1 # Lytic transglycosylases RlpA and MltC assist in *Vibrio cholerae*
2 daughter cell separation
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13 ## ABSTRACT

14    The cell wall is a crucial structural feature in the vast majority of bacteria and

15 comprises a rigid, covalently closed, mesh-like network of peptidoglycan (PG) strands.

16 While PG synthesis is important for bacterial survival under many conditions, the cell wall

17 is also a dynamic structure, undergoing degradation and remodeling by so-called

18 "autolysins", enzymes that break bonds in the PG network.  Cell division, for example,

19 requires extensive PG remodeling and separation of daughter cells, which depends

20 primarily upon the activity of amidases.  However, in *V. cholerae*, we have found that

21 amidase activity alone is insufficient for daughter cell separation and that the lytic

22 transglycosylases RlpA and MltC both contribute to this process.  MltC and RlpA both

23 localize to the septum and are functionally redundant under normal laboratory conditions;

24 however, only RlpA can support normal cell separation in low salt media.  The division-

25 specific activity of lytic transglycosylases has implications for the local structure of septal

26 PG, suggesting that there may be glycan bridges between daughter cells that cannot be

27 resolved by amidases.  We propose that lytic transglycosylases at the septum serve as a

28 back-up mechanism to cleave rare, stochastically produced PG strands that are

29 crosslinked beyond the reach of the highly spatio-temporally limited activity of the

1    amidase and to clear PG debris that may block the completion of outer-membrane

2    invagination.

3

## INTRODUCTION

5    The cell wall is a crucial structural feature for the vast majority of bacteria and is

6    mainly composed of a rigid, yet elastic, covalently-bound network of peptidoglycan (PG)

7    strands. PG has an oligomeric glycan backbone that is assembled by

8    glycosyltransferases (GTs, RodA/FtsW and class A Penicillin Binding Proteins [aPBPs])

9    (Cho et al., 2016; Leclercq et al., 2017; Zhao et al., 2017; Taguchi et al., 2019) through

10    the polymerization of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM)

11    heterodimers. These PG strands are crosslinked to adjacent strands primarily by the

12    transpeptidase domains of aPBPs and bPBPs via short peptides attached to the NAM

13    residues, resulting in the strong, mesh-like sacculus. While the rigidity functions to resist

14    bacterial cells' high internal pressure (Osawa & Erickson, 2018), it restricts processes

15    such as cell growth, division, and insertion of multiprotein trans-envelope complexes such

16    as the flagellum and secretion systems (Nambu et al., 1999; Santin & Cascales, 2017).

17    The cell wall must therefore be a dynamic structure, and indeed undergoes constant

18    remodeling and recycling by PG degradation enzymes collectively known as "autolysins"

19    (T. K. Lee & Huang, 2013).

20    Autolysins are numerous and diverse, in part owing to the complexity of the

21    substrate on which they act. Almost all different types of covalent bonds that are found

22    within PG can be cleaved by autolysins, and many of these enzymes are functionally

23    redundant under standard laboratory growth conditions. Functional redundancy has

2

1   stymied the elucidation of the physiological role of many autolysins, as it makes them

2   inaccessible to many traditional means of assessing gene-phenotype associations, such

3   as analysis of single gene knockouts. The lytic transglycosylases (LTGs), for example,

4   have been exceptionally well-characterized biochemically (Dik et al., 2017), but still

5   relatively little is known about their individual physiological functions. LTGs target PG at

6   the glycosidic bond between NAG and NAM residues and the primary mechanism for this

7   cleavage is a non-hydrolytic, intramolecular cyclization of NAM to form 1,6-

8   anhydroMurNac ([anh]NAM) (Holtje et al., 1975; Dik et al., 2017; Williams et al., 2018). At

9   least in well-understood model organisms, this signature anhydro "cap" is assumed to be

10  at the end of almost all peptidoglycan strands *in vivo* (Kraft et al., 1998; Heidrich et al.,

11  2002a). Members of the LTG class have been implicated in many cellular processes,

12  including the termination of GT-mediated PG polymerization (Tsui et al., 2016; Yunck et

13  al., 2016), insertion of secretory apparati and flagella (Herlihey & Clarke, 2017; Santin &

14  Cascales, 2017), pathogenesis (Chan et al., 2012), and PG recycling (Cloud & Dillard,

15  2002).

16      One process where PG remodeling is particularly important is cell division. Our

17  current understanding of bacterial cell division includes a step in which lateral PG must

18  be remodeled to allow for insertion of a septal wall between daughter cells, followed by

19  cleavage of that septal wall to facilitate daughter cell separation (Potluri et al., 2012; Egan

20  & Vollmer, 2013). Septal PG cleavage by amidases, which cleave off the dipeptide side

21  stem from the NAM residue, is tightly controlled spatiotemporally to ensure that PG

22  degradation is exclusively localized to where it is needed (Heidrich et al., 2002a; Uehara

23  et al., 2010; D. C. Yang et al., 2011; Peters et al., 2011; Möll et al., 2014; Tsang et al.,

1    2017). The amidases are generally assumed to be the main enzymes mediating daughter

2    cell separation, though there is evidence that other autolysins, including LTGs, are

3    pleiotropically involved (Heidrich et al., 2001, 2002a; Priyadarshini et al., 2006). *E. coli*

4    strains lacking LTGs MltABCDE and Slt70, for example, have mild cell separation defects

5    (Heidrich et al., 2002a). In addition, Jorgenson, et. al. identified a highly conserved LTG,

6    RlpA, which exhibits septum-specific cleavage activity in *Pseudomonas aeruginosa.* RlpA

7    is required under low salt conditions, but not during growth in standard laboratory media,

8    suggesting *P. aeruginosa* encodes at least one redundant septal LTG (Jorgenson et al.,

9    2014). *Salmonella enterica* similarly appears to require LTGs MltC and MltE for proper

10   cell daughter cell separation in low salt conditions (Wall et al., 2011) and in *Neisseria*

11   *gonorrhea*, mutating the LTG LtgC results in daughter cell separation defects (Cloud &

12   Dillard, 2004). Thus, LTGs appear to play important and often redundant, but poorly-

13   understood roles in septal cleavage in diverse bacteria. In particular, it is unclear whether

14   the separation defects observed in LTG mutants are due to the lack of a septum-specific

15   function of these autolysins, or a general consequence of pleiotropic cell wall damage.

16          Here we show that in the cholera pathogen, *Vibrio cholerae,* the two LTGs MltC

17   and RlpA are collectively required for daughter cell separation. Their inactivation results

18   in the generation of cell chains reminiscent of amidase mutants and additional deletion of

19   *V. cholerae*'s sole amidase exacerbates this chaining defect. Our data suggest that these

20   LTGs fulfill specialized roles in daughter cell separation and have important implications

21   for septal PG architecture in the cholera pathogen.

22

23   **RESULTS**

**Simultaneous inactivation of seven LTGs induces a lethal cell separation defect**

Autolysins are often functionally redundant. *V. cholerae*, for example, can tolerate the inactivation of its sole amidase or simultaneous deletion of 5 out of its 6 M23 family endopeptidases (Dörr et al., 2013; Dörr, Davis, et al., 2015), suggesting that redundant autolysins can substitute for each other to sustain at least basic growth processes. In addition to the amidase and the endopeptidases, *V. cholerae*'s genome encodes 8 predicted LTGs that were identified based on their homology to *E. coli* LTGs. Whether these enzymes fulfill unique or redundant roles within *V. cholerae'*s life cycle is unknown. To find new phenotypes associated with LTG deficiency, we endeavored to make sequential deletions in all eight genes. Interestingly, we were able to inactivate six LTGs (*mltA, mltB, mltC, mltD, mltF* and *slt70*, leaving only *rlpA* and *mltG* intact) with the resulting strain exhibiting only slight morphological aberrations, such as a mild division defect with a corresponding increase in cell length (**Fig. 1A, Fig. S1A**). Thus, in *V. cholerae*, RlpA and MltG are cumulatively capable of performing all potentially essential functions of LTGs, at least under laboratory conditions.

Though single knockouts of *mltG* and *rlpA* could be readily obtained in a wild-type background, (**Fig. S1B**) we were unable to further delete *mltG* or *rlpA* from the Δ6 LTG strain, suggesting that these two represent a minimal set of LTG functions required for viability. To test the phenotypic consequences of loss of RlpA in the Δ6 LTG background, we constructed a strain that expressed its sole copy of *rlpA* under control of an arabinose-inducible promoter. Growing this strain in the absence of arabinose resulted in the formation of long chains of unseparated cells, many of which had lysed (**Fig. 1B**). This lysis was also evident in the ~$10^6$-fold reduction in plating efficiency after RlpA depletion

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1    and dramatic color change when plated on the cell-impermeable β-galactosidase

2    substrate, chlorophenol red-β-D-galactopyranoside (CPRG) (**Fig. 1CD**), an established

3    readout of cell lysis (Paradis-bleau et al., 2014). The lethal chaining defect seen in the Δ6

4    LTG strain depleted of RlpA suggests that at least one of the roles of LTGs is an essential

5    function related to septal PG remodeling and/or daughter cell separation that cannot be

6    adequately fulfilled by other autolysins under native conditions. Since a similar depletion

7    strain for MltG did not exhibit a phenotype, we here focused on RlpA for further study; the

8    reason for MltG's essentiality in the Δ6 background is subject of future work.

