Insights into the role of lipoteichoic acids in *Bacillus subtilis*- a new function for MprF

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Running title: MprF alters lipoteichoic acid production

Abstract

1 In *Bacillus subtilis*, the cell is protected from the environment by a cell envelope, which
 2 comprises of layers of peptidoglycan that maintain the cell shape and anionic teichoic acids
 3 polymers whose biological function remains unclear. In *B. subtilis*, loss of all Class A
 4 Penicillin-Binding Proteins (aPBPs) which function in peptidoglycan synthesis is conditionally
 5 lethal. Here we show that this lethality is associated with an alteration of the lipoteichoic acids
 6 (LTA) and the accumulation of the major autolysin LytE in the cell wall. We provide the first
 7 evidence that the length and abundance of LTA acts to regulate the cellular level of LytE.
 8 Importantly, we identify a novel function for the aminoacyl-phosphatidylglycerol synthase
 9 MprF which acts to modulate LTA biosynthesis in *B. subtilis* and in the pathogen
 10 *Staphylococcus aureus*. This finding has implications for our understanding of antimicrobial
 11 peptide resistance (particularly daptomycin) in clinically relevant bacteria and MprF-
 12 associated virulence in pathogens, such as methicillin resistant *S. aureus*.

Keywords: *Bacillus*/ LTA/ LytE/ MprF/ PBP1

Introduction

Gram-positive bacteria have a common cell envelope architecture that contributes to maintaining cell shape and protects the cell from environmental changes. The best characterised component of the cell envelope is the peptidoglycan (PG) which forms a mesh like structure enclosing the cell membrane. Anionic polymers are either covalently attached to PG N-acetylmuramic acids and known as wall-teichoic acids (WTA) or are tethered to the external face of the membrane and named lipoteichoic acids (LTA) (Hanson and Neely, 2012, Percy and Grundling, 2014, Schneewind and Missiakas, 2016, Rajagopal and Walker, 2017). It is unclear whether LTA are localized in a periplasm-like space between the membrane and PG or extend through the PG layers (Neuhaus and Baddiley, 2003, Erickson, 2021). Peptidoglycan is synthesised by glycosyltransferases (GTases) which catalyse the addition of a disaccharide pentapeptide precursor (lipid II), to the nascent PG chain while transpeptidase (TPase) enzymes cross-link a proportion of the pentapeptides to adjacent PG strands (Pazos and Vollmer, 2021, Straume et al., 2021). In the absence of the bifunctional transmembrane GTase and TPase enzymes, known as class A Penicillin-Binding Proteins (aPBPs), *B. subtilis* cells are viable but the cells are longer and thinner and are prone to lysis (McPherson and Popham, 2003, Kawai et al., 2009b). The viability of *B. subtilis* lacking all aPBPs (referred as $\Delta 4$) depends on the functionality of the essential protein RodA, that was recently identified to function as a PG glycosyltransferase (Meeske et al., 2016, Emami et al., 2017). The $\Delta 4$ strain is conditionally lethal on PAB agar medium unless supplemented with magnesium (Kawai et al., 2009b). It is thought that this divalent cation alters the structure of the wall by binding to teichoic acids or PG and by doing so, might alter the activity of cell wall hydrolases, the enzymes that modify and sculpt synthesised PG to allow the cell to elongate and divide (Neuhaus and Baddiley, 2003, Do et al., 2020, Wilson and Garner, 2021).

In rod-shaped *B. subtilis* 168, LTA and WTA are composed of polyglycerolphosphate (polyGroP) generated by specific biosynthetic pathways (**Fig 1A**) (Neuhaus and Baddiley, 2003, Percy and Grundling, 2014, Schneewind and Missiakas, 2016, Rajagopal and Walker, 2017). Importantly, *B. subtilis* is not able to survive the loss of both LTA and WTA synthesis (Schirner et al., 2009) and these teichoic acids have roles in cell separation-division (Schirner et al., 2009, Wormann et al., 2011) and cell elongation (Soldo et al., 2002), respectively. The net anionic charge of these polymers can be altered by D-alanylation, a process proposed to regulate autolysin activity and cell wall ion homeostasis (Neuhaus and Baddiley, 2003, Schneewind and Missiakas, 2016).

The teichoic acids located in the wall and the membrane phospholipids produced by MprF both impact on the negative net charge of the bacterial cell envelopes (**Fig 1A**) (Peschel et al., 2001, Slavetinsky et al., 2017). The transmembrane protein MprF is the only enzyme known to synthesise and translocate aminoacyl-phosphatidylglycerol (aaPGol), in particular lysyl-PGol (**Fig 1A**) (Staubitz and Peschel, 2002, Johnson and Grossman, 2016, Atila et al., 2016, Ernst and Peschel, 2019). MprF plays a role in the virulence of bacterial pathogens (Ernst and Peschel, 2019, Nowak et al., 2021) and in *B. subtilis*, it confers resistance against positively charged antimicrobial peptides such as nisin and daptomycin through mechanisms that are not yet characterized (Salzberg and Helmann, 2008, Hachmann et al., 2009).

Phosphatidylglycerol is also consumed by LTA-synthases LtaS ('house-keeping'), YfnI ('stress') and YqgS ('sporulation') (Schirner et al., 2009, Wormann et al., 2011) (**Fig 1A**). These transmembrane LTA-synthases have an extracellular catalytic domain that uses PGol to generate a polyGroP polymer on a lipid anchor (Percy and Grundling, 2014, Schneewind and Missiakas, 2016). The lipid anchor is usually a diglucosyldiacylglycerol (Glc₂DAG) produced by UgtP on the internal surface of the cell membrane (Jorasch et al., 1998) (**Fig 1A**). As a *ugtP* mutant still produces LTA (Lazarevic et al., 2005), it suggests that polyGroP polymers are

anchored to the lipid diacylglycerol (DAG) as shown in *Staphylococcus aureus* *AugtP* (Grundling and Schneewind, 2007a, Hesser et al., 2020b).

In *S. aureus*, LTA is essential and seems to promote autolytic activity (Oku et al., 2009), while WTA is dispensable but appears to restrict autolytic activity (Hanson and Neely, 2012, Rajagopal and Walker, 2017). Contrary to *B. subtilis*, *S. aureus* has only one LTA-synthase (LtaS) whose absence is (conditionally) essential (Coe et al., 2019) and can be complemented by *B. subtilis* *ltaS* (Grundling and Schneewind, 2007b). A recent *in vitro* study showed that *S. aureus* LtaS is processive and that the LTA polymer elongation is directly regulated by the identity and concentration of lipid starter units. The use of Glc₂DAG as a preferred starter unit led to the formation of shorter polymer (Hesser et al., 2020b) while the use of PGol resulted in the synthesis of longer polymers. It is not clear if this mechanism might be conserved in *B. subtilis* however, in absence of *B. subtilis* LtaS, YfnI expression and LTA length increase (Wormann et al., 2011, Hashimoto et al., 2013).

Through characterising *B. subtilis* strains lacking peptidoglycan GTase activity (Emami et al., 2017), we aimed to understand the cell wall stresses causing aPBP mutants to lyse on glucose rich media. Collectively, our data supports the idea that in *B. subtilis* LTA length acts to moderate the activity of the major autolysin LytE. The presence of glucose increases the expression of LtaS, YfnI, and their activity results in an alteration of LTA production in absence of major PBP1. Crucially, we identify a new role for MprF in altering LTA polymerisation in *B. subtilis* and the pathogen *S. aureus*. Our results reveal that MprF acts to change the cell envelope in a more dramatic way than simply increasing the positive charge on the cell membrane which has implications for understanding the virulence associated to MprF in Gram-positive bacteria.

Results

MprF alters cell viability in absence of *ponA* or *mbl*

In *Bacillus subtilis*, the absence of all aPBPs ($\Delta 4$ for $\Delta ponA \Delta pbpD \Delta pbpF \Delta pbpG$ mutant) is conditionally lethal on glucose-rich PAB agar medium unless supplemented with magnesium (Kawai et al., 2009b, Emami et al., 2017). The suppressive effect of divalent magnesium has been observed in other cell-wall deficient mutants (Murray et al., 1998, Claessen et al., 2008, Kawai et al., 2009b, Schirner et al., 2009) and it is not well understood. To identify the cell wall factors able to suppress $\Delta 4$ lethality, we applied a random transposition mutagenesis to a strain deleted for the vegetative aPBPs (**Tables EV1-3**). Mapping of the transposon in strain SWA11a revealed its insertion in the gene *mprF*, encoding a lysyl-phosphatidylglycerol (LysPGol) synthase known to participate in phospholipids metabolism (**Fig 1A**) (Staubitz and Peschel, 2002, Salzberg and Helmann, 2008). Introduction of a null *mprF* deletion into $\Delta 4$, confirmed that $\Delta 4 \Delta mprF$ was viable on nutrient agar (NA) with glucose and on PAB agar (**Fig 1B, Appendix Fig S1A**). In absence of *ponA* encoding the major aPBP (PBP1), the single mutant is viable and yet its growth and the colony morphology is seen to improve in absence of *mprF* (**Fig 1B, Fig EV1**).

We reasoned that if MprF activity is detrimental to $\Delta 4$, its overexpression should be deleterious to $\Delta ponA$. Indeed, overexpression of MprF (MprF $\uparrow$) was lethal to *ponA*-deleted strains on NA-glucose (**Fig 1B**). On NA, both $\Delta ponA$ and $\Delta 4$ strains grew slowly (**Fig 1B**) but no obvious effect was observed for 168CA and $\Delta 3$ aPBPs (i.e. $\Delta pbpD \Delta pbpF \Delta pbpG$). Consistently, the overexpression of MprF caused the cells of $\Delta ponA$ and $\Delta 4$ to lyse in nutrient broth (NB) (**Appendix Fig S1B**).

Figure 1. MprF alters cell viability of *B. subtilis* class A PBPs and *mbl* mutants.

A A schematic representation of the biosynthetic links between lipoteichoic acid, phospholipids and glucolipids in *B. subtilis*, adapted from previous studies (Matsuoka et al., 2011, Schneewind and Missiakas, 2016). Lipoteichoic acid (LTA) consist of glycerolphosphate units anchored to the membrane by a glycolipid. In absence of cytoplasmic UgtP, LTA polymer is linked to the membrane by a diacylglycerol (DAG), instead of a diglucosyldiacylglycerol (Glc₂DAG). When grown in LB at 37°C, the majority of *B. subtilis* phospholipids are phosphatidylglycerol (PGol) (37.5% ± 6.2), phosphatidylethanolamine PE (30.5% ± 3.8), Glc₂DAG (9.6% ± 4.7) and lysyl-phosphatidylglycerol (LysPGol, 7.8% ± 3.7) produced by MprF (Matsuoka et al., 2011). Recently, L- and N- succinyl-LysPGol, and also L- and D-AlaPGol were identified in *B. subtilis* (Atila et al., 2016). Grey arrow indicates a reaction step that might be bidirectional.

B Ten-fold spot growth assays of strains *B. subtilis* wild type 168CA, AG157 (*AponA ΔpbpD ΔpbpF ΔpbpG* renamed *Δ4*), AG223 (*Δ4 ΔmprF*), SWA11a (*AponA ΔpbpD ΔpbpF mprF::TnYLB-1 lacA*), AG417 (*ΔpbpD*

ΔpbpF ΔpbpG renamed *Δ3*), RE101 (*ΔponA*), AG193 (*ΔponA ΔmprF*) and AG181 (*ΔmprF*). Plates (here Glc 1%) were incubated at 37°C for 24 h and images taken.

C The effect of *mprF* overexpression (MprF ↑) is assay in different strain backgrounds using an IPTG-inducible *mprF* construct. Plates (here Glc 1%) were incubated at 37°C for 24 h and scanned. Strains tested were AG304 (MprF ↑), AG317 (*Δ4* MprF ↑), AG311 (*ΔponA* MprF ↑), AG421 (*Δ3* MprF ↑) and AG322 (*Δmbl* MprF ↑).

D Phase-contrast and membrane dye (FM5-95) microscopy images of *B. subtilis* 168CA and AG322 (*Δmbl* MprF ↑) strains grown in NB ± IPTG (0.1 mM) to mid-exponential phase at 37°C. Scale bar indicates 5 μm.

E-H Transmission electron microscopy (TEM) images show cross-sections of (F) wild type 168CA, (G) AG181 (*ΔmprF*) and (H-I) AG304 strain (MprF ↑) grown in nutrient broth (and IPTG for AG304). Arrows indicate abnormal cell wall structures that seem specific to the strain MprF ↑. All images were taken at 15,000x magnification and processed in Fiji with scale bars attached.

Data information. Strains phenotype was tested at least three times, TEM experiment was carried out one time. Supporting information for this figure is presented in **Appendix Figures S1-S3**

The identification of *ΔmprF* as a suppressor suggested that increased negative charge on the membrane could contribute to the rescue of *Δ4* growth. This seemed to contradict the long-standing hypothetical role of magnesium in altering the cell envelope by neutralizing anionic polymers and/or autolysis activity (Neuhaus and Baddiley, 2003, Hanson and Neely, 2012). To determine if electrostatic change in the wall was the suppressive effect we deleted *dltAB* in *Δ4*, as the Dlt proteins add positive charges to the LTA polymers by D-alanylation (Perego et al., 1995, Wood et al., 2018). However, the new mutant was unable to grow on glucose rich media (our unpublished observations).

To understand if the effect of MprF was specific to *Δ4*, we altered MprF expression in known magnesium dependent mutants. The *B. subtilis* *Δmbl* mutant requires an elevated level of magnesium for growth compared to *ΔmreB* (**Appendix Fig S2**). MreB, Mbl and MreBH are actin-like isologues which help maintain cell shape by controlling the cell wall synthesis (Kawai et al., 2009a) and the major autolytic enzymes CwlO and LytE (Mbl for CwlO and MreB/BH for LytE) (Dominguez-Cuevas et al., 2013). Interestingly, we identified that MprF overexpression rescued the growth of *Δmbl* on NA-glucose and NA (**Fig 1C**). In addition, morphology defects were less pronounced, changing towards a wide rod-like shape and cell chains were less often twisted (**Fig 1D**).

To explain our genetic findings in *Δmbl* background, we had to consider that MprF might play a role in cell wall metabolism. This idea seemed to be supported by our observations made by

transmission electron microscopy (TEM) in a wild type background overexpressing MprF (**Fig 1GH, Appendix Fig S3**). Here cells exhibited an increase in cell wall thickness (e.g. cell poles with a ‘dented’ shape, lateral walls with septum misplacement), a defect similar to that observed in cell division mutants. However the change in cell morphology of the strain deleted for *mprF* was less significant (**Fig 1F**).

Lethality of *B. subtilis* Δmbl is suppressed by the deletion of the major LTA-synthase LtaS (Schirner et al., 2009) and in a single mutant $\Delta ltaS$, the LTA-synthase YfnI synthesises longer LTA polymers (Wormann et al., 2011). This suggested that the overexpression of MprF in Δmbl has contributed to diminish cellular level of PGol (through a different pathway, **Fig 1A**) (Johnson and Grossman, 2016) or MprF possibly functions in LTA synthesis.

Conditional essentiality of UgtP in the absence of MprF and the Class A PBPs

To comprehend the effect of MprF-loss in a strain lacking aPBPs, we carried out a transposon screen in $\Delta 4 \Delta mprF$ pLOSS-*P_{spac}-ponA* strain (Emami et al., 2017) (AG200, **Table EV2**). Strain AG200BK#42 which stabilized the plasmid carried a transposon inserted in the gene *gtab*. GtaB catalyses a cytoplasmic conversion of Glucose-1-P to UDP-Glc, where the latter ultimately contributes to LTA synthesis and WTA modification (**Fig 1A**). Subsequent mutagenesis in backgrounds $\Delta 4$ and $\Delta 4 \Delta mprF$ confirmed the lethal (NA-glucose) and sick (NA) phenotypes in absence of GtaB (**Fig 2A**).

To determine if this was related to LTA synthesis or WTA modification, we deleted sequentially, in the $\Delta 4$ or $\Delta 4$ pLOSS-*P_{spac}-ponA* backgrounds, each enzyme whose function is immediately upstream or downstream of GtaB (**Fig 1A**). Characterisation of the resulting strains (**Table S2**) showed that only deletion of *ugtP*, the enzyme which produces the Glc₂DAG lipid anchor for LTA, had a similar effect to that of *gtab*. Strain $\Delta 4 \Delta mprF \Delta ugtP$ was unable to grow on NA glucose (**Fig 2A**), a phenotype common to $\Delta ponA \Delta gtaB$ and $\Delta ponA \Delta ugtP$ (**Appendix Fig S4**). On NA, $\Delta 4 \Delta gtaB$ and $\Delta 4 \Delta ugtP$ grew very slowly (**Fig 2A**, right panel).

151 LysPGol content increases in *ΔugtP* cells (Johnson and Grossman, 2016) which could explain
152 the similarity of phenotype of *Δ4 ΔugtP* and *Δ4 MprF* ↑ (i.e. slow growing on NA) but not the
153 dominant effect of *ΔugtP* on *Δ4 ΔmprF*. In *S. aureus*, it has been observed that *ΔugtP* (*ypfP*)
154 and *ΔgtaB* mutants produce long LTA (Grundling and Schneewind, 2007a). Thus this context
155 and our results reinforced the idea that MprF may have a role in LTA synthesis.

Figure 2. LtaS causes cell morphogenesis defect of an all-class A PBP mutant.

A Absence of *gtaB* or *ugtP* causes severe growth defect in absence of class A PBPs and MprF. Strains were grown exponentially in NB with 20 mM MgSO₄, then were washed and diluted in NB prior to preparing a 10-fold serial dilution in NB spotting on the NA and NA glucose (0.5%) plates which were incubated at 37°C and imaged after 24 h and 45 h. Strains were also spotted on same media supplemented with 10 mM MgSO₄ to confirm all the strains dilution spots could grow, at 17 h of incubation only *Δ4 ΔgtaB* and *Δ4 ΔugtP* displayed colonies of smaller size at that time point (data not shown). Strains tested: *B. subtilis* wild type (168CA), PG253 (*ΔugtP*), SM08 (*ΔgtaB*), AG157 (*Δ4*), AG290NopLOSS (*Δ4 ΔgtaB*), AG443 (*Δ4 ΔugtP*), AG223 (*Δ4 ΔmprF*), AG636 (*Δ4 ΔmprF gtaB::TnYLB-1*) and AG632 (*Δ4 ΔmprF ΔugtP*).

B Spot dilution growth assays for the following strains: *B. subtilis* wild type 168CA, AG157 (*Δ4*), AG342 (*Δ4 ΔltaS*), AG343 (*Δ4 ΔyfnI*), AG344 (*Δ4 ΔyqgS*), AG370 (*Δ4 ΔltaS ΔyfnI*), AG372 (*Δ4 ΔltaS ΔyqgS*), AG377 (*Δ4 ΔyfnI ΔyqgS*) and AG380 (*Δ4 ΔltaS ΔyfnI ΔyqgS*). Samples were inoculated onto NA ± Glc 1% and, incubated at 37°C for 24 h.

C Ten-fold serial dilutions were prepared for *B. subtilis* wild type 168CA, AG223 (*Δ4 ΔmprF*), AG389 (AG223 *ΔltaS ΔyqgS*), AG390 (AG223 *ΔyfnI ΔyqgS*), AG399 (AG223 *ΔltaS ΔyfnI*) and AG400 (AG223 *ΔyqgS ΔltaS ΔyfnI*) strains. Plates (here Glc 1%) were incubated at 37°C and imaged after 24h.

D Representative microscopy images of cells of strains of (A) grown in NB at 37°C for 120 min and stained with a FM5-95 dye. Scale bar represents 5 µm. The average of cells diameter extracted from **Appendix Figure S5** is indicated. * The cells measurement was done on the straight regions of the cell chain.
Data information: Strains phenotype was tested at least three times. Supporting information is presented in **Appendix Figure S4-S6**. **Appendix Figures S4, S6** displayed results obtained in *ΔponA* background.

Growth of a strain lacking all aPBPs mutant improves in absence of LtaS

To verify that LTA synthesis alters *Δ4* growth, we analysed the phenotype of strains lacking aPBPs and the LTA-synthase genes individually or in combination (**Fig 1A**). Surprisingly, the *Δ4 ΔltaS* mutant was viable on NA-glucose as well as with the additional deletion of *yfnI* or *yqgS* (**Fig 2B**). Thus, LtaS activity is also linked to the glucose-associated lethality of *Δ4*. Interestingly, the absence of MprF is more advantageous to *Δ4*, as it results in a faster growth and larger colonies formed on NA-glucose compared to *Δ4 ΔltaS* (**Fig EV1**). This phenotype difference indicated that slightly different mechanisms helped restore the growth of *Δ4*. To test this, LTA-synthase genes were systematically deleted in the *Δ4 ΔmprF* (AG223) background and the viability of the strains generated determined on NA with or without glucose. All the strains remained viable on NA glucose (**Fig 2C**) which indicated the effect of MprF loss could be related to LTA. In support of this, in the *Δ4 ΔmprF* background the combined absence of *ltaS* and *yfnI* delayed growth on NA glucose resulting in a phenotype similar to that observed on NA (**Fig 2C**). Thus, in these specific genetic backgrounds LTA synthesis is not essential but desirable for cell growth.

Neither absence of LtaS or MprF has an effect on the filamentous thin cell morphology of *Δ4* (**Appendix Fig S5**) but cell lysis is significantly decreased in NB. In the absence of all LTA-synthases, *Bacillus* is viable but the strain forms filamentous and clumpy cells (Schirner et al., 2009). We observed that the combined absence of LtaS, YfnI and/or YqgS in *Δ4*, *ΔponA* or *Δ4 ΔmprF* genetic backgrounds lead to an increase in cell diameter and cells remained attached at the site of division (**Fig 2D, Appendix Fig S5-6**). However, when LtaS was the only functional LTA-synthase (e.g. *Δ4 ΔyqgS ΔyfnI*, **Fig 2D**), cells were thin and comparable to *Δ4* and the

ΔponA background (**Appendix Fig S6**). Collectively, our results indicate that LtaS activity accounts for the conditional lethality of *Δl*.

Impact of glucose, PBP1 and MprF on LTA synthesis.

To comprehend the effect of LtaS, MprF and UgtP in the absence of aPBPs we decided to analyse LTA production. We developed a Western blot protocol relying on commercially available Gram-positive monoclonal LTA and polyclonal PBP2B antibodies. First we determined the LTA profile for 168CA and the LTA-synthases mutants (**Fig 3A** and **Appendix Fig S7**) grown in parallel in NB and in NB glucose (0.2%). The wild type LTA signal corresponded to a mobility comparable to 7-17 kDa as previously identified (Wormann et al., 2011). Interestingly, our media comparison showed that LTA-signal in *ΔltaS* cells grown in NB-glucose (> 15 kDa) (**Fig 3A** left panel, 2nd lane) was distinct from that seen for growth in NB (7-32 kDa) (**Fig 3A** right panel, 2nd lane). However, the LTA pattern was different when YfnI was the only functional LTA-synthase (**Fig 3A**). From this analysis, short LTA (<10 kDa) were more abundantly produced in NB-glucose presumably through LtaS activity, as we observed an increase in short LTA in *ΔyfnI* and *ΔyfnI ΔyqgS* mutants compared to 168CA (**Fig 3A** left panel, 3rd & 5th lanes). Collectively, our results show that the regulation of LTA synthesis is more complex than thought (Wormann et al., 2011) as it alters in presence of glucose.

As the *mprF* mutation suppressed the *ΔponA* phenotype (**Fig 1B**, **Fig EV1**, **Appendix Fig 6A**) we reasoned that any alteration of LTA synthesis would be detectable in a strain where only the major aPBP was deleted. Moreover, *ΔponA* is impaired but viable on NA-glucose (0.5%), whereas the multiple aPBP deletion strain was very lytic and did not grow under these conditions (**Fig EV1**). Thus, we settled to work in *ΔponA* background and using NB glucose 0.2% for growth of samples for the Western blot analysis. This revealed that there was an

increase of short LTA in *ΔponA* cells grown in the presence of glucose (**Fig 3B** left panel, 3rd lane, **Appendix Fig S8**). The LTA signal of *ΔponA ΔmprF* decreased compared to *ΔponA* (**Fig 3B** left panel, 5th lane) and the *ΔmprF* mutant in NB-glucose (**Fig 3B** left panel, 4th lane) decreased compared to 168CA. However, we found that *mprF* overexpression (MprF ↑) had a significant effect, increasing LTA polymer length (6th lane in **Fig 3B**). This effect was independent of YfnI, as a similar LTA profile was observed in a strain *ΔyfnI* MprF ↑ (our unpublished observations, strain AG353 in **Table EV2**). The overexpression of *mprF* in *ΔponA* cells, which resulted in lysis on NA-glucose (**Fig 1C**), resulted in LTA-signals that corresponded to the sum of the effects of the two mutations (7th lane in **Fig 3B**).

Interestingly, our analysis showed that LTA production is altered in *Δmbl*, as we observed a weak, short LTA-signal (second to last lane **Fig 3B**). Previously, we found that overexpression of MprF rescued *Δmbl* growth (**Fig 1C**) and here we identified that it is associated to an increase in LTA length and abundance (last lane **Fig 3B**). Consistently, we observed the production of long LTA polymers in the suppressor strain *Δmbl ΔltaS* (our unpublished observations) (Schirner et al., 2009) similar to that of *ΔltaS* (**Fig 3A**). Thus an increase in LTA length seems to characterize the effect of these two *Δmbl* suppressor mutations.

Analysis of the suppressor *ΔltaS ΔponA* revealed the dominant effect of *ltaS* deletion, in that the LTA exhibited a retarded migration (3rd lanes in **Fig 3C**, **Appendix Fig S9**), similar to that observed in *ΔltaS* (**Fig 3C**). In absence of UgtP which produces a lipid anchor Glc₂DAG (**Fig 1A**), we observed an increase in LTA length in both media conditions, but the shift was more distinct in glucose (>12 kDa, left panel 4th lane in **Fig 3C**). The latter is comparable to the LTA signal detected in the *S. aureus* *ΔugtP* mutant grown in TSB, a glucose rich medium (Grundling and Schneewind, 2007a). Analysis of *ΔponA ΔugtP*, which was conditionally lethal on NA-glucose (**Appendix Fig S4**), revealed an increase of the LTA-signals and length (7-25 kDa, left

For each set of cultures (NB ± Glc 0.2%), samples were separated on the same Bis-Tris gradient gel. Membrane was split at ~ 60 kDa position. The lower part of the membrane was probed using a monoclonal LTA antibody and an HRP-linked anti-mouse antibody. The other membrane part was probed using polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies, to provide a sample loading control.

A Samples were prepared from strains *B. subtilis* wild type (168CA), 4285CA (*AltaS*), 4289CA (*ΔyfnI*), 4292CA (*ΔyqgS*), AG595 (*ΔyfnI ΔyqgS*), AG593 (*AltaS ΔyqgS*), AG594 (*AltaS ΔyfnI*), AG600 (*AltaS ΔyfnI ΔyqgS*) and 4620 (*AltaS ΔyfnI ΔyqgS ΔyvgJ*, noted here *ΔLTA*).

B Strains analysed: *B. subtilis* wild type, AG417 (*ΔpbpD ΔpbpF ΔpbpG*), RE101 (*ΔponA*), AG181 (*ΔmprF*), AG193 (*ΔponA ΔmprF*), AG304 (MprF ↑), AG311 (*ΔponA* MprF ↑), 4261CA (*Δmbl*), AG322 (*Δmbl* MprF ↑).

C Samples were extracted from *B. subtilis* 168CA, 4285CA (*AltaS*), AG383 (*AltaS ΔponA*), PG253 (*ΔugtP*) and AG444 (*ΔugtP ΔponA*) strains.

Data information: Representative of one of three independent experiments shown in **Appendix Figures S7-S9**. At each section A, B, C, samples were derived from the same experiment.

Overexpression of major autolysin LytE is lethal in absence of PBP1 or LtaS

Our results inferred that the conditional lethality of aPBPs mutant might be linked to an interplay between LTA length and autolysin activity. Autolysins or cell wall hydrolases help shape and recycle the peptidoglycan during cell elongation and division. *B. subtilis* expresses a larger number of them but only the loss of both PG D,L-endopeptidases CwlO and LytE is lethal (Hashimoto et al., 2012, Salzberg et al., 2013, Dobihal et al., 2019, Do et al., 2020, Wilson and Garner, 2021). Our interest in LytE derived from the facts that this major autolysin is secreted and thought to be anchored to the lateral cell wall and the septum (Dominguez-Cuevas et al., 2013). LytE abundance also increased in *AltaS* (Kasahara et al., 2016) and both LtaS and LytE affect colony development (Mamou et al., 2017) and cell diameter (Schirner et al., 2009, Dominguez-Cuevas et al., 2013).

We reasoned that if an imbalance of LytE activity existed in the aPBPs mutants (*Δ4* and *ΔponA*) grown in glucose-rich medium, then a deliberate increase in LytE expression should exacerbate the strain phenotype, leading to cell death. Interestingly, LytE overexpression (by IPTG induction of a *P_{hyspank}-lytE* allele) was found to be lethal to *ΔponA* on NA-glucose and it significantly delayed the growth of *Δ4* and *ΔponA* on NA but not that of *Δ3 ponA*+ (**Fig 4A**, **Appendix Fig S10**). This result confirmed that an imbalance in autolytic activity was detrimental to the cell that had lost PBP1. Importantly, the overexpression of *lytE* in the *Δ4*

250 $\Delta ltaS$, $\Delta ponA \Delta ltaS$ and $\Delta ltaS$ strains was lethal, while the $\Delta 4 \Delta mprF$ and $\Delta ponA \Delta mprF$ strains
251 grew slowly (**Fig 4A**) but no change was observed for the $\Delta mprF$ background.

Figure 4. LytE overexpression is conditionally lethal in absence of PBP1 or LtaS.

A $lytE$ overexpression was analysed in strains which integrated at the $amyE$ locus an (0.1 mM) IPTG-inducible $P_{hyspant}$ - $lytE$, represented here LytE $\uparrow$. The strains tested had the following relevant features: AG484 ($\Delta ponA$ LytE $\uparrow$), AG1460 ($\Delta 3 ponA^+$ LytE $\uparrow$), AG501 ($\Delta 4$ LytE $\uparrow$), AG502 ($\Delta 4 \Delta mprF$ LytE $\uparrow$), AG1465 ($\Delta 4 \Delta ltaS$ LytE $\uparrow$), AG1478 ($\Delta mprF$ LytE $\uparrow$), AG1479 ($\Delta ponA \Delta mprF$ LytE $\uparrow$), AG1462 ($\Delta ltaS$ LytE $\uparrow$), AG1480 ($\Delta ponA \Delta ltaS$ LytE $\uparrow$), AG1497 ($\Delta ltaS \Delta yfnI$ LytE $\uparrow$), AG1498 ($\Delta ltaS \Delta yqgS$ LytE $\uparrow$) and AG1499 ($\Delta yqgS \Delta yfnI$ LytE $\uparrow$). The following strains that did not display any obvious phenotype are not represented here: AG475 (168CA LytE $\uparrow$), AG1461 ($\Delta lytE$ LytE $\uparrow$), AG1463 ($\Delta yfnI$ LytE $\uparrow$), AG1492 ($\Delta yqgS$ LytE $\uparrow$). Plates (here Glc 0.5%) were incubated at 37°C and scanned after 24 h (this figure) and 48 h (**Appendix Figure S10**).

B Strains AG1481 ($\Delta mprF$ pLOSS- P_{spac} - $lytE$) and AG551 (168CA pLOSS- P_{spac} - $lytE$) were streaked on NA and NA glucose (Glc 0.5%), supplemented with IPTG (1 mM) and X-Gal from a fresh colony on NA spectinomycin. Plates were incubated at 37°C and scanned after 24 h. Blue colonies indicate the stabilisation of plasmid pLOSS- P_{spac} - $lytE$ in $\Delta mprF$. Grey scale images are here to help visualize white colonies.

C Detection of LytE-His₁₂ and PBP2B by Western blots. For simplification on this figure only the relevant background feature is displayed for the following strains (expressing LytE-His₁₂ under the control of its native promoter): wild-type-like AG565 (WT*), AG587 ($\Delta ponA$), AG1486 ($\Delta mprF$), AG1487 ($\Delta ponA \Delta mprF$), AG1489

(*ΔltaS*), AG1488 (*ΔponA ΔltaS*), AG1535 (*ΔugtP*) and AG1541 (*ΔcwlO*). In addition, *B. subtilis* 168CA was grown in parallel (without antibiotic) and used here as a negative control for the LytE-blot. The membrane was split and incubated either with a monoclonal Penta-His and an HRP-linked anti-mouse antibodies or with a polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies. Note: a ‘ladder’ sample loaded after the NB-Glc (0.2%) sample set is not displayed and the image editing is symbolised by the vertical black line (see original on **Appendix Fig S11B**).

D Detection of LTA-synthases LtaS/YfnI and PBP2B by Western blots. The samples used in experiment A were loaded on a new SDS-gel and the Western blot was carry out with polyclonal anti-LtaS (able to detect YfnI, unpublished Errington’s lab), anti-PBP2B antibodies, and HRP-linked anti-rabbit antibody.

Data information: One representative set of three independent Western Blot experiments is shown here and in **Appendix Figures S11-12**. Additional supporting information is presented in **Fig EV2-3** and **Appendix Figure S13**. At each section samples were derived from the same experiment.

This finding suggested that *ΔponA ΔltaS* and *ΔponA ΔmprF* mutants, which have distinct LTA length, were differently sensitive to LytE activity. Therefore, we examined the double LTA-synthase mutant backgrounds, here we found that only the strain expressing *ltaS* was viable when LytE was overexpressed (**Fig 4A**, last strain). In these genetic backgrounds, the absence of *mprF* or *ugtP* had no effect (our unpublished observations). Interestingly, *ΔltaS* LytE ↑ was viable on NA (**Fig 4A**, 8th strain) but not on NA-glucose while we previously identified that *ΔltaS* produces distinct LTA length on each of these media (**Fig 3A**). Thus, it is clear that the activity of LtaS, presumably the presence of a specific LTA length, is required for the cell to balance the effect of overexpression of *lytE*.

To comprehend the apparent tolerance of LtaS⁺ and *ΔmprF* cells to LytE, we constructed an unstable replicon pLOSS-*P_{spac}-lytE* plasmid which expresses *lytE* at a level lower than *P_{hyspank}-lytE* (**Table EV1**). Introducing this plasmid into *ΔmprF* resulted in better stability of the plasmid in all media conditions tested (AG1481 strain), including elevated magnesium. This was indicated by the fact that the colonies remained blue on IPTG XGal supplemented media (**Fig 4B**) whereas other strains tested showed no effect (e.g. 168CA, *ΔponA* and LTA-synthases mutants, **Table EV2**). At this stage, it is unclear if the *ΔmprF* strain’s tolerance for *lytE* overexpression is due to the loss of aaPGols or the production of shorter LTA or both.

LTA influences LytE activity

The conditional lethality observed when overexpressing *lytE* prompted us to investigate the cellular levels of this autolytic enzyme in our strains. First, we engineered strains that could express a recombinant protein LytE-LEMGRSH₁₂ (using pMUTin- '*lytE*-his, **Table EV1**) and then analysed total lysates by Western blot using anti-histidine (for LytE) (**Fig 4C, Appendix Fig S11**) or an anti-LtaS antibody raised against LtaS (74 kDa) that cross reacts with YfnI (73 kDa) (**Fig 4D, Appendix Fig S12**). Importantly, our results showed that LytE and LtaS/YfnI accumulate in *ΔponA* cells, particularly when grown in presence of glucose (**Fig 4C-D**). Using a set of strains that has integrated either pMUTin- '*ltaS*-his or pMUTin- '*yfnI*-his, we could confirm that both LtaS and YfnI are more abundant in *ponA* cells (**Fig EV2, Tables EV1-3**). In *ΔponA ΔmprF*, LytE and LtaS/YfnI protein levels decreased compared to *ΔponA* (**Fig 4C-D**). No obvious change was observed in the wild-type background (WT*) and *ΔmprF* strains. *ΔltaS* and *ΔponA ΔltaS* exhibited an increase in LytE and YfnI when grown in NB-glucose, an effect more pronounced in the *ΔponA ΔltaS* mutant (**Fig 4C-D**, left panel). In addition, comparative analysis of the whole set of LTA-synthase mutants (**Fig EV3**) revealed that LtaS/YfnI signal increased in presence of glucose. In the absence of YfnI, LtaS increased significantly and more importantly LytE abundance increased in the absence of LtaS.

To corroborate that the altered abundance of these proteins, identified in *ΔponA* cells, were associated with the sick phenotype, we analysed the strains that were lytic under the same growth conditions as **Fig 4C**. Our result show that there is an excess of LtaS/YfnI in *ΔponA ΔugtP* and *ΔponA MprF* ↑ cells in NB and NB-glucose (**Appendix Fig S13 B-C**, e.g. 6-7th lanes) and that LytE signal is higher than the wild type.

Finally, we looked at the effect of magnesium in presence of glucose as this cation suppresses the growth defect of aPBP mutants (Kawai et al., 2009b). Our analysis showed that magnesium decreased the abundance of LytE significantly in *ΔponA* and *ΔponA ΔmprF* (**Appendix Fig**

S13) but not as much in *ΔponA ΔltaS*. Perhaps, the unknown mechanism by which exogenous magnesium helps the growth of some cell wall mutants might partially require LtaS activity to tune LytE (Wilson and Garner, 2021).

Taken together, our results show a correlation between LTA length and LytE abundance in the cell which suggest that LTA acts to moderate cell wall metabolism. In *ponA* mutants, this function is unbalanced such that cell wall degradation is predominant.

LTA production is altered in *S. aureus* *AmprF* and *Bacillus* daptomycin resistant strains

MprF is conserved in the *Firmicutes* and has been shown to contribute to *S. aureus* pathogenicity (Peschel et al., 2001, Slavetinsky et al., 2017). Importantly, studies of clinical isolates have found that methicillin-resistant *S. aureus* strains (MRSA) resistant to daptomycin (Dap^R) frequently carry nucleotide polymorphisms (SNPs) in *mprF* (Friedman et al., 2006, Ernst et al., 2018). The active Ca²⁺-Dap lipopeptide antibiotic is often used as last resort in the treatment of MRSA infections and acts to interfere with membranes containing phosphatidylglycerol (Muller et al., 2016, Ernst and Peschel, 2019, Gray and Wenzel, 2020, Grein et al., 2020). Although some *mprF* SNPs were associated with a MprF gain of function (increased Lys-PGol and decreased PGol contents), others had no clear correlation between the phospholipids composition and their relative cell surface charge (Ernst and Peschel, 2019). In that context, we asked whether the role of MprF in LTA production is conserved in *S. aureus* and could shed new light in Dap^R MRSA. Analysis of LTA production in methicillin sensitive *S. aureus* strains showed that the LTA from the *mprF* mutant migrated faster than that of the wild type SA113 (**Fig 5A, Fig EV4A**). Thus, a role of MprF in modulating LTA production is conserved across the studied *Firmicutes* species.

We, therefore, turned our interest to the Dap^R *B. subtilis* strain that carries a *pgsA* A64V allele (Hachmann et al., 2011). This mutation of the essential gene *pgsA* encoding the PGol-synthase

(Fig 1A), is shown to reduce cellular PGol content and was suggested to modify LTA production. Thus, we analysed LTA of *B. subtilis* 168 1A1 wild type (WT), Dap^R and Dap^R *pgsA*^{WT} strains grown overnight in NB (Fig 5B, Fig EV4 B-D). Our assays revealed that no LTA signal was detected below 30 kDa in Dap^R strains whereas LTA signals migration detected in the strains complemented for *pgsA* (Dap^R *pgsA*^{WT}) were similar to that of the wild type. Thus, PgsA A64V accounts for the severe reduction in LTA production. Finally, we verified that the two major LTA-synthases were still produced in Dap^R strains by Western blot (Fig 5C, Fig EV4 B-D). A strong LtaS/YfnI signal was detected in Dap^R strains which proved that one or both LTA-synthases were still expressed. In addition to the altered membrane composition, the severe reduction in LTA production could also act to reduce susceptibility to daptomycin.

Figure 5. Altered LTA in *S. aureus* SA113 $\Delta mprF$ and in *B. subtilis* daptomycin resistant strains.

A Samples of methicillin-sensitive *Staphylococcus aureus* strains SA113 and SA113 $\Delta mprF$ (Peschel et al., 2001) were probed for LTA by Western Blot.

B Researchers identified that a modified allele of *pgsA* (A64V) was the major determinant of daptomycin resistance of *B. subtilis* Dap^R1 and Dap^R20 strains selected by serial daptomycin antibiotic passages (Hachmann et al., 2011). The strains 168 1A1 (wild type, WT), Dap^R1, Dap^R20, HB15516 (Dap^R1 *pgsA*^{WT}) and HB15507 (Dap^R20 *pgsA*^{WT}) were grown in NB overnight at 30°C and LTA was detected along with PBP2B productions by Western Blots. Analysis of the samples grown exponentially at 37°C using these overnight cultures is presented in Fig EV4.

C shows the cellular abundance of the LTA-synthase with anti-LtaS (able to detect YfnI, unpublished Errington's lab), an anti-PBP2B and an HRP-linked anti-rabbit antibodies.

Data information: One representative set of three experiments is shown here and in Fig EV4.

Discussion

In absence of the class A PBPs ($\Delta 4$), *B. subtilis* is viable due to the presence of RodA, the essential PG glycosyltransferase (controlled by the cell envelope stress regulator σ^M (Meeske et al., 2016, Emami et al., 2017)) and yet lethal on glucose rich medium (**Fig 1B, Fig EV1**). By studying either $\Delta 4$ or $\Delta ponA$ single mutant, we now understand that glucose presence led to an increase in abundance of LtaS, YfnI (LTA-synthases) presumably causing the observed change in LTA production that results in the accumulation of major autolysin LytE (**Fig 3-4**). The end result being an environment unable to regulate the activity of autolysins leading to cell lysis. Indeed, we have found that $\Delta 4$ can grow on NA-glucose in the absence of LtaS or the absence of MprF (**Fig 1B, 2B**). In $\Delta ponA$, loss of LtaS lead to the production of very long LTA polymers similar to the single *ltaS* mutant (**Fig 3C**). The *ltaS* mutation is not as beneficial for the growth of $\Delta 4$ strain compared to $\Delta mprF$ (**Fig EV1**), which we observed reduced LTA production in $\Delta ponA$ background (**Fig 3B**). This is consistent with our finding that an increase in length and abundance of LTA (overexpression *mprF*, *AugtP*) and/or an increase in LytE coincide with glucose-mediated lethality in $\Delta ponA$ (**Fig 1C, 3, 4A, Appendix Fig S13 B-C**). The analysis of LTA-synthases mutants indicates that LtaS activity and a distinct LTA length (7-17kDa) and/or abundance similar to the wild type LTA is required to prevent cell lysing when *lytE* is artificial overexpressed (**Fig 4A**). Indeed, a LTA signal (7-20 kDa) is present in the mutant $\Delta ltaS$ in NB but not in NB-glucose (long LTA > 20 kDa) (**Fig 3A**) and it happens that the strain $\Delta ltaS$ LytE $\uparrow$ is unable to grow on NA-IPTG-glucose (**Fig 4A**). Moreover, the mutants $\Delta ltaS \Delta yqgS$ (long LTA-only) and $\Delta ltaS \Delta yfnI$ (weak short LTA signal, **Fig 3A**) were unable to grow on both media when *lytE* was overexpressed (**Fig 4A**).

Previous studies have suggested that LTA could inhibit autolysins activity (Neuhaus and Baddiley, 2003, Hanson and Neely, 2012) but the key factors involved have not been identified.

Recent work in *S. aureus* showed a *ΔugtP* mutant producing long LTA is susceptible to PG hydrolases (Hesser et al., 2020a) and it has been suggested that major autolysin LytA is sequestered by LTA in exponential growth (Flores-Kim et al., 2019). Our results indicate that LytE in *B. subtilis* exhibits similar characteristics and that the length of LTA has a role in modulating LytE abundance or presumably its activity. However it is unclear how this mechanism may work in relation to the localisation of the LTA in the wall relative to active autolysins, particularly LytE, and remain an important question to answer.

In *B. subtilis* 168, expression of *lytE* is quite complex and coordinated by regulators such as σ^I and WalR (Dobihal et al., 2019, Patel et al., 2020). The transcriptional regulation of *mprF* is not well defined, but interestingly *B. subtilis* BSB1 *mprF* and *pgsA* expression is positively correlated with that of *walkR*, the two-component response system that controls cell wall metabolism, and notably *lytE* (Nicolas et al., 2012, Zhu and Stulke, 2018) (cluster A418). Expression of *walkR* and *sigI* is not significantly altered in LB-glucose (Nicolas et al., 2012) or in the absence of aPBPs (Emami et al., 2017) but these regulators might play a post-transcriptional role in $\Delta 4$ that interferes with the σ^M cell wall stress response (Emami et al., 2017, Patel et al., 2020). Some of the cell wall associated genes mentioned (*sigM*, *ponA*, *ltaS*, *yfnI*, *mprF*, *pgsA*) are differently expressed in cells of *B. subtilis* BSB1 in LB-glucose (Nicolas et al., 2012, Zhu and Stulke, 2018). Thus *B. subtilis* might need to adapt its cell wall metabolism in response to glucose, this might account for the partial redundancy of function of *B. subtilis* LTA-synthases. Altogether, our study brings a new perspective on the importance of LTA in the cell envelope stability.

In this study, we have identified a new function for MprF, an enzyme known to synthesise aminoacyl-phosphatidylglycerol (aaPGol) phospholipids and translocate them across the membrane (**Fig 1A**). We show that MprF activity impacts the biosynthesis of LTA and this in turn alters cell wall metabolism. The LTA profiles observed in the absence of *B. subtilis* MprF

or *S. aureus* MprF (faster mobility and/or decreased signal) and the overexpression of *B. subtilis* MprF (smear & retarded mobility) (**Fig 3B, 5A**) suggest that MprF positively regulates LTA production. Other studies have indicated that MprFs may have relaxed substrate specificities across species (Slavetinsky et al., 2017, Song et al., 2021) and even synthesise lysyl-glucosyl-DAG glycolipid in *Streptococcus agalactiae* (Joyce et al., 2021). Recently, it has been shown that when *S. aureus* LtaS uses PGol as an alternative anchor to the glycolipid produced by UgtP, the concentration of these free lipid starter units regulates the length of the LTA polymerised *in vitro* (Hesser et al., 2020b). Future works could investigate whether MprF produces a substrate that contributes to LTA initiation/processivity. Alternatively, MprF might act to synthesise positively charged aaPGols to provide an electrostatic environment that either impacts on LTA-synthase(s) activity and/or directly contributes to altering the abundance of hydrolases in the wall (e.g. LytE, **Fig 4**). This might help maintain the nascent anionic LTA polymers adjacent to the membrane and facilitate D-alanylation of LTA (Schneewind and Missiakas, 2014), hence leading to a modification in the cell envelope charge.

Gain of function in MprF has been identified in methicillin-resistant *S. aureus* (MRSA) clinical isolates that have acquired resistance to daptomycin (Dap), an antibiotic used as a last resort to treat MRSA infections (Ernst et al., 2018, Ernst and Peschel, 2019). Over the years, MprF-mediated resistance to Dap (Dap^R) has been investigated but a consensus model has yet to link Dap^R to a particular domain of MprF and/or to a particular Lys-PGol content in one or both membrane leaflets (Ernst and Peschel, 2019, Thitiananpakorn et al., 2020). In *B. subtilis*, our analysis shows the severe decrease of LTA produced in Dap^R *pgsA* A64V (**Fig 5B**), a strain with a reduced PGol content (Hachmann et al., 2011). The function of MprF in LTA synthesis and the new understanding of *S. aureus* LtaS processivity (Hesser et al., 2020b) can now be linked to the identification of other MRSA Dap^R isolates (*cls* encoding a cardiolipin synthase, *pgsA*, *dlt* operon) (Gray and Wenzel, 2020). Together, these findings support the idea that a

403 modification in LTA polymerisation and D-alanylation of teichoic acid (Bertsche et al., 2013,
404 Mishra et al., 2014) could result in a cell envelope less permeable to Dap, as such not
405 necessarily linked to cell wall thickness (Yang et al., 2010). Future research simultaneously
406 analysing LTA production and cell wall composition will help redefine MprF-mediated Dap^R
407 mechanism and help appreciate the exact role of MprF in the net cell surface charge, the
408 protection against cationic antimicrobial peptides and the contribution to bacterial virulence.

Material and Methods

Plasmids, bacterial strains, and primers

Plasmids and strains used in this study are listed in **Table EV1 and EV2**. Strains constructions are described below or in **Table EV2** (last column). Primers used are listed in **Table EV3**.

Growth conditions and general methods.

B. subtilis strains were grown routinely on nutrient agar (NA, Oxoid) or in nutrient broth (NB, Oxoid). For some assays, strains were grown in liquid Difco antibiotic medium 3 (PAB, Oxoid) and PAB agar (15 g/l agar, Oxoid). *B. subtilis* transformations were carried out as previously described using SMM defined minimal medium (Anagnostopoulos and Spizizen, 1961, Hamoen et al., 2002). *B. subtilis* was usually incubated at 30°C (overnight), next day cells were diluted and grown at 37°C (unless specified differently). *Escherichia coli* and *Staphylococcus aureus* strains were incubated at 37°C on NA plate or in Luria-Bertani (LB). PCR, plasmid manipulations, and *E. coli* transformation were carried out using standard methods. Strains were selected on NA supplemented with ampicillin (for *E. coli*, 200 µg/ml), spectinomycin (60 µg/ml), kanamycin (5 µg/ml), phleomycin (1 µg/ml), erythromycin (0.5 µg/ml), and chloramphenicol (5 µg/ml). Various supplements were also used: IPTG (isopropyl-β-D-thiogalactopyranoside, 0.1 or 1 mM), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 100 µg/ml), MgSO₄ (between 5 mM to 25 mM as required), and glucose (final concentrations up to 1%). For NA plates supplemented with 20 mM MgSO₄, the concentration of spectinomycin, kanamycin and erythromycin antibiotics was doubled to avoid growth of false positive strains. Strains with the pMUTin-*lytE-his* integration were always grown in presence of erythromycin.

Plasmids constructions

To obtain pDR111-*P_{hyspank}-mprF* plasmid, the *mprF* (BSU_08425) coding sequence was amplified using primers oAG271-272, which changed the start codon (from TTG to ATG). The PCR product and pDR111 plasmid (**Table EV1**) were digested by *SalI* and *SphI* restriction enzymes, ligated together and transformed into *E. coli* DH5 α . Digestion and PCR sequencing using primers oAG300-301 verified the presence of the insert.

To construct pDR111-*P_{hyspank}-ltaS*, pDR111-*P_{hyspank}-yfnI* and pDR111-*P_{hyspank}-yqgS*, each LTA-synthase gene was amplified by PCR with primers pairs oAG308-309 (2064 bp), oAG310-311 (2078 bp) and oAG312-313 (1990 bp), respectively. PCR products were digested by *NheI* and *SphI*, each ligated with the plasmid pDR111 digested with the same restriction enzymes. The next steps were carried out as described for pDR111-*P_{hyspank}-mprF*.

To construct pDR111-*P_{hyspank}-mprF-lytE* and pDR111-*P_{hyspank}-lytE-mprF* plasmids for coexpression of *mprF* and *lytE*, the gene *lytE* was amplified from the chromosome by PCR with oAG911-912 or oAG917-918 primers, respectively. PCR reactions were set up with plasmid pDR111-*P_{hyspank}-mprF* and with one or the other PCR-*lytE* product (PrimeSTAR GXL Polymerase, TaKaRa). Recombinant plasmid-inserts were obtained by overlapping PCR and *DpnI* digestion and transformed into *E. coli* DH5 α . The resulting plasmids (with duplicate RBS site, 24 bp) were verified by PCRs and by plasmid sequencing using primers oAG913-916, oAG919-20, oAG292 and oAG294.

For pLOSS-*P_{spac}-lytE*, *lytE* was amplified by PCR with oAG348-349 (1143 bp) primers, and the PCR product inserted in pLOSS* (**Table EV1**) as a *NotI* and *BamHI* fragment. After transformation, the resulting plasmid was checked by PCRs with primers oAG18-19 and finally checked by sequencing.

To construct the plasmids pMUTin-*lytE-his*, pMUTin-*ltaS-his* and pMUTin-*yfnI-his* the 3'-terminus part of gene of interest gene (except for the stop codon) was amplified with primer

pairs oAG354-355, oAG366-367 and oAG360-361, respectively to obtain a PCR products ranging from 288 to 309 bp. The PCