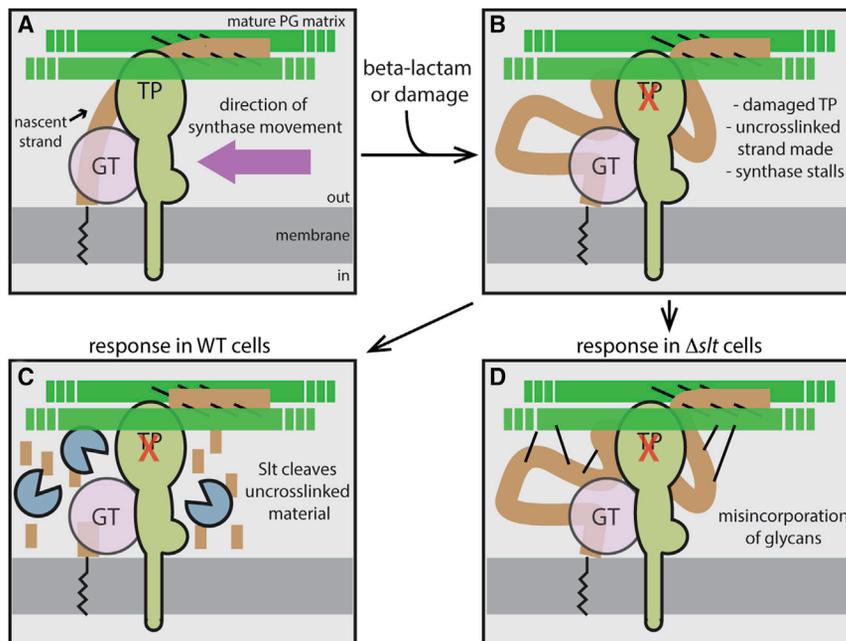


Figure 7. Role of Slt in Maintaining Coordination between GT and TP Activity during PG Biogenesis

Shown is a schematic highlighting the role of Slt in PG biogenesis. (A) The normal synthetic process with properly coupled GT and TP activities. The new glycan strands (brown) are polymerized from lipid-linked precursors (black zig-zag line) by an enzyme with GT activity (pink) that may either be the canonical domain of an aPBP or an as yet unidentified GT enzyme. The newly polymerized glycans are rapidly crosslinked into the mature matrix (green) by an associated TP activity shown here as a bPBP, but it could also be the TP domain of an aPBP. Other components of the putative synthetic complex including cytoskeletal elements were omitted for simplicity. When the TP active site is damaged or targeted by a beta-lactam (B), GT activity continues to produce glycan chains that are no longer crosslinked into the matrix. In WT cells (C), such strands are targeted for destruction by Slt. Conversely, in cells inactivated for Slt (D), the uncrosslinked glycans produced by the damaged/targeted machinery are not degraded, but are instead aberrantly incorporated into the matrix by an alternative crosslinking enzyme. In cells with normal FtsZ levels, the resulting morphological changes are lethal, leading to beta-lactam hypersensitivity of Slt-defective cells.

A Potential Role for Slt as a Quality Control Enzyme in PG Biogenesis

In addition to revealing novel features of the beta-lactam killing mechanism, our findings also shed significant new light on the process of PG biogenesis. Pulse-labeling studies of PG assembly indicate that nascent PG material is rapidly crosslinked into the mature matrix (Burman and Park, 1983; Glauner and Höltje, 1990), indicating a tight coupling between GT and TP activities. Such an orderly insertion mechanism is likely critical for the maintenance of proper cell shape and integrity. Here, we show that the inhibition of TP activity by beta-lactams functions to uncouple glycan polymerization from crosslinking, leading to the generation of uncrosslinked glycan strands (Figure 7). Our results indicate that this nascent material is rapidly degraded in an Slt-dependent manner, setting up a futile cycle of synthesis and degradation (Figure 7). Mutants defective for other PG cleaving enzymes were not isolated in selections for mecillinam resistance. Additionally, nascent PG turnover was largely eliminated in the absence of Slt. We therefore conclude that Slt is likely to be directly responsible for destroying the uncrosslinked material produced by the beta-lactam-targeted synthetic machinery.

How Slt is activated to degrade uncrosslinked PG is not known. Importantly, affinity chromatography studies identified a potential interaction of Slt with PBPs 1b, 2, and 3 in *E. coli* (von Rechenberg et al., 1996; Romeis and Höltje, 1994), suggesting that the turnover we observe may be stimulated by association with the beta-lactam-targeted synthases. Additionally, Slt is known to have a doughnut-like structure with the active site oriented toward a central hole wide enough to accommodate only a single uncrosslinked glycan chain (Thunnissen et al., 1994). Thus, the enzyme may have a strong substrate preference for uncrosslinked glycan chains, a possibility that is sup-

ported by the observation that pretreatment of purified cell wall sacculi with an endopeptidase stimulates the release of soluble material by Slt (Romeis and Höltje, 1994). This apparent substrate preference may contribute to its role in the futile cycle in addition to, or independent of, an interaction with the PBPs. Notably, Slt functions primarily as an exoglycosidase (Beachey et al., 1981) with some detectable endoglycosidase activity (Lee et al., 2013). Although it has been argued that Slt degrades glycan chains from an anhydro-MurNAc end (van Asselt et al., 1999), the structural data are also consistent with Slt processing glycan strands from the nonreducing end as has been demonstrated biochemically. We therefore envision that Slt may either recognize an existing, free nonreducing end of an uncrosslinked glycan, or, in the case of partially crosslinked material, it could create such an end with its weak endoglycosidase activity, followed by the degradation of the remaining uncrosslinked glycan chain.

