Role of EnvC and the phosphoenolpyruvate:sugar phosphotransferase system in resistance to MreB disruption

Short title: EnvC and the PTS in resistance to MreB disruption

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Abstract

Cell wall synthesis in bacteria is determined by two protein complexes: the elongasome and divisome. The elongasome is coordinated by the actin homolog MreB while the divisome is organized by the tubulin homolog FtsZ. While these two systems must coordinate with each other to ensure that elongation and division are coregulated, this cross talk has been understudied. Using the MreB depolymerizing agent, A22, we found that multiple gene deletions result in cells exhibiting increased sensitivity to MreB depolymerization. One of those genes encodes for EnvC, a part of the divisome that is responsible for splitting daughter cells after the completion of cytokinesis through the activation of specific amidases. Here we show this increased sensitivity to A22 works through two known amidase targets of EnvC: AmiA and AmiB. In addition, suppressor analysis revealed that mutations in the phosphoenolpyruvate:sugar phosphotransferase system (PTS) can suppress the effects of A22 in both wild-type and envC deletion cells. Together this work helps to link elongation, division, and metabolism.
Introduction

Bacteria come in a variety of shapes and sizes that are determined by the peptidoglycan cell wall. One of the more common shapes is a bacillus, or rod shape. Rod shape is regulated by the actin homolog, MreB. In addition to MreB many proteins are needed to form and maintain a rod shape. Together these proteins are termed the elongasome and are made of penicillin binding proteins (PBP), SEDS proteins (shape, elongation, division, and sporulation) and different accessory factors, such as MreC, MreD, and RodZ (1-8). MreB is thought to act as a scaffold to organize and direct the localization of the cell wall synthesis enzymes to maintain rod shape (9-12).

MreB is highly conserved among bacteria and can be found in both Gram-positive and Gram-negative species. MreB forms short polymers on the inner surface of the cytoplasmic membrane (8, 13, 14). Loss of mreB leads to spherical cells that eventually lyse, unless grown very slowly or with suppressor mutations, such as the overexpression of the cell division genes, ftsZAQ (15). To avoid accumulating suppressor mutations, the MreB depolymerizing agent A22 is often used to study the role of MreB in cell shape and physiology (16, 17).

We have previously explored conditions in which Escherichia coli cells are better able to withstand the loss of MreB function. Using A22 as a method for disrupting MreB we have shown that MreB and the elongasome in general are dispensable for growth but not cell shape once cells reach a threshold density (18). In addition, we have shown that mutations in metabolic genes that lead to an increase in cell wall precursors allow cells to better tolerate A22 treatment (19). To further understand the role of MreB in cell physiology we sought to find mutants that lead to an increase in sensitivity to A22 treatment.

Here, we used the Keio collection to find mutants that grow poorly in low levels of A22 and found that deletion of five genes result in reduced growth when MreB is disrupted (20). One of these genes, envC, is an activator of the cell wall amidases, AmiA and...
AmiB, which are needed for cell separation after division (21, 22). EnvC is part of the divisome, a separate cell wall synthesis complex used during cell division that is distinct from the MreB elongasome (23). We show that the increased A22 sensitivity is due to the lack of AmiA and AmiB activity and can be suppressed by mutations in MreB or deletion of enzyme 1 of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). This work further connects central metabolism and cell size.

Results

Deletions of diverse genes lead to A22 sensitivity

MreB is an essential gene for determining rod shape in many bacteria. It is conditionally essential for viability, as mutations that upregulate the cell division genes ftsZAQ or very slow growth can suppress the lethality of mreB deletions without restoring rod shape (15). It is unknown if there are genetic conditions that make cells more susceptible to disruption of MreB. It is also unclear if MreB plays other roles in the cell, independent from regulating rod shape. We have previously reported that two gene deletions, envC and tusE, result in cells with increased sensitivity to A22, although the mechanism for this is unknown (18). Interestingly, deletion of either of these genes leads to slower growth, a condition normally thought to promote growth without MreB (Fig S1) (18).

