Mechanisms of Bacterial Envelope Stress From Within and Without

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th>Citable link</th>
<th><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:37944997">http://nrs.harvard.edu/urn-3:HUL.InstRepos:37944997</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
The undersigned, appointed by the Department of Chemistry & Chemical Biology have examined a dissertation entitled:

Mechanisms of bacterial envelope stress from within and without

presented by: Ghee Chuan Lai

candidate for the degree of Doctor of Philosophy and hereby certify that it is worthy of acceptance.

Signature

Typed name: Professor Thomas G. Bernhardt

Signature

Typed name: Professor Johan M. Paulsson

Signature

Typed name: Professor David Z. Rudner

Signature

Typed name: Professor Daniel E. Kahne

Date: 14 December 2016
Mechanisms of Bacterial Envelope Stress from Within and Without

A dissertation presented

by

Ghee Chuan Lai

to

The Department of Chemistry and Chemical Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Chemical Physics

Harvard University

Cambridge, Massachusetts

December 2016
Mechanisms of Bacterial Envelope Stress from Within and Without

Abstract

The bacterial cell envelope is the frontline protective barrier between the cell and the environment. It is a complex multilayered structure essential for maintaining cellular integrity and plays a crucial defensive role against diverse environmental challenges. Bacteria can encounter stress in the envelope either spontaneously or from external insults such as drug treatments. The mechanism by which drug-induced stress leads to cell death is still poorly understood. How often and to what degree bacteria encounter stresses from within is also not described. Here, I investigate both processes and the results reveal new functions for cell wall biogenesis enzymes whose roles have not yet been clearly defined.

Specifically, I attempt to define the downstream events following β-lactam inhibition of PG synthases (PBPs) by understanding how cell wall biogenesis overcomes β-lactam stress. β-lactams not only inhibit PBPs essential for growth, but also induce a lethal malfunctioning of their target cell wall biosynthetic machinery. I used a genetic system to uncouple these two processes by isolating mecillinam suppressors that overcome the toxic Rod machinery under conditions where the target PBP2 is not essential. Subsequent functional profiling uncovered a stress response-independent suppressor encoding an inactivating mutation of a protease, that was implicated to degrade a cell wall hydrolase Spr. Spr cleaves crosslinks between strands in the cell wall matrix to
allow for cell wall expansion during growth, but how that is achieved is unclear. Further investigation of Spr and similar enzymes suggests these hydrolases can stimulate PBP activity to promote cell wall synthesis during normal growth. Our results provide evidence for a 'cut-and-insert' strategy mediated by hydrolases and PBPs during cell wall expansion.

Cell wall expansion during growth requires coordinated concurrent expansion of its surrounding envelope layers. It is not clear how often envelope biogenesis run into problems during normal growth and what these problems might be. Because envelope stress responses are intrinsically tuned to cell envelope physiology, studying them can unveil mechanisms that regulate cell envelope homeostasis. I harnessed envelope stress responses to detect stresses from within by tracking their spontaneous activation using transcriptional fluorescent reporters in unperturbed cells maintained in a constant environment. Rare spikes in activity of the Rcs stress response were observed and correlated with the appearance of membrane blebs in the affected cells. Further characterization demonstrates that these spikes are bona fide indicators of envelope biogenesis errors, and this monitoring system is robust in identifying factors involved in quality control or repair, which have been difficult to detect otherwise.

Many gaps remain in our understanding of the bacterial cell envelope, owing to its complexity in both structure and function. While I have used β-lactams and envelope stress responses as chemical and biological probes to interrogate poorly understood
aspects of bacterial cell wall regulation in *E. coli*, these approaches can be extended to address other similar questions in cell envelope biogenesis.
“It’s a magical world… Let’s go exploring!”

- Calvin and Hobbes
# Table of Contents

Title Page i
Copyright Page ii
Abstract iii
Table of Contents vii
Acknowledgements x

## Chapter 1:
**Introduction to cell envelope biogenesis and envelope stress responses in Gram-negative bacteria**

1.1: Bacterial Cell Envelope 2

1.2: Bacterial Cell Wall 5
   1.2.1: Peptidoglycan synthesis 6
   1.2.2: Peptidoglycan synthases 8
   1.2.3: Multiprotein complexes during peptidoglycan synthesis 13
   1.2.4: Peptidoglycan hydrolases 15
   1.2.5: Peptidoglycan turnover and recycling 18
   1.2.6: β-lactam mechanism of action 20
   1.2.7: Outstanding questions on β-lactam mechanism of action to be addressed 22

1.3: Envelope Stress Responses 24
   1.3.1: Extracytoplasmic function σ factor 26
   1.3.2: Two component systems and envelope stress responses 27
   1.3.3: Challenges in envelope stress responses 30
   1.3.4: Outstanding questions on envelope stress responses to be addressed 31

1.4: References 33

## Chapter 2:
**Investigating the killing mechanism of mecillinam reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis** 47
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7</td>
<td>What is the physiological function of the Rcs stress response during spontaneous envelope defects?</td>
<td>136</td>
</tr>
<tr>
<td>4.8</td>
<td>Applying our imaging screen to other PG biogenesis questions</td>
<td>138</td>
</tr>
<tr>
<td>4.9</td>
<td>Conclusion</td>
<td>139</td>
</tr>
<tr>
<td>4.10</td>
<td>References</td>
<td>142</td>
</tr>
</tbody>
</table>
Acknowledgements

I am grateful to many people for helping me grow as a person and scientist and for helping me grow E coli.

To Tom, for your constant support and encouragement. You have shaped my development as a scientist in ways great and small, some direct and some by example. I am incredibly grateful for your blind investment in me, a student with no wet-lab experience and little knowledge of molecular biology and genetics when I first entered. You have an uncanny ability to ask simple questions that get to the heart of an interesting scientific problem; that has only inspired and motivated me to work harder. You put up with my wacky ideas at times, and my occasional bouts of neuroses. You also taught me never to pipette through your mouth.

To Johan, for giving me the freedom to explore. Your enthusiasm for science is infectious. You are always armed with a bag of crazy anecdotes. Thank you for the encouragement and support. You are incredibly sharp and creative, and I have learnt a great deal from you.

To my committee members, past and present, David Rudner, Daniel Kahne and Adam Cohen, for traveling on this road with me, and providing me scientific guidance and encouragement.
To former and current members of the Bernhardt, Paulsson and Rudner labs, you have been a source of encouragement during my time in graduate school. The labs have evolved considerably over the years. Thank you everyone. In particular, thank you Andrew for always having an open ear. You are one of the most grounded and warm person I know. Desiree, you taught me everything I currently know about E coli husbandry and all the good food there is in Boston when I first arrived. Thank you Rachel and Mary-Jane for joining the lab with me and entering this journey with me. Rachel and Thao, you both have been wonderful bay mates. I only wish I can raise to the same level as you have. Chris R., thank you for the coffee and pastry breaks. Thank you Assya for keeping my science sharp.

Thank you to the night-owl members, basically most of the mother machine crew in the Paulsson lab. I'm not a late night person, but I will gladly have coffee with you. Thank you Burak for being patient and training me the basics of microscopy and microfluidics and the late Christmas holiday nights trying out crazy experiments. Thank you Somenath for always willing to help troubleshoot my failures with me. Thank you Tsusyohi, Nick, Desiree, Catherine for welcoming me to the Bernhardt lab, and Dann, Dirk and Burak on the other end.

To the younger graduate students. Thank you for inspiring me with your passion for science.
To my friends outside of lab. Thank you Lindsay and Sixun for your sass and endless support. Thank you Sanyu for our impromptu meals. You help keep my neuroses in check. Thank you Richard for being a terrific housemate and keeping me sane and grounded. To all my friends in Boston, and beyond, thanks for celebrating with me, and all the good times during graduate school.

To my parents and brothers, thank you for feeding and loving me and for occasionally feigning interest in E coli. Your support and perspective was often all that stood between me and madness, and I am grateful to have been raised by you.
CHAPTER 1:
Introduction to cell envelope biogenesis and envelope stress responses in Gram-negative bacteria
CHAPTER 1:
Introduction to cell envelope biogenesis and envelope stress responses in Gram-negative bacteria

1.1: BACTERIAL CELL ENVELOPE

The bacterial cell envelope is a complex multilayered structure consisting of membrane(s) and peptidoglycan that surround and protect the cytoplasm against external insults [1]. Nearly all bacteria can be classified into two large groups based on their cell envelope structure. Gram-negative bacteria have an envelope made up of outer and inner membranes and a thin layer of peptidoglycan sandwiched between them. Gram-positive bacteria compensate for their lack of an outer membrane with a much thicker peptidoglycan layer. The eponymous Gram staining facilitates this distinction by specifically detecting exposed peptidoglycan in Gram-positive bacteria [2]. Corynebacterineae possess another distinct envelope class. In addition to an inner membrane and a peptidoglycan layer, they contain a second polysaccharide layer known as the arabinogalactan which is covalently attached to peptidoglycan. The arabinogalactan is in turn covalently attached to long hydrophobic mycolic acids which are thought to constitute the equivalent of the Gram-negative outer membrane [3].

The outer membrane is the first outermost layer of the cell envelope, and is the distinguishing feature of Gram-negative bacteria. It is an asymmetric lipid bilayer containing glycolipids, primarily lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet [4,5]. It primarily serves as a permeability barrier
against antimicrobial peptides while allowing entry of nutrients. Proceeding inwards, the peptidoglycan is a tough cell wall exoskeleton that confers bacterial cell shape and protects the cells against osmotic lysis. The outer membrane is stapled to the underlying peptidoglycan by the Braun’s lipoprotein or Lpp, with the amino terminus embedded in the outer membrane [6]. Finally, the inner membrane is a symmetric phospholipid bilayer that surrounds the cytoplasmic space. It is the site of many critical processes: energy production, lipid biosynthesis, protein secretion and transport of nutrients and waste. (Fig 1)

**Fig1. The Gram-negative bacterial cell envelope.**

Diagram of a rod-shaped bacterium with distinct envelope layers. Gram-negative cells have a relatively thin PG layer compared to their Gram-positive cousins. The red box on the left contains a close-up representation of the Gram-negative cell envelope consisting of the IM, inner membrane; OM, outer membrane; and PG, peptidoglycan cell wall layer within the periplasmic space between the two membranes. The green box in the right is a schematic detailing the structure of PG, which spans in all directions to envelope the cell (green arrows). Alternating units of N-acetyl-glucosamine (G) and N-acetyl-muramic acid (M) make up the glycan strands, while the dots represent the attached peptides.
All the components of the Gram-negative cell envelope are synthesized either in the cytoplasm or at the inner leaflet of the inner membrane. These components must be translocated across or flipped to the outer leaflet of the inner membrane where they reside, or be transported to their respective final destinations. Most of the essential envelope biogenesis systems have now been identified in *Escherichia coli*, including: the Sec system that translocates proteins across or inserts them into the inner membrane, the Bam system for outer membrane protein assembly, the Lol system for lipoprotein transport to the outer membrane, the Lpt system for LPS transport to and assembly in the outer membrane, and finally the diverse repertoire of cell wall enzymes that construct and remodel the peptidoglycan layer [7]. All these intricate construction apparently takes place outside the cytoplasm that lacks an obvious energy source. It is still unclear how these different processes are regulated individually and coordinated with one another to maintain a uniform contiguous multi-layered envelope during bacterial growth and division.
1.2: **BACTERIAL CELL WALL**

The major conserved component for almost all bacterial cell envelopes is the peptidoglycan (PG), also referred to as murein. PG is a heteropolymer consisting of long glycan chains interconnected by short stem peptides. Specifically, it contains strands of alternating N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) sugars linked by β-1,4-glycosidic bonds. These glycan strands are further cross linked by short stem peptides extending from MurNAc sugars (Fig 1). In *E. coli*, the primary sequence of the attached peptide of newly synthesized strands is (L-alanine)—(D-glutamate)-(γ)-(meso-diaminopimelic acid or m-DAP)—(D-alanine)—(D-alanine), with the terminal D-Ala being subsequently removed. Most peptide cross links connect D-Ala at position 4 of one stem peptide to the free amino group of m-DAP at position 3 on an adjacent stem peptide. Together, this meshwork forms a continuous net-like shell that acts as a force-bearing structure against the high intracellular osmotic pressure, maintaining cell integrity and establishing cell shape [8].

The PG cell wall is believed to be the major determinant of cell shape because of the following observations. Sacculi isolated from SDS-treated bacterial cells still retain the overall shape and dimensions of the original cell from which it was isolated [8]. Additionally, treating cells with lysozyme, an enzyme that cleaves the glycosidic bond between the GlcNAc and MurNAc units, causes a rapid loss of rod shape and eventual lysis. These lysozyme treated cells can avoid lysis and retain a stable spherical shape if they are maintained on an isotonic medium [9].
1.2.1: Peptidoglycan synthesis

Since PG surrounds bacteria in one continuous net, bacteria must dynamically expand and remodel the cell wall during growth and division. Synthesis of the cell wall can be thought of as a multi-step pathway involving synthesis of PG precursor molecules, transport of these precursors across the inner membrane to the periplasmic space, and finally incorporation into the pre-existing cell wall matrix (Fig 2).

Fig 2. Schematic of PG biosynthesis.
(1) UDP-GlcNAc is converted to UDP-MurNAc through a series of reaction steps. (2) UDP-MurNAc is attached to undecaprenyl phosphate, giving rise to lipid I. (3) Addition of GlcNAc to lipid I yields lipid II. (4) Lipid II is flipped across the membrane, and (5) undergoes polymerization mediated by glycosyltransferases (GT), and cross linking into the existing PG meshwork by transpeptidases (TP).
**PG precursor synthesis and transport**

The basic building blocks of peptidoglycan are first synthesized in the cytoplasm to yield the final substrate used in polymerization reactions, a lipid-linked disaccharide pentapeptide known as lipid II. The relevant enzymes involved in each step is listed in brackets. To achieve this, nucleotide activated N-acetylmuramic acid (UDP-MurNAc) is generated from UDP N-acetylglucosamine (UDP-GlcNAc) [by MurA and MurB] [10-12]. This is the first committed step in this pathway. MurNAc contains a lactyl group unlike GlcNAc, and this is the point of attachment of the five amino acids by specific and dedicated ligases. The order of addition is L-Ala [by MurC] [13,14], γ-D-Glu [by MurD] [15,16], (L)-meso-diaminopimelic acid (DAP) [by MurE] [17,18], and finally D-Ala-D-Ala [by MurF] [19]. Both D-Glu and D-Ala-D-Ala amino acids are unique to PG [20-22]. The resulting UDP-MurNAc-pentapeptide product is attached to the membrane anchoring lipid undecaprenyl phosphate [by MraY], giving rise to lipid I [23]. Undecaprenol is made via the non-mevalonate pathway of isoprenoid synthesis [24,25]. Finally, attachment of GlcNAc to lipid I [by MurG] yields lipid II, the basic PG substrate [26,27]. Lipid II is subsequently flipped from the inner face of the inner membrane to the outer face into the periplasm [by MurJ], prior to PG polymerization [28,29]. (Fig 2)

**Incorporation of PG substrate**

The last step of PG synthesis is the incorporation of PG substrates into the pre-existing matrix. This requires two sets of chemical reactions: the formation of a glycosidic and a peptide/amide bond [30]. These are mediated by enzymes with glycosyltransferase (GT) and transpeptidase (TP) activities. GTs use flipped lipid II to synthesize long glycan
polymers, while TPs crosslink these polymers to the existing meshwork via their stem peptides. Amidst these processes, existing bonds within the meshwork are hydrolyzed, so that new PG material can be incorporated through crosslinks without compromising cell integrity. Therefore, growth and expansion of the cell wall structure requires coordination of two opposing enzymatic activities: PG synthesis via glycan polymerization and cross linking, and PG hydrolysis of the pre-existing mesh [8]. (Fig 2)

1.2.2: Peptidoglycan synthases

The major classes of PG synthetic enzymes is comprised of penicillin-binding proteins (PBPs) and shape elongation division and sporulation (SEDS) proteins. (Fig 3)

Fig 3. aPBP and SEDS proteins are major essential PG synthetic systems. SEDS proteins work within cytoskeletal machineries during PG synthesis. RodA, a SEDS protein with GT activity works together with PBP2 (with TP activity) and MreB (actin homologue) to form one of the major PG synthetic machineries during cell elongation. The other is assumed by class A PBPs (aPBP) with bifunctional GT and TP activities. There is some degree of coordination between both systems, although the mechanistic details are unknown.
**High molecular weight PBPs are PG synthases**

PBPs are named for their ability to bind penicillin and other $\beta$-lactams [31]. PBPs can be classified into high molecular weight (HMW) and low molecular weight (LMW) enzymes. HMW PBPs are involved in PG synthesis, and these enzymes can further be subdivided into class A and B PBPs. Class A PBPs are bifunctional enzymes, with both GT and TP activities. In *E. coli*, there are three class A PBPs: PBP1a, PBP1b and PBP1c [32-35]. Class A PBPs are generally indispensable for growth in many organisms. Either PBP1a or PBP1b must be present in *E. coli* to survive, but cells are dead if both PBPs are removed simultaneously [36-37]. This phenomenon is known as synthetic lethality. The physiological importance of PBP1c is still unclear; it contains both GT and TP amino acid motifs, of which only the GT activity has been demonstrated [35]. MtgA is the only known non-PBP that is capable of polymerizing glycan strands in *E. coli* [38]. It is a monofunctional GT that is believed to be involved during septal PG synthesis [39].

Class B PBPs, on the other hand, are monofunctional enzymes containing TP activity. In *E. coli*, the class B synthases are PBP2 [40] and PBP3 [41]. Both PBP2 and PBP3 are essential for proper growth and division. Inactivation of PBP2 generates spherical cells that eventually lyse [42]. This shape phenotype suggests that PBP2 is specifically required for cell wall elongation. In contrast, depletion of PBP3 forms long filamentous cells indicative of cell division defects [42]. This suggests that PBP3 is important for septal PG synthesis. Together, these results imply that *E. coli* is governed by two independent modes of cell wall growth: lateral PG synthesis during elongation and
septal PG synthesis during division. Both activities have to be coordinated in order to maintain proper rod shape and cell length in *E. coli* and ensure its survival.