The physiological role of Slt has remained mysterious for some time. It has been implicated in the turnover of mature cell wall material and cell separation (Heidrich et al., 2002; Kraft et al., 1999). However, these functions have been ascribed to Slt based on the phenotypes of mutants lacking multiple PG processing enzymes, suggesting that they represent secondary activities. Clues to the likely primary physiological function of Slt were provided by our analysis of the response of Δ slt cells to mecillinam treatment. In the absence of Slt, PG produced by mecillinam-targeted synthetic complexes was found to be aberrantly incorporated into the PG matrix via the formation of alternative (3-3) crosslinks by non-PBP transpeptidases. Additionally, the morphology of Δ slt cells was dramatically altered following exposure to low doses of mecillinam, while the same treatment had little effect on the shape of wild-type cells. Because these drug

concentrations are well below the IC_{50} for PBP2 (Curtis et al., 1979), only a fraction of the cellular PBP2 pool is predicted to be inhibited in cells treated with this level of mecillinam. Thus, the morphological changes displayed by $\Delta s/t$ cells exposed to low concentrations of mecillinam are unlikely to be due to general inhibition of the Rod system. Instead, we conclude that misincorporation of uncrosslinked PG produced by mecillinam-targeted machines is likely to be responsible for the shape change of the mutant, most likely because the aberrant material creates a local defect in the matrix that interferes with the proper assembly or remodeling of PG by other (untargeted) machines. By extension, we infer that a major function of Slt is to prevent the stable accumulation of uncrosslinked glycan strands so that they are not aberrantly crosslinked into the mature matrix. Such an activity is consistent with a novel “quality control” or “repair” function that helps maintain cell shape and integrity.

In our experimental system, low drug concentration most likely serves to mimic environmental damage to the synthetic machinery or other natural conditions resulting in the uncoupling of GT and TP activities. For example, uncrosslinked glycan strands might accumulate if synthetic complexes enter an area of the PG matrix with few peptides available to accept crosslinks. In these cases, we envision that glycan chain degradation by Slt might facilitate the exchange of a damaged TP for a functional one, and/or allow the release of stalled complexes tethered to a partially crosslinked glycan strand so that they can restart synthesis at other locations. In support of the latter possibility, recent studies indicate that bPBPs are likely to be dynamically associated with the synthetic machinery (Lee et al., 2014).

An additional phenotype consistent with a quality control function for Slt is the rapid lysis displayed by $\Delta s/t$ cells treated with cephalixin (Templin et al., 1992). PBP3 inactivation normally results in a division block and the formation of filamentous cells that lyse many mass doublings after cephalixin addition (Spratt, 1975). However, cells defective for Slt lyse rapidly following cephalixin treatment (Templin et al., 1992). Rapid lysis under these conditions is known to require the activity of the cell separation amidases (Heidrich et al., 2001). By analogy with mecillinam-treated cells, addition of cephalixin to $\Delta s/t$ cells likely results in the production of stable uncrosslinked glycan strands that can be misincorporated by alternative crosslinking enzymes. Lysis may result because this misincorporated material mimics productive PG synthesis by the divisome such that the cell separation enzymes are activated to process the malformed material resulting in a localized breach in the PG. Thus, in addition to shape maintenance, the proposed quality control function of Slt may also enforce the coupling of GT and TP activities to prevent the misactivation of PG hydrolases by damaged PG synthases. Taking this line of reasoning a step further, it is interesting to speculate that it may be the failure of Slt to process certain types of glycan strands (e.g., those with high pentapeptide content) that underlies the ability of some beta-lactam drugs to induce rapid cell lysis via the misactivation of autolytic PG hydrolases.