In order to determine if MreB has other cellular roles we performed a screen of the Keio collection, an ordered library of E. coli mutants, to find strains that are more sensitive to growth in low levels of A22, an MreB depolymerizing drug (16, 17, 20). The Keio collection was grown in either LB medium or LB supplemented with a non-lethal amount of A22 (1 µg/ml). At this concentration of A22, wild-type (WT) cells grow close to the same rate as in LB alone; therefore, we looked for deletion strains that have reduced growth in A22 compared to LB alone to determine which, if any, gene deletions lead to increased sensitivity to MreB disruption. After three trials, any deletion that had reduced growth in A22 compared to LB in more than one trial was transduced from the Keio collection into MG1655, the WT E. coli strain used in our lab.
Five gene deletions were found to have an increased sensitivity to A22 (Table S1, Fig.1A). To further characterize this sensitivity, these strains were grown in low (1 µg/ml) or high (10 µg/ml) levels of A22 for six hours. As the different mutant strains may have reduced growth rate in LB alone, the O.D.₆₀₀ of cells grown in A22 was compared to growth in LB medium alone to produce a growth ratio. In this way we can measure the specific effect of A22 on growth for each strain. WT MG1655 cells grow to nearly the same density in six hours in LB or low A22, producing a growth ratio ~0.9, but have about a 50% reduction in growth when grown in high levels of A22 (Fig. 1A). All five strains from our screen display lower growth in high levels of A22 with three stains also having reduced growth in low levels of A22. In support of our findings (∆secB), defects in the Sec system have been shown to cause increased sensitivity to A22 (24). The envC mutant displays the most dramatic phenotype, with almost no growth in high levels of A22.

EnvC is an activator of cell wall hydrolases that are needed to separate daughter cells after division (21, 25). Due to the role of EnvC in cell division and therefore possible connection to MreB and the severe growth defect seen in both low and high levels of A22, we focused on determining why deletion of this gene leads to increased sensitivity to MreB disruption. To confirm that deletion of envC is responsible for the observed A22 sensitivity, we complemented envC on an arabinose inducible plasmid. Induction of plasmid-born envC fixes the growth defect in A22 (Fig. S1).

A22 sensitivity in the envC mutant is caused by the loss of AmiAB activity

EnvC works by activating two cell wall hydrolases, AmiA and AmiB (21). To determine if the A22 sensitivity of the envC mutant is due to the lack of activation of AmiA and/or AmiB we made deletions of both genes individually and together. Deletion of either amiA or amiB alone has no effect on sensitivity to low levels of A22 (Fig. 1B). However, the double deletion strain is even more sensitive to A22 than the envC deletion and did not grow at low levels of A22 (Fig. 1B). This suggests that the sensitivity of the envC mutant to A22 is due to the lack of AmiAB activation and not a secondary role of EnvC.
AmiA and AmiB are not the only cell wall hydrolases in *E. coli*. AmiC is a third hydrolase that is activated by the protein NlpD (21, 22). Because loss of *amiA* and *amiB* causes cells to become sensitive to A22, we tested whether loss of AmiC has any effect. Cells lacking AmiC show a mild increase in sensitivity to A22 (Fig. 1B). We further analyzed double mutants of *amiAC* and *amiBC* as well as the triple *amiABC* mutant for A22 sensitivity. The *amiAC* mutant is even more sensitive than the *amiC* mutant alone, but not nearly as sensitive as either the *envC* deletion or *amiAB* mutant. As expected, the triple mutant shows an extreme sensitivity phenotype similar to the double *amiAB* mutant and did not grow in A22 (Fig. 1B). These results suggest that activation of the hydrolases AmiA and AmiB is most important when MreB is disrupted.

As might be expected, the *envC* and *amiAB* deletions result in similar phenotypes of elongated and chained cells (Fig. 2A) (21). As both strains are sensitive to A22 we examined all the strains under the microscope when grown with and without A22 to determine what effect A22 has on cell shape. All cells were grown in LB for two hours before A22 (10 µg/ml) was added and cells were allowed to grow for another two hours. An LB only control was allowed to grow for four hours in total. As expected, WT cells become spherical upon A22 treatment. A similar change in shape was observed for all strains (Fig. 2A). The *amiAB* double deletion exhibits a similar phenotype to the *envC* mutant both with and without A22. Interestingly, the *amiAC* deletion strain exhibits a more pronounced chaining phenotype than the *amiAB* mutant when grown in LB (Fig. 2A) (26). This suggests that the increased sensitivity of the *envC* and *amiAB* mutants to A22 is not due to those cells being chained or elongated, as the *amiAC* strain is more resistant to A22 than either of those strains and forms longer chains (Fig. 1B and 2A).