**SEDS proteins and class B PBPs work together**

Working together with class B PBPs are SEDS proteins. Both class B PBPs and SEDS proteins are thought to work together as a subcomplex [43] for the following observations. Both PBP2 and RodA are required for cell elongation [44], while cell division requires PBP3 and FtsW [45,46]. RodA and FtsW are members of the SEDS family of proteins [47]. Additionally, both *rodA* and *pbpA* (PBP2), *ftsW* and *ftsI* (PBP3) are encoded in the same operons in *E. coli* [48,49]. The genetic linkage between SEDS proteins and PG synthases has recently been demonstrated in a diverse set of bacterial taxa [50].

**SEDS proteins are glycosyltransferases**

Although SEDS proteins have been implicated in PG biogenesis for decades, their precise physiological function in this process was ill-defined until very recently. RodA and FtsW were previously thought to be lipid II transporting flippases, although that was also recently disproved [28,29]. Recent genetic and biochemical evidence demonstrates that RodA is the primary GT for PG synthesis in *Bacillus subtilis* during cell elongation [50]. *B. subtilis* cells lacking all known class A PBPs are still viable and proficient in cell elongation [51]. By extension, FtsW is proposed to be providing PG polymerase activity during cell division [50]. This is a paradigm shift in our understanding of bacterial PG
synthesis and challenged the well-accepted textbook knowledge that polymerization of lipid II precursors is mediated extensively by class A PBPs.

**Class A PBPs and SEDS proteins are distinct polymerase systems**

This is further supported by a recent complementary study on class A PBPs in *E. coli* using *in vivo* biochemical and microscopy approaches [52]. Class A PBPs do not account for all GT activity during cell elongation, and are observed to function outside the Rod system, a multiprotein complex involved in cell elongation. In contrast, RodA and PBP2 display similar circumferential motion to MreB, all of which are members of the Rod complex. Inactivation of either polymerase systems greatly reduces, but does not abolish PG synthesis. Given both class A PBP activity and SEDS/class B PBP system are essential, efficient and maximal PG synthesis can only be achieved in the presence of both systems [52]. It is still unclear why these polymerase systems are partially-dependent on each other for efficient PG synthesis, and how they are coordinated to achieve that. (Fig 3)

**Transpeptidases come in two flavors**

HMW PBPs possess TP activity, and they are responsible for the majority of crosslinks between glycan strands in *E. coli* [8]. These 4-3 crosslinks are mediated by D,D peptide bonds between D-Ala and DAP of adjacent glycan polymers [8]. During transpeptidation, PBPs form a PBP-substrate intermediate with the precursor pentapeptide before releasing the terminal D-Ala on the pentapeptide. This intermediate stage is where penicillin, a structural analog of D-Ala-D-Ala, interferes with PG
synthesis [53,31]. Penicillin functions as a suicide substrate and forms a strong irreversible covalent bond to the incipient PBP, hence the name penicillin-binding protein [53].

In addition to D,D transpeptidation, *E. coli* is also capable of generating L,D crosslinks between two DAP moieties. L,D (or 3-3) crosslinks only represent about 3% of the total crosslinks in cells during exponential growth, but increases to 10% during stationary phase [8]. L,D transpeptidation is also required for attaching Lpp to the peptidoglycan layer, as mentioned earlier. Lpp is one of most highly expressed proteins in *E. coli*. Despite its abundance and covalent linkage to peptidoglycan, Lpp is dispensable to proper rod shape in *E. coli* [54], and its precise physiological role *in vivo* has yet been determined.

In *E. coli*, there are five L,D transpeptidases: ErfK, YbiS, YcfS, YnhG, YcbB [55,56]. These L,D transpeptidases are penicillin-insensitive because they recognize different substrates [57]. A quadruple mutant lacking four of these enzymes can still perform L,D transpeptidation, but is unable to attach Lpp to PG [55]. Three of the five enzymes (ErfK, YbiS and YcfS) can restore Lpp attachment [55]. L,D transpeptidases were previously found to bypass PBPs during β-lactam stress in *Enterococcus faecium*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, and recent work indicates that YcbB can also bypass β-lactam inhibited PBPs in *E.coli* when overexpressed [58].
1.2.3: Multiprotein complexes during peptidoglycan synthesis

Multiprotein complexes are a group of 2 or more proteins linked together by non-covalent protein-protein interactions and often play key roles in many cellular processes. Rod-shaped bacteria typically use two essential PG multiprotein complexes to grow and divide. Cell elongation is mediated by the Rod system, consisting of RodA (SEDS), PBP2 (class B PBP), RodZ, MreB (an actin homologue), MreC and MreD (proteins of undefined function) [59]. The Rod system is thought to direct PG synthesis and maintain its characteristic rod shape [60-65]. Cell division is promoted by the division machinery, consisting of but not limited to FtsW (SEDS), PBP3 (class B PBP) and FtsZ (a tubulin homologue) [59,66]. (Fig 4)

Fig 4. Essential multiprotein complexes during cell growth and division.
The Rod system is essential for cell elongation. It is a cytoskeletal machinery comprising of RodA (SEDS), PBP2 (bPBP), MreBCD proteins. An equivalent SEDS-bPBP pair, FtsW and PBP3 respectively, and FtsZ forms the divisome necessary for cell division.

One defining property of multiprotein complexes is that inactivation of individual protein constituents often exhibit similar phenotypes, since many complexes require all
individual components in order to assemble and function properly. This is also true for the Rod system. Inactivation of any components of the Rod system causes bacterial cells to 1. grow and divide as small spheres under slow growth rates, 2. form spheres that fail to divide and eventually lyse under fast growth rates, and finally, 3. mutants of the Rod system can be rescued by a modest increase in the production of FtsZ, which allows them to grow and divide as small spheres at any growth rate [67]. This makes it incredibly difficult to discern the unique function of each component in vivo. In vitro reconstitution methods to demonstrate a protein’s function within a complex is extremely challenging, and must still be corroborated with in vivo data to demonstrate biological relevance. Regardless, these findings suggest that over expression of FtsZ suppresses the essentiality of the Rod system. (This will be relevant in Chapter 2)

*E. coli* also employ nonessential multiprotein complexes during PG synthesis. An example would be major class A PBPs and their cognate Lpo cofactors [68,69]. As mentioned previously, PBP1a and 1b are synthetic lethal. This synthetic lethal phenotype extends to their cognate Lpo activators and all possible cross-combinations (LpoA-PBP1b and LpoB-PBP1a) [68,69]. Further biochemical, structural and genetic analyses support the multiprotein complex model. In the case of LpoB and PBP1b, LpoB is shown to directly stimulate the GT activity of PBP1b by interacting with the non-catalytic UB2H domain, which indirectly stimulates its TP activity [70,71]. This is further reinforced by the recent discovery of suppressor mutations in *ponB* that bypass LpoB-mediated activation [72].
1.2.4: Peptidoglycan hydrolases

Growth and division of the bacterial cell requires the concurrent growth and division of the cell wall structure. Bacteria must expand, remodel and degrade PG while maintaining the integrity of this essential force-bearing structure. Cell wall remodeling is enabled by a diverse set of PG hydrolases that hydrolyze different bonds within the PG meshwork. To date, close to 40 PG hydrolases have been identified, and they fall into one of the 12 classes, each representing a unique cleavage site specificity [73].

PG hydrolases have even more flavors

PG hydrolases can be broadly classified into peptidases/amidases and glycosidases. Peptidases and amidases cleave the PG glycan side chains or crosslinks, while glycosidases cleave the glycan polymers. Within the peptidase family, endopeptidases and carboxypeptidases hydrolyze the various peptide bonds within the stem peptide. On the other hand, amidases cleave the amide bond between MurNAc and L-Ala, separating the stem peptide from the glycan strand. Finally, glycosidases can be further subdivided into the types of linkages they cleave: glucosaminidases hydrolyze GlcNAc-(1→4)-MurNAc bonds while acetylmuramidases cleave MurNAc-(1→4)-GlcNAc bonds. Lytic transglycosylases (LTs) belong to the family of acetylmuramidases. Specifically, they carry out an intramolecular transglycosylation reaction, generating 1,6-anhydro-MurNAc sugars [74-76]. These sugars, or anhydro-muropeptides are either liberated from the PG matrix during cell wall turnover and recycling, or assimilated into the cell wall. The evidence for direct integration of anhydro-muropeptides into PG is supported
by the presence of non-reducing 1,6-Anhydro-MurNAc caps for all glycan strands within the PG matrix [74].

**PG hydrolases are functionally redundant**

Although PG hydrolases are important enzymes in bacterial physiology, it has been difficult to assign a precise physiological role to a specific enzyme or classes of enzymes because bacteria possess a large number of functionally redundant hydrolases [73]. Most cell wall mutants do not exhibit any growth or morphological defects. Systematic inactivation of multiple PG hydrolases is often necessary to uncover phenotypes and to assess the protein’s physiological function *in vivo*, albeit with limited success. For example, in the context of lytic transglycosylases, a mutant lacking up to six of the eight total LTs in *E. coli* is viable and only displays a mild chaining phenotype [77-78]. Consequently, it is still unclear how different classes of PG hydrolases contribute to bacterial cell growth and division, and how these hydrolytic enzymes are regulated during the process.

**PG hydrolases are involved in cell separation**

There are a few successes nonetheless. The most clearly defined function for PG hydrolases is the cleavage of shared PG material between daughter cells during cell division [73,79]. In *E. coli*, the septal PG is processed by LytC-type amidases (AmiA, AmiB, and AmiC) in order to shape the new polar caps and allow the formation of two separate daughter cells. Mutants lacking all three amidases are able to complete the
constriction of the inner membrane but fail to split the septal PG layer, forming long chains connected by unsplit septal PG [77,79,80].

**PG hydrolases are involved in cell elongation**

**Fig 5. Growth of PG requires coordination of PG hydrolases and synthases.**

The PG cell wall layer is a continuous meshwork that encases the cytoplasmic membrane and needs to be expanded to allow cell growth. The box contains a diagram highlighting the need for both PG synthesis and hydrolysis for the expansion of the cell wall. PG hydrolases are required to make space within the cell wall structure to allow the incorporation of new glycan strands (indicated by the green arrow) that are produced by the PG synthases. How that is achieved is still unclear.

It has long been appreciated that cell elongation requires cleavage of the PG matrix to make space for insertion of new cell wall material, but only recently have the critical
enzymes been identified (Fig 5). In *E. coli*, these space makers are endopeptidases that cleave the D-Ala-mDAP crosslinks in the lateral PG wall. The minimally redundant cluster of endopeptidases required for viability are Spr (MepS), YebA (MepM) and YdhO (MepH) [81]. When all three endopeptidases are inactivated, cells can no longer cleave peptide crosslinks, and consequently stop growing before lysing. This study is the first demonstration that PG hydrolases are critical for growth in *E. coli*, even though such a role had been proposed in the “three-for-one” model and the “make-before-break” strategy many years before [8, 82-85]. As illustrated by these two examples, PG hydrolases play an essential role in growth and division.

1.2.5: PG turnover and recycling

The diversity of PG hydrolases in *E. coli* is remarkable. This has led to the widespread belief that their enzymatic activity must be tightly controlled to avoid unwarranted degradation of the cell wall. How this is accomplished is still unclear. Regardless, the combined activity of all PG hydrolases generates nearly 50% of PG turnover per generation [86] Because the outer membrane is a semi-permeable layer, nearly 90% of the PG turnover products are trapped in the periplasm by size exclusion, and are thus recycled and inserted back into the cell wall matrix [87,88].

Briefly, recycling proceeds in three successive stages (Fig 6), and the relevant enzymes involved are listed in brackets:
Fig 6. Schematic of PG synthesis and recycling pathways.
Anhydro-muropeptides generated from degradation by LTs are translocated into the cytoplasm by AmpG. Through a series of cytoplasmic reactions, anhydro-muropeptides are broken down into their component parts and reincorporated into PG precursors.

First, anhydro-muropeptides released by LTs in the periplasm are translocated into the cytoplasm [by AmpG] [89]. Second, these PG turnover products are further degraded upon entering the cytoplasm. Specifically, PG dissacharides are hydrolyzed [by NagZ] to yield GlcNAc and 1,6-anhydro-MurNAc-peptides [90,91]. 1,6-anhydro-MurNAc-peptides are further degraded into D-Ala [by LdcA] [92], L-Ala-γ-D-Glu-L-mDAP and 1,6-anhydro-MurNAc [by AmpD] [93]. Additional enzymes convert 1,6-anhydro-MurNAc to GlcNAc. Finally, all the degraded products are re-channeled to other enzymes for reuse [88,94]. Both AmpG and AmpD are specific for anhydro-muropeptides, and this allows the recycling pathway to distinguish incoming PG sugars destined for recycling from outgoing PG precursors that are subsequently polymerized by GTs [89,93].
some bacteria with the exception of *E. coli* and *Shigella* species, PG recycling is closely tied to the induction of antibiotic resistance mechanisms during β-lactam treatment [95].

1.2.6: β-lactam mechanism of action

Penicillin and related β-lactam drugs are one of our oldest and most widely used antibacterial therapies [96]. These drugs target cell wall biogenesis by inhibiting the activity of PBPs that participate in PG biogenesis [102]. It is long known that β-lactams covalently modify and inhibit the TP site of HMW PBPs necessary for PG cross linking [31]. The classical paradigm for β-lactam mode-of-action has been attributed to straightforward PG misregulation, and its bactericidal effect comes from the loss of cell wall integrity that is accompanied by cell lysis. It is believed that these drugs function as simple enzyme inhibitors of PG synthases, leaving the PG hydrolases unaffected. The continued action of PG hydrolases cleaving the matrix in the absence of PG cross linking tips the balance between PG synthesis and degradation, eventually causing the wall to lose its structural integrity [97-99].

Given the long history of β-lactam research, this is surprisingly sparse as a mechanistic framework. It is still unknown which PG hydrolases are responsible for disrupting the cell wall following drug treatment, and whether the activity of these factors is suppressed, only to surface during drug perturbation. On top of that, not all β-lactams promote rapid cell lysis, and there are examples of lysis-independent lethality in β-lactams that have yet been characterized [100].
The downstream consequences of PBP inhibition by β-lactams have been difficult to elucidate because the effect of drug treatment on cell growth and morphology varies, depending on the study organism, the number of different PBPs inhibited by the particular β-lactam derivative used, and their corresponding binding affinities [101]. It is also hard to uncouple the direct effects of target inhibition from the many physiological changes dying cells undergo.

To learn more about the mode-of-action of β-lactams, our lab has been re-investigating the killing activity of the β-lactam mecillinam in E. coli. Mecillinam specifically targets PBP2, a critical PBP involved in cell elongation [101]. Our lab has recently demonstrated that mecillinam not only inhibits PBP2 activity, but also causes the activity of the Rod system it associates with to become toxic [103]. The toxic activity of the Rod system in the presence of mecillinam is caused by the inactivation of PBP2 and the corresponding failure to crosslink newly synthesized PG glycans into the wall. The uncrosslinked glycans are rapidly degraded by the lytic transglycosylase (LT) Slt, resulting in a futile-cycle of cell wall synthesis and degradation by the drug-targeted Rod complex (Fig7). In vivo biochemical turnover experiments with other β-lactams cephalexin and cefsulodin show that these drugs also promote nascent PG degradation by the PG synthase systems they target. This suggests that futile-cycle induction is a common activity of this drug class in E. coli and potentially other gram-negative bacteria. How LTs (or Slt specifically) are activated during β-lactam stress remains an open question.
Fig 7. β-lactams inhibits target PBPs and induce a lethal malfunction of the cell wall synthesis machinery associated with the target. Shown is a schematic highlighting the role of Slt during mecillinam treatment. Under normal growth, both GT and TP are properly coupled for efficient PG synthesis. When the TP (PBP2) is inhibited by β-lactam (mecillinam), RodA GT activity is unhindered and continues to polymerize new glycan strands which cannot be crosslinked into the existing PG meshwork. Slt functions as a quality control factor by cleaving these glycan strands to restore the proper coupling between GTs and TPs. In the absence of Slt, the potentially less efficient L,D transpeptidases crosslink the glycan strands into the existing matrix.

1.2.7: Outstanding questions on β-lactam mechanism of action to be addressed

The steps downstream of the futile cycle induced by β-lactams leading to cell death and lysis are not defined. We reasoned that mecillinam suppressors would provide more insight into the nature of the toxic futile cycle and how bacteria might overcome this
stress. Mecillinam suppressors have previously been identified in *E. coli*, but these mutants were selected under conditions where the Rod system was essential. Such conditions would not have identified mutants that can suppress the futile cycle or its downstream toxic effects but fail to survive in a non-functional Rod system.

We decided to specifically select for suppressors in conditions where the Rod system is not essential. Since induction of the stringent response or envelope stress responses are known to provide protection from mecillinam lethality, we also performed suppressor analyses in genetic backgrounds defective for each of these responses. One of the identified stress response-independent suppressors is loss of function of Prc, a periplasmic protease that degrades the cell wall endopeptidase MepS. MepS, together with MepM and MepH, were previously implicated to be important space makers during cell growth. Further genetic analysis and cell wall turnover assays suggest that endopeptidases, in addition to their space maker function, are also capable of stimulating PBP activity to promote PG synthesis.
1.3: ENVELOPE STRESS RESPONSES

Bacteria live in fluctuating environments and must constantly sense and respond to diverse environmental stresses. The envelope demarcates the interface between the cell and its environment. Envelope stress response sensors dynamically monitor the envelope status and transmit signals to induce an adaptive transcriptional response. Many different envelope stress response (ESR) systems have been described, and several have been studied in detail. The role of ESRs in gram-negative bacteria has received the most attention [104], but similar response systems have also been identified in gram-positive bacteria [105]. This is despite the fundamental difference in cell envelope architecture (Refer Chapter 1.1), and therefore the definition of stress between gram-negative and -positive bacteria. This overview and subsequent sections will focus on ESRs in the biology of *E. coli*, a gram-negative bacterium.

In *E. coli*, at least five extracytoplasmic response signaling pathways are induced in response to envelope stress. This is orchestrated by one extracytoplasmic function (ECF) σ factor, three two-component systems and the Phage-shock protein (Psp) response [106,107], of which the σE [108], Conjugative plasmid expression (Cpx) [109] and Regulator of colanic acid capsule synthesis (Rcs) [110] systems are most well-characterized (Fig 8). The remaining two ESRs: Bacteria adaptive response (Bae) [111,112] and Psp response will not be discussed in the review. These stress response pathways respond to heat, alkaline pH, ethanol, antimicrobial compounds, stress, misfolded proteins, high osmolarity, attachment to abiotic surfaces, loss of the proton motive force and intracellular signals from other bacteria [104,113]. Many of these
Nonetheless, several regulated mechanisms for redecorating the Gram-negative cell surface to monitoring and responding to protein-folding problems in the PP and OM, respectively with the study of the Cpx and Gram-positive bacteria. Historically, the use of the term repair the damage caused.

response, they alter the expression of adaptive functions that modify the envelope, rid the cell of the toxic entity, and/or including antibiotics, toxic metabolites, bacteriocins, attack by type VI secretion systems (T6SSs) and bacteriophage. In bacteria. These include the Cpx, Figure I. Gram-Negative Envelope Stress Responses (ESRs).

Fig 8. Major well-characterized Gram-negative envelope stress responses (ESRs) in E. coli. (adapted from Raivio et al. [149])

The three most well characterized ESRs in E. coli are σE, Cpx and the Rcs stress responses. All three of them display very different molecular signaling architectures, and it is still unclear what is the molecular nature inducing the Cpx and Rcs responses. These ESRs are induced by a variety of envelope stresses, including antibiotics, toxic metabolites, surface adhesion, misfolded proteins and more. Activation of these stress responses result in a transcriptional rewiring of cellular pathways to mitigate the stress.

1.3.1: Extracytoplasmic function σ factor

The essential ECF σ factor σE is the best-studied ESR in E. coli [117]. It is induced in the presence of misfolded outer membrane proteins (OMPs) in the periplasm that can accumulate during elevated temperatures [118]. It regulates a large set of genes whose
products either act directly on these misfolded proteins, or are involved in the synthesis, assembly and/or insertion of OMPs and lipopolysaccharide (LPS) [119].

Under non-inducing conditions, the inner membrane (IM) anti-σ factor RseA sequesters σE in an inactive conformation [120]. Unfolded OMPs are sensed by the IM protease DegS [121]. DegS is typically kept inactive by its periplasmic PDZ domain [118]. Accumulated misfolded OMPs in the periplasm competitively bind to the PDZ domain via their carboxyl termini, relieving the auto-inhibition [118]. Active DegS cleaves the periplasmic domain of RseA, which is now sensitive to secondary cleavage by RseP [122]. Degradation of RseP releases the soluble component of RseA, still bound to σE into the cytoplasm [123]. The σE-RseA fragment is finally degraded by ClpXP to liberate σE [123].

The σE system has a second negative regulator, RseB which binds to RseA, preventing cleavage by DegS [108,124,125]. LPS can bind to RseB and dissociate it from RseA [127]. In vitro results demonstrate that RseA degradation in the presence of RseB requires both OMPs and LPS [126]. This is further supported by in vivo data: mutations that disrupt LPS transport or alter LPS structure, in combination with activated DegS, are necessary for maximal induction of σE [126]. Therefore, full activation requires sensing both unfolded OMPs and periplasmic LPS.

1.3.2: Two-component systems and envelope stress responses
Two-component signal transduction systems are commonly used by bacteria to monitor and respond to extracytoplasmic conditions. A typical two-component system is composed of a transmembrane sensor histidine kinase and a cytoplasmic response regulator that is activated through phosphorylation [127].

**Cpx two-component system**

The Cpx system is a classical two-component system (TCS) with CpxA as the histidine kinase and CpxR as the cognate response regulator [128]. Cpx responds to a large number of stimuli that include alkaline pH, copper ions, adhesion to abiotic surfaces, and disruptions of the secretory apparatus, perturbations in IM lipid composition and PG cell wall defects [109]. Cpx regulates periplasmic foldases, chaperones and proteases to directly mitigate envelope stress. On top of that, the Cpx regulon includes genes associated with biofilm formation, amino acid biosynthesis, toxin elimination and peptidoglycan enzymes.

CpxA is a transmembrane protein with a periplasmic loop that acts as the sensor domain. In the presence of an inducing signal, CpxA cytoplasmic histidine kinase is autophosphorylated, which subsequently phosphorylates the receiver domain of CpxR [129]. Phosphorylated CpxR binds to target promoters on the chromosome to regulate gene expression. CpxA has both kinase and phosphatase activity, allowing it to rapidly control the extent of CpxR phosphorylation and the level of Cpx induction [129].
Two auxiliary proteins add additional layers of regulation to the Cpx TCS: CpxP and NlpE. CpxP is a periplasmic protein that binds to CpxA and prevents its autophosphorylation \[130\]. CpxP is also a chaperone that clears misfolded pillins from the periplasm. In the presence of misfolded pillins, CpxP is titrated away from CpxA, and delivers the pillins to DegP such that both driver and cargo are degraded \[131\]. \textit{cpxP} is also highly up-regulated during Cpx induction \[132\], which in turn exerts a negative feedback loop on CpxA to halt the induction \[133\]. On the other hand, NlpE is an OM lipoprotein that activates CpxA in response to surface attachment \[134\]. It has been proposed that NlpE is unfolded to directly contact CpxA from the OM to activate the signaling cascade \[135\].

\textbf{Rcs phosphorelay}

The Rcs system was first identified for its role in regulating colanic acid synthesis in \textit{E. coli}. \textit{lon} mutants were originally discovered to form mucoid colonies \[136\]. The mucoid phenotype was subsequently shown to be dependent on functional RcsA (a Lon substrate) and RcsB proteins, which form a heterodimer to activate colanic acid synthesis \[137\]. The Rcs system responds to alterations in the cell envelope caused by osmotic stress, peptidoglycan biogenesis defects and cationic antimicrobial peptides \[110, 113-116, 139, 141, 144\].

In contrast to the Cpx two-component system, the Rcs system relies on a complex phosphorelay involving RcsC, RcsD and RcsB proteins. RcsC is a transmembrane protein containing both a histidine kinase and a receiver domain. The histidine kinase
domain is thought to autophosphorylate itself in response to environmental signals [110], which subsequently phosphorylates the receiver domain within the same protein. The phosphoryl group is then relayed to a second IM protein RcsD, and then finally to RcsB, the cytoplasmic response regulator. Phosphorylated RcsB, either alone or in combination with another auxiliary transcription factor RcsA, activates transcription of a large set of genes, including those for capsule synthesis, cell division, biofilm formation. Genes repressed by the Rcs phosphorelay are related to structures on the surface of the cell, including flagella, curli and pili, which are all important for locating and attaching to surfaces [110,137,139].

Additionally, the Rcs phosphorelay requires other auxiliary proteins for normal functioning of the system. The essential IM protein IgaA is a negative regulator that inhibits RcsC activation, and might play a role in sensing IM or periplasmic stresses [138]. The OM lipoprotein RcsF is the sensor for stresses on the OM and in the periplasm, and transduces signals to RcsC, thus initiating the signaling cascade [110,140-144] It is proposed that RcsF stress sensing allows the protein to interact with IgaA and thereby relieve the inhibition of RcsC [142].

**Rcs and Cpx systems are activated by PG stress**

Both Cpx and Rcs stress responses are activated upon β-lactam treatment [114] and in a variety of mutants lacking certain cell wall enzymes [115], but it is still unclear how they are involved in response to peptidoglycan damage. While only Cpx activation is known to directly regulate some peptidoglycan biosynthetic enzymes [145], the Rcs
system is required for de novo shape recovery post-lysozyme treatment [146], and Rcs inactivation results in β-lactam hypersensitivity [114]. How Rcs activation suppress the toxic effects of β-lactams is still unknown.

1.3.3: Challenges in ESRs

One of the major questions in the field is how the physiological changes that accompany stress activate envelope stress responses on the molecular level. We still do not know the molecular nature of the inducing signal, and the mechanism of activation for ESRs, except σE. External and genetic stimuli often induce multiple stress response pathways, making it difficult to discern the precise function of each pathway [111, 114-116]. Even though we can induce specific ESR pathways using genetic stimuli, it is unclear if these stimuli generate one common inducing signal, or distinct independent signals that could still be recognized by different components within the signaling cascade [106, 110, 111].

To complicate things further, there is some degree of cross-regulation between envelope stress responses, with multiple systems controlling the same genes. Additionally, some ESRs directly regulate the transcription of signaling proteins involved in other ESRs, and are themselves mediated by other stress responses. For example, both σE and Cpx responses, when induced, up-regulate the chaperone-protease DegP, presumably to enhance protein turnover within the envelope, but the Cpx master regulator CpxR directly represses the rpoE-rseA-rseB operon, which encodes σE [132, 147]. In another instance, RprA, a small RNA (sRNA), was recently found to be
induced by both Rcs and Cpx activation, and yet exerts a negative feedback loop on the
Cpx response in a CpxR-dependent manner [114,148]. These cross-connections
between ESRs make it incredibly hard to distinguish upstream stimuli from downstream
effects, let alone understand the physiological function of a particular response. It is also
unclear why such inter-ESR signaling mechanisms exist, but it has been proposed that
these mechanisms could be important when cells face multiple stresses simultaneously,
such as during an infection.

1.3.4: Outstanding questions on envelope stress responses to be addressed
ESRs have traditionally been studied in the context of external stresses or following
genetic inactivation or over-expression of envelope components. Although useful, these
conditions do not necessarily reflect the native biological states in which stress
response systems are harnessed. Given the dynamic and complex multilayered nature
of the cell envelope, it is reasonable to postulate that ESRs play a role in the
maintenance of envelope homeostasis during bacterial growth and division. However,
we do not know how frequent envelope biogenesis machinery malfunctions during
normal balanced growth, what these problems might manifest as, and which ESRs are
recruited to alleviate the stress resulting from these problems. At the same time, we
have not identified the quality control factors in cell envelope biogenesis responsible for
suppressing such spontaneous malfunction events or ‘errors’.

To learn more about the spectrum and frequency of spontaneous errors, we used a
microfluidic setup to track unperturbed cells for extended duration during balanced
growth, and screened for the rare occasion of cells with spontaneous activated ESRs. As described in Chapter 3, this imaging screen revealed rare spikes of Rcs stress response activity and correlated with membrane blebs in affected cells. Further genetic analysis suggest that our monitoring system is a robust tool for identifying quality control factors that promote proper cell wall assembly by reducing the error rate.
1.4 REFERENCES


35


domain of a bacterial lytic transglycosylase defines a novel class of lysozymes.

multiple deletions of murein hydrolases on viability, septum cleavage, and
sensitivity to large toxic molecules in Escherichia coli. Journal of Bacteriology,
184(22), 6093–6099.

Lactamase Induction in Murein Hydrolase Mutants of Escherichia coli.
Antimicrobial Agents and Chemotherapy, 49(4), 1404–1409.

79. Heidrich, C., Templin, M. F., Ursinus, A., Merdanovic, M., Berger, J., Schwarz, H.,
et al. (2001). Involvement of N-acetylmuramyl-l-alanine amidases in cell
separation and antibiotic-induced autolysis of Escherichia coli. Molecular
Microbiology, 41(1), 167–178.

amidases in the development and morphology of the division septum in

murein endopeptidases catalyse an essential cleavage step in peptidoglycan

82. Holtje, J. V. (1996). A hypothetical holoenzyme involved in the replication of the
murein sacculus of Escherichia coli. Microbiology (Reading, England), 142(8),

Guarantees a Precise Copy of the Thin, Rod-Shaped Murein Sacculus of
Escherichia coli. In Bacterial Growth and Lysis (pp. 419–426). Boston, MA:
Springer US.


Bacteriology, 163(1), 305–310.

medium by exponentially growing Escherichia coli. Journal of Bacteriology,
162(1), 391–397.


CHAPTER 2:

Analysis of the *Escherichia coli* mecillinam resistome reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis
ATTRIBUTIONS

The work presented in this chapter was performed primarily by Ghee Chuan Lai and Hongbaek Cho. Hongbaek Cho performed turnover experiments and explored the generality of endopeptidases capable of suppressing mecillinam toxicity. All remaining experiments were performed by Ghee Chuan Lai. The manuscript was written by Ghee Chuan Lai and Thomas Bernhardt.
CHAPTER 2:

Analysis of the *Escherichia coli* mecillinam resistome reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis

Ghee Chuan Lai¹*, Hongbaek Cho¹*, Johan Paulsson², and Thomas G. Bernhardt¹

¹Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115
²Department of Systems Biology, Harvard Medical School, Boston, MA 02115

Bacterial cells are typically surrounded by an essential net-like macromolecule called the cell wall constructed from the heteropolymer peptidoglycan (PG). Biogenesis of the PG matrix is the target of many of our most effective antibiotic therapies, including penicillin and related β-lactams. These drugs inhibit the transpeptidase activity of cell wall synthases called penicillin-binding proteins (PBP) and prevent the crosslinking of nascent PG glycans into the existing network. The β-lactam mecillinam specifically targets the PBP2 enzyme in the cell elongation machinery of *Escherichia coli*. Selections for mecillinam resistance have provided a wealth of information about the mechanisms underlying the process of cell wall biogenesis and the killing mechanism of β-lactam antibiotics. Here, we used transposon-sequencing (Tn-Seq) to comprehensively identify all mecillinam resistance loci in the *E. coli* genome. Previous studies have implicated the stringent response and the Rcs envelope stress response in promoting survival of mecillinam challenge. We, therefore, also performed the Tn-Seq analysis in mutants defective for these responses to determine the stress response dependence of each resistance allele. We reasoned that the stress response independent loci would most likely have a direct effect on the cell wall biogenesis process. Characterization of one of these
alleles led to the discovery that PG endopeptidase enzymes which cleave peptide crosslinks in the PG matrix are rate limiting for cell wall biogenesis and their activity stimulates cell wall synthesis by the PBPs.
2.1 INTRODUCTION

Bacterial cells are typically surrounded by an essential net-like macromolecule called the cell wall. This structure is constructed of peptidoglycan (PG), a unique bacterial heteropolymer consisting of glycan chains of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) repeating units with attached stem-peptides used to form the matrix crosslinks [1]. Many of our most effective antibiotic therapies target cell wall biogenesis, and much of what we know about the cell wall assembly process was facilitated using these antibiotics as functional probes. In both respects, penicillin and related β-lactam drugs are standouts. They are the most frequently prescribed antibiotics worldwide, and their use in basic research has provided major insights into the structure of the wall and how it is built.

β-lactams inhibit their targets by covalently modifying their active sites [2], a property that facilitated the identification of the penicillin-binding proteins (PBPs) as key cell wall biogenesis factors. The PBPs are subdivided into class A (aPBPs), class B (bPBPs), and class C (cPBPs) enzymes [3]. aPBPs are bifunctional and possess both glycosyltransferase (GT) activity for polymerizing the glycan strands of PG and transpeptidase (TP) activity for crosslinking them. bPBPs, on the other hand, are only known to possess TP activity [3]. cPBPs cleave PG and either break crosslinks (endopeptidases) or tailor the peptide stem by removing the terminal D-Ala residue (carboxypeptidases) [3]. β-lactams block the TP active site of the synthetic PBPs and inhibit PG hydrolysis by the cPBPs. Depending on the type and concentration of β-lactam used, cells treated with these drugs either rapidly lyse or undergo
significant morphological changes before lysing several generations after drug addition [4]. Despite years of study, molecular details of the events downstream of PBP inhibition that elicit these dramatic effects are only beginning to be uncovered.

β-lactams that are highly specific for a single target PBP have been particularly useful probes for understanding PG biogenesis and the β-lactam killing mechanism. Among them, mecillinam has probably stimulated the greatest number of discoveries. It specifically targets the bPBP PBP2 in *Escherichia coli* and causes the loss of rod shape and the formation of large spherical cells that eventually lyse [3]. Early selections for mecillinam resistance in *E. coli* led to the identification of loss-of-function mutations in the *pbpA* (PBP2), *rodA*, and *mreCDE* genes [4-6]. These mutants paved the way for the discovery of the cell wall biogenesis machinery called the Rod system (elongasome) [1]. This system promotes the elongation of rod-shaped cells and is organized by dynamic filaments of the actin homolog MreB. Within the complex, the SEDS-family protein RodA supplies the PG polymerase function while PBP2 uses its TP activity to crosslink the new material into the PG matrix [7,8]. An analogous multi-protein machine called the divisome mediates PG synthesis during cell division [1]. It is organized by the tubulin-like FtsZ protein, which brings together a subset of PG biogenesis factors similar to those in the Rod system, including the SEDS-family protein FtsW and PBP3, a bPBP related to PBP2.

The Rod system is normally essential in *E. coli* [9,10]. However, when this essentiality was initially discovered, it conflicted with the original reports describing
the isolation of mecillinam-resistant mutants defective for cell shape and Rod system activity [4,5]. It was subsequently shown that these mutant isolates had secondary mutations that increased the production of FtsZ to suppress Rod system essentiality [6,9,10]. The reason why extra FtsZ (designated FtsZ\textsuperscript{up}) results in suppression is not clear. Nevertheless, it suggested that the original selections for mecillinam resistance were more complicated than initially appreciated. If mecillinam works simply by inactivating the Rod system, why isn't FtsZ overproduction alone sufficient to bypass drug action and promote resistance? Why were double mutants that both overproduce FtsZ and inactivate the Rod system isolated?

This genetic conundrum recently led us to reinvestigate the mode-of-action of mecillinam. We discovered that mecillinam not only inhibits the TP activity of PBP2 but also causes the activity of the Rod system to become toxic [11]. Thus, to gain mecillinam-resistance, cells must both inactivate the Rod system and acquire mutations that render the system non-essential for growth. The toxic activity of the Rod system in the presence of mecillinam is caused by the inactivation of PBP2 and the failure to crosslink nascent PG material into the wall. The un-crosslinked glycans produced by the machine are rapidly degraded by the lytic transglycosylase (LT) Slt, resulting in a futile-cycle of PG synthesis and degradation by the drug-targeted Rod complex [11]. Experiments with the \(\beta\)-lactams cephalixin and cefsulodin showed that they also promote nascent PG degradation by the PG synthase systems they target, indicating that futile-cycle induction is a common activity of this drug class in \textit{E. coli} and likely many other gram-negative bacteria [11].
The downstream steps via which the futile-cycle of PG synthesis and degradation induced by β-lactams results in cell death and lysis have not been clearly defined. We reasoned that mutants resistant to the toxic effects of mecillinam should shed light on this lethal mode-of-action. Such mutants should also provide new insights into drug-resistance mechanisms and the process of cell wall biogenesis in general. Many mecillinam-resistant *E. coli* mutants have been isolated and characterized previously, including several from the extensive studies of D’Ari and co-workers [4-6, 12-16]. However, these mutants were selected under conditions where the Rod-system was essential. Thus, they were required to survive both the crippling of the Rod system by PBP2-inactivation and the downstream toxic effects of the futile-cycle. These conditions likely limited the spectrum of mutants isolated.