Slt, Beta-Lactamase Induction, and Potential Combination Therapies

Many gram-negative bacteria encode a beta-lactamase (AmpC) produced in response to beta-lactam treatment (Fisher and Mo-

bashery, 2014). In some organisms, expression of the *ampC* gene is controlled by the transcriptional regulator AmpR (Jacobs et al., 1997). Mutants defective for the AmpD amidase involved in cell wall recycling were found to overexpress *ampC* even in the absence of beta-lactams (Jacobs et al., 1994). This finding along with supporting in vitro transcription experiments has led to a model in which beta-lactam treatment leads to the accumulation of ^{anh}MurNAC-peptide turnover products in the cytoplasm that serve as a signal for *ampC* induction (Fisher and Mobashery, 2014; Jacobs et al., 1997). It has been widely assumed that these recycling intermediates increase in concentration because beta-lactams induce general cell wall damage. Here, we have shown that targeting PG synthetic machines with beta-lactams causes nascent PG to be degraded by Slt, generating high levels of ^{anh}MurNAC-peptides. Thus, rather than generic insults to the PG matrix, the *ampR-ampC* regulatory system is perfectly tuned to detect the futile cycle of PG synthesis and degradation induced upon TP inhibition. Importantly, based on the critical role observed for Slt in *ampC* induction (Kraft et al., 1999) and the hypersensitivity of Slt-defective mutants to beta-lactams (Templin et al., 1992), it has been proposed that Slt inhibitors may be useful in combination therapies with beta-lactams (Kraft et al., 1999). Our results provide a mechanistic picture of what such combinations would likely accomplish. The downside of blocking Slt activity simultaneously with beta-lactam treatment would be loss of the toxic effects of the futile cycle and subsequent precursor depletion. However, what would be gained is an alternative malfunction of PG biogenesis caused by the accumulation of uncrosslinked glycan strands that can cause lysis or lethal shape changes as described above. Thus, inhibiting Slt function should indeed be an effective way to stymie beta-lactamase induction systems in gram-negative pathogens while still allowing beta-lactams to derange the PG biogenesis machinery to promote cell death.

CONCLUSION

Beta-lactams are arguably one of the most important therapeutic classes in the history of medicine. In this report, we present data that changes the view of how these drugs work. They are not simple inhibitors of cell wall synthesis as is commonly believed. Instead, they derange the activity of the PG biogenesis machinery to induce a futile cycle of PG synthesis and degradation that depletes cellular resources. While investigating the nature of the futile cycle, we uncovered what appears to be a new role for PG-cleaving enzymes as quality control factors that intervene when problems arise during PG synthesis. Overall, these new insights provide a mechanistic foundation that will not only help us better understand the process of cell wall biogenesis, but also how best to interfere with it for the development of novel antibacterial treatments.

EXPERIMENTAL PROCEDURES

Media, Bacterial Strains, Plasmids, and Culture Conditions

Cells were grown in LB or minimal M9 medium supplemented with 0.2% casamino acids and carbon source (0.4% glycerol or 0.2% maltose) as indicated. The bacterial strains and plasmids used in this study are listed in Tables S2 and S3, respectively.

Measurement of the Turnover of Newly Synthesized PG

The effect of beta-lactams and A22 on the turnover of nascent PG was monitored by using $\Delta lysA \Delta ampD$ strains essentially as described previously (Uehara and Park, 2008). Cells of the relevant strains were grown overnight in M9-glycerol medium with 0.2% casamino acids. The resulting cultures were then diluted to an OD₆₀₀ of 0.04 in the same medium and grown to an OD₆₀₀ between 0.3–0.4 at 30°C. For experiments measuring PG synthesis and turnover by the Rod complex, *suIA* expression was induced from a chromosomally integrated plasmid (pHC739) for 30 min before drug treatment. The cultures were then treated with the indicated beta-lactams and/or A22 for 5 min after adjusting the culture OD₆₀₀ to 0.3. Following drug treatment, [³H]-mDAP (1 μCi) was added to 1 ml of each culture for 10 min (1/10 of the doubling time) to label the newly synthesized PG and its turnover products. After the labeling, cells were pelleted, resuspended in 0.7 ml water, and heated at 90°C for 30 min to extract water-soluble compounds. After the hot water extraction, insoluble material was pelleted by ultracentrifugation. The resulting supernatant was then removed, lyophilized, and resuspended in 0.1% formic acid for HPLC analysis. To determine [³H]-mDAP incorporated into the PG matrix, the pellet fraction was washed and treated with lysozyme. The suspensions were incubated overnight at 37°C. Insoluble material was then pelleted by centrifugation, and the resulting supernatant was subjected to scintillation counting. Similar procedures were used for measuring PG synthesis and turnover by the divisome following cephalixin treatment except that Rod system activity was suppressed by treating the cultures with A22. See [Supplemental Information](#) for a detailed protocol.

Muropeptide Analysis of PG Sacculi Isolated from Drug-Treated Cells

Cultures of MG1655 and its Δslt derivative, HC408, were diluted in 1 l of LB or LB containing 10 μg/ml mecillinam to OD₆₀₀ of 0.025 and incubated at 30°C for 3 hr with shaking. The OD₆₀₀ of each culture reached approximately 0.6 at the end of the growth period. PG was then purified from each culture and analyzed by LC/MS as described in [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.11.017>.

AUTHOR CONTRIBUTIONS

H.C. designed, performed, and interpreted all experiments. T.U. designed, performed and interpreted critical foundational studies for this work. T.G.B. designed and interpreted the experiments. The paper was written and edited by T.G.B., H.C., and T.U.

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