9

10   **Simultaneous inactivation of RlpA and MltC results in a cell separation defect**

11          Since cell separation defects are not lethal in other *V. cholerae* autolysin mutants

12   (*e.g.,* amidase mutants [Möll et al., 2014]), we hypothesized that the lethal phenotype of

13   the Δ6 LTG/*rlpA* depletion strain was in principle separable from the chaining defect. To

14   dissect this further, we assayed different combinations of LTG mutants for cell

15   morphology defects **(Fig. S2A).** LTGs were generally inactivated by replacing their open

16   reading frame with a scar sequence.  However, the gene for RlpA is located within a

17   genomic region containing other important cell wall factors, including *rodA* and *dacA,* and

18   so we instead inserted a premature stop codon in position 133 to reduce potential polar

19   effects.  Visual inspection of different mutant combinations revealed that a strain deleted

20   in both *mltC* and *rlpA* (Δ2 LTG) exhibited a pronounced cell separation defect, manifest

21   as long chains in a culture grown to ~$OD_{600}$ 0.8 (**Fig 2A**).   Imaging these chains

22   expressing cytoplasmic GFP from a constitutive promoter revealed clearly separated

23   cytoplasmic spaces, indicating that cytokinesis was complete in cell chains. In contrast to

1     the Δ2 LTG chaining phenotype, the *rlpA*::stop and Δ*mltC* single mutants exhibited no

2     morphological differences from the WT in either LB or minimal medium (**Fig. S2B**).

3     Therefore, MltC and RlpA collectively fulfill a crucial role in daughter cell separation.

4     Despite visible chaining, the Δ2 LTG mutant grew as well as the WT in batch culture (**Fig.**

5     **S2C**); however, we did observe a strong motility defect that appeared to be largely due

6     to RlpA inactivation (**Fig. S2D**).

7       It has been previously reported that a mutant *Pseudomonas aeruginosa* lacking

8     RlpA forms unseparated chains of cells when grown in media with low osmolarity

9     (Jorgenson et al., 2014). Similarly, the *V. cholerae rlpA::stop* single mutant, but not the

10     Δ*mltC* single mutant, formed chains of cells when grown in low-salt LB medium and

11     exhibited a 1000-fold plating defect on low-salt LB plates (**Fig. 2BC**). Interestingly, none

12     of these phenotypes were replicated in *Escherichia coli*. An *E. coli* Δ*rlpA* mutant exhibits

13     no obvious morphological defects in LB or low-salt medium (Jorgenson et al., 2014),

14     though this is unsurprising as *E. coli* RlpA, despite being well-conserved, is predicted to

15     be non-functional as a LTG (Jorgenson, 2014). Informed by our *V. cholerae* Δ2 LTG

16     mutant and the *S. enterica* Δ*mltC* Δ*mltE* mutant phenotypes (Wall et al., 2011), we made

17     additional, simultaneous mutations in *E. coli mltC* and *mltE*, but still observed only a very

18     mild morphological defect **(Fig S3)**. Thus, there are species-specific differences in the

19     utilization of these autolysin homologs.

20

21     **Δ2 LTG mutations result in outer membrane permeability perturbations**

22       Given that daughter cell separation-defective chains are sensitive to osmotic

23     stress, we also hypothesized that the outer membrane (OM) may be compromised in the

1 Δ2 LTG mutant, as the OM is an important structural and load-bearing component of

2 Gram-negative bacteria (Rojas et al., 2018). Lysozyme typically cannot permeate the

3 OM in sufficient quantities to cause damage to the Gram-negative cell wall. Consistent

4 with this, WT *V. cholerae* tolerated exposure to 5 mg mL$^{-1}$ lysozyme without exhibiting

5 signs of morphological defects (**Fig. 2D**). In contrast, a significant portion of Δ2 LTG

6 mutant cells exhibited a loss of rod shape after just a brief exposure to the same

7 concentration of lysozyme (**Fig. 2D**). This suggests that delayed cell separation may

8 present a barrier to proper OM invagination and results in OM permeabilization.

9

10 **Δ2 LTG and Δ*amiB* chaining phenotypes are growth phase dependent**

11 The Δ2 LTG chaining phenotype is reminiscent of a mutant deleted in *V. cholerae'*s

12 sole amidase, AmiB, which also exhibits chain formation (Möll et al., 2014) due to a well-

13 understood daughter cell separation defect (Heidrich et al., 2001, 2002b; Priyadarshini et

14 al., 2007; Uehara et al., 2009, 2010). We thus explored other similarities between the two

15 mutants. During our imaging experiments, we noticed that overnight cultures of Δ2 LTG

16 and Δ*amiB* mutants were devoid of any cell chains, suggesting that chaining was a growth

17 phase-specific phenotype. Single cells of stationary phase Δ2 LTG and Δ*amiB* were not

18 spontaneous suppressor mutants arising at a high frequency, as redilution into

19 exponential phase always resulted in renewed chain formation and subsequent resolution

20 in stationary phase (**Fig. S4**). To more precisely define the growth stage that promotes

21 cell chain resolution in these mutants, we imaged the mutant strains versus WT over their

22 growth cycle and quantified the cells per chain at each time point (**Fig. 3A**). Both Δ2 LTG

23 and Δ*amiB* exhibited peak chain lengths in mid- to late exponential phase (OD$_{600}$ ~0.8,

1    median chain length of 8 cells for Δ2 LTG and 24 cells for Δ*amiB*), followed by a gradual

2    decline in chain length as the cells entered stationary phase.

3         The recurring shift between an enrichment of chains in exponential phase and

4    single cells in stationary phase could be explained by stationary phase-specific events,

5    e.g., growth-phase specific lysis cycles or the induction of stationary phase-specific PG

6    remodeling pathways.  Alternatively, cell separation could be due to a stochastic process,

7    i.e., reduced septal cleavage activity is initially outpaced by septal PG synthesis during

8    rapid growth, but becomes sufficient for daughter cell separation as division rates slow in

9    stationary phase.  Growth phase-dependent chaining has been previously reported for *E.*

10   *coli* amidase mutants and it was postulated that single cells were likely generated by lysis

11   of cells within a chain (Heidrich et al., 2001).  We tested this hypothesis in *V. cholerae* by

12   plating autolysin mutants on CPRG.  The Δ2 LTG mutant failed to indicate significant

13   CPRG degradation (**Fig. 3A**), suggesting that lytic elimination of long chains, either by

14   mechanical sheering or cell death, is an unlikely explanation for stationary phase cell

15   separation. The absence of excessive cell debris in microscope images corroborated this

16   observation, as did the WT-equivalent growth rate of this mutant (**Fig. S2C**).  In contrast,

17   the Δ*amiB* mutant did exhibit a slight color change on CPRG, indicative of higher

18   background lysis, which is consistent with previous observations in *E. coli* amidase

19   mutants (Heidrich et al., 2001; Priyadarshini et al., 2007). Additionally,  this mutant in *V.*

20   *cholerae*  suffers a slight defect in growth rate when compared to WT (Möll et al., 2014)

21   and Δ2 LTG, suggesting that at least some of the chain resolution of the Δ*amiB* mutant

22   may depend on lysis (**Fig. 3A**, **Fig. S2C**).  Thus, while cell lysis may somewhat contribute

1    to apparent chain "resolution" in the amidase mutant, it is unlikely to facilitate the same in

2    the Δ2 LTG mutant.

3

4    **Δ2 LTG chain resolution is stochastic**

5          We conducted several experiments to help distinguish between chain resolution

6    by stationary phase-induced PG remodeling and stochastic activity of other hydrolases.

7    First, we performed a chemostat-like experiment where Δ2 LTG cells were kept in

8    prolonged exponential phase through periodic back-dilution to maintain an $OD_{600}$ < 0.6.

9    In a simple scenario, if chain resolution were mediated by a stationary phase-exclusive

10   factor, chains would be expected to elongate *ad infinitum* when kept in perpetual

11   exponential phase and single cells would no longer be present in the population.

12   However, if other autolysins could stochastically resolve shared septal PG, albeit with a

13   lower efficiency, we would expect an increase in the variation of chain lengths rather than

14   infinite chains.  What we observed was more indicative of stochastic resolution. There

15   was some increase in maximum chain length (**Fig. 3B**), but chains must also have

16   achieved some degree of resolution, as short chains and single cells were still present

17   even after five back-dilutions. These results tentatively suggest that an equilibrium

18   between division and subsequent separation can be achieved in a Δ2 LTG mutant without

19   entering stationary phase.

20         To test this further, we also surveyed other possible stationary phase-specific

21   factors that could affect cell separation.  Culturing Δ2 LTG in the filter-sterilized

22   supernatant of a saturated WT culture failed to prevent chaining (**Fig. S5A**) as would have

23   been expected should chain resolution be modulated by a secreted compound, for

10

1   example D-amino acids, which in *V. cholerae* are exclusively produced in stationary

2   phase (Cava, De Pedro, et al., 2011; Cava, Lam, et al., 2011).  We also inactivated the

3   global stationary phase transcriptional regulator, RpoS, in the Δ2 LTG background and

4   found that chain formation during exponential phase and chain resolution during

5   stationary phase were unaffected (**Fig. S5B**).  Finally, we blocked all new protein

6   synthesis in Δ2 LTG grown to exponential phase ($OD_{600}$~0.4) with chloramphenicol (10µg

7   $mL^{-1}$, ~15 x minimum inhibitory concentration [MIC]).  The majority of chloramphenicol-

8   treated Δ2 LTG chains resolved to single or double cells within an hour of chloramphenicol

9   treatment while untreated chains elongated in this time (**Fig. 3C**).  This indicates that the

10  cells have already translated the necessary enzymes for chain resolution by $OD_{600}$ 0.4

11  and adds further support that chain resolution in Δ2 LTG can be achieved by reducing the

12  division rate.

13      These experiments collectively indicate that the enzymes responsible for septal

14  resolution in the absence of MltC and RlpA are expressed in exponential phase and do

15  not depend on stationary phase-specific signals or factors.  Therefore, it is likely that

16  redundant housekeeping PG hydrolases or LTGs can mediate daughter cell separation,

17  albeit at a lower efficiency, when the main separation systems are inactivated. We thus

18  hypothesize that chain resolution during transition into stationary phase is the

19  consequence of reduced division rate at this growth stage, which allows other, less

20  efficient, autolysins to "catch up" and separate daughter cells.

21

22  **Alternative septal resolution factors are insufficient in the cumulative absence of**

23  **RlpA, MltC, and AmiB**

1    Given the similarities between the Δ2 LTG and Δ*amiB* mutants and the established

2    dependence of cell separation on the tight spatiotemporal regulation of AmiB

3    (Priyadarshini et al., 2006, 2007; Uehara et al., 2010; D. C. Yang et al., 2011; Peters et

4    al., 2011; Möll et al., 2014), we tested the formal hypothesis that RlpA and MltC directly

5    or indirectly contributed to AmiB recruitment and that the Δ2 LTG chaining defect might

6    thus be due to lack of AmiB localization.  A functional AmiB-mCherry fusion localized to

7    septal rings in Δ2 LTG chains, suggesting that this cell separation defect occurs despite

8    proper AmiB localization (**Fig. S6AB**).

9    We then investigated the possibly redundant autolytic roles of AmiB, RlpA, and

10   MltC by generating a Δ2 LTG Δ*amiB* triple mutant and quantifying growth phase-

11   dependent chaining.  While this strain was viable, it grew much more slowly than the Δ2

12   LTG or Δ*amiB* mutants (**Fig. S2C**) and perhaps more strikingly, chain resolution was

13   incomplete even after 24 hours and gave visual evidence of strong lysis under the

14   microscope as well as on CPRG plates (**Fig. 3D**, **Fig. S4**). This increased lysis and failure

15   to completely resolve chains suggest that the amidase and septal LTGs are the principle

16   daughter cell separation systems.

17   We were interested to learn whether the additive, deleterious effects of the Δ2 LTG

18   and Δ*amiB* mutations were due to the unique potential functions of LTGs versus amidases

19   at the septum, or if over-expression of AmiB, or other classes autolysins for that matter,

20   could complement the Δ2 LTG chaining defect.  Overexpression of *V. cholerae*'s primary

21   housekeeping endopeptidases, ShyA or ShyC (Dörr et al., 2013), was unable to

22   appreciably reduce chaining in the Δ2 LTG mutant (**Fig. 4A**) (functional expression of

23   these constructs was validated by their ability to complement a mutant defective in

1    multiple endopeptidases (Dörr, Davis, et al., 2015)). Similarly, overexpression of *V.*

2    *cholerae*'s only amidase, AmiB (co-overexpressed with its activators NlpD and EnvC [Möll

3    et al., 2014; Uehara et al., 2009; Yang et al., 2011]), demonstrated functionality by

4    complementing the Δ*amiB* mutant (**FigS6B**), but could not prevent chaining in the Δ2 LTG

5    mutant. Thus, daughter cell separation likely specifically requires LTG activity, rather than

6    increased general PG hydrolysis.

7         Interestingly, we found that heterologous expression of the *E. coli* LTG MltE was

8    highly effective at facilitating septal resolution in the Δ2 LTG mutant (**Fig. 4A**). MltE has

9    a relatively broad spectrum of PG substrate specificity, shown *in vitro* to generate

10   products indicative of both endo- and exolytic cleavage on denuded or un-crosslinked

11   muropeptides (M. Lee et al., 2013; Dik et al., 2017; Byun et al., 2018), and has no known

12   homolog in *V. cholerae.* Overexpression of *mltE* was not toxic in a WT background,

13   suggesting that it primarily digests septal PG in the *V. cholerae* Δ2 LTG mutant (**Fig. S7**).

14   MltE has a strong preference for uncrosslinked PG (M. Lee et al., 2013; Dik et al., 2017;

15   Byun et al., 2018), providing a possible explanation for its lack of general toxicity, since

16   the main body of the cell's PG is generally crosslinked (Desmarais et al., 2015).

17   Incidentally, this also suggests that septal PG in Δ2 LTG is largely uncrosslinked; which

18   would be consistent with completed or concurrent amidase or endopeptidase activity.

19   MltE was also able to rescue Δ2 LTG growth in low-salt media (**Fig. 4B**). In combination,

20   these observations suggest that some characteristic of the division septum requires the

21   activity of LTGs over other autolysins to allow for septal resolution and daughter cell

22   separation.

23   **RlpA and MltC are late division proteins**

13

1    Division proteins are often recruited specifically to midcell.  We generated stable,

2    functional **(Fig. S8AB)** translational fusions of MltC and RlpA to mCherry to track

3    localization throughout growth and division.   Both RlpA-mCherry and MltC-mCherry

4    clearly localized to the midcell and co-expression of FtsZ-YFP or YFP-FtsN further

5    revealed that both LTGs arrive closer to or after FtsN recruitment to the septum, indicating

6    that RlpA and MltC are likely late division proteins (**Fig. 5**).  RlpA midcell localization is

7    consistent with the published roles of its highly conserved homologues in *E. coli* and *P.*

8    *aeruginosa* (Gerding et al., 2009; Jorgenson et al., 2014; Yahashiri et al., 2015), however,

9    the subcellular localization pattern of MltC homologs in any organism has not been

10    previously reported.

11

12    **Outer membrane insertion is not required for MltC and RlpA LTG activity**

13    Both LTGs are predicted outer membrane proteins with well-conserved outer

14    membrane target lipoboxes LXGC (**Fig. S9A)** (Babu et al., 2006), so we postulated that

15    this localization may be important for either their recruitment to the midcell or for their

16    function as septal cleavage enzymes. To test this, we substituted the outer membrane

17    targeting signal sequences of MltC and RlpA N-terminal mCherry fusions with the

18    periplasmic signal sequence of thiol disulfide oxidoreductase, DsbA.  Surprisingly, these

19    soluble versions of both proteins were able to complement the Δ2 LTG chaining

20    phenotype in LB (**Fig. 6A**) yet did so with what appeared to be reduced localization to the

21    midcell (**Fig. S9B**). Thus, outer membrane attachment is not essential to the function of

22    RlpA and MltC in LB.  However, the functionality of DsbA$^{ss}$-RlpA$_{[18-263]}$ was not absolute

23    under all conditions; expression of this construct in a Δ2 LTG background during growth

14

1     in low salt LB resulted in the formation of severe morphological aberrations, including

2     short filaments and bulging at the midcell (**Fig. 6B**). Induction of this defect was not

3     dominant as the DsbA$^{ss}$-RlpA$_{[18-263]}$ mutant did not affect the morphology of WT in low

4     salt LB, nor did over-expression of RlpA-mCherry retaining its native signal sequence

5     affect the morphology of the Δ2 LTG mutant in low salt LB (**Fig. S9CD**). Despite the

6     apparent division defect induced by soluble DsbA$^{ss}$-RlpA$_{[18-263]}$ expression, the mis-

7     localized protein could still restore growth of the Δ2 LTG mutant on low salt LB (**Fig. 6C**).

8     The phenotype induced by the DsbA$^{ss}$-RlpA$_{[18-263]}$ mutant in low-salt medium was more

9     similar to filamentous division mutants than to other chaining autolysin mutants. Though

10     we did not explore this further, we suspect that RlpA may have conserved roles in division

11     other than septal resolution, roles that may explain why *E. coli* retains an LTG-deficient

12     homologue of RlpA.

13

## DISCUSSION

### LTG activity plays a conserved role in septal PG resolution

16     We report here that two LTGs, RlpA and MltC, are septum-specific enzymes with

17     crucial roles in daughter cell separation in *V. cholerae.* To our knowledge, this is the first

18     report of a physiological role for any housekeeping LTG in the cholera pathogen and the

19     second direct report of a physiological role for MltC (Wall et al., 2011). Follow-up studies

20     will be required to determine how septal LTGs interact with the divisome and the PG

21     structural reasons for why MltC and RlpA specifically contribute so significantly to the

22     separation of daughter cells. RlpA and MltC are well-conserved amongst proteobacteria

23     (Artola-Recolons et al., 2014; Yahashiri et al., 2015) yet despite this conservation,

1    mutations in either RlpA (Jorgenson et al., 2014), MltC, or both fail to produce phenotypes

2    in the classic model system *E. coli.* This study serves to highlight the importance of variety

3    in microbial perspective to understand key processes in bacterial physiology. At the same

4    time, the high conservation of RlpA, especially amongst Gram-negatives (Yunck et al.,

5    2016), and the aberrant morphology of a Δ2 LTG mutant overexpressing *dsbA^{ss}-rlpA* in

6    low salt suggests that septal LTGs may also have other unappreciated roles in bacterial

7    cell division and daughter cell separation in other Gram-negative pathogens.

8

9    **A "Cleave and Clear" LTG-dependent model of daughter cell separation**

10   With the addition of RlpA and MltC to the division complex, we can add more detail

11   to the molecular landscape of the septal cell wall.  We observed that the chaining defect

12   of the Δ*amiB* mutant was slightly more severe than the Δ2 LTG mutant, suggesting that

13   the majority of PG shared between daughter cells at the septum is connected by peptide

14   crosslinks that can be removed by amidase activity.  However, the chaining phenotype of

15   the Δ2 LTG mutant implies that there is shared septal PG that cannot be resolved by

16   amidase activity.  It is ideally expected, from the close association of septal PG synthesis

17   with rotating FtsZ filaments, that PG strands are inserted perpendicular to the long cell

18   axis (Daniel & Errington, 2003; X. Yang et al., 2017; Bisson-Filho et al., 2018).  Our

19   findings lead us to speculate that the cell division machinery stochastically generates PG

20   strands that are deposited and/or elongated at a non-ideal angle such that they transect

21   the septal plane to create a bridge between daughter cells (**Fig. 7**).  Since amidase activity

22   is precisely controlled by activators NlpD and EnvC (Möll et al., 2014), the radius of

23   amidase activity is likely highly restricted.  Some aberrantly deposited glycan strands

16

1     might then be crosslinked outside of the range of amidase activity, requiring LTGs as

2     backup enzymes to cleave connecting PG left behind after amidase activity is complete.

3     Consistent with this idea, RlpA in *P. aeruginosa* acts endolytically on denuded glycan

4     strands (Jorgenson et al., 2014; M. Lee et al., 2017) and may follow AmiB to cleave

5     remaining bridging strands. MltC may function to resolve septal PG in two ways. It might

6     also perform a similar function to RlpA, as some endolytic activity has been attributed *E.*

7     *coli* MltC (Artola-Recolons et al., 2014). One might also envision that long PG strands

8     between daughter cells, even those that are no longer covalently linked to the PG

9     network, might still present an obstacle to outer-membrane invagination. MltC, which in

10     *E. coli* has been shown to also be processive and exolytic, but inactive on crosslinked PG

11     strands, could be responsible for clearing such debris from the path of outer-membrane

12     invagination (Artola-Recolons et al., 2014).

13     We suggest that a function to remove aberrant septal PG strands would be

14     particularly important for *V. cholerae*, which completes the final stages of cell division with

15     astonishing speed (Galli et al., 2017). While the model bacteria *E. coli*, *B. subtilis,* and *C.*

16     *crescentus* have mature divisomes by 50% of the cell cycle (Aarsman et al., 2005; Gamba

17     et al., 2009; Goley et al., 2011) and *E. coli and B. subtilis* both encode multiple amidases

18     to assist in daughter cell separation (Heidrich et al., 2001; Firczuk & Bochtler, 2007), *V.*

19     *cholerae* encodes a single amidase and septation occurs only in the final 10% of its cell

20     cycle (Galli et al., 2017) This is likely to increase the chance for errors to occur, which

21     would create a need to efficiently remove spatial obstructions, including PG aberrantly

22     crosslinked or uncrosslinked PG debris. The absence of a strong chaining phenotype in

23     *E. coli* Δ*mltC* Δ*rlpA* Δ*mltE* (and the need to delete further LTGs to elicit a weak version of

1  such a phenotype) may indeed reflects this species' slower division process, resulting in

2  fewer errors. It should also be noted that repression of $P_{tac}$ is somewhat leaky (Rosano &

3  Ceccarelli, 2014) and that complementation of either the $\Delta 2$ LTG or $\Delta amiB$ chaining

4  defects occurred readily without induction, which suggests that very little autolytic activity

5  is actually required for separation and/or that obstruction of OM invagination by PG is

6  rare.

7      The exponential phase-dependent manner of the $\Delta amiB$ and $\Delta 2$ LTG chaining

8  phenotype suggests that AmiB and RlpA/MltC functions are redundant with at least one

9  other autolysin whose activity alone is insufficient to sustain daughter cell separation at

10  the fast rate imposed by exponential growth. We hypothesize that RlpA/MltC and AmiB

11  maintain separate functions and that chain resolution in stationary phase is a reflection of

12  each system requiring a distinct autolytic substitute (or group of autolysins) to mediate

13  chain resolution in its absence. For the amidase, this substitute could be D,D or L,D

14  endopeptidases, for LTGs the substitute could be other remaining LTGs. Thus, RlpA,

15  MltC, and AmiB compose the primary group of autolysins responsible for septal resolution

16  and other LTGs or endopeptidases that are secondary to RlpA/MltC or AmiB,

17  respectively, cannot support daughter cell separation in the absence of all three primary

18  septal autolysins. Future work will address the intricate redundancy relationships between

19  these diverse groups of autolysins.

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21

22  **EXPERIMENTAL PROCEDURES**

23  **Strains, Media, and Growth Conditions**

18

1      All *V. cholerae* strains in this study are derivatives of *V. cholerae* WT El Tor strain

2   N16961 (Heidelberg et al., 2000) and *E. coli* strains are derivatives of *E. coli* K-12 strain

3   MG1655 (Blattner et al., 1997).

4      Strains were grown at 30°C or 37°C in Luria-Bertani (LB) broth with or without 1.5%

5   NaCl, or in M9 medium containing 0.2% glucose ("M9 Salts," 2006; "M9 minimal medium

6   (standard)," 2010). When required for selection or plasmid retention, growth media were

7   supplemented with 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-gal, 40 µg mL$^{-}$

8   $^{1}$) streptomycin (200 µg mL$^{-1}$), kanamycin (5 µg mL$^{-1}$), carbenicillin (100 µg mL$^{-1}$), and/or

9   chloramphenicol (5 µg mL$^{-1}$ for *V. cholerae* and 20 µg mL$^{-1}$ for *E. coli*). Sucrose counter-

10   selection was performed on 10% sucrose LB medium without NaCl. Genes under $P_{tac}$ or

11   $P_{BAD}$ control were induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) or

12   0.2% L-arabinose, respectively. For lysis assays, growth media were supplemented with

13   chlorophenol red-β-D-galactopyranoside (CPRG, 20 µg mL$^{-1}$, Sigma Aldrich #

14   10884308001).

15      At least two replicates were completed per strain and condition for growth curves

16   and growth dependent chaining experiments. For growth curves, strains were inoculated

17   1:100 into 200 µL of medium from an overnight culture and grown in a Bioscreen growth

18   plate reader (Growth Curves America) at 37°C with random shaking of medium amplitude,

19   and OD$_{600}$ readings at 5-minute intervals. For chaining experiments with more than three

20   time points, overnight cultures were diluted 1:100 into 200 mL of medium in a 500 mL

21   non-baffled flask and incubated at 37°C with shaking. For experiments with three or fewer

22   time points, overnight cultures were diluted 1:100 into 5 mL of medium in culture tubes.

23

**Construction of Plasmids and Strains**

All strains are derivatives of *V. cholerae* El Tor N16961 or *E. coli* MG1655. Strains, plasmids, and primers are summarized in **Table S1-S4**. *E. coli* DH5$\alpha$ $\lambda$pir was used for general cloning while *E. coli* SM10 *$\lambda$pir* or MFD *$\lambda$pir* (Ferrières et al., 2010) were used for conjugation into *V. cholerae*. Plasmids were constructed using Gibson assembly (Gibson et al., 2009).

*V. cholerae* chromosomal in-frame deletions were generated by amplifying 500bp up- and downstream of the gene of interest by PCR, cloning into suicide vector pCVD442 (Donnenberg & Kaper, 1991), and conjugating into *V. cholerae* N16961 followed by sucrose counter-selection. Briefly, conjugation was performed by mixing and pelleting equal volumes LB overnight culture of plasmid donor *E. coli* SM10 *$\lambda$pir* or MFD *$\lambda$pir* strains with recipient *V. cholerae*, spotting mixed pellet onto an LB (+ 600µM diaminopimelic acid for MFD *$\lambda$pir*), and incubating at 37°C for 3 hrs. Single colonies were first selected for on LB + sm200 + vector selection, followed by counter-selection on salt-free LB + 10% sucrose and PCR verification of the deletion.

Ectopic, inducible chromosomal expression of proteins was achieved by cloning the gene(s) of interest open reading frame with either the native 20bp upstream sequence, a strong, consensus ribosome binding site (RBS), or no ribosome binding site into pTD101, a derivative of pJL1 (Miyata et al., 2013) engineered to carry the lac promoter, *lacIq*, and a multiple cloning site for integration of expression constructs at the native *lacZ* locus. In particular, pAW51 for polycistronic co-expression *amiB, nlpD,* and *envC*, was designed such that *amiB* was translated from a strong consensus RBS and the 20bp upstream regions containing the native RBS's of *nlpD* and *envC* gene were

20

1    included between *amiB* and *nlpD* or *nlpD* and *envC*, respectively, to generate a 3-gene

2    transcriptional fusion under $P_{tac}$ regulation. pTD101 derivatives were conjugated into *V.*

3    *cholerae* as described above for pCVD442.

4        Depletion of RlpA was accomplished by using suicide vector pAM299 (Möll et al.,

5    2015) to place *rlpA* under $P_{BAD}$ control at its native locus.  pAM299 was conjugated into

6    *V. cholerae* as a single crossover, without counterselection.  For fluorescent localization

7    or detection of proteins by Western blot, genes were cloned into pBAD33 (Guzman et al.,

8    1995) or pHL100 (Dörr, Alvarez, et al., 2015)

9        *E. coli* chromosomal in-frame deletions were generated using a combination of $\lambda$

10    Red and FLP recombinase systems as previously described (Cherepanov &

11    Wackernagel, 1995; Wanner, 2000; Murphy & Campellone, 2003).  Briefly, plasmids

12    pKD3 and pKD4 were used as templates for amplifying *cmR* and *kanR* cassettes flanked

13    by FLP recombinase sites with 50bp homology to the up- and downstream regions of the

14    gene of interest.  Electrocompetent MG1655 carrying the $\lambda$ Red recombinase system on

15    pKM208 was transformed with the PCR product amplified from pKD3/4 and recombinants

16    selected for on LB + kan50 or cm20.  The gene replacements were moved into a clean

17    MG1655 background by P1 phage transduction (Thomason et al., 2007) and the resulting

18    strain transformed with pCP20 carrying the FLP recombinase.  Finally, pCP20 was

19    induced and cured at 37°C and candidates screened for loss of antibiotic resistance and

20    PCR verification of the chromosomal gene deletion.

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**Microscopy**

Cells from liquid culture were imaged without fixation on 0.8% agarose pads containing the same medium from the relevant experiment using a Leica DMi8 inverted microscope. Phase contrast images of chaining mutants were analyzed manually to calculate the number of cells per chain in >100 chains. Raw phase and fluorescent images were analyzed in Oufti (Paintdakhi et al., 2016) using the pre-set *E. coli* M9 subpixel parameters for calculation of cell width and length and for generation of fluorescent signal localization "demographs."