To overcome the complications of prior genetic analyses, we initiated selections for mecillinam resistance using FtsZ<sup>+</sup> cells, in which the Rod system is non-essential. Thus, in order to grow, mutants are only required to survive the futile-cycle of PG synthesis and degradation. Under these conditions, mecillinam-resistant mutants arise at a high frequency, indicating that there are many ways to either inactivate the futile cycle or ameliorate its consequences. Therefore, to identify the full spectrum of resistance loci, we employed transposon sequencing (Tn-Seq) [17] of large pools of mutants capable of growth on either low, intermediate, or high doses of drug. Furthermore, because induction of the stringent response or envelope-stress responses are known to provide protection from mecillinam lethality [6, 18], we
additionally performed the analysis of mecillinam resistance in genetic backgrounds
defective for these responses. This approach allowed us to identify loci that most
likely provide resistance via induction of the stress response systems. The results
provide a useful dataset of mutants that are likely to be constitutively activated for
the Rcs and stringent responses.

For further biological studies, we were especially interested in mecillinam resistant
mutants that appeared to be stress-response independent. We suspected that such
loci are more likely to identify factors directly involved in modulating cell wall
biogenesis to affect drug sensitivity. Among this class of mutants were those
inactivated for the periplasmic protease Prc, which was recently implicated in the
degradation of the cell wall endopeptidase Spr [19,20]. We therefore hypothesized
that elevated Spr concentration might provide mecillinam resistance. Strikingly, we
found that overproduction of Spr and several other endopeptidases conferred
mecillinam resistance. This was a surprising result because PG degrading enzymes
are typically associated with the induction of cell lysis following β-lactam treatment,
not with promoting survival [21]. Spr-mediated resistance required the class aPBP,
PBP1b, and radiolabeling experiments indicated that Spr overexpression stimulates
PG synthesis. Our results are therefore consistent with a model in which
endopeptidases are not only required for creating space in the matrix for the
insertion of new material [19], but that they are also capable of stimulating PBP
activity to promote PG synthesis.
2.2 RESULTS

Identification of mecillinam resistance loci using Tn-Seq.

For our analysis, we used wild-type *E. coli* MG1655 cells producing extra FtsZ from a low-copy number plasmid containing the *ftsQAZ* operon (pTB63) [11]. When these FtsZ<sup>+</sup> cells were selected for spontaneous mecillinam resistance at concentrations between 1-10 µg/ml, survivors arose at a frequency of 1-5 x 10<sup>-4</sup>. This is at least 10 fold higher than was obtained in previously described mecillinam selections. The increased frequency of survival conferred by pTB63 indicated that previous selections for mecillinam resistance without elevated FtsZ levels were likely to have missed a significant number of resistance loci. To identify the full set of mecillinam resistance determinants, MG1655/pTB63 cells were mutagenized with the EZTn-Kan transposome to generate a library consisting of approximately 2 x 10<sup>5</sup> independent insertions. The library was then plated on LB agar with 0, 1.0, 2.5, or 10 µg/ml mecillinam. Survivors on mecillinam arose at a frequency of 2-6 x 10<sup>-3</sup>. This frequency was ten times greater than for unmutagenized cells, indicating that the vast majority of the isolates gained resistance due to a transposon insertion. Given the high frequency of resistance, we expected the number of loci involved to be large. Therefore, rather than mapping individual alleles in isolated clones, we pooled the survivors at each mecillinam concentration and simultaneously mapped the location of all transposon insertions in the population using Tn-Seq methods [22]. Genes with an elevated frequency of transposon insertions in the mecillinam-treated samples relative to the untreated library were identified as likely resistance loci. They are listed in Table 1 along with their fold enrichment in mecillinam versus the no drug
control condition. Representative Tn-Seq profiles of several of the identified resistance loci are shown in Figure 1. As an indication that the analysis was working as expected, several known mecillinam resistance loci were clearly identified, including *pbpA*, *rodA*, *mreBCD*, and *slt* [4,5,11] Fig. 1). Many novel alleles were also uncovered, including *sspA*, *crr*, and *ptsI*. In all, over 90 different resistance loci were identified in all mecillinam concentrations.

**Fig 1. Tn-seq for uncovering the mecillinam resistome.**

**A.** Tn insertion profiles of the Rod system components and *slt* under LB and mecillinam (MEC) conditions

**B.** Tn insertion profiles of previously known MEC suppressors

**C.** Tn insertion profiles of several discovered MEC suppressors
To confirm that inactivation of the identified genes confers mecillinam resistance, relevant deletion-insertion mutants from the Keio collection [23] were transduced into the MG1655/pTB63 background and their mecillinam resistance was assessed. Loci corresponding to a range of different enrichment levels in the Tn-Seq analysis were chosen for validation. Overall, the level of enrichment observed for transposon insertions in a given gene roughly correlated with the degree of mecillinam-resistance displayed by the corresponding deletion-insertion mutant (Fig. 2). Inactivation of all genes with an 26-fold enrichment or higher by Tn-Seq clearly confirmed resistance (Fig. 2). Genes with insertions found at lower enrichment values yielded mixed results and typically conferred only partial resistance when inactivated (Fig. 2). We conclude that the Tn-Seq analysis has faithfully identified the majority of, if not the complete, mecillinam "resistome" of *E. coli*. 
Fig 2. Mecillinam selection is both accurate and sensitive.
Spot dilutions of MG1655/pTB63 mecillinam (MEC) suppressors ranked in descending fold change enrichment. Strains are spotted on 1ug/mL MEC and left at 30°C for 2 days. Fold change enrichment of Tn insertions relative to LB is a satisfactory proxy for MEC suppression.

Stress response dependence of the mecillinam resistome.
Induction of the stringent response is known to confer mecillinam resistance, and the Rcs envelope stress response pathway has been implicated in the protection of cells from β-lactam stress [6,18]. Consistent with these findings, a number of loci identified in the Tn-Seq analysis have previously been associated with constitutive production of guanosine tetraphosphate (ppGpp) to induce the stringent response (e.g. tufA, efp) or constitutive Rcs activation (e.g. lpp, rfaP, nlpD). To identify all loci that require induction of either the stringent response or Rcs to confer mecillinam
resistance, the Tn-Seq analysis was repeated in either a ΔrelA or ΔrcsB background, respectively. RelA is the major ppGpp synthase in *E. coli*, and RcsB is the response regulator required to modulate the expression of Rcs-responsive genes. When transposon libraries generated in the ΔrelA or ΔrcsB backgrounds were plated on mecillinam agar, survivors arose at a frequency of 0.5-1 x 10^{-3} and 4-6 x 10^{-4}, respectively. The reduced level of survivors in each case relative to WT cells, indicates that many loci identified in the original Tn-Seq analysis are stress response-dependent for resistance. **Table 2** lists the RelA- and RcsB-dependent resistance loci as well as loci found to be stress-response independent.

Representative Tn-Seq profiles for each gene class are shown in **Figure 3**. As expected, the RelA-dependent alleles are enriched for genes implicated in translation elongation, tRNA modification, or amino acid metabolism (**Table 2**), indicating that they likely induce ppGpp production when they are inactivated due to adverse effects on protein synthesis. Similarly, many of the RcsB-dependent resistance loci are genes associated with cell envelope biogenesis (**Table 2**), defects in which are among the primary signals that result in Rcs activation.

To confirm the RelA- or RcsB-dependence of several representative mutants to confer mecillinam resistance we plated lawns of the mutants and assessed mecillinam killing using MIC test strips impregnated with a concentration gradient of mecillinam. Resistance due to inactivation of the LPS biogenesis factor WaaP was found to be RcsB-dependent in the Tn-Seq analysis (**Fig. 3A**). Consistent with this
analysis, the single Δ\textit{waaP} mutant displayed resistance in the test-strip assay, whereas sensitivity was restored in the double Δ\textit{waaP} Δ\textit{rcsB} derivative (Fig. 3B).

**Fig 3. Tn-seq to uncouple stress response dependencies for mecillinam resistance.**

A. Tn insertion profile of a Rcs-dependent, RelA-dependent and stress response-independent mecillinam resistant loci.

B. Confirmation of Tn-seq results via mecillinam MIC test strip assay. MIC test strips are applied on plates inoculated with strain of interest and left at 30°C for 18hrs.
Similarly, inactivation of *tufA* encoding translation elongation factor EF-Tu promoted RelA-dependent mecillinam resistance in the Tn-Seq analysis, and this result was confirmed using the test strips (Fig. 3). Finally, as expected, blocking the futile cycle of PG synthesis and degradation by Slt inactivation showed stress response-independent mecillinam resistance in the Tn-Seq profiles (Fig. 3). This stress response independence was confirmed in the MIC test strip assay in which the Δslt strain showed similar levels of resistance whether or not they possessed a functional RelA or Rcs response (Fig. 3). Several other mutants in each category displayed the expected phenotype in the test strip assay based on the observed stress response dependence of resistance from the Tn-Seq analysis (data not shown). We therefore conclude that the Tn-Seq analysis in the different genetic backgrounds correctly defined the stress response dependence of most mecillinam resistance loci. Further study of stress response induction in the various mutants may reveal new information about the precise signals stimulating these important global response systems.
Effect of the Rcs and stringent responses on cell wall synthesis and the futile cycle.

We have previously shown that β-lactams inhibit PBPs to cause the formation of un-crosslinked glycans that are rapidly degraded by Slt [11]. The resulting futile-cycle of PG synthesis and degradation by the drug-targeted Rod complex contributes significantly to the killing activity of mecillinam. We were interested in determining how the stringent response and Rcs responses affect mecillinam-induced degradation of nascent PG to promote survival. Do they antagonize Rod system activity to limit nascent PG degradation, or do the changes in gene expression allow cells to cope with the toxic side-effects of the futile cycle? To investigate these possibilities, we generated constructs that overexpress either relA or rcsF to stimulate ppGpp production or the Rcs response, respectively. RcsF is an outer membrane lipoprotein that functions as an inducer of the Rcs system when it is improperly localized in the envelope [24]. The RelA produced from our construct was a truncated form (RelA*) predicted to be hyperactive for ppGpp production. As expected from prior genetic analyses and the results presented above, both factors were sufficient to promote mecillinam resistance when overproduced to induce their respective responses (Fig. 4A).

Mecillinam-induced PG turnover was monitored using a previously established radiolabeling protocol [11]. In this assay, cells were first blocked for divisome function to focus the PG synthesis measurements on cell elongation activity by the Rod system. Cells with or without drug treatment were then pulse labeled with the
radiolabeled PG precursor $[^3]H$-diaminopimelic acid ($[^3]H$-DAP). After only an additional 1/10th of a generation of growth, the distribution of the label between the PG matrix, PG turnover products, or the PG precursor UDP-MurNAc-pentapeptide (UDP-MurNAc-pep$_5$) was determined. In the absence of mecillinam, cells harboring the vector control incorporated most of the label into the PG matrix with very little material being converted to degradation products (Fig. 4B). As observed previously [11], mecillinam treatment resulted in the conversion of most of the PG material into turnover products (Fig. 4B). Prior studies have shown that this induction of turnover is blocked by the MreB-antagonist A22, indicating that the synthesis and degradation detected is carried out by the Rod system [11]. Overproduction of RcsF did not significantly affect the level of mecillinam induced turnover compared to the vector control (Fig. 4B). For RelA* producing cells, the overall levels of PG synthesis and turnover were lower due to the reduced growth rate imposed by ppGpp accumulation. However, the relative level of turnover to synthesis in mecillinam treated cells was similar to cells with the empty vector control (Fig. 4C). We conclude the resistance promoted by the Rcs and stringent response is not due to an inhibition of the futile cycle. Instead, the Rcs response is most likely helping cells deal with the consequences of the futile cycle, whereas the effects of the stringent response most likely stem from a reduced growth rate, which generally limits PG synthesis and may allow cells to cope better with an active futile cycle.
Fig 4. Activation of the Rcs or stringent response suppresses mecillinam toxicity, but does not inhibit the futile cycle.

A. Spot dilutions of strains over-expressing \( \text{relA}^* \) or \( \text{rcsF} \), with the relevant empty vector controls. Strains are spotted on 1ug/mL MEC and left at 30°C for 2 days. Suppression of mecillinam toxicity via RcsF overproduction requires an functional Rcs stress response.
B. Cells of TU278(attHKpHC859)(att\(\lambda\)pGL69) and TU278(attHKpHC859)(att\(\lambda\)pGL68) \(\Delta\)lysA \(\Delta\)ampD \(P_\text{tac}::\text{sulA} P_\text{tac}::\text{null}\) and \(\Delta\)lysA \(\Delta\)ampD \(P_\text{tac}::\text{sulA} P_\text{tac}::\text{rcsF}\) respectively) producing SulA to block cell division were pulse labelled with \([^3\text{H}]\)-mDAP following treatment with the indicated compound(s). Turnover products were extracted with hot water and quantified by HPLC and in-line radiodetection. PG incorporation was determined by digesting the pellets resulting from the hot water extraction with lysozyme and quantifying the amount of label released into the supernatant by scintillation counting. Mecillinam concentrations used: mecillinam (10 \(\mu\text{g/mL}\)). Results are the average of three independent experiments. Error bars represent the standard error of the mean (s.e.m.).

C. Similar procedure was applied for cells of TU278(attHKpHC859)(att\(\lambda\)pGL70) and TU278(attHKpHC859)(att\(\lambda\)pGL65) \(\Delta\)lysA \(\Delta\)ampD \(P_\text{tac}::\text{sulA} P_\text{ara}::\text{null}\) and \(\Delta\)lysA \(\Delta\)ampD \(P_\text{tac}::\text{sulA} P_\text{ara}::\text{rcsF}\) respectively), except that the cells were pre-incubated with both 0.2\%arabinose and 1mM IPTG for 30min prior to adding MEC or DMSO.

Overproduction of PG endopeptidases promotes mecillinam resistance

Genes coding for components of the Rod system \([mreB, mreC, rodZ, mrdA]\) (encoding PBP2), and \(mrdB\) (encoding RodA)] along with \(slt\) encoding the LT responsible for mecillinam-induced PG turnover were identified as stress response independent mecillinam resistance loci.

Thus, other genes included in this class may also encode factors that directly or indirectly alter PG biogenesis. We were particularly interested in \(prc\) given that it encodes a protease recently shown to be involved in the turnover of Spr [20], a PG endopeptidases implicated in PG matrix expansion [19]. This was intriguing because inactivation of Spr has the opposite phenotype. It was found to result in mecillinam hypersensitivity in a high-throughput chemical genomic screen of the \(E.\ coli\) Keio
collection [25], a result that we confirmed (Fig. 5A). Thus, the mechanism by which
Prc inactivation suppresses mecillinam toxicity might be through the overproduction
of Spr. Indeed, overexpression of spr from a multicopy plasmid was capable of
promoting mecillinam resistance (Fig. 5B).

To determine if suppression by spr overexpression required the endopeptidase
activity of Spr, we generated an overexpression vector encoding Spr(C68A), in
which the active site Cys was replaced by Ala. Surprisingly, overproduction of
Spr(C68A) was also capable of promoting growth in the presence of mecillinam
(Fig. 5B). One possible explanation for this result is that Spr catalytic activity is not
required for mecillinam suppression. Alternatively, the overproduction of Spr(C68A)
in otherwise wild-type cells might overwhelm the Prc protease and thereby stabilize
and increase the levels of the native and active Spr protein. In support of the latter
possibility, overproduction of Spr(C68A) was unable to suppress mecillinam toxicity
in a strain deleted for the native copy of the spr gene (Fig. 5B). To determine if the
suppression activity was specific to Spr, we tested the effect of overproduction of
other E. coli PG endopeptidases on mecillinam killing activity. Remarkably,
overproduction of several additional endopeptidases was capable of promoting
growth on mecillinam agar: YebA and MepA, which are LAS-family metallo-
endopeptidases, and PBPG (PBP7), which is a cPBP-type endopeptidase, both
distinct families from each other and the NlpC/P60 family to which Spr belongs
(Fig. 6). We conclude that elevated PG endopeptidase activity promotes survival
upon mecillinam treatment. This observation is surprising because cell wall
hydrolase activity is typically thought to promote the lethal and lytic effects of β-lactam antibiotics, not to counteract them [21].

Fig 5. Mecillinam suppression is mediated by Spr endopeptidase activity.

A. Δspr is hypersensitive to mecillinam. Addition of sub-inhibitory concentration of mecillinam causes rapid lysis in both Δspr and ΔponB cells. Overnight cultures are diluted to OD₆₀₀ = 0.05 and incubated at 30°C. OD measurements were taken every half hour. Mecillinam was added to the exponentially growing cultures after 4.5 hours such that the final concentration is 0.0625ug/mL (MIC = 0.25ug/mL). OD measurements were taken every 15min for the next 90min.

B. Spr overproduction suppresses mecillinam toxicity, and is dependent on the catalytic activity of Spr. Strains were spotted on LB plates containing 1ug/mL of mecillinam and incubated at 30°C for 24hrs.
Fig 6. Elevated PG endopeptidase activity promotes survival upon mecillinam treatment.
Strains are spotted on LB plates containing 1μg/mL mecillinam and various concentrations of IPTG driving the expression of endopeptidase, and incubated at 30°C for 24hrs. Ptac::s-ydhO represents a ydhO over-expression variant with a strong ribosome binding site.

Spr overproduction suppresses the mecillinam induced futile cycle by boosting PG synthesis

To investigate the mechanism by which endopeptidase overproduction suppresses mecillinam toxicity, we monitored the effect of Spr overproduction on the mecillinam-induced futile cycle of cell wall synthesis and degradation. Importantly, the the incorporation of label into the PG matrix was increased in mecillinam-treated cells overproducing Spr relative to the vector control, and this was accompanied by a decrease in the level of PG turnover (Fig. 7A). Thus, increased Spr endopeptidase activity appears to promote survival by limiting the mecillinam-induced futile cycle. A possible explanation for this activity is that Spr overproduction stimulates the activity
of PG synthases functioning outside of the Rod complex thereby redirecting PG precursors from the crippled Rod complex to functional synthetic machinery.

We recently showed that the SEDS protein RodA serves as the PG polymerase in the Rod system [7] and that the aPBP synthases can operate independently of the cytoskeletally-organized PG synthesis complexes [8]. Based on this observation, we hypothesized that mecillinam suppression by Spr overproduction might be mediated by activation of PG synthesis by the aPBPs. To test this, we took advantage of our in vivo PG labeling system in which we can independently measure the PG biogenesis activity of the Rod system or the aPBPs. For these assays, we use a strain producing a modified PBP1b, referred to as MS\textsuperscript{PBP1b}, that has a Ser247Cys substitution in its PGT domain rendering it sensitive to inhibition by treatment with the cysteine-reactive reagent MTSES (2-sulfonatoethyl methanethiosulfonate) [8]. When cells of this strain are inhibited for cell division by expression of the FtsZ antagonist SulA, total [\textsuperscript{3}H\textsuperscript{-DAP}} incorporation into the PG matrix represents a combination of the activities of the Rod system and MS\textsuperscript{PBP1b} [8]. Upon treatment with the Rod system inhibitor A22, the remaining level of [\textsuperscript{3}H\textsuperscript{-DAP}} incorporation reflects the activity of MS\textsuperscript{PBP1b}, whereas the level of PG synthesis detected in MTSES treated cells is a measure of Rod system activity [8]. Importantly, and as expected based on this line of reasoning, co-treatment with A22 and MTSES completely inhibits all detectable [\textsuperscript{3}H\textsuperscript{-DAP}} incorporation [8].
Overproduction of Spr resulted in a small but reproducible increase in \[^3\text{H}\]-DAP incorporation into the PG matrix in untreated cells relative to those harboring the vector control (Fig. 7B). Strikingly, however, PG synthesis in A22 treated cells more than doubled in cells overproducing Spr, suggesting that elevated endopeptidase activity greatly enhances PG synthesis by MS\text{PBP1b}. Accordingly, this elevated level of incorporation was completely inhibited by simultaneous treatment with A22 and MTSES (Fig. 7B). The observed activation appeared to be specific for aPBP synthase function as Spr overproduction did not enhance label incorporation in cells treated with MTSES alone where PG is primarily being synthesized by the Rod system (Fig. 7B). We therefore infer that elevated PG endopeptidase activity can stimulate PG synthesis by the aPBPs and that crosslink cleavage in the matrix is likely rate limiting for aPBP-mediated PG synthesis (see Discussion).
Fig 7. Spr overproduction stimulates PG synthesis by activating aPBPs.

A. PG matrix assembly and turnover were measured as in Fig 4B and C, using the strain TU278(attHKpHC859)(attλpGL66), with TU278(attHKpHC859)(attλpGL69) as a control. pGL66 encodes spr under IPTG-inducible control.

B. HC533(attHKpHC859)(attλpGL66) (HC533 = ΔlysA ΔampD ΔponA::frt ΔpbpC::frt ΔmtgA::frt MSponB) cells were used to test if Spr activity stimulates PG synthesis through PBP1b activity. Compound concentrations used: mecillinam (10 μg/ml), A22 (10 μg/ml), MTSES (1 mM).
3.3 DISCUSSION

In addition to serving as powerful therapeutics, β-lactams have served as useful probes for uncovering the mechanisms underlying the process of cell wall biogenesis in bacteria. Here, we present the first comprehensive genetic analysis of mecillinam resistance in \textit{E. coli}. Using Tn-Seq, we simultaneously mapped all loci in the genome where gene disruption by transposon insertion promotes survival upon mecillinam challenge. Moreover, we performed the analysis in genetic backgrounds defective for stress responses known to confer mecillinam resistance when they are induced. Thus, we were able to classify all resistance loci according to their stress response dependence, identifying those alleles that most likely promote resistance by activating a stress response and those that confer resistance independent of the responses. We reasoned that many of these stress response independent alleles are likely to provide mecillinam resistance by directly affect the cell wall biogenesis. Accordingly, this class includes mutants inactivated for components of the Rod system and the cell wall cleaving enzyme Slt known to be required for the futile cycle of cell wall synthesis and degradation observed following mecillinam treatment. Further study of mutants in \textit{prc}, another stress response independent allele, led to the discovery that the cleavage of cell wall crosslinks by PG endopeptidases results in the activation of PG synthesis by the aPBP\(_s\).

Because the cell wall matrix is a continuous structure surrounding the cell, it has long been understood cleavage of bonds in the matrix is likely to be required for the insertion of newly synthesized PG to promote surface expansion and cell growth.
However, it was only recently that candidate “space-maker” enzymes required for PG matrix expansion were identified. In *E. coli*, these enzymes are the PG endopeptidases YebA and Spr [19]. Neither enzyme is essential individually, but cells lacking both endopeptidases are inviable on rich medium. Cells depleted of Spr in the absence of YebA stop elongating and eventually lyse. The also show reduced incorporation of radiolabeled PG precursors into the matrix. Thus, it has been clearly established that Spr and YebA are required for cell wall biogenesis. What has remained unclear is how endopeptidase activity is coordinated with cell wall synthase function and whether or not crosslink cleavage by the endopeptidases is rate limiting for cell wall synthesis. Our findings address both of these outstanding issues.

The key result was the demonstration that the overproduction of a variety of different PG endopeptidases from three different protein families promoted resistance to mecillinam. This was a surprising finding because cell wall hydrolase activity is typically thought to promote the lethal and lytic effects of β-lactam antibiotics, not to counteract them [21]. We hypothesized that the increased cleavage of cell wall crosslinks was promoting mecillinam resistance by activating cell wall synthase activity outside of the Rod complex. This activation would effectively redirect cell wall synthesis away from the futile cycle of synthesis and degradation promoted by the mecillinam-targeted Rod system and thereby dampen its toxic effects. Consistent with this idea, Spr overproduction was shown to increase productive PG synthesis in mecillinam treated cells and to reduce the turnover of nascent PG material.
Furthermore, radiolabeling studies monitoring the activity of either the Rod system or the aPBPs showed that Spr overproduction specifically enhanced PG biogenesis by the aPBP enzymes.

The coordination of PG synthase activity with the space making enzymes is commonly believed to be mediated by the formation of a multi-enzyme complex that includes both PG synthase and PG hydrolase activities [26]. However, there is limited support for the existence of such complexes save for a few co-precipitation studies for which the physiological relevance of the detected interactions remain undetermined. Furthermore, our results support an alternative model in which endopeptidases can stimulate PG synthase function without a direct protein-protein interaction. Overproduction of catalytically inactive Spr(C68A) remained capable of stimulating mecillinam resistance provided cells also encoded native functional Spr. We interpret this result to indicate that the overproduced Spr(C68A) overwhelms the Prc protease that degrades Spr, thus elevating the levels of the active protein. In this scenario, the periplasm is flooded with excess Spr(C68A) that would presumably occupy the binding sites of most Spr interacting partners. Thus the active Spr in this context is unlikely to be functioning in complex with a cell wall synthase to promote productive PG assembly in during mecillinam challenge. Similarly, the fact that the overproduction of three different endopeptidases each from a distinct protein family are all capable of promoting mecillinam resistance argues against a specific PG synthase binding partner for the hydrolase.
How then might crosslink cleavage and PG synthase activity be coupled if not via a direct protein-protein interaction? One attractive possibility is via the regulation of the aPBP by their cognate outer membrane lipoproteins. Several years ago, it was discovered that the *E. coli* aPBP, PBP1a and PBP1b, each require a cognate outer membrane lipoprotein activator, LpoA and LpoB, respectively for their in vivo function. It was also shown that these Lpo factors can stimulate the PG synthase activity of their cognate aPBP in vitro. At the time, it was proposed that the Lpo-PBP interaction might function as a “sensor” for the detection of loosely crosslinked areas in the PG matrix. In this scenario, it would be these areas of the matrix where an Lpo protein and partner PBP could span the matrix from opposite membranes to interact, promoting synthesis exactly where it is most needed. Such a mechanism may be at the heart of the activation of aPBP activity we observe upon endopeptidase overproduction.

In conclusion, we have used high-throughput genetic methods to map the mecillinam resistome of *E. coli*. Using several different genetic backgrounds for this analysis, we were able to rapidly identify resistance loci that promote resistance without requiring a functional stringent response or Rcs envelope stress response. Characterization of one of these alleles led to a new fundamental understanding of the cell wall biogenesis process: that crosslink cleavage can stimulate the activity of aPBP synthases and that the synthases need not work in direct physical contact with the endopeptidases to properly coordinate PG synthesis with cleavage. Further studies of other loci identified in the mecillinam resistome should shed further light on the
mechanism of cell wall biogenesis and how best to target the process for the development of new antibiotics capable of defeating resistance.
2.4 MATERIALS AND METHODS

**Media, bacterial strains and plasmids:**

Cells were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Unless otherwise indicated, antibiotics were used at 25 (chloramphenicol; Cm), 25 (kanamycin; Kan), or 5 (tetracycline; Tet) ug/mL.

The bacterial strains used in this study are listed in **Table S1**. All *E. coli* strains used in the reported experiments are derivatives of MG1655 [28]. Plasmids used in this study are listed in **Table S2**. PCR was performed using KOD polymerase (Novagen) for cloning purposes and *Taq* DNA polymerase (NEB) for diagnostic purposes, both according to the manufacturer’s instructions. Unless otherwise indicated, MG1655 chromosomal DNA was used as the template. Plasmid DNA and PCR fragments were purified using the Zyppy plasmid miniprep kit (Zymo Research) or the Qiaquick PCR purification kit (Qiagen), respectively.

**Table S1. Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F− hsdR17 deoR recA1 endA1 phoA supE44 thi-1</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td></td>
<td>gyrA96 relA1 Δ(lacZYA-argF)U169 φ80dlacZΔM15</td>
<td></td>
</tr>
<tr>
<td>TB10</td>
<td>MG1655 λΔcro-bio nad::Tn10</td>
<td>[29]</td>
</tr>
<tr>
<td>MG1655/pTB63</td>
<td>rph-1 ilvG rfb-50 / PftsQAZ::ftsQAZ</td>
<td>[28]</td>
</tr>
<tr>
<td>TB28</td>
<td>MG1655 ΔlacZYA::frt</td>
<td>[30]</td>
</tr>
<tr>
<td>GL38/pTB63</td>
<td>MG1655 ΔrcsB::frt / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Source/ Reference</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>GL44/pTB63</td>
<td>MG1655 ΔrelA::frt / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL74</td>
<td>MG1655 ΔrfaP::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL76</td>
<td>MG1655 ΔrfaH::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL82</td>
<td>MG1655 Δprc::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL112</td>
<td>MG1655 Δmnt::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL120</td>
<td>MG1655 Δefp::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL122</td>
<td>MG1655 ΔsspA::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>AG04</td>
<td>MG1655 Δlpp::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>HC408</td>
<td>MG1655 Δslt::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL101</td>
<td>MG1655 ΔarcA::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL90</td>
<td>MG1655 ΔgcvR::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL92</td>
<td>MG1655 Δcrr::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL70</td>
<td>MG1655 ΔlepA::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL72</td>
<td>MG1655 ΔtrxB::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL124</td>
<td>MG1655 ΔcysE::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL78</td>
<td>MG1655 Δpgm::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL84</td>
<td>MG1655 Δnpl::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL80</td>
<td>MG1655 ΔopgH::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL206</td>
<td>MG1655 ΔnlpD::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL66</td>
<td>MG1655 ΔtufA::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL120</td>
<td>MG1655 Δefp::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL204</td>
<td>MG1655 ΔrcsB::frt ΔrfaP::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL205</td>
<td>MG1655 ΔrcsB::frt ΔnlpD::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL203</td>
<td>MG1655 ΔrcsB::frt Δlpp::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL207</td>
<td>MG1655 ΔrelA::frt ΔtufA::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
</tbody>
</table>

79
### Table S2. Plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL243</td>
<td>MG1655 ΔrelA::frt Δefp::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL62</td>
<td>MG1655 ΔrcsB::frt Δslt::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL180</td>
<td>MG1655 ΔrelA::frt Δslt::KanR / PftsQAZ::ftsQAZ</td>
<td>This study</td>
</tr>
<tr>
<td>GL68</td>
<td>MG1655 Δspr::frt</td>
<td>This study</td>
</tr>
<tr>
<td>GL67</td>
<td>MG1655 ΔponB::frt</td>
<td>This study</td>
</tr>
<tr>
<td>TU278</td>
<td>TB28 ΔlysA::frt ΔampD::frt</td>
<td>[11]</td>
</tr>
<tr>
<td>HC533</td>
<td>TU278 ΔponA::frt ΔpbpC::frt ΔmtgA::frt M8ponB</td>
<td>This study</td>
</tr>
</tbody>
</table>

#### Table S2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Origin</th>
<th>Source / Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSC101</td>
<td>tetA</td>
<td>pSC101</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pTB63</td>
<td>tetA PftsQAZ::ftsQAZ</td>
<td>pSC101</td>
<td>[31]</td>
</tr>
<tr>
<td>pGL69</td>
<td>attλ cat lac² P₂lac</td>
<td>R6K</td>
<td>This study</td>
</tr>
<tr>
<td>pGL70</td>
<td>attλ cat araC P₃ara</td>
<td>R6K</td>
<td>This study</td>
</tr>
<tr>
<td>pGL65</td>
<td>attλ cat araC P₃ara::relA*</td>
<td>R6K</td>
<td>This study</td>
</tr>
<tr>
<td>pGL66</td>
<td>attλ cat lac² P₂lac::spr</td>
<td>R6K</td>
<td>This study</td>
</tr>
<tr>
<td>pGL67</td>
<td>attλ cat lac² P₂lac::spr(C68A)</td>
<td>R6K</td>
<td>This study</td>
</tr>
<tr>
<td>pGL68</td>
<td>attλ cat lac² P₂lac::rcsF</td>
<td>R6K</td>
<td>This study</td>
</tr>
<tr>
<td>pHC859</td>
<td>attHK tetA lac² P₂lac::sulA</td>
<td>R6K</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Suppressor selection**

The strains interrogated were mutagenized with the Ez-Tn5 <Kan-2> transposome (Epicentre) as previously described [30]. Mutants were selected for kanamycin.
resistance at 30°C, yielding libraries ranging from ~100,000 to ~400,000 independent transposon insertions. These mutant libraries were harvested, plated on LB agar with 0, 1.0, 2.5, or 10 µg/ml mecillinam and incubated at 30°C to isolate mecilliam suppressors. The frequency at which survivors arose ranged from $10^{-4}$ to $10^{-3}$ for the transposon libraries, depending on the genetic background, and the frequency of spontaneous suppressors is consistently about 10 fold lower. Suppressor libraries were harvested for transposon sequencing.

**Transposon Sequencing**

Genomic DNA was extracted from the suppressor libraries using Wizard Genomic DNA Purification Kit (Promega). Tn-seq sequencing libraries were prepared by a modified version of a published protocol [17]. Genomic DNA was digested using NEBNext dsDNA Fragmentase (NEB) for 25min at 37°C. Fragmented DNA was purified with 1.8× volume Agentcourt AMPure XP beads (Beckman Coulter, Inc.) and eluded into 32uL water.

Purified fragmented DNA was then treated with terminal deoxynucleotidyl transferase (TdT; Promega) in a 20uL reaction with 1uL 9.5mM dCTP/0.5mM ddCTP, 4uL 5x TdT reaction buffer and 0.5uL rTdT at 37°C for 1h, then at 75°C for 20min. TdT-treated DNA was purified with Performa DTR Gel Filtration Cartridge (EdgeBio). Purified, TdT-treated DNA was used as a template in a PCR reaction to amplify the transposon junctions using the Easy-A Hi-Fi Cloning System (Agilent Technologies). The primers used are
PolyG-1st-1 5’- 
GTGACTGGAGGATGCCAGTGTGCTCTTCGATCTGGGAGGGGGGGGGGGGGGGG-3’
and Tn5-1st-1 5’-ACCTGCAGGATGCAAGCTTCAGGG-3’
A second nested PCR was next performed to further amplify the transposon 
junctions and append the sequencing barcode. The primers used are generic 
NEBNext Multiplex Oligos for Illumina (NEB) and 
Tn5-2nd-1 5’- 
AATGATACGGCGACCACCAGATCTACACTCTTTTCAGGGTTGAGATGTGTATAA 
GAGA-3’.
The final library was run on a 2% agarose gel, size-selected between 200-500bp, 
and gel purified using QIAquick Gel Extraction Kit (Qiagen). Libraries were 
sequenced at the Tufts University Core Facility on a HiSeq 2500 (Illumina) on a 
1× 100 single end run.

Reads were mapped to the E. coli MG1655 genome (NCBI NC_000913), and genes in 
which reads were overrepresented were identified by calculating the fold change 
enrichment under mecillinam conditions relative to LB conditions. Genes that were at 
least 4-fold enriched in reads are listed in Tables 1 & 2. Visual inspection of transposon 
insertion profiles was performed with the Sanger Artemis Genome Browser and 
Annotation tool.

Measurement of PG synthesis and turnover
The effect of Spr overproduction on PG synthesis and turnover in β-lactam-treated E. coli cells was examined essentially as described previously [8]. HC533(attλHC739), a ΔlysA ΔampD strain, was grown overnight in M9-glycerol medium supplemented with 0.2% CAA. The overnight culture was diluted to an optical density at 600 nm (OD_{600}) of 0.04 in the same medium and grown to an OD_{600} of between 0.26 and 0.3. Divisome formation was then blocked by inducing sulA expression for 30 min from a chromosomally integrated P_{lac}::sulA construct (pHC739) by adding IPTG to 1 mM. After adjusting the culture OD_{600} to 0.3, MTSES (1 mM), A22 (10 μg/ml), mecillinam (10 μg/ml) and/or cefsulodin (100 μg/ml) were added to the final concentrations indicated and cells were incubated for 5 min. Following drug treatment, 1 μCi of [³H]-meso-2,6-diaminopimelic acid (mDAP) was added to 1 ml of each drug-treated culture and incubated for 10 min to label the newly synthesized PG and its turnover products. After labelling, cells were pelleted, resuspended in 0.7 ml water, and heated at 90 °C for 30 min to extract water-soluble compounds. After hot water extraction, insoluble material was pelleted by ultracentrifugation (200,000g for 20 min at 4 °C). The resulting supernatant was then removed, lyophilized and resuspended in 0.1% formic acid for HPLC analysis and quantification of turnover products as described previously. To determine the [³H]-mDAP incorporated into the PG matrix, the pellet fraction was washed with 0.7 ml buffer A (20 mM Tris-HCl, pH 7.4, 25 mM NaCl) and resuspended in 0.5 ml buffer A containing 0.25 mg lysozyme. The suspensions were incubated overnight at 37 °C. Insoluble material was then pelleted by centrifugation (21,000g for 30 min at 4 °C) and the resulting supernatant was mixed with 10 ml EcoLite (MP
Biomedicals) scintillation fluid and quantified in a Microbeta Trilux 1450 liquid scintillation counter (Perkin-Elmer).

**MIC determination**

Overnight culture was normalized to an optical density $OD_{600}$ of 0.1, inoculated in warm H-top agar and then applied on LB plates as a thin layer. MIC Test Strip (Liofilchem) was then applied on the inoculated agar surface and incubated for 18h.
2.5 REFERENCES


CHAPTER 3:

Visualization of rare spontaneous defects in bacterial envelope assembly
ATTRIBUTIONS

The work presented in this chapter was performed primarily by Ghee Chuan Lai. Somenath Bakshi contributed imaging tools and assisted initial microscopy experiments. All remaining experiments were performed and generated by Ghee Chuan Lai. The manuscript was written by Ghee Chuan Lai, Thomas Bernhardt and Johan Paulsson.
CHAPTER 3:

Visualization of rare spontaneous defects in bacterial envelope assembly

Ghee Chuan Lai$^{1,2}$, Somenath Bakshi$^2$, Johan Paulsson$^2$, and Thomas G. Bernhardt$^1$

$^{1}$Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115
$^{2}$Department of Systems Biology, Harvard Medical School, Boston, MA 02115

Bacteria live in fluctuating environments and have evolved various stress responses to endure diverse insults. The bacterial cell envelope is the first and major line of defense against the environment, and envelope stress responses dynamically monitor and respond to perturbations in the cell envelope. While envelope stress responses have been studied in the context of exogenous insult or genetic mutation, the basal rate at which envelope defects arise during normal growth of wild-type cells has not been measured. Moreover, the quality control factors that limit defect formation are largely unknown. We therefore used fluorescent protein reporters driven by stress response promoters and a microfluidic device to follow the spontaneous induction frequencies of envelope stress responses in unperturbed *Escherichia coli* cells growing over many generations in balanced growth. Rare spikes in activity of the Rcs stress response were observed (1/1000 generations) and correlated with the appearance of membrane blebs in the affected cells. The induction events were largely dependent on a functional sensor RcsF and were abolished upon inactivation of the core signaling proteins RcsC and RcsB. Finally, several cell wall synthesis or remodeling proteins with no observable morphological phenotypes or clearly defined roles in cell morphogenesis were inactivated and shown to increase the frequency of Rcs activation events. Among these factors was the lytic transglycoslayse Slt, which was previously implicated as a quality
control protein for cell wall biogenesis. The results thus suggest our monitoring system is a powerful tool for the identification of additional quality control factors that facilitate proper cell wall assembly by reducing the error rate.
3.1 INTRODUCTION

The bacterial cell envelope marks the interface between the cell and its environment. This complex multilayered structure is essential for maintaining cellular integrity and protects the cell against external insults. In gram-negative bacteria, the cell envelope consists of the outer and inner membranes and a thin layer of peptidoglycan sandwiched between them [1]. The outer membrane, an asymmetric lipid bilayer comprised of LPS and phospholipids, forms a permeability barrier against antimicrobial substances while allowing entry of nutrients [2]. The inner membrane phospholipid bilayer is the site of many essential cellular processes, including energy production and transport of nutrients and waste products in and out of the cytoplasm [3]. Finally, the peptidoglycan layer, also known as the cell wall, determines cell shape and fortifies the cell against osmotic lysis [4].

Bacteria have evolved to survive in variable and at times extreme environments and must sense and effectively respond to perturbations in the cell envelope to maintain its integrity. In gram-negative bacteria such as *Escherichia coli*, five envelope stress responses (ESRs) have been identified: the σE, Rcs, Cpx, Bae and Psp responses [5-12]. These systems monitor the envelope status and mediate adaptive changes by concomitantly activating large sets of genes that primarily encode extracytoplasmic factors [12,13]. However, mounting a costly response in the absence of stress will negatively impact cellular fitness, since the resources wasted on unnecessary adaptations could otherwise be used for growth and division [14]. For example, *cpxA* mutants constitutively activated for the Cpx stress responses suffer from growth defects.
As such, envelope stress responses systems have to be highly regulated so that they are activated only when necessary and to the degree sufficient for alleviating the stress.

$\sigma^E$, Rcs (Regulator of colanic acid capsule synthesis) and Cpx (Conjugative plasmid expression) responses are most well-characterized amongst the envelope stress responses in *E. coli*. The $\sigma^E$ pathway is regulated via sequestration by a membrane bound anti-sigma factor that is proteolytically inactivated in response to specific signals. It is known to maintain homeostasis of the outer membrane by sensing concomitant defects in outer membrane protein (OMP) and lipopolysaccharide (LPS) assembly [15-17,32] and controlling the levels of LPS and OMP production as well as the production of the corresponding assembly machineries accordingly [33].

Both Cpx and Rcs responses are two-component systems activated by cell envelope and cell wall stress [18,19,27-31]. A typical two-component system is comprised of a transmembrane sensor histidine kinase and a cytoplasmic response regulator that is activated through phosphorylation. The Cpx system is a classical two-component system with CpxA as the histidine kinase and CpxR as the cognate response regulator [7]. In contrast, the Rcs system relies on a complex phosphorelay involving RcsC, RcsD and RcsB proteins. RcsC is an inner membrane histidine kinase that is thought to autophosphorylate itself in response to environmental signals [6]. The phosphoryl group is then relayed to a second inner membrane protein RcsD, and then finally to RcsB, a cytoplasmic response regulator. Phosphorylation of RcsB homodimers allows it to bind
to target promoters on the chromosome via DNA-binding domains and regulate gene expression. Additionally, the Rcs phosphorelay requires other auxiliary proteins for normal functioning of the system. RcsF is an outer membrane lipoprotein sensor. When it detects envelope defects, it inactivates the IgaA inhibitor of RcsC, thus initiating the signaling cascade [6,28,34-38] . Another auxiliary factor is RcsA, which forms heterodimers with phosphorylated RcsB to regulate a subset of genes in the Rcs regulon, including those involved in capsule biogenesis [39, 6].

Both Cpx and Rcs stress responses are activated by cell wall stress caused by beta-lactam drugs that target cell wall syntheses called penicillin-binding proteins (PBPs) [18] and in a variety of mutants lacking certain cell wall enzymes [19]. However, it remains unclear how they sense and respond to peptidoglycan damage. While only Cpx activation is known to directly regulate some peptidoglycan biosynthetic enzymes [30], the Rcs system is required for de novo shape recovery post cell wall destruction by lysozyme treatment [29], and Rcs inactivation results in beta-lactam hypersensitivity [18].

Envelope stress responses have typically been studied in the context of externally applied insults (e.g. heat or ethanol) or following genetic inactivation or overexpression of envelope components. Although useful, many of these stimuli often induce multiple stress response pathways, making it difficult to discern the precise function of each pathway [8,18-20]. Moreover, these conditions are unlikely to reflect the normal biological context in which the stress response systems are engaged. Instead, a more
plausible scenario given the complexity of coordinately assembling multiple envelope layers is that spontaneous damage arises as a result of the biogenesis machinery malfunctioning. However, the frequency with which such problems are encountered during normal balanced growth has not been measured. It is also not known which response system(s) may be principally tasked with alleviating the stress resulting from these problems or what the cellular repertoire of quality control factors is that may act to reduce the rate of spontaneous damage.

Given that envelope biogenesis is such a critical cellular process, the occurrence of spontaneous envelope defects in the absence of applied stress is likely to be rare. Therefore, a method capable of detecting a very low frequency of envelope problems is required. We reasoned that a microfluidic device called the “mother machine” [25] provides the perfect platform to detect these rare events using the spontaneous induction of envelope stress responses as a proxy for damage. This device facilitates long-term observation of hundreds of individual cells for many generations in a stable nutrient-rich environment (Figure 1). To monitor envelope damage, strains producing fluorescent reporters driven by σE, Rcs, or Cpx responsive promoters were generated and monitored in the mother machine for spontaneous induction events. The Rcs system was the only one that displayed rare sporadic spikes of induction. Importantly, these events were correlated with the formation of membrane blebs emanating from the affected cells. The induction events were largely dependent on a functional sensor RcsF and were abolished upon inactivation of the core signaling proteins RcsC and RcsB. Finally, several cell wall synthesis or remodeling proteins with no observable
morphological phenotypes or clearly defined roles in cell morphogenesis were inactivated and shown to increase the frequency of Rcs activation events. Among these factors was the lytic transglycoslayse Slt, which was previously implicated as a quality control protein for cell wall biogenesis. The results thus suggest our monitoring system is a powerful tool for the identification of additional quality control factors that facilitate proper cell wall assembly by reducing the error rate.
3.2 RESULTS

Visualizing rare spontaneous defects in envelope assembly

To monitor the spectrum and frequency of spontaneous envelope defects, we used a variant of the “mother machine” [25] to track cells for extended durations during balanced growth. The device is adapted to accommodate multiple strains in parallel with each cell channel well separated from its neighbors to minimize fluorescent halo effects [Fig1A]. To aid cell segmentation, our host strain of wild-type *E. coli* MG1655 was engineered to constitutively express mCherry-mKate2 hybrid marker from a chromosomal locus. The flagellin gene from this strain was also deleted to prevent cells from swimming out of the channels. We focused our analysis of envelope stress response induction to the three most well characterized systems: $\sigma^E$, Rcs, and Cpx. A set of strains, each with a different stress responsive promoter driving the expression of a rapidly maturing YFP variant was therefore constructed. For the $\sigma^E$ response, we chose the P3 promoter of the *rpoH* gene ($P_{rpoH}$-p3::yfp) encoding the heat-shock sigma factor $\sigma^{32}$. To follow induction of Rcs, we used the promoter of the *rprA* sRNA gene ($P_{rprA}$::yfp), and for the Cpx response, the *cpxP* promoter ($P_{cpxP}$::yfp) was used.

Each reporter strain was grown in the microfluidic device in EZRDM (EZ Rich Defined Media, Teknova) at 37°C and approximately 200 cells were monitored for ~50 generations to detect spontaneous induction events of the respective stress responses. Rare pulses of YFP expression were only observed for the $P_{rprA}$::yfp reporter strain [Fig1B] at a frequency of roughly one induction event per 1000 generations. Because the spikes in *rprA* transcription were rare and sporadic, we first tested whether these
Fig 1A. Tracking spontaneous envelope defects in *Escherichia coli*. Schematic of the microfluidic channels in which individual bacteria are held. Mother cell that experience envelope stress will appear as yellow as shown above.

Fig 1B. Rare pulses of *rprA* induction in unperturbed cells. Representative envelope stress response activity traces of cells for 12h. We use $P_{\text{rpoH}}\cdot p_3::yfp$, $P_{\text{cpxP}}::yfp$ and $P_{\text{rprA}}::yfp$ transcriptional reporters to follow induction of $\sigma^E$, Cpx and Rcs response respectively.
spikes were due to noisy gene expression inherent to the rprA promoter or were driven by an activated stress response. To do so, we constructed a dual reporter strain encoding identical rprA promoters driving either YFP or CFP (P_{rprA}::yfp P_{rprA}::cfp) and measured the transcription activities of both reporters simultaneously. If the pulses of YFP and CFP expression in the dual reporter strain are uncorrelated, the results would suggest the pulses are the result of transcriptional noise. Conversely, if the reporters are highly correlated, this would suggest that the pulses are driven by an activated stress response. Strikingly, the pulse profiles in both the YFP and CFP channels of the dual reporter strain were virtually identical and correlated in time [Fig1C].

Another indication that P_{rprA}::yfp activity was reflecting real envelope stress induction events rather than transcriptional noise is that it was correlated with the appearance of envelope blebs in the affected cells [Fig1D]. All cells with a bleb had high P_{rprA}::yfp activity, and half of the induced cells exhibited blebs. The latter correlation is likely an underestimate because the imaging will only reliably detect blebs emerging from the sides of the cell, while blebs on the top and bottom surfaces are likely to escape detection. Based on the linked activity of the dual P_{rprA} reporter constructs and the high correlation of P_{rprA}::yfp activity with envelope blebs, we conclude that the observed induction events are likely to reflect rare problems in envelope assembly encountered during steady-state growth.
Fig 1C. rprA induction spikes are driven by an activated stress response. Transcriptional activity traces of dual reporter $P_{rprA}::yfp$ $P_{rprA}::cfp$ strain (125 cells, 30h) are perfectly correlated.
Fig 1D. *rprA* induction events correlate with appearance of membrane blebs. Kymograph showing a single mother cell (top cell in each frame) spontaneously blebbing and activating *rprA* transcription. Top kymograph corresponds to the cytoplasmic segmentation channel, bottom kymograph corresponds to the *rprA* transcriptional activity or YFP channel. Time between frames is approximately 7min.

**Investigating the origins of spontaneous envelope stress response activation**

Induction of RprA sRNA expression has recently been shown to be activated by either the Rcs or Cpx stress responses [18,24], despite it being a canonical reporter for Rcs activation [6]. To determine which of these systems is responsible for the observed pulses of *rprA* activation, we monitored *rprA* promoter activity in cells of *rcsB* and *cpxR* deletion mutants. While *cpxR* deletion mutant still displayed *rprA* pulses [Fig2A], pulses were never observed in cells lacking RcsB, confirming that the spikes of *rprA* transcription are driven by the Rcs stress response.

We wondered if the inducing signal for Rcs activation is channeled through the entire signaling cascade, or if it is mediated by an abbreviated or alternative pathway. To this end, we monitored *rprA* promoter activity in deletion mutants inactivated for RcsF, RcsC, and RcsA. RcsF, the outer membrane sensor was found to be largely required for the observed pulses in *rprA* transcription. Proper transport of RcsF to the outer membrane and its assembly into complexes with porin proteins is required to prevent its inhibition of IgA and activation of the Rcs cascade. Thus, the observed activation events are likely to result from a defect in outer membrane or peptidoglycan assembly that prevents proper RcsF transport. In the *rcsC* mutant, *rprA* pulsing was abolished and the
Fig 2A. *rprA* induction spikes are driven by the Rcs stress response. *rprA* transcriptional activity traces of WT, ΔrcsB and ΔcpxR deletion mutants.

Fig 2B. Chasing the signal down the Rcs phosphorelay pathway. *rprA* transcriptional activity traces of WT, ΔrcsC, ΔrcsF and ΔrcsA deletion mutants.
mean rprA transcription level was constitutively high [Fig2B], suggesting that RcsC primarily acts as a phosphatase of RcsB to reduce basal rprA expression. RcsA inactivation did not appreciably affect the frequency of rprA expression pulses observed, suggesting that that the auxiliary transcriptional activator does not exert indirect effect on rprA expression by titrating phosphorylated RcsB away from its homodimeric form. Based on the results from the Rcs inactivation mutants, we conclude that the observed P_{rprA::yfp} activation events result from a signaling stimulated by the RcsF sensor and require both the histidine kinase RcsC and the response regulator RcsB. Thus, all indications are that the pulses of rprA expression reflect spontaneous induction of the Rcs response mediated by “unforced” errors in envelope biogenesis.

**Increased frequency of P_{rprA::yfp} induction in mutants defective in peptidoglycan remodeling functions**

Like many bacteria, *E. coli* encodes dozens of enzymes that synthesize, cleave, or otherwise modify the cell wall. Mutants inactivated for any single enzyme with a known or predicted activity for peptidoglycan synthesis or remodeling typically do not display any overt growth or morphological defects. Multiple mutations are normally required to reveal problems in shape maintenance or wall integrity. This paucity of phenotypes has made it difficult to assign a clear physiological function to peptidoglycan modifying enzymes.

In a recent study of the killing mechanism of beta-lactam antibiotics, a role for the peptidoglycan cleaving enzyme Slt in degrading uncrosslinked glycans produced by
drug targeted cell wall synthesizing complexes was revealed [58]. Mutant defective for
Slt undergo lethal morphological changes at low doses of beta-lactams and are
hypersensitive to these drugs. Analysis of the cell wall composition in these mutants
showed that the uncrosslinked glycans formed following beta-lactam treatment were
misincorporated into the cell wall matrix by alternative crosslinking enzymes. This
observation led to the proposal that Slt may be functioning as a quality control enzyme
that intervenes when the normally tight coupling between peptidoglycan polymerization
and crosslinking is disrupted.

The assignment of a quality control function in cell wall biogenesis for Slt is based solely
on the behavior of drug treated mutant cells. Mutants defective for Slt do not display any
problems with cell wall synthesis under normal growth conditions. Thus, if Slt is truly
needed to deal with defects in the coupling of peptidoglycan polymerization and
crosslinking, these problems must arise infrequently and may be difficult to detect.
Given its demonstrated ability to detect rare spontaneous envelope defects, we thought
our P_{prA::yfp} monitoring system would be ideal for testing the hypothesis that Slt
functions as a quality control factor for peptidoglycan biogenesis even in the absence of
drug treatment.

Strikingly, we observed that slt mutant display more frequent and often larger YFP
pulses in our monitoring system. [Fig3A]. Our finding is therefore consistent with the
model in which Slt operates as a quality control enzyme to resolve problems arising
**Fig 3A.** Δslt mutant display more frequent and often larger YFP pulses. rprA transcriptional activity traces of WT, Δslt deletion mutants.

from rare uncoordinated peptidoglycan polymerization and crosslinking events during unstressed balanced growth conditions.

Encouraged by our results on Slt, we decided to test other mutants in cell wall biogenesis factors that lack observable phenotypes. We surveyed a representative collection of cell wall enzyme mutants from different enzymatic classes and monitored their rprA transcriptional activity in unperturbed cells. Of the ten mutants we sampled, seven (ponA, ponB, slt, mltG, mepS, dacA, dacB, amiA) exhibited more frequent rprA induction events, while the remaining two (yebA, ynhG) are appreciably similar to the wild-type control [Fig3B]. PBP, lytic transglycosylase, endopeptidase, carboxypeptidase, and amidase mutants were represented amongst these seven mutants, confirming that most if not all enzymes classes interrogated have a nonessential enzyme that play a quality control function at normal balanced growth.
Fig 3B. Surveying frequency of rprA induction events in a panel of cell wall enzymes.
Enzyme classes include bifunctional PBPs (PonA, PonB), endopeptidases (Spr, YebA), carboxypeptidases (DacA, DacB), lytic transglycosylases (Slt - in Fig 3A, MltG), amidase AmiA and L,D transpeptidase YnhG.
One notable cell wall mutant of mention that display more frequent \textit{rprA} induction events is \textit{mltG}. MltG was recently identified as a potential terminase during peptidoglycan polymerization in bacteria [59]. Mutants lacking MltG activity were shown to have longer glycans in their PG relative to wild-type cells, suggesting that MltG controls glycan strand length during normal growth, which might have an impact on preserving cell wall integrity. However, it has been difficult to show a functional physiological significance for MltG in the absence of stress, given that loss of MltG function is neither lethal nor associated with a morphology or growth defect. This also holds true for double mutants of MltG in combination with other LTs. Our monitoring system was sensitive enough to identify MltG as a potential quality control factor to facilitate proper cell wall assembly, therefore consistent with the model that MltG suppresses problems arising from unregulated peptidoglycan polymerization.

Cell wall enzymes that were previously implicated to play more important roles in cell wall biogenesis than their redundant counterparts consistently displayed more frequent and higher levels of spontaneous Rcs induction. This was evident from our \textit{rprA} promoter activity measurements in cell wall mutants that exhibit synthetic lethal phenotypes. PBP1a and PBP1b, encoded by \textit{ponA} and \textit{ponB} respectively, operate as a synthetic lethal pair of PG synthases necessary for cell growth [60-61]. Although both enzymes appear to be largely interchangeable to support growth, \textit{ponB} mutants are known to be hypersensitive to beta-lactams and synthetic lethal with many genes coding for factors involved in septal PG biogenesis unlike their \textit{ponA} cousins, suggesting that PBP1b has additional functional roles \textit{in vivo} yet been determined. We
detected more frequent and larger spikes of rprA transcription in ponB cells, while ponA cells display considerably similar frequency and levels of rprA promoter activity as wild-type cells. The same trend was also observed in PG hydrolase synthetic lethal pair MepS and MepH. Together, these findings support the model that cell wall synthase and hydrolase synthetic lethal pairs might not be truly redundant, since their individual loss leads to difference consequences.

Similar to wild-type cells, rprA induction events are also dependent on a functional sensor RcsF in PG remodeling enzyme mutants, as demonstrated by the ponB mutant [Fig3C]. ponB cells also exhibit more frequent blebs with corresponding Rcs activation, indicating disruption in cell wall integrity. While we are not able to determine the exact molecular origin of the inducing signal in both ponB mutant and wild type cells, our findings further support the model that rprA induction is a bona fide readout for envelope biogenesis errors, and this inducing signal is largely detected by RcsF. Overall, our combined results from a wide spectrum of cell wall mutants suggest our P_rprA::yfp monitoring system is robust in identifying factors likely to be involved in quality control or repair, which have been difficult to detect otherwise.
Fig 3C. *rprA* induction is a bona fide readout for PG biogenesis errors which are also sensed by RcsF.
3.3 DISCUSSION

Construction of the cell envelope of gram-negative bacteria involves the coordinate activity of many multi-protein complexes. Similar to chromosome replication, cells have evolved mechanisms to ensure that this process proceeds with high fidelity. The availability of sophisticated genetic selections and screens combined with DNA sequencing has enabled the detection of rare errors in DNA replication and the identification of repair and quality control activities that limit them. However, similar methods to detect rare errors in envelope assembly have not been available, and this deficit has hampered our understanding of the process and what makes it so robust. Here, we report a method combining the stable environmental control in microfluidics systems with fast-maturing fluorescent protein reporters driven by stress response promoters that are intrinsically sensitive to cell envelope status to detect rare errors in envelope biogenesis, which manifest as transient but distinct bright cells in a dark sea of isogenic cousins.

Peptidoglycan quality control factors

Using this assay, we observed infrequent spontaneous rprA induction spikes in unperturbed wild-type cells, confirming that envelope biogenesis errors arising from uncoordinated envelope machineries do happen, albeit rarely, as expected for such a critical cellular process. More frequent spikes of the similar nature were detected in cell wall mutants whose function were previously implicated to be quality control factors facilitating growth and division. Moreover, Rcs activation in these bleb-containing cells is consistent with previous findings that the Rcs phosphorelay responds to peptidoglycan
stress. Together, these results support the model that \textit{rprA} induction is a bona fide readout for envelope biogenesis errors, and we were able to take advantage of this interesting phenotype to identify important but under-appreciated cell wall enzymes from a diverse set of enzyme classes in nutrient-rich balanced conditions.

**Genesis of the rapid kinetics of \textit{rprA} transcriptional pulses**

The pulse-like profile of \(P_{\text{rprA}}::yfp\) activation events is striking. Induction is extremely rapid, occurring within less than a generation, during which the YFP signal sharply increases to more than five times its basal level. This induction time-scale is likely an underestimate due to the maturation delay of YFP. The shut-off of transcription appears equally fast, with the long tail of fluorescence signal decay being largely due to dilution of the stable YFP by division. It is likely that the true decay rate of the relatively unstable \textit{rprA} sRNA is much faster and potentially as rapid as the observed induction. To our knowledge, such rapid kinetics of sRNA induction and decay have not been observed previously. We therefore investigated whether this behavior is a general property of spontaneous rRNA induction events and what factors might be responsible for the rapid kinetics of the observed pulses. We tested reporter fusions to the promoters of the sRNA genes: \textit{dsrA}, \textit{oxyS} and \textit{micL}. DsrA, like \textit{rprA}, alters the secondary structure of the \textit{rpoS} leader sequence, thereby stabilizing the mRNA for subsequent translation of the stationary phase and general stress sigma factor \textit{RpoS} [50]. OxyS is produced during oxidative stress to protect cells against oxidative damage [51]. One of its targets is also \textit{rpoS}, but it has been suggested that OxyS represses \textit{RpoS} mRNA translation by sequestering the sRNA chaperone Hfq rather than by directly binding to \textit{rpoS} mRNA.
Finally, MicL is recently shown to specifically repress translation of outer membrane lipoprotein Lpp, one of the most highly expressed proteins in E. coli [53]. Thus, although MicL regulates a cell envelope factor, it does not affect the expression of RpoS. When we examined promoter activities of these sRNAs in unperturbed cells during balanced growth for extended durations, no pulses of activation were observed. Thus, under the conditions used, the responses governing expression of these additional sRNAs must not be induced even at a low frequency. Therefore until such conditions are identified, we are unable to make any definitive comparisons to the induction behavior of \textit{rprA}.

We also explored the role of other potential regulators of RprA in the sharp induction and deactivation of \textit{rprA} transcription. It is known that RpoS and RprA levels increase sharply during stationary phase [56, 57]. We therefore reasoned that RprA might be involved in a positive feedback loop, with its target RpoS further activating RprA expression following its initial induction. This model could explain the sharp induction kinetics of \( P_{\text{rprA}::\text{yfp}} \) activation. To test this, we measured the induction kinetics in wild-type and an \textit{rpoS} mutant. Strong differences in the \( P_{\text{rprA}::\text{yfp}} \) transcriptional profile were not observed when RpoS was inactivated [Fig 4]. Thus, other factors must be responsible for the extremely rapid induction kinetics of \( P_{\text{rprA}::\text{yfp}} \), and we are in the process of testing several additional candidates.

Nonetheless, our monitoring system is an effective tool for studying low frequency errors in envelope assembly, and will be useful to further definition of quality control and
repair factors in PG biogenesis and assembly of other envelope layers. The cell envelope in gram-negative bacteria is extremely complex, both in structure and function. While most essential envelope biogenesis systems have been identified and characterized, there are still significant gaps in our knowledge of how the individual players fit together and how they are controlled and coordinated to maintain a uniform contiguous envelope during bacterial growth and division [63]. Given that over a quarter of the protein-coding genes is predicted to have either a signal peptide or at least one transmembrane region [64], and over a third of these have yet to be functionally characterized [65], there remain many factors important for coordinating envelope biogenesis waiting to be discovered. Because envelope stress responses are highly sensitive to the state of the cell envelope, studying them can often unveil mechanisms that regulate cell envelope homeostasis. Even though we have specifically used rprA promoter pulsing or Rcs induction as a readout for interrogating cell wall enzymes, we believe this assay can be readily extended to identify quality control factors of envelope biogenesis in E. coli and other organisms with established stress response reporters.
3.4 MATERIALS AND METHODS

Media, bacterial strains and Plasmids

Cells were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl), or EZ Rich Defined Media (Teknova) supplemented with 0.2% glucose and 0.8% Pluronic F108 (Sigma Aldrich, included as a surfactant). Unless otherwise indicated, antibiotics were used at 25 (chloramphenicol; Cm), 25 (kanamycin; Kan), 50 (ampicillin; Amp), 5 (tetracycline; Tet) ug/mL.

The bacterial strains used in this study are listed in Table S1. All *E. coli* strains used in the reported experiments are derivatives of MG1655 [67]. Plasmids used in this study are listed in Table S2. Plasmids were constructed either using standard restriction enzyme-based cloning methods, or by the isothermal (Gibson) assembly method [68]. PCR was performed using Q5 High-Fidelity DNA Polymerase (NEB) for cloning purposes and *Taq* DNA polymerase (NEB) for diagnostic purposes, both according to the manufacturer’s instructions. Unless otherwise indicated, MG1655 chromosomal DNA was used as the template. Plasmid DNA and PCR fragments were purified using QIAprep Spin Miniprep Kit (Qiagen) and Qiaquick PCR purification kit (Qiagen) respectively.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype(^a)</th>
<th>Source/Reference(^b,c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>(F^{-}\ hsdR17\ deoR\ recA1\ endA1\ phoA\ supE44\ thi-1\ gyrA96\ relA1\ Δ(lacZYA-argF)U169\ φ80lacZΔM15)</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>MG1655</td>
<td>(rph-1\ ilvG\ rfb-50)</td>
<td>[67]</td>
</tr>
<tr>
<td>TB10</td>
<td>MG1655 (\lambdaΔcro-bio\ nad::Tn10)</td>
<td>[69]</td>
</tr>
<tr>
<td>RY34</td>
<td>TB10 (ΔmltG::Cm)</td>
<td>[59]</td>
</tr>
<tr>
<td>NDL156</td>
<td>TB10 (glmS::P_{RNA1}-mCherry/mKate2-frt-Kan(^R)-frt)</td>
<td>Gift from Nathan Lord</td>
</tr>
<tr>
<td>GL201</td>
<td>MG1655 (glmS::P_{RNA1}-mCherry/mKate2-frt)</td>
<td>P1(NDL156) x MG1655</td>
</tr>
<tr>
<td>GL208</td>
<td>GL201 (ΔfliC::frt)</td>
<td>P1(ΔfliC::Kan(^R)) x GL201</td>
</tr>
<tr>
<td>GL211</td>
<td>GL208 attTn7::(P_{cpxP}-mVenus)</td>
<td>GL208/pGL54</td>
</tr>
<tr>
<td>GL215</td>
<td>GL208 attTn7::(P_{rprA}-mVenus)</td>
<td>GL208/pGL58</td>
</tr>
<tr>
<td>GL220</td>
<td>GL208 attTn7::(P_{null}-mVenus)</td>
<td>GL208/pGL63</td>
</tr>
<tr>
<td>GL221</td>
<td>GL208 attTn7::(P_{poH-P3}-mVenus)</td>
<td>GL208/pGL64</td>
</tr>
<tr>
<td>GL250</td>
<td>GL208 attTn7::(P_{cpxP}-mVenus\ (ΔcpxA::Kan(^R)))</td>
<td>P1(ΔcpxA::Kan(^R)) x GL211</td>
</tr>
<tr>
<td>GL258</td>
<td>GL215 (ΔponB::frt)</td>
<td>P1(ΔponB::Kan(^R)) x GL215</td>
</tr>
<tr>
<td>GL277</td>
<td>GL215 (ΔrcsF::frt)</td>
<td>P1(ΔrcsF::Kan(^R)) x GL215</td>
</tr>
<tr>
<td>GL281</td>
<td>GL215 (ΔrcsF::frt\ (ΔponB::Kan(^R)))</td>
<td>P1(ΔponB::Kan(^R)) x GL215</td>
</tr>
<tr>
<td>GL291</td>
<td>GL208 attTn7::(P_{poH-P3}-mVenus\ (ΔrseA::Kan(^R)))</td>
<td>P1(ΔrseA::Kan(^R)) x GL221</td>
</tr>
<tr>
<td>GL293</td>
<td>GL215 (ΔrcsB::frt)</td>
<td>P1(ΔrcsB::Kan(^R)) x GL215</td>
</tr>
<tr>
<td>GL294</td>
<td>GL215 (ΔrcsC::frt)</td>
<td>P1(ΔrcsC::Kan(^R)) x GL215</td>
</tr>
<tr>
<td>GL296</td>
<td>GL215 (Δslt::frt)</td>
<td>P1(Δslt::Kan(^R)) x GL215</td>
</tr>
<tr>
<td>GL315</td>
<td>GL208 attTn7::(P_{rprA}-mVenus\ (P_{rprA}-mSCFP))</td>
<td>GL208/pGL74</td>
</tr>
<tr>
<td>GL316</td>
<td>GL208 attTn7::(P_{lacUV5}-mVenus\ (P_{lacUV5}-mSCFP))</td>
<td>GL208/pGL75</td>
</tr>
<tr>
<td>GL318</td>
<td>GL208 attTn7::(P_{lacUV5}-mVenus\ (P_{rprA}-mSCFP))</td>
<td>GL208/pGL77</td>
</tr>
<tr>
<td>GL321</td>
<td>GL208 attTn7::(P_{micL}-mVenus)</td>
<td>GL208/pGL83</td>
</tr>
<tr>
<td>GL322</td>
<td>GL208 attTn7::(P_{dsrA}-mVenus)</td>
<td>GL208/pGL84</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Source/Reference&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>GL323</td>
<td>GL208 attTn7::P&lt;sub&gt;oxyS&lt;/sub&gt;-mVenus</td>
<td>GL208/pGL85</td>
</tr>
<tr>
<td>GL325</td>
<td>GL208 attTn7::P&lt;sub&gt;Null&lt;/sub&gt;-mVenus P&lt;sub&gt;Null&lt;/sub&gt;-mSCFP</td>
<td>GL208/pGL90</td>
</tr>
<tr>
<td>GL329</td>
<td>GL215 Δspr::ftr</td>
<td>P1(Δspr::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL330</td>
<td>GL215 ΔrcsA::ftr</td>
<td>P1(ΔrcsA::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL360</td>
<td>GL215 ΔdacA::ftr</td>
<td>P1(ΔdacA::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL361</td>
<td>GL215 ΔdacB::ftr</td>
<td>P1(ΔdacB::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL362</td>
<td>GL215 ΔycbB::ftr</td>
<td>P1(ΔycbB::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL363</td>
<td>GL215 ΔynhG::ftr</td>
<td>P1(ΔynhG::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL364</td>
<td>GL215 ΔrpoS::ftr</td>
<td>P1(ΔrpoS::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL366</td>
<td>GL215 ΔmltG::ftr</td>
<td>P1(RY34) x GL215</td>
</tr>
<tr>
<td>GL367</td>
<td>GL215 ΔamiA::ftr</td>
<td>P1(ΔamiA::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL368</td>
<td>GL215 ΔcpxR::ftr</td>
<td>P1(ΔcpxR::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL369</td>
<td>GL215 ΔponA::ftr</td>
<td>P1(ΔponA::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL370</td>
<td>GL215 Δprc::ftr</td>
<td>P1(Δprcs::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
<tr>
<td>GL371</td>
<td>GL215 ΔyebA::ftr</td>
<td>P1(ΔyebA::Kan&lt;sup&gt;R&lt;/sup&gt;) x GL215</td>
</tr>
</tbody>
</table>

<sup>a</sup> The Kan<sup>R</sup> and Cm<sup>R</sup> cassettes are flanked by ftr sites for removal by FLP recombinase.

<sup>b</sup> Strain constructions by P1 transduction are described using the shorthand: P1(donor) x recipient. Unless otherwise listed as a strain, all donors are deletion alleles either sourced from the Keio knockout collection [70] or constructed to resemble those in the collection. Transductants were selected on LB Kan, LB Cm or LB Tet plates where appropriate. Unless otherwise indicated in the genotype, all strains were removed of the cassette using FLP expressed from pCP20, leaving a ftr scar. Diagnostic PCR was performed to confirm no inversion during recombination in strains with multiple ftr scars.

<sup>c</sup> Strains resulting from Tn7 integration are indicated as: Parental strain/plasmid (see Tn7 integration for details)
Table S2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Origin</th>
<th>Source / Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCP20</td>
<td><em>bla</em> <em>cat</em> cI857&lt;sup&gt;ts&lt;/sup&gt; <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; P&lt;sub&gt;AR::flp&lt;/sub&gt;</td>
<td>pSC101</td>
<td>[69]</td>
</tr>
<tr>
<td>pGRG37</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attR</em> (Gateway cassette) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>[71]</td>
</tr>
<tr>
<td>pNDL1</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attB</em> (Gateway cassette) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>Gift from Nathan Lord</td>
</tr>
<tr>
<td>pGL54</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;cpxP·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL58</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;rprA·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL63</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;null·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL64</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;pohH·P3·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL71</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;psl·mVenus&lt;/sub&gt; P&lt;sub&gt;psl·mSCFP&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL74</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;prrA·mVenus&lt;/sub&gt; P&lt;sub&gt;prrA·mSCFP&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL75</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;lacUV5·mVenus&lt;/sub&gt; P&lt;sub&gt;lacUV5·mSCFP&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL77</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;lacUV5·mVenus&lt;/sub&gt; P&lt;sub&gt;prrA·mSCFP&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL83</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;micL·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD·tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL84</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;daRA·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD·tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL85</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;oxyS·mVenus&lt;/sub&gt;) P&lt;sub&gt;BAD·tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
<tr>
<td>pGL90</td>
<td><em>bla</em> <em>oriT</em> <em>repA</em>&lt;sup&gt;ts&lt;/sup&gt; <em>mTn7::attL</em> (P&lt;sub&gt;null·mVenus&lt;/sub&gt; P&lt;sub&gt;null·mSCFP&lt;/sub&gt;) P&lt;sub&gt;BAD-tnsABCD&lt;/sub&gt;</td>
<td>pSC101</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> *flp* encodes for FLP recombinase.
Tn7 integration

Tn7 integrations were performed as follow [71]. Briefly, the target *E. coli* strain was transformed with the integration plasmid and grown overnight at 30°C on LB Amp plates. Transformants were restreaked on fresh LB Amp and grown again at 30°C. The integration plasmid was then cured by growing overnight at 37°C and confirming sensitivity to ampicillin. Successful integration was then verified by colony PCR.

Mother machine device preparation

Chips were prepared as described previously [72], with little modifications. Briefly, dimethylsiloxane monomer (Sylgard 184) was mixed in a 10:1 ratio with curing agent, poured onto the silicon wafer master, degassed and cured at 65°C. Holes to connect the feeding channels to fluidics were then punched using a biopsy punch, and individual chips were cut and bonded onto KOH-cleaned cover slips using oxygen plasma treatment (30s at 50W with O2 at 170 mTorr) on the day of the experiment. Bonded chips were baked at 95°C for 30min before use.

Cell preparation for imaging

*E coli* cells were grown to late stationary phase in EZRDM to decrease their size and improve their efficiency of loading in the cell channels. They were then centrifuged at 500g for 30s, resuspended in one tenth their original volume, and injected into the feeding lane. After filling all feeding lanes with appropriate cell suspensions, the device was mounted into a standard benchtop microcentrifuge using a custom adaptor, and cells were spun into the cell channels by centrifuging at 5000g for 5min. The device was
then connected to peristaltic pumps using Tygon tubing, which were in turn linked to bottles containing EZRDM media. Both pumps and media containing bottles were encased in a custom-built incubator maintained at 37°C, linked to the temperature-controlled chamber encasing the microscope at the same temperature. Fresh media was pumped into the device initially at 50uL/min for 1h to flush the unloaded cells in the feeding lanes, and subsequently reduced to 5-8uL/min.

**Microscopy and image acquisition**

Imaging was performed on a Eclipse Nikon Ti inverted microscope equipped with a temperature-controlled chamber, automated xy-stage (Ludl), a 60X Plan Apo oil objective (numerical aperture 1.4, Nikon), an Orca R2 CCD camera (Hamamatsu) and a light engine LED excitation source (Lumencor). The following filter sets were used for acquisition: RFP (Semrock mCherry-A), YFP (Semrock YFP-2427A) and CFP (Semrock CFP-2432A). All experiments were performed at 37°C. Image acquisition was performed using MATLAB scripts interfacing with μManager. Typical exposure was kept at low illumination intensities and acquisition periods of 100ms to reduce photobleaching. Cells were allowed to equilibrate in the device for several hours before starting imaging, and all data prior to the first pulse in each lineage was ignored subsequent analysis. Images were taken every 6-8min and saved as 16-bit TIFFs. Focal drift was controlled via the Nikon PerfectFocus system, as well as a custom autofocus routine based on z-stack images of a sacrificial position in each feeding lane.
Image Analysis and Lineage Tracking

Adaptive thresholding was first used to identify cells from the grayscale images of segmentation channel, followed by the marker-controlled watershed algorithm to separate connected masks of adjacent cells.

The mother machine consists of parallel channels, each containing cells derived from a single mother cell resting at the bottom of the channel. First, we used a clustering algorithm to identify the cell clusters residing in individual channels. Within each cell cluster, we identified the mother cell from its centroid position (cell at the bottom) and create a segmentation mask of the mother cell. Using these raw masks as an initial guess, we refined the mask quality by performing local segmentation operation using active contouring to optimize the segmentation boundaries. This creates a mother cell mask with sub pixel smoothness and accuracy.

The operations as described were performed on every mother cell in each frame/time point. A simple particle tracking algorithm based on nearest neighbor each was applied to connect related masks across different time points, thereby obtaining lineage information for each mother cell during the entire experiment.
3.5 REFERENCES


CHAPTER 4:

Discussion
4.1 SUMMARY OF RESULTS

Chapter 2

The downstream events following β-lactam inhibition of PBPs are not clearly defined. We reasoned that suppressors will provide more insight on how cell wall biogenesis might overcome β-lactam stress. β-lactams not only inhibit essential PBPs, but also induce a lethal malfunctioning of their target cell wall biosynthetic machinery. To this end, we uncoupled the two processes by identifying all genetic determinants of mecillinam resistance under conditions where the Rod system is not essential, and functionally dissected these suppressors based on their dependence on various stress responses for resistance. Amongst the stress response-independent suppressors, we isolated a loss of function mutation in prc, which encodes a protease that was previously implicated to degrade an important cell wall endopeptidase Spr. This suggests that mecillinam resistance arising from Prc inactivation might be operating through the excess Spr substrates available. Overproduction of Spr confirms this hypothesis and mecillinam resistance is dependent on the catalytic activity of Spr. Subsequent genetic analyses and PG turnover assays support the model that endopeptidases can stimulate class A PBP (aPBP) activity to promote cell wall synthesis during normal growth, and that crosslink cleavage by endopeptidases (EP) is rate limiting. EPs were previously implicated as important space makers during cell growth, but the mechanism by which cell wall expansion is achieved is not understood. Our findings provide evidence and additional molecular details for a ‘cut-and-insert’ strategy mediated by EPs and aPBPs during cell wall expansion.
Cell wall expansion during growth requires coordinated concurrent expansion of its surrounding envelope layers. Like DNA replication, cells have evolved strategies to ensure this crucial process is carried out with the greatest fidelity. But no process is perfect. DNA replication is extraordinarily accurate, but rare errors have been detected from genetic screens and selections coupled with DNA sequencing as a tractable readout. No similar readouts for envelope biogenesis errors have been described. Because envelope stress responses are intrinsically tuned to cell envelope physiology, we employed them as biological probes to detect stresses from within by tracking their spontaneous activation using transcriptional fluorescent reporters in cells maintained in a constant stress-free environment. Rare spikes of \textit{rprA} promoter activity were observed and correlated with the appearance of membrane blebs in the affected cells. Dual reporter measurements confirmed that these spikes are driven by an activated stress response. These activation events require a functional Rcs signaling pathway and are largely dependent on a functional RcsF sensor. More frequent spikes of a similar nature were detected in cell wall mutants exhibiting no observable growth and morphological phenotypes, but were previously implicated to be quality control factors facilitating growth and division. Together, our findings establish \textit{rprA} induction as a bona fide readout for cell envelope biogenesis errors, and demonstrate that this system is robust in identifying elusive quality control factors in cell envelope biogenesis that suppress rare errors.
4.2 Endopeptidases cuts PG crosslinks before aPBPs insert new PG

During growth, bacteria must carry out the delicate task of expanding, remodeling and degrading PG, while maintaining the integrity of this essential force-bearing structure to avoid lysis. There are two primary strategies in which PG synthases and hydrolases can cooperate to expand the cell wall. Hydrolases can first cleave existing bonds within the PG matrix to provide sites for subsequent insertion of new PG material by synthases. This is the essence of the ‘cut-and-insert’ strategy proposed by Park [1,2]. Alternatively, new PG material can first be patched and temporarily crosslinked to the PG matrix by synthases before cleaving the crosslinks of the old intermediate glycan strand along the boundaries of the patch. Release of the old strand will pull the docked patch into the existing PG layer, since the cell wall is always under high lateral stress due to the cell’s high internal osmotic pressure. The latter strategy is often referred to as the “make-before-break” strategy first proposed by Holtje [3-5].

These models have been proposed decades ago, but there is little experimental evidence to distinguish them. Both require a similar core set of enzymes operating in different order, and there are currently no means to resolve such a dynamic and connected process. Our combined genetic and PG turnover results provide evidence that bond cleavage by Spr is rate limiting during cell wall expansion mediated by aPBPs. Increasing the activity of aPBPs via over-expression of PBP1b or its LpoB-bypass variant only partially suppresses mecillinam at best (data not shown), consistent with the cut-and-insert strategy.
4.3 EPs and aPBPs function as separate but highly efficient entities for a common purpose

Besides distinguishing the models, our findings also shed significant new light on the process of cell wall expansion. Previous protein-protein interaction studies via affinity chromatography revealed a group of PG synthases and hydrolases interacting with each other, potentially as a multi enzyme complex [6,7]. Furthermore, this group of enzymes embodies the common set of enzymatic specificities outlined by either cell expansion models, leading to the proposal of a murein replication holoenzyme model [8]. Here, we show that Spr does not appear to require a cofactor for its activity, nor operate as a stable multiprotein complex with other PG enzymes during cell growth. Even though coordination of PG synthases and hydrolases is paramount to ensure survival during growth, it appears that efficient coordination between multiple enzymes can still be achieved without the formation of multiprotein complexes.

Such a setup is plausible for the following reasons. First, Spr is highly expressed according to ribosome profiling and western blot results [9,10], and is therefore not a limiting factor during cell wall expansion. Second, aPBPs have been demonstrated to be highly dynamic even at limiting concentrations [11]. The Lpo activators for the corresponding aPBPs might only traverse the loosely cross linked areas in the PG matrix to be quickly recognized by aPBPs. The diffusive property of aPBPs and the role of Lpo activators as “markers” could allow aPBPs to efficiently detect loosely crosslinked areas for subsequent gap filling. If both Spr and PBP1b activities are not limiting, coordinated cell expansion can still be achieved without physical linkage.
between multiple PG enzymes. This is also consistent with the model proposed by Koch [12], that the crosslink status of PG might be regulating Spr activity indirectly, be it the local crosslink density or the conformational accessibility of crosslinks for efficient Spr cleavage. Therefore, it appears that a highly intricate process involving multiple individual enzymes can be maintained if it is not limited by enzymatic activities but is regulated by crosslink ('substrate') availability.

It is possible that Spr and PBP1b might not act as a dedicated hydrolase-synthase pair under normal physiological conditions, given that most of our results were obtained under the condition where Spr is overproduced. However, we favor the idea that these proteins are most likely working together during normal growth for the following reason: both ponB and spr mutants are not only mecillinam hypersensitive, they also display rapid lysis when sub-inhibitory concentrations of mecillinam is added (data not shown). This strongly implies that Spr and PonB are operating together, and that PBP1b is not merely functioning as a repair enzyme to cope with the increased hydrolytic activity of over-expressed Spr.

4.4 Investigating the extent of endopeptidase-Rod/aPBP system coordination during cell elongation

Cell wall expansion is mediated by SEDS proteins working within cytoskeletal machineries and aPBPs functioning semi-autonomously [11]. The Rod system is one such cytoskeletal machinery responsible for maintaining proper rod shape in E. coli. spr cells are slightly wider and shorter (data not shown), suggesting that Spr might be
involved in stimulating the activities of both the Rod system and aPBP activities. Accordingly, we also observed a mild increase in PG synthesis when Spr is overproduced in a division-inhibited system devoid of aPBP activity. The only mild increase in PG synthesis observed could indicate that the Rod system is less proficient in sensing or reaching cleavage sites when they are generated in excess. In support of this hypothesis, the Rod complex moves in a directed circumferential motion, and is therefore less dynamic than aPBP [11]. Regardless, it is unknown to what extent Spr is responsible for stimulating the Rod system or aPBP under normal conditions. It would be interesting to directly measure Spr contribution to each system using in vivo PG polymerization assays coupled with a rapid Spr depletion system, such as an engineered MSspr allele that is disrupted of its catalytic activity upon MTSES treatment. We have also noticed that many endopeptidases, for example YebA, can suppress mecilliam when overexpressed. Both YebA and Spr are synthetic lethal under fast growth conditions [13], indicating that these enzymes are major space-makers during growth. Along the same lines, we can start to address the contributions of YebA to the Rod system and aPPB activity using PG polymerization assays and an equivalent YebA depletion system.

4.5 Interrogating additional layers of endopeptidase-aPBP coordination

Cutting the existing matrix and inserting new PG material requires the concerted action of endopeptidases (EPs), glycosyltransferases (GTs), transpeptidases (TPs) and lytic transglycosylases (LTs). We have identified the specific enzymes (Spr and PBP1b) for the first three enzyme classes, but it is not clear which lytic transglycosylase is
responsible for terminating glycan strands polymerized by PBP1b. Previous work in our lab has shown that MltG works in proximity to PBP1b based on bacterial two hybrid assays [14]. MltG might work with Spr and PBPB1b to coordinate PG synthesis and hydrolysis during growth. We can confirm this hypothesis or identify other LTs of unknown physiological functions in a ‘sensitized’ strain overproducing Spr. Already, overproduction of Spr requires PBP1b activity for viability. We will test if overproduction of Spr requires MltG activity or other LTs for viability.

We can also extend this approach to identify the minimal set of PG enzymes working together with Spr and PBP1b during cell wall expansion by performing a negative Tn-seq selection in a strain containing two integrated inducible copies of Spr. In this system, we will identify inactivating mutations that kill the cell when Spr is overproduced. We expect to get \textit{lpoB} and \textit{ponB} as candidates, and they will serve as our positive controls. These candidates should act downstream of Spr activity, and could play a role facilitating efficient polymerization and crosslinking by PBP1b during cell wall expansion.

Finally, we can perform suppressor analysis in a non-viable strain devoid of PBP1b and overproducing two integrated copies of Spr to identify quality control factors mitigating dysregulated crosslink cleavage. We might expect to obtain quality control factors affecting or directing Spr activity, gain-of-function PG synthases that complement the absent PBP1b activity, or important envelope stress response activated repair pathways.
4.6 How are rprA transcriptional spikes generated?

As mentioned previously in Chapter 3, the pulse-like profile of rprA promoter activation events is striking. Conventional bulk methods measuring sRNA transcript levels would have failed to detect these rare rprA inductions in single cells. The general short-time dynamics of sRNA in response to internal stimuli has not been described. We therefore investigated whether this behavior is a general property of spontaneous rRNA induction events. We have examined promoter activities of several sRNA candidates but did not observe any distinct pulse-like transcriptional profiles. Screening for conditions under which sRNAs are spontaneously induced might be prohibitively tedious. However, we can examine the promoter activities of Rcs-regulated genes to test if they also display a similar induction profile. If they do, this would suggest that the pulse-like transcription activation and shut-off is general in Rcs signaling. If they do not, this implies the induction behavior could be unique to rprA (or sRNA, since we cannot make any definitive conclusions with our sRNA data). In all experiments, we will examine the transcriptional profiles based on their rate and duration of induction before transcriptional shutoff, and the magnitude of these inductions.

4.7 What is the physiological function of the Rcs stress response during spontaneous envelope defects?

Given that we only observed spontaneous Rcs activation in unperturbed cells amongst the surveyed envelope stress responses, the Rcs stress response must serve a
physiological purpose to cope with envelope biogenesis errors. The Rcs system could play an active role by up-regulating proteins that directly repair these errors and/or undertake a more passive role by temporarily buffering the cell against the envelope defects while other repair enzymes or quality control factors are employed via other means to resolve these errors. We believe that the Rcs system is more likely to be assuming a passive role for the following reasons. First, we did not observe significantly different rprA pulse features in our diverse panel of cell wall mutants. If the Rcs system were to directly employ a dedicated PG repair enzyme via transcriptional up-regulation, it should have been reflected in one of our candidates as an altered rprA transcription induction profile, perhaps a sharp induction over a significantly longer delay before transcriptional shutdown. However, it is certainly possible that this missing Rcs-regulated PG enzyme lies beyond our panel of test candidates. It should be noted that the repair enzymatic function can also be relieved by redundant PG enzymes. Second and more importantly, transcriptional regulation by stress response systems may be too slow to effectively resolve the stress due to transcription and translation delays. Rather, it is more plausible that activated envelope stress responses issue slow but broad transcriptomic changes to dynamically adjust cellular processes and therefore buffer the envelope while the stress is actively resolved by other effector protein(s).

The Rcs system regulates the expression of a large set of genes, and indirectly controls RpoS, the master regulator of the general stress response [15]. We have previously tested an rpoS mutant and observed appreciably similar rprA induction frequencies and spike profiles. We also did not detect significant differences in a rcsA mutant, which
suggests that the repair/buffer pathway might not be RcsA-dependent. We will next test to see if RprA itself is part of the effector repair/buffer pathway by monitoring \textit{rprA} induction events in the deletion mutant. There is a distinct possibility that there is ultimately no single effector pathway during Rcs activation; all the pathways of the Rcs regulon could be integrated to mitigate the stress. Regardless, it would be exciting to identify the repair/buffer pathway downstream of Rcs induction, if it exists, and we have been reasonably judicious in our choice of candidate mutants.

4.8 Applying our imaging screen to answer other PG biogenesis questions

The mother machine is a powerful and versatile platform that allows high-throughput observation of individual cells for quantitative conclusions of a cell’s physiology. Here, we took advantage of its high-throughput nature and harnessed it as an imaging screen to detect rare envelope biogenesis errors reported by stress response reporters. Armed with the right probes, the same approach can be easily extended to address other cell wall biogenesis questions.

One longstanding question is with regards to PG turnover during growth and division. A remarkable 60\% of the lateral cell wall is degraded and recycled each generation [16]. In contrast, PG at the cell pole is completely resistant to turnover [17,18]. It is not clear why polar PG is stable, and which factors are required for polar PG stability. We can start to address this question by using fluorescent D-amino acids to label the sites of active PG synthesis in single cells and track cell wall synthesis dynamics in the mother
machine. Within the microfluidic device, mother cells are trapped at the bottom of cell channels while newborn cells grow, divide and are eventually pushed out of the cell channels. This means that each mother cell will always possess an old cell pole touching the bottom of the residing cell channel. Since polar PG is stable to hydrolysis, these old cell poles will not be labeled by fluorescent D-amino acids. Therefore, mother cells exposed to fluorescent D-amino acids will always have a dark cell pole facing the bottom of each well, unless the cell pole is not inert.

As a first pass test, we can pre-label cells with fluorescent D-amino acids and screen for rare events where the mother cell possess an unstable and therefore labeled cell pole. We can also apply the same monitoring strategy on different cell wall mutants to identify enzymes required for polar PG stability, which will display increased unstable/bright cell poles when inactivated. It is likely that these enzymes are LD-transpeptidases, given that these enzymes have been shown to be involved in septal PG synthesis [19], and no LD-endopeptidases have been reported so far. This is certainly a very exciting experiment to attempt.

4.9 Conclusion

Although PG biogenesis has been extensively studied over the years, we are only beginning to uncover the mechanisms involved in cell wall expansion and distinguish models proposed by researchers decades ago. At the same time, we are also starting to uncover important physiological functions in highly redundant enzymes that display no observable growth or morphological phenotypes. We expect the future of PG biogenesis
research to be an exciting one. A better understanding of cell wall metabolism will not only advance our knowledge of this fundamental biological process, but will also uncover new vulnerabilities in the cell wall for the development of new classes of antibacterial therapies.
"I wonder what's on TV now."

- Calvin and Hobbes.
4.10 REFERENCES