**Loss of amidase activity increases sensitivity to envelope targeting antibiotics.**

It is possible that the above phenotypes are due to a general mechanism increasing cell permeability rather than a specific MreB mechanism. It has been shown that deletion of *envC* causes an increase in permeability as seen by sensitivity to vancomycin and bacitracin, cell wall targeting antibiotics that normally do not pass through the outer
membrane (27). This permeability is thought to work through the amidases as mutants in different amidases also lead to increases in cell permeability (27-29).

To test if the envC mutant is more sensitive to all antibiotics, we tested for growth in a low and high amount of ampicillin, tetracycline, and chloramphenicol. The low amount is below the inhibitory concentration for WT cells and the high amount shows a significant decrease in growth of WT cells. The envC mutant only displays an increased sensitivity to ampicillin, the cell well targeting drug, but not the ribosomal targeting antibiotics (Fig. S2A). These data suggest that loss of amidase activity leads to increased sensitivity to envelope targeting antibiotics in E. coli, but not a generalized sensitivity to all antibiotics; therefore, we do not believe cell permeability is the cause of the increased sensitivity.

**Disruption of MreB reduces chaining caused by loss of amidase activity**

It is well established that envC or amidase mutants form chains of cells when grown in LB (22, 25, 26, 30). This chaining phenotype is not recapitulated in A22. After A22 treatment, WT, envC, and amiAB strains all have ~70% of their cells alone or in pairs, with a small percentage of cells found in clusters of larger groups. However, the amiAC mutant has ~30% of its cells found in large (>7 cells) clusters (Fig. 2B). These data suggest some crosstalk between MreB and the cell separation machinery that requires AmiA. When MreB is polymerized (LB), the lack of AmiA and AmiB leads to chained cells, but when MreB is depolymerized (A22 treatment) there is no additional chaining. However, in either a polymerized or depolymerized state of MreB cells that lack AmiA and AmiC form chains. This suggests that the loss of AmiA in parallel with the loss of another amidase produces different phenotypes depending on the polymerization state of MreB, but if AmiA is functional chaining is reduced independent of the state of MreB, explaining why there is no chaining in an amiBC mutant.

We further characterized the effects of A22 on each of these mutants by measuring the change in rod shape and size of the cells. Using the metric, coefficient of variation of intracellular diameter deviation (IDD), we can measure the “rodness” of cells (4, 12, 18).
A centerline is drawn through the long axis of the cell and the diameter is measured across the cell body. The change in the standard deviation of these diameters is used to determine “rodness”, as a sphere will have a greater standard deviation than a rod. All strains tested become spherical upon A22 treatment (increased IDD) (Fig. 2C). As cell length will affect the IDD value (longer cells reduce the influence of the poles) and envC cells are longer (Fig. S3) we did not attempt to statistically compare IDD values across strains. Not only do all cells treated with A22 become spherical, but there is a concurrent increase in cell area (Fig. 2D). Interestingly, the increase in area upon A22 treatment is smaller in ∆envC cells than WT cells (Fig. 2E).

**A22 sensitivity caused by loss of cpxR is not due to changes in amidase expression**

The CpxR/CpxA two-component system has been shown to positively regulate the expression of both amiA and amiC (31). As a mutant of cpxR was found to be more sensitive to A22 (Fig. 1A) and an amiAC double mutant is more sensitive to A22 (Fig. 1B) we asked if the sensitivity of the ∆cpxR deletion strain was due to loss of amiA and/or amiC expression. We cloned amiA, amiB, and amiC into the vector pTrc99A under the control of a leaky lac promoter and attempted to suppress the A22 sensitivity of the ∆cpxR strain through ectopic expression of any of the amidases. While there does appear to be some effect of the empty vector on A22 sensitivity, expression of any amidase was unable to restore growth in A22 to WT levels in the cpxR mutant (Fig. S4). This suggests a distinct mechanism of A22 sensitivity in the ∆cpxR strain.