**Western Blot Analysis**

Expression of translational mCherry fusions was induced in WT *V. cholerae* with 0.2% arabinose for pBAD33 or 1 mM IPTG for pHL100 and *lacZ::P$_{tac}$* in LB and grown to OD$_{600}$~0.6. Cells were harvested by centrifugation (9500 *g*, 15 min) at room temperature and resuspended in 1% SDS + 10 mM DTT lysis buffer. Resuspended cells were incubated at 95°C for 3 min, then sonicated 4 x 5 seconds at 20% amplitude. Standard Western blots against mCherry were performed using polyclonal mCherry antibody (Genetex #GTX59788) and detection by IRDye 800CW secondary antibody (Li-cor #926-32211). After imaging for mCherry, the same blots were then re-incubated with monoclonal RpoA antibody (BioLegend # 663104) detected by IRDye 800CW secondary antibody on an Odyssey CLx imaging device (Li-cor).

22

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## AUTHOR CONTRIBUTIONS

AIW and TD have made major contributions to the design of the study, the collection, analysis, and interpretation of data, and writing of the manuscript. VJR, ST, and BR have made major contributions to the collection and analysis of data. KW has made major contributions to the analysis of data.

## FIGURE LEGENDS

**Fig 1. Lytic transglycosylases are collectively required for survival of *V. cholerae.***

**A)** WT and Δ6 LTG cultures grown LB at 37°C and imaged on an agarose pad. **B)** RlpA was depleted in the Δ6 LTG background by placing its native promoter under control of arabinose induction and growing in the absence or presence of arabinose (ara). Cells were imaged on an agarose pad and **C)** spot-plated onto LB+/-0.2% ara, followed by incubation at 30°C for 24hrs. Grid lines = 1cm. **D)** Lysis was visualized by culturing strains overnight in LB + ara at 37°C, spotting 10µL directly onto an LB+20 µg mL$^{-1}$ CPRG plate, and incubating 18hrs at 30°C. Scale bars = 5µm. All experiments are representatives of at least two biological replicates.

1

**Fig 2. A mutant defective in *rlpA* and *mltC* exhibits a chaining defect.**

 **A)** WT and Δ2 LTG (Δ*mltC rlpA::*stop) cultures were grown to OD$_{600}$ ~0.8 in LB at 37°C and imaged on agarose pads.  Cytoplasmic GFP was expressed constitutively from the native *lacZ* locus. **B)** The indicated strains were grown for 4 hours in low salt LB (0 mM NaCl) at 37°C and imaged on agarose pads. **C)** Overnight cultures of the indicated strains grown in LB were diluted in LB and spot-plated on LB and low salt LB and incubated for 18hrs at 30°C. Grid lines = 1cm. **D)** Exponential phase cultures of WT and Δ2 LTG were exposed to 5 mg/mL lysozyme for 10 min. Scale bars = 25 µm.  All data are representative of at least two biological replicates.

**Fig 3. Chaining defects of septal autolysin mutants depend on fast growth and stochastic resolution.**

**A)** WT, Δ2 LTG (Δ*mltC rlpA::*stop), and Δ*amiB* were grown in LB at 37°C and imaged on agarose pads. Cells per chain where manually counted (n >100). Circles represent raw data points of cells/chain (gold bar = median), line graph shows OD$_{600}$. **B)** A Δ2 LTG culture was back-diluted every 2 hrs into pre-warmed LB at 37°C to maintain exponential phase and imaged on agarose pads. Analysis was conducted as described for Fig. 3A **D)** Δ2 LTG Δ*rpoS* was grown in LB at 37°C and imaged on agar pads. Analysis was conducted as described for Fig. 3A **E)** Chloramphenicol (10 µg/mL, ~10 x MIC) was added to cultures after growth to OD$_{600}$~0.45 in LB at 37°C and imaged on agarose pads after 1 hr. Analysis was conducted as described for Fig. 3A. **D)** Δ2 LTG Δ*amiB* was grown in LB at 37°C and imaged on agarose pads. Analysis of chain length and CPRG lysis assays

24

1  were performed as described for Fig. 3A. All data are representative of at least two

2  biological replicates.

3

4  **Fig 4. Lytic transglycosylase activity is required for septal PG resolution.**

5  **A)** Expression of chromosomal $P_{tac}$: *shyC,* $P_{tac}$: *shyA,* $P_{tac}$: *amiB nlpD envC, or* $P_{tac}$: *mltE$_{E.}$*

6  *$_{coli}$* was induced with 1 mM IPTG in a $\Delta$2 LTG background, grown in LB at 37°C to ~$OD_{600}$

7  0.6, and imaged on agarose pads.  Analysis was conducted as described for Fig. 3A

8  **B)**  An overnight culture of $\Delta$2 LTG $P_{tac}$: *mltE$_{E. coli}$* grown in LB was diluted in LB and spot-

9  plated on low salt LB +/- 1mM IPTG and incubated for 18hrs at 30°C. Grid lines = 1cm.

10  All data are representative of at least two biological replicates.

11

12  **Fig 5.  RlpA and MltC are recruited to the septum during late stages of division.**

13  WT carrying pBAD33 *yfp-ftsN* or *yfp-ftsZ* and pHL100 *rlpA-mCherry* or *mltC-mCherry*

14  was grown in M9 + 0.2% glucose supplemented with the appropriate antibiotics at 30°C,

15  induced with 0.2% arabinose and 1mM IPTG after 2hrs, and imaged on agarose pads at

16  $OD_{600}$~0.15.  Demographs of **A)** RlpA/FtsZ co-localization, **B)** RlpA/FtsN co-localization,

17  **C)** MltC/FtsZ co-localization, and **D)** MltC/FtsN co-localization were generated using

18  Oufti. All data are representative of at least two biological replicates.

19

20  **Fig 6.  OM insertion of RlpA and MltC is not required for septal resolution.**

21  **A)** Expression of chromosomal $P_{tac}$: *dsbA$^{ss}$-rlpA$_{[18-263]}$* or *dsbA$^{ss}$-mltC$_{[42-396]}$* was induced

22  with 1 mM IPTG in a $\Delta$2 LTG background, grown in LB at 37°C to ~$OD_{600}$ 0.6, and imaged

23  on agarose pads.  Analysis was conducted as described for Fig. 3A **B)** $\Delta$2 LTG $P_{tac}$:

1    *dsbA^ss^-rlpA*[18-263] was grown in low salt LB +/- 1 mM IPTG at 37°C for 4 hrs and imaged

2    on agarose pads. **C)** Overnight culture of Δ2 LTG P$_{tac}$: *dsbA^ss^-rlpA*[18-263] grown in LB at

3    37°C was spot-plated onto low salt LB +/- 1mM IPTG and incubated overnight at 30°C.

4    Grid lines = 1cm. All data are representative of at least two biological replicates.

5

6    **Fig 7. Model of RlpA and MltC requirement for daughter cell separation**. Septal

7    LTGs may remove PG debris that cannot be processed by other autolysins and that

8    impedes the completion of OM invagination.

9

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# REFERENCES

Aarsman, M. E. G., Piette, A., Fraipont, C., Vinkenvleugel, T. M. F., & Nguyen-distèche, M. (2005). *Maturation of the Escherichia coli divisome occurs in two steps*. *55*, 1631–1645. https://doi.org/10.1111/j.1365-2958.2005.04502.x

Artola-Recolons, C., Lee, M., Bernardo-García, N., Blázquez, B., Hesek, D., Bartual, S. G., … Hermoso, J. A. (2014). Structure and cell wall cleavage by modular lytic transglycosylase MltC of Escherichia coli. *ACS Chemical Biology*, *9*(9), 2058–2066. https://doi.org/10.1021/cb500439c

Babu, M. M., Priya, M. L., Selvan, A. T., Madera, M., Gough, J., Aravind, L., & Sankaran, K. (2006). A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. *Journal of Bacteriology*, *188*(8), 2761–2773. https://doi.org/10.1128/JB.188.8.2761-2773.2006

Bisson-Filho, A. W., Hsu, Y.-P., Squyres, G. R., Kuru, E., Wu, F., Jukes, C., … Garner, E. C. (2018). Treadmilling by FtsZ Filaments Drives Peptidoglycan Synthesis and Bacterial Cell Division. *Science*, *355*(6326), 739–743. https://doi.org/10.1126/science.aak9973.Treadmilling

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., … Shao, Y. (1997). The complete genome sequence of Escherichia coli K-12. *Science*, *277*(5331), 1453–1474. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18430135

Byun, B., Mahasenan, K. V., Dik, D. A., Marous, D. R., Speri, E., Kumarasiri, M., … Mobashery, S. (2018). Mechanism of the Escherichia coli MltE lytic transglycosylase, the cell-wall-penetrating enzyme for Type VI secretion system assembly. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-22527-y

Cava, F., De Pedro, M. A., Lam, H., Davis, B. M., & Waldor, M. K. (2011). Distinct pathways for modification of the bacteria. *EMBO Journal*, *30*(16), 3442–3453. https://doi.org/10.1038/emboj.2011.246

Cava, F., Lam, H., De Pedro, M. A., & Waldor, M. K. (2011). Emerging knowledge of regulatory roles of d-amino acids in bacteria. *Cellular and Molecular Life Sciences*, *68*(5), 817–831. https://doi.org/10.1007/s00018-010-0571-8

Chan, Y. A., Hackett, K. T., & Dillard, J. P. (2012). *The Lytic Transglycosylases of Neisseria gonorrhoeae*. *18*(3). https://doi.org/10.1089/mdr.2012.0001

Cherepanov, P. P., & Wackernagel, W. (1995). Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene*, *158*(1), 9–14. https://doi.org/10.1016/0378-1119(95)00193-A

Cho, H., Wivagg, C. N., Kapoor, M., Barry, Z., Rohs, P. D. A. A., Suh, H., … Bernhardt, T. G. (2016). Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-Autonomously. *Nature Microbiology*, *1*, 1–21. https://doi.org/10.1038/nmicrobiol.2016.172

Cloud, K. A., & Dillard, J. P. (2002). *A Lytic Transglycosylase of Neisseria gonorrhoeae Is Involved in Peptidoglycan-Derived Cytotoxin Production*. *70*(6), 2752–2757. https://doi.org/10.1128/IAI.70.6.2752

Cloud, K. A., & Dillard, J. P. (2004). Mutation of a Single Lytic Transglycosylase Causes

Aberrant Septation and Inhibits Cell Separation of Neisseria gonorrhoeae. *Journal of Bacteriology*, *186*(22), 7811–7814. https://doi.org/10.1128/JB.186.22.7811

Daniel, R. A., & Errington, J. (2003). Control of Cell Morphogenesis in Bacteria: Two Distinct Ways to Make a Rod-Shaped Cell termining cell shape. The relative roles of these closely- related proteins are partially resolved in this paper. The precursor for PG synthesis is a disaccharide pen. *Cell*, *113*, 767–776. Retrieved from http://www.cell.com/cell/pdf/S0092-8674(03)00421-5.pdf

Desmarais, S. M., Tropini, C., Miguel, A., Cava, F., Monds, R. D., de Pedro, M. A., & Huang, K. C. (2015). High-throughput, Highly Sensitive Analyses of Bacterial Morphogenesis Using Ultra Performance Liquid Chromatography. *Journal of Biological Chemistry*, *290*(52), 31090–31100. https://doi.org/10.1074/jbc.m115.661660

Dik, D. A., Marous, D. R., Fisher, J. F., & Mobashery, S. (2017). Lytic transglycosylases: concinnity in concision of the bacterial cell wall. *Critical Reviews in Biochemistry and Molecular Biology*, *52*(5), 503–542. https://doi.org/10.1080/10409238.2017.1337705

Donnenberg, M. S., & Kaper, J. B. (1991). Construction of an eae Deletion Mutant of Enteropathogenic Escherichia coli by Using a Positive-Selection Suicide Vector. *Infection and Immunity*, *59*(12), 4310–4317.

Dörr, T., Alvarez, L., Delgado, F., Davis, B. M., Cava, F., & Waldor, M. K. (2015). A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. *Proceedings of the National Academy of Sciences*, *113*(2), 404–409. https://doi.org/10.1073/pnas.1520333113

Dörr, T., Cava, F., Lam, H., Davis, B. M., & Waldor, M. K. (2013). Substrate specificity of an elongation-specific peptidoglycan endopeptidase and its implications for cell wall architecture and growth of Vibrio cholerae. *Molecular Microbiology*, *89*(5), 949–962. https://doi.org/10.1111/mmi.12323

Dörr, T., Davis, B. M., & Waldor, M. K. (2015). Endopeptidase-Mediated Beta Lactam Tolerance. *PLoS Pathogens*, *11*(4), 1–16. https://doi.org/10.1371/journal.ppat.1004850

Egan, A. J. F., & Vollmer, W. (2013). The physiology of bacterial cell division. *Annals of the New York Academy of Sciences*, *1277*(1), 8–28. https://doi.org/10.1111/j.1749-6632.2012.06818.x

Ferrières, L., Nham, T., Gue, A., Mazel, D., Beloin, C., & Ghigo, J. (2010). *Silent Mischief : Bacteriophage Mu Insertions Contaminate Products of Escherichia coli Random Mutagenesis Performed Using Suicidal Transposon Delivery Plasmids Mobilized by Broad-Host-Range*. *192*(24), 6418–6427. https://doi.org/10.1128/JB.00621-10

Firczuk, M., & Bochtler, M. (2007). Folds and activities of peptidoglycan amidases. *FEMS Microbiology Reviews*, *31*(6), 676–691. https://doi.org/10.1111/j.1574-6976.2007.00084.x

Galli, E., Paly, E., & Barre, F.-X. (2017). Late assembly of the Vibrio cholerae cell division machinery postpones septation to the last 10% of the cell cycle. *Scientific Reports*, *7*(December 2016), 1–11. https://doi.org/10.1038/srep44505

Gamba, P., Veening, J., Saunders, N. J., Hamoen, L. W., & Daniel, R. A. (2009). Two-Step Assembly Dynamics of the Bacillus subtilis Divisome. *Journal of Bacteriology*,

*191*(13), 4186–4194. https://doi.org/10.1128/JB.01758-08

Gerding, M. A., Liu, B., Bendezú, F. O., Hale, C. A., Bernhardt, T. G., & De Boer, P. A. J. (2009). Self-enhanced accumulation of FtsN at division sites and roles for other proteins with a SPOR domain (DamX, DedD, and RlpA) in Escherichia coli cell constriction. *Journal of Bacteriology*, *191*(24), 7383–7401. https://doi.org/10.1128/JB.00811-09

Gibson, D. G., Young, L., Chuang, R., Venter, J. C., Iii, C. A. H., Smith, H. O., & America, N. (2009). *Enzymatic assembly of DNA molecules up to several hundred kilobases. 6*(5), 12–16. https://doi.org/10.1038/NMETH.1318

Goley, E. D., Yeh, Y., Hong, S., Fero, M. J., Abeliuk, E., Mcadams, H. H., & Shapiro, L. (2011). Assembly of the Caulobacter cell division machine. *Molecular Microbiology*, *80*(May), 1680–1698. https://doi.org/10.1111/j.1365-2958.2011.07677.x

Guzman, L. M., Belin, D., Carson, M. J., & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Journal of Bacteriology*, *177*(14), 4121–4130. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7608087%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC177145

Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., … Fraser, C. M. (2000). DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. *Nature, 406*(6795), 477–483. https://doi.org/10.1038/35020000

Heidrich, C., Templin, M. F., Ursinus, A., Merdanovic, M., Berger, J., Schwarz, H., … Höltje, J. V. (2001). Involvement of N-acetylmuramyl-L-alanine amidases in cell separation and antibiotic-induced autolysis of Escherichia coli. *Molecular Microbiology*, *41*(1), 167–178. https://doi.org/10.1046/j.1365-2958.2001.02499.x

Heidrich, C., Ursinus, A., Berger, J., Schwarz, H., & Höltje, J.-V. (2002a). Effects of 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. https://doi.org/10.1128/JB.184.22.6093-6099.2002

Heidrich, C., Ursinus, A., Berger, J., Schwarz, H., & Höltje, J. V. (2002b). Effects of 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. https://doi.org/10.1128/JB.184.22.6093-6099.2002

Herlihey, F. A., & Clarke, A. J. (2017). Controlling Autolysis During Flagella Insertion in Gram-Negative Bacteria. In M. Z. Atassi (Ed.), *Protein Reviews: Volume 17* (pp. 41–56). https://doi.org/10.1007/5584_2016_52

Holtje, J. V, Mirelman, D., Sharon, N., & Schwarz, U. (1975). *Novel Type of Murein Transglycosylase in Escherichia coli. 124*(3), 1067–1076.

Jorgenson, M. A. (2014). A tale of two RLPAs : studies of cell division in Escherichia coli and Pseudomonas aeruginosa. *Iowa Research Online*. Retrieved from https://ir.uiowa.edu/etd/1342.

Jorgenson, M. A., Chen, Y., Yahashiri, A., Popham, D. L., & Weiss, D. S. (2014). *The Bacterial Septal Ring Protein RlpA is a Lytic Transglycosylase that Contributes to Rod Shape and Daughter Cell Separation in <i>Pseudomonas aeruginosa<i>. 93*(1), 113–128. https://doi.org/10.1002/bmb.20244.DNA

Kraft, A. R., Templin, M. F., & Biochemie, A. (1998). *Membrane-Bound Lytic*

*Endotransglycosylase in Escherichia coli. 180*(13), 3441–3447.

Leclercq, S., Derouaux, A., Olatunji, S., Fraipont, C., Egan, A. J. F., Vollmer, W., … Terrak, M. (2017). Interplay between Penicillin-binding proteins and SEDS proteins promotes bacterial cell wall synthesis. *Scientific Reports*, *7*(November 2016), 1–13. https://doi.org/10.1038/srep43306

Lee, M., Hesek, D., Dik, D. A., Fishovitz, J., Lastochkin, E., Boggess, B., … Mobashery, S. (2017). From Genome to Proteome to Elucidation of Reactions for All Eleven Known Lytic Transglycosylases from Pseudomonas aeruginosa. *Angewandte Chemie - International Edition*, *56*(10), 2735–2739. https://doi.org/10.1002/anie.201611279

Lee, M., Hesek, D., Llarrull, L. I., Lastochkin, E., Pi, H., Boggess, B., & Mobashery, S. (2013). Reactions of all escherichia coli lytic transglycosylases with bacterial cell wall. *Journal of the American Chemical Society*, *135*(9), 3311–3314. https://doi.org/10.1021/ja309036q

Lee, T. K., & Huang, K. C. (2013). The role of hydrolases in bacterial cell-wall growth. *Current Opinion in Microbiology*, *16*, 760–766. https://doi.org/10.1016/j.mib.2013.08.005

M9 minimal medium (standard). (2010). *Cold Spring Harbor Protocols* , *2010*(8), pdb.rec12295. https://doi.org/10.1101/pdb.rec12295

M9 Salts. (2006). *Cold Spring Harbor Protocols*, *2006*(1). https://doi.org/10.1101/pdb.rec614

Miyata, S. T., Unterweger, D., Rudko, S. P., & Pukatzki, S. (2013). Dual Expression Profile of Type VI Secretion System Immunity Genes Protects Pandemic Vibrio cholerae. *PLoS Pathogens*, *9*(12), 1–18. https://doi.org/10.1371/journal.ppat.1003752

Möll, A., Dörr, T., Alvarez, L., Chao, M. C., Davis, B. M., Cava, F., & Waldor, M. K. (2014). Cell separation in Vibrio cholerae is mediated by a single amidase whose action is modulated by two nonredundant activators. *Journal of Bacteriology*, *196*(22), 3937–3948. https://doi.org/10.1128/JB.02094-14

Möll, A., Dörr, T., Alvarez, L., Davis, B. M., Cava, F., & Waldor, M. K. (2015). A D, D-carboxypeptidase is required for Vibrio cholerae halotolerance. *Environmental Microbiology*, *17*(2), 527–540. https://doi.org/10.1111/1462-2920.12779

Murphy, K. C., & Campellone, K. G. (2003). Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic E. coli. *BMC Molecular Biology*, *4*, 1–12. https://doi.org/10.1186/1471-2199-4-11

Nambu, T., Minamino, T., & Macnab, R. M. (1999). *Peptidoglycan-Hydrolyzing Activity of the FlgJ Protein , Essential for Flagellar Rod Formation in Salmonella typhimurium. 181*(5), 1555–1561.

Osawa, M., & Erickson, H. P. (2018). Turgor Pressure and Possible Constriction Mechanisms in Bacterial Division. *Frontiers in Microbiology*, *9*(January), 1–7. https://doi.org/10.3389/fmicb.2018.00111

Paintdakhi, A., Parry, B., Campos, M., Irnov, I., Elf, J., Surovtsev, I., & Jacobs-wagner, C. (2016). Oufti : an integrated software package for high-accuracy , high-throughput quantitative microscopy analysis. *Molecular Microbiology*, (2015), 767–777. https://doi.org/10.1111/mmi.13264

Paradis-bleau, C., Kritikos, G., Orlova, K., Typas, A., & Bernhardt, T. G. (2014). *A*

*Genome-Wide Screen for Bacterial Envelope Biogenesis Mutants Identifies a Novel Factor Involved in Cell Wall Precursor Metabolism. 10*(1). https://doi.org/10.1371/journal.pgen.1004056

Peters, N. T., Dinh, T., & Bernhardt, T. G. (2011). A Fail-safe mechanism in the septal ring assembly pathway generated by the sequential recruitment of cell separation amidases and their activators. *Journal of Bacteriology, 193*(18), 4973–4983. https://doi.org/10.1128/JB.00316-11

Potluri, L. P., Kannan, S., & Young, K. D. (2012). ZipA is required for FtsZ-dependent preseptal peptidoglycan synthesis prior to invagination during cell division. *Journal of Bacteriology, 194*(19), 5334–5342. https://doi.org/10.1128/JB.00859-12

Priyadarshini, R., De Pedro, M. A., & Young, K. D. (2007). Role of peptidoglycan amidases in the development and morphology of the division septum in Escherichia coli. *Journal of Bacteriology, 189*(14), 5334–5347. https://doi.org/10.1128/JB.00415-07

Priyadarshini, R., Popham, D. L., & Young, K. D. (2006). Daughter Cell Separation by Penicillin-Binding Proteins and Peptidoglycan Amidases in Escherichia coli. *Journal of Bacteriology, 188*(15), 5345–5355. https://doi.org/10.1128/JB.00476-06

Rojas, E. R., Billings, G., Odermatt, P. D., Auer, G. K., Zhu, L., Miguel, A., … Huang, K. C. (2018). The outer membrane is an essential load-bearing element in Gram-negative bacteria. *Nature, 559*(7715), 617–621. https://doi.org/10.1038/s41586-018-0344-3

Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in Escherichia coli: Advances and challenges. *Frontiers in Microbiology, 5*, 1–17. https://doi.org/10.3389/fmicb.2014.00172

Santin, Y. G., & Cascales, E. (2017). Domestication of a housekeeping transglycosylase for assembly of a Type VI secretion system. *EMBO Reports, 18*(1), 138–149. https://doi.org/10.15252/embr.201643206

Taguchi, A., Welsh, M. A., Marmont, L. S., Lee, W., Sjodt, M., Kruse, A. C., … Walker, S. (2019). FtsW is a peptidoglycan polymerase that is functional only in complex with its cognate penicillin-binding protein. *Nature Microbiology, 4*(April). https://doi.org/10.1038/s41564-018-0345-x

Thomason, L. C., Costantino, N., & Court, D. L. (2007).  E. coli Genome Manipulation by P1 Transduction . *Current Protocols in Molecular Biology*, (July), 1.17.1-1.17.8. https://doi.org/10.1002/0471142727.mb0117s79

Tsang, M. J., Yakhnina, A. A., & Bernhardt, T. G. (2017). NlpD links cell wall remodeling and outer membrane invagination during cytokinesis in Escherichia coli. In *PLoS Genetics* (Vol. 13). https://doi.org/10.1371/journal.pgen.1006888

Tsui, H.-C. T., Zheng, J. J., Magallon, A. N., Ryan, J. D., Yunk, R., Rued, B. E., … Winkler, M. E. (2016). Suppression of a deletion mutation in the gene encoding essential PBP2b reveals a new lytic transglycosylase involved in peripheral peptidoglycan synthesis in <i>Streptococcus pneumoniae<i> D39. *Molec, 100*(6), 1039–1065. https://doi.org/10.1111/mmi.13366

Uehara, T., Dinh, T., & Bernhardt, T. G. (2009). LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in Escherichia coli. *Journal of Bacteriology, 191*(16), 5094–5107. https://doi.org/10.1128/JB.00505-09

Uehara, T., Parzych, K. R., Dinh, T., & Bernhardt, T. G. (2010). Daughter cell separation

1       is controlled by cytokinetic ring-activated cell wall hydrolysis. *EMBO Journal*, *29*(8),
2       1412–1422. https://doi.org/10.1038/emboj.2010.36
3 Wall, C., Monteiro, C., Fang, X., Ahmad, I., Gomelsky, M., & Ro, U. (2011). Regulation
4       of Biofilm Components in Salmonella enterica Serovar Typhimurium by Lytic
5       Transglycosylases Involved in. *Journal of Bacteriology*, *193*(23), 6443–6451.
6       https://doi.org/10.1128/JB.00425-11
7 Wanner, K. A. D. and B. L. (2000). *Escherichia One-step inactivation of chromosomal
8       genes in. xx*(xx), 1–7. https://doi.org/10.1016/j.jbiosc.2015.08.006
9 Williams, A. H., Wheeler, R., Rateau, L., Malosse, C., Chamot-Rooke, J., Haouz, A., …
10       Boneca, I. G. (2018). A step-by-step in crystallo guide to bond cleavage and 1,6-
11       anhydro-sugar product synthesis by a peptidoglycan-degrading lytic
12       transglycosylase. *Journal of Biological Chemistry*, *293*(16), 6000–6010.
13       https://doi.org/10.1074/jbc.RA117.001095
14 Yahashiri, A., Jorgenson, M. A., & Weiss, D. S. (2015). Bacterial SPOR domains are
15       recruited to septal peptidoglycan by binding to glycan strands that lack stem
16       peptides. *Proceedings of the National Academy of Sciences*, *112*(36), 11347–
17       11352. https://doi.org/10.1073/pnas.1508536112
18 Yang, D. C., Peters, N. T., Parzych, K. R., Uehara, T., Markovski, M., & Bernhardt, T. G.
19       (2011). An ATP-binding cassette transporter-like complex governs cell-wall
20       hydrolysis at the bacterial cytokinetic ring. *Proceedings of the National Academy of
21       Sciences of the United States of America*, *108*(45), E1052–E1060.
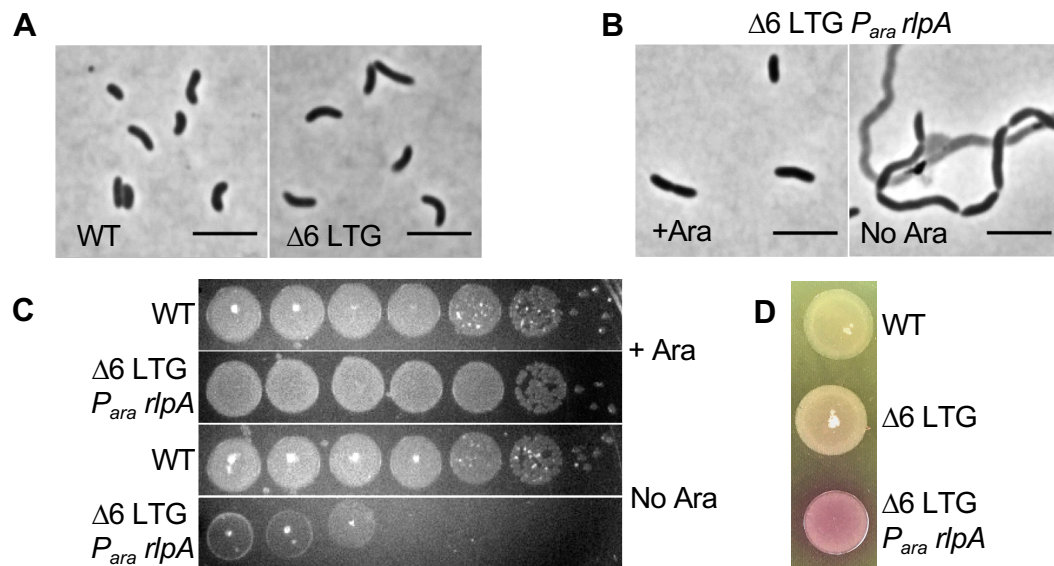22       https://doi.org/10.1073/pnas.1107780108
23 Yang, X., Lyu, Z., Miguel, A., McQuillen, R., Huang, K. C., & Xiao, J. (2017). GTPase
24       activity–coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall
25       synthesis. *Science*, *355*(February), 744–747.
26       https://doi.org/10.1126/science.aak9995
27 Yunck, R., Cho, H., & Bernhardt, T. G. (2016). Identification of MltG as a potential
28       terminase for peptidoglycan polymerization in bacteria. *Molecular Microbiology*,
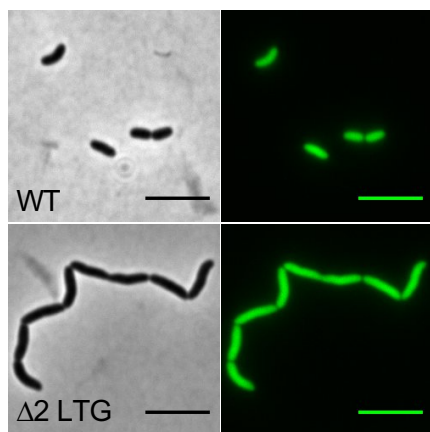29       *99*(4), 700–718. https://doi.org/10.1111/mmi.13258
30 Zhao, H., Patel, V., Helmann, J. D., & Dörr, T. (2017). Don't let sleeping dogmas lie:
31       new views of peptidoglycan synthesis and its regulation. *Molecular Microbiology*,
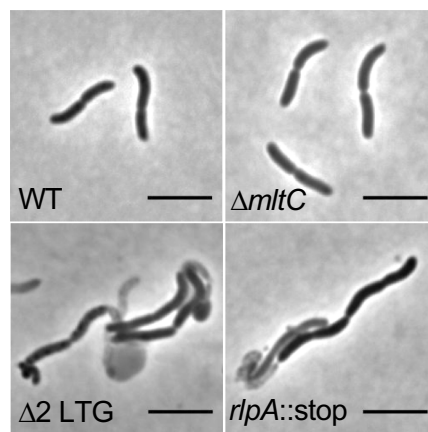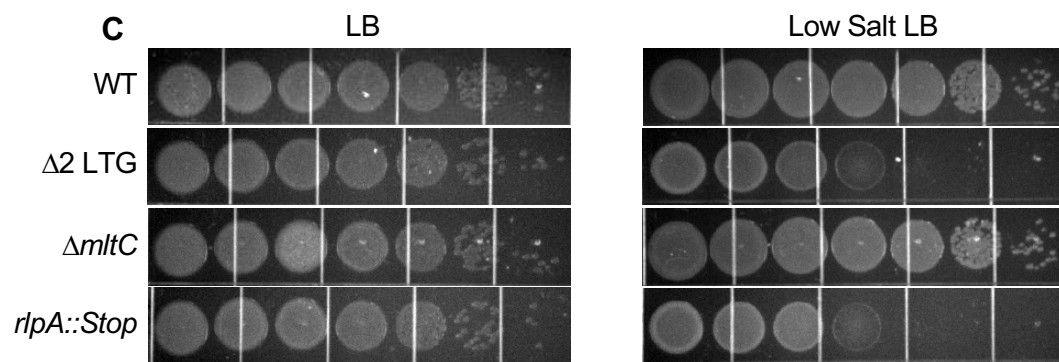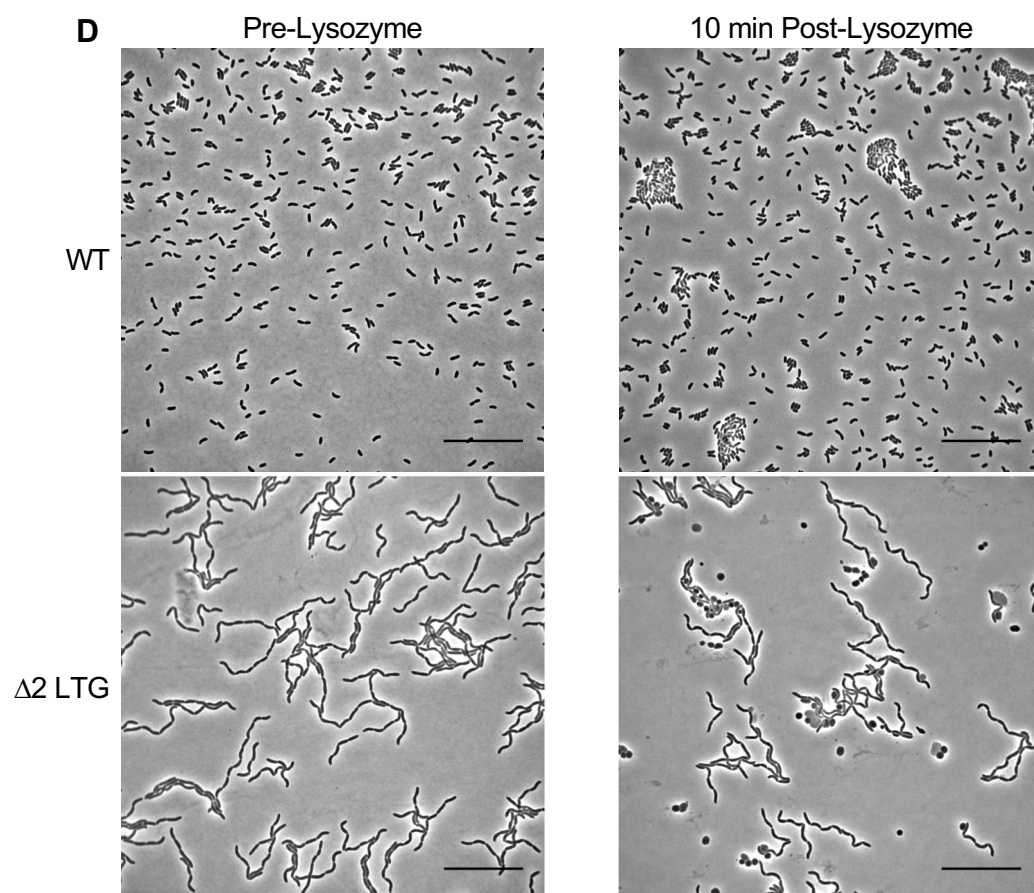32       *106*(6), 847–860. https://doi.org/10.1111/mmi.13853
33

**Fig 1**

**A**



WT      Δ6 LTG

**B**     Δ6 LTG $P_{ara}$ *rlpA*



+Ara      No Ara

**C**



WT

Δ6 LTG $P_{ara}$ *rlpA*     + Ara

WT

Δ6 LTG $P_{ara}$ *rlpA*     No Ara

**D**



WT

Δ6 LTG

Δ6 LTG $P_{ara}$ *rlpA*

**Fig 2**

**A**



WT

Δ2 LTG

**B**

WT

ΔmltC

Δ2 LTG

rlpA::stop

**C**

| | LB | | Low Salt LB |
|---|---|---|---|
| WT | | | |
| Δ2 LTG | | | |
| ΔmltC | | | |
| rlpA::Stop | | | |

**D**

Pre-Lysozyme    10 min Post-Lysozyme

WT

Δ2 LTG

# Fig 3

**A**

### WT



**B**

### Δ2 LTG "Chemostat"



### Δ2 LTG



**C**

### Δ2 LTG



|  | 1.5hrs | 2.5hrs | 1.5hrs | 2.5hrs |
|--|--------|--------|--------|--------|
|  | No cm | | + cm10 | |

### Δ*amiB*



**D**

### Δ2 LTG
### Δ*amiB*

**Fig 4**

**A**



**B**



Δ2 LTG *lac::P_tac Ec mltE*

Low Salt No IPTG

Low Salt + IPTG

# Fig 5

**A**



**B**



**C**



**D**

**Fig 6**

**A**

Δ**2 LTG**



**B**

Δ2 LTG
*lacZ::P_tac dsbA^ss-rlpA*

Low Salt | Low Salt + IPTG



**C**

Δ2 LTG
*lacZ::P_tac dsbA^ss-rlpA*

Low Salt

Low Salt
+ IPTG

**Fig 7**



GlcNAC-MurNAC

Peptide Crosslink

Amidase

Zone of Amidase Activity

MltC

RlpA

Endopeptidase