**Suppressor mutations in the envC deletion strain can grow in A22**

We have shown that an envC deletion is more sensitive to A22 treatment in an AmiAB dependent manner; however, it is still unclear why the loss of AmiAB function results in increased A22 sensitivity. The envC mutant does not grow in high levels of A22, whereas WT cells are able to grow (Fig. 1). We performed a random suppressor screen, by plating the envC mutant on A22 plates to find mutants that restore growth. Colonies
that grew on A22 plates were streaked onto fresh A22 plates before being grown in liquid cultures with A22, at 37°C. These experiments were performed independently three times. As a control of the acquisition of non-allele specific mutations that make the cells resistant to A22, we moved a known A22 resistant MreB point mutation (MreB_{S14A}) into the envC deletion (12). This resistant envC strain is able to grow in A22 and has an elongated/chained phenotype when grown in LB, although the poles appear to taper. Any colony that grew throughout this selection process was tested for its A22 growth ratio in low and high levels of A22.

We classified the suppressor mutations into three categories based on A22 resistance. Group 1 consists of four mutants (#s 2, 3, 5, 27) that display a high level of resistance, most similar to the WT or ΔenvCmreB_{S14A} strains. Group 2 mutants (#s 7, 15) do not show much change in low levels of A22 but are able to grow in A22 10 µg/ml, unlike the parental envC deletion. Group 3 (#s 8, 10, 38) consists of mutants that grow very well in low A22 with only show a small increase in growth at higher A22 levels (Fig. 3).

To help determine the function of these suppressor mutations we imaged all strains in LB to compare their cell shapes with WT, ΔenvC, and ΔenvCmreB_{S14A} stains, and sequenced the MreB. Five of the nine suppressors have MreB point mutations (Table S2). Interestingly, all group 1 mutants have MreB point mutations, although mutants #5 and #27 have unique shapes that deviate from the shape of the envC mutant, suggesting that these mutations have a function distinct from generic A22 resistance. The other MreB point mutant is in group 3 (#8) and results in loss of the elongation and chaining seen when envC is removed. This mutant and mutant #27 have a missense mutation in residues close to each other yet have different A22 resistance profiles (Table S2).

We performed whole-genome sequencing on all mutants without an MreB mutation (#s 7, 15, 10, 38) and the three mutants with MreB point mutations with shapes that deviate from the MreB_{S14A} control (#s 5, 27) or are in a different resistance group (#8) (Table
Due to their similar shape and resistance profile to \( \Delta envCmreB_{S14A} \), we did not perform whole-genome sequencing on strain 2 and 3.

Both strains in group 2 have an insertion in \( ptsI \), encoding for enzyme 1 of the PTS (32).

The three mutants in group 3 have no mutation in common. Mutant #8 has an MreB point mutation, while the sequencing of mutant #38 did not reveal any changes. Mutant #10 has a \( \sim 13 \)kb deletion of many genes involved in chemotaxis and flagella rotation.

Due to known connections between metabolism and cell shape we will focus on the role of \( ptsI \) on A22 resistance (19, 33-35). It is of note that mutants #3 and #5 have mutations in adjacent residues of MreB which are predicted to be in the A22 binding pocket, yet produce different cell shapes (Table S2) (36). As this screen was not saturating it is possible that mutations in other genes can suppress \( \Delta envC \) growth on A22.

**Loss of functional PtsI suppresses A22 sensitivity to \( \Delta envC \)**

\( ptsI \) encodes for enzyme 1 in the PTS, which is used to transport and phosphorylate sugars, such as glucose, as they enter the cell (37). As all the described experiments were performed in LB without the addition of any sugar, it is surprising to find suppressor mutations in \( ptsI \). The suppressors have the same two base pair insertion in the gene; therefore, we tested whether a deletion of \( ptsI \) also increases A22 resistance.

Deletion of \( ptsI \) in a WT background leads to increased resistance to high levels of A22 and can recapitulate the suppressor phenotype in an \( \Delta envC \) background (Fig. 4A), supporting the hypothesis that the original suppressor phenotype came about from the loss of \( ptsI \).

To further confirm the role of PtsI in A22 resistance we complemented the \( ptsI \) and \( envCptsI \) deletion strains with either a WT allele of PtsI or an allele that is unable to be phosphorylated (PtsI\(^{H189A}\)) (38, 39). If PtsI phosphorylation is linked to its role in A22 sensitivity than the H189A mutant should mimic the deletion strain and provide increased resistance to A22. We found that complementation with the WT allele makes
both the ptsI and envCptsI deletion strains more sensitive to A22 than when complemented with the nonphosphorylatable allele (Fig. S5), supporting our hypothesis that the loss of PtsI activity increases the resistance of cells to disruption of MreB.

One of the common mechanisms for suppression of the lethality of mreB disruption is the upregulation of the ftsZAQ operon. It is possible that deletion of ptsI leads to increased levels of the ftsZ operon providing resistance to A22. We transduced a functional fluorescent copy of FtsZ into the native site of the chromosome as the sole copy of FtsZ in the cell (19). A ptsI deletion strain expressing this construct is still smaller than WT cells (Fig. S6). These strains allowed us to use fluorescence intensity to determine if there are changes in FtsZ levels caused by deleting ptsI. We found similar albeit slightly lower levels of FtsZ-GFP in the ΔptsI strain when accounting for cell size (3.88 x 10^5 ± 2.77 x 10^5 versus 3.03 x 10^5 ± 2.25 x 10^5 AU/pixel) suggesting that the suppressive effect of ptsI deletion is not due to increases in ftsZ levels. We also noticed that cells deleted for ptsI are more likely to lack an FtsZ structure. Although this was rare in both WT (4.5%) and ΔptsI (8.5%) cells the lack of FtsZ structures would not account for smaller cells (Fig. S6 white arrows).

In addition to A22, we found that ΔenvC cells are sensitive to ampicillin. To determine if the deletion of ptsI is specific to A22 treatment or more general cell wall stresses, we tested the sensitivity of the envCptsI double mutant in ampicillin and found that the loss of ptsI is able to suppress the sensitivity to ampicillin at high concentrations, similar to that seen with A22 (Fig. S2B).

**Loss of ptsI suppresses A22 sensitivity to ΔenvC but not ΔamiAB cells**

We have shown that the sensitivity to A22 seen in envC cells works through amiAB (Fig. 1B); therefore, we tested if deletion of ptsI would also be able to suppress the A22 sensitivity in an amiAB background. Interestingly, while deletion of ptsI in an envC background leads to an 11X fold increase in growth ratio in A22 10 µg/ml, in an amiAB deletion background there is only a 0.02X fold change and growth is most likely below
the accuracy level of the spectrophotometer (Fig. 4AB). This suggests that the
suppressive effect of losing PtsI works through AmiA or AmiB. This datum also
suggests that the loss of PtsI does not change membrane permeability, as we would
expect all strains that have increased A22 sensitivity to become more resistant if
permeability of A22 was affected.

Loss of ptsI suppresses the cell elongation phenotype of an envC mutant

One of the initial observations of an envC mutant was that it forms elongated and
chained cells (Fig. 3A, Fig. 4C, Fig. S3) (25). Because deletion of ptsI suppresses the
A22 sensitivity phenotype through AmiA/AmiB, we asked whether cell size is also
affected. Cells were grown in LB without additional sugar, to mid log phase and imaged.
To account for the chaining effect of the envC mutant, we measured both individual and
chained cells. As expected, ΔenvC cells are longer than WT; interestingly, the lack of
ptsI reduces the cell length of WT cells and ΔenvC cells (Fig. 4C). Since deletion of ptsI
is unable to provide amiAB cells A22 resistance, we examined if deletion of ptsI would
reduce the cell size of the amiAB mutant. Deletion of ptsI is able to reduce cell length in
the absence of amiAB (Fig. 4D). These results show that loss of ptsI can suppress both
the A22 sensitivity and cell length phenotype resulting from the loss of envC, but only
the cell length phenotype of an amiAB mutant, suggesting that the role of PtsI in A22
resistance can be separated from its role in cell size regulation and supporting a role for
AmiAB in A22 sensitivity.

Discussion

MreB is a highly conserved protein essential for the regulation of rod shape and viability,
making it a potentially good target for antibiotic development (1, 15). While we have
previously reported on conditions that help cells grow without MreB, there is little known
about how cells can become hypersensitive to the disruption of MreB (18, 19). Here, we
performed a screen of the Keio collection to identify gene deletions that lead to the
inhibition of growth in sublethal levels of A22, an MreB depolymerizing agent. Five
strains were found to have increased sensitivity to A22, one of which, envC, is involved in cell division (Fig. 1A).

There are two distinct cell wall synthesis complexes in rod-shaped bacteria, the MreB-regulated elongasome controlling cell elongation and growth, and the FtsZ-regulated divisome controlling cell division. If and how these two systems talk with each other is underexplored. While it is well established that MreB localizes to the Z-ring during division in Caulobacter crescentus, it is more controversial in E. coli (40, 41). While our lab and others have not been able to see an enrichment of MreB at the division site when using a functional fluorescent sandwich fusion of MreB as the only copy in the cell, others have reported that N-terminal fusions to MreB and the MreB interaction partner, RodZ, localize to the midcell (42-44). This study shows that there is some crosstalk between these two systems as loss of envC (divisome) makes cells more sensitive to disruption of MreB (elongasome) with A22.

The “adder-model” of cell size regulation says that rather than reach a critical size before dividing, cells add a constant volume before dividing (45, 46). It would reason that there is coordination between lateral wall synthesis controlled by MreB and cell division. Why this system would work at the level of cell separation after division is unclear, but it may suggest the role of proteins upstream or downstream of EnvC, such as FtsN (25, 47).

**Loss of amiA and amiB phenocopy envC’s A22 sensitivity**

EnvC activates two cell wall hydrolases, AmiA and AmiB, needed to separate daughter cells after division has occurred. To determine if the sensitivity of the envC deletion is due to a secondary role of EnvC or its role in activating AmiA and AmiB, we deleted both proteins alone and together. The double amiAB deletion was even more sensitive to A22 than the envC deletion (Fig. 1). This is most likely due to latent activity of either enzyme in the envC deletion that is absent when the proteins are deleted. It will be interesting in future work to determine if AmiA and/or AmiB have a secondary role in the
cell that when absent leads to cells being more sensitive to changes in MreB polymerization.

The activity of the amidases during cell division produces denuded glycan chains (missing peptide side chains) which signal to activate FtsN (48, 49). When active, FtsN activates PBP1B at the division site (50). PBP1B mutants are sensitive to A22, and PBP1B is necessary to overcome the loss of the elongation machinery (18, 19, 51). One possible reason for the increased sensitivity of an envC mutant is a lowering of the presence of denuded glycans leading to less active FtsN and therefore less active PBP1B. Without a highly active PBP1B the cell would not be able to overcome the loss of the elongasome.

Point mutations in mreB suppress effects of envC

In order to further understand the connection between MreB and EnvC we performed an A22 resistance suppressor screen. Of the nine suppressor strains found in this screen, five have mutations in MreB. Interestingly, while 4/5 of the envCmreB mutants have increased A22 resistance over WT cells, they also have unique cell shapes in LB medium (Fig. 3). Mutants #5 and #27 are both shorter than the envC parent strain with tapered tails and do not appear to chain. Furthermore, #3 and #5 have mutations in adjacent residues that are predicted to interact with ATP, yet cause different cell shapes (36). Molecular dynamic simulations suggest that ATP binding of the MreB monomer is necessary for polymerization and that polymerization induces structural changes in MreB leading to hydrolysis; therefore, we suggest that these two mutations may help stabilize ATP-binding leading to increased filamentation and A22 resistance (52). Alternatively, these mutations may decrease the exchange between ADP and ATP, stabilizing filaments and leading to A22 resistance. The nucleotide-bound state of MreB polymers may also affect membrane binding or protein interactions, as seen with eukaryotic actin (53, 54).
Residues 72 (#8) and 79 (#27) are near each other on the MreB protein but are not predicted to have a structural role in filamentation and lead to different A22 resistance profiles (Fig. S7). These residues may represent an MreB-protein interaction site. It will be interesting to study the effects of these MreB mutations in a background with EnvC to see if cell shape effects are due to the loss of envC.

**PtsI regulates cell size even without excess sugars**

Cells can sense their metabolic environment and adjust their size accordingly. The fact that cells grow larger in rich medium and smaller in nutrient poor medium is termed the “Growth Law” (55). One mechanism for connecting nutrient levels with cells size links the inhibition of FtsZ with UDP-glucose levels (56, 57). Our experiments reveal that deletion of *ptsI* results in shorter cells in WT, ∆*envC*, and ∆*amiAB* backgrounds (Fig. 5C). Interestingly, these cells were grown in LB medium without the addition of sugar. As the PTS system is involved in sugar uptake, it is surprising to find such a dramatic change in cell length in these conditions.

PtsI is the first protein in the PTS which transfers a phosphate from phosphoenolpyruvate (PEP) and shuttles it to enzyme 2 of the PTS, where it can be added to sugars upon entry into the cell. Lack of PtsI will affect the PEP:pyruvate ratio of the cell (58, 59). We have previously proposed that increased pyruvate levels in cells can activate gluconeogenesis and increase the levels of cell wall precursors providing cells with increased tolerance to A22 (19). In *Bacillus subtilis*, deletion of pyruvate kinase (*pyk*) effects Z-ring formation (60). We propose that deletion of *ptsI* results in a change in the PEP:pyruvate ratio in the cell, resulting in increased resistance to A22 and changes in cell size. This mechanism appears to connect with AmiAB as the deletion of *ptsI* does not result in A22 resistance in this strain. However, it is also possible that changes in the PTS affect cAMP levels, which has been shown to regulate cell shape (35).

**Experimental Procedures**
Bacterial Growth. Bacteria were grown using standard laboratory conditions. Cultures were grown overnight in LB medium (10 g L\(^{-1}\) NaCl, 10 g L\(^{-1}\) tryptone, 5 g L\(^{-1}\) yeast extract) and subcultured in the morning 1:1000 and grown to exponential phase (O.D.\(600\) 0.2-0.5) at 37\(^\circ\)C in a shaking incubator. Keio collection mutants were moved in MG1655 using P1 transduction. Phage were propagated in a WT MG1655 strain before being grown with PCR verified mutants from the Keio collection. Transducing phage were then grown on MG1655 and transductants were selected on kanamycin (30 \(\mu\)g ml\(^{-1}\)) and confirmed by PCR. See Table S1 for a list of strains used in this study.

Growth Ratios
Overnight cultures of cells were subcultured 1:1000 into media with 1 or 10 \(\mu\)g ml\(^{-1}\), or no A22. Cultures were grown shaking at 37\(^\circ\)C for 6 hours before measuring the O.D.\(600\). Growth ratios were determined for each strain comparing the A22 cultures to the no drug culture. Error bars were determined by taking the standard deviation of the growth ratio across multiple experiments.

Suppressor Screen
A22 suppressors of the \(\Delta envC\) mutant were isolated by growing \(\Delta envC\) cells overnight in LB media lacking A22. Cells were plated on LB + A22 (10 \(\mu\)g ml\(^{-1}\)) and incubated for 16 hours at 37\(^\circ\)C. Colonies were streaked onto fresh A22 plates and incubated for 16 hours at 37\(^\circ\)C. Colonies were then grown in liquid A22 media for 16 hours.

Mutants that grew in liquid media were subjected to growth ratio analysis in A22 1 and 10 \(\mu\)g ml\(^{-1}\) and imaged from exponential phase in LB medium. MreB was PCR amplified and sequenced before strains were sent to MiGS for whole-genome Illumina sequencing. Both the \(\Delta envC\) parent and suppressors were sequenced. Indicated mutations are the only differences. Variants were found using breseq on census/mixed base mode (61). The consensus mutation E-value cutoff and polymorphism E-cutoff was set to 10 with a frequency cutoff of 0.8 and a polymorphism frequency cutoff of 0.2.
Microscopy

For all imaging, cells were grown at 37°C in LB medium. Imaging was done on 1% M63-glucose agarose pads at room temperature. Phase contrast and fluorescent images were collected on a Nikon Ni-E epifluorescent microscope equipped with a 100X/1.45 NA objective (Nikon), Zyla 4.2 plus cooled sCMOS camera (Andor), and NIS Elements software (Nikon).

Cell area and IDD were calculated using the matlab software Morphometrics (62), and custom software as described previously. Only single non-diving cells were used for analysis unless stated otherwise.

MreB Mutation Modeling

MreB mutations were modeled using Swiss model server (SWISS-MODEL (expasy.org)) using Caulobacter crescentus MreB (4czg.1.pdb) (https://swissmodel.expasy.org/templates/4czg.1) as a template. The mutations were mapped using PymoL Version 2.5.2.
References


5. Bendezu FO, Hale CA, Bernhardt TG, de Boer PAJ. RodZ (YfgA) is Required for Proper Assembly of the MreB Actin Cytoskeleton and Cell Shape in E. coli. EMBO. 2009;28(3):193-204; PMCID: PMC2637328.


Figure 1. A22 sensitivity is increased in multiple gene deletions. Cells were grown for six hours in LB or LB + A22. The ratio of O.D.₆₀₀ between the two conditions was determined as a growth ratio. A) The growth ratio was determined for WT (MG1655) and five gene deletions found through a screen of the Keio collection. Mutations were transduced into MG1655. B) Growth ratios were determined for different strains related to cell separation after division. A-B) Error bars are standard deviation from three independent trials. * p <0.05, ** p <0.01, *** p <0.001.
Figure 2. Cell shape characteristics of mutants involved in cell separation with and without A22.

A) Cells were grown in LB for four hours or LB for two hours and then A22 (10 µg/ml) for two hours before being imaged. Scale bar = 4 µm.

B) Cell cluster sizes were counted from images of cells grown in A22 as in A. Shown is pooled data from three independent trials.

C-D) Data is pooled from three independent experiments. Cells were grown for 2 hours in LB before the addition of A22 for two hours. C) Coefficient of variation diameter deviation is a metric to...
measure how ‘rodlike’ cells are. The higher the number the less rod and more spherical a cell is.

D) Cell area measurements of cells grown in A. Only individual cells were counted, and chains of
cells were ignored in the analysis. All statistical comparisons were made between WT LB and
mutants LB or WT A22 and mutants A22 and have a p value < 0.001 unless noted. ** p < 0.01.

See table S3 for number of cells. C-D) error bars are 95% CI. E) Percent change of cell area
between cells grown in LB or with A22. Error bars are standard deviation from three
independent experiments. All comparison are to WT and not significant unless noted. * p <0.5.
Figure 3. Suppressor screen for mutants of ΔenvC cells that can grow in A22. Suppressors were selected with multiple rounds of growth on A22 10 µg/ml. Top- Cells are separated into four groups. The leftmost is a group of control strains, including WT, ΔenvC, and ΔenvC + an MreB point mutation resistant to A22 (MreB_{S14A}). Group 1 contains four strains with high levels of A22 resistance. Group 2 has two strains with increased growth in high levels of A22 and group 3 has three strains with high growth in low A22 and increased growth in high levels. Error bars are standard deviation from three independent experiments. Bottom- representative images of cells grown in LB medium. Scale bar = 4 µm.
Figure 4. Deletion of ptsI suppresses both A22 and cell elongation phenotypes of ΔenvC cells. A-B) Average growth ratio from three experiments of cells grown in LB or LB + A22. Error bars are standard deviation. * p <0.05, ** p <0.01. C-D) Cell length of WT and indicated mutant cells. Cells in chains were included to capture the effect of chaining in different backgrounds. Error bars are 95% CI and scale bar = 4 µm. ** p < 0.01, *** p < 0.001.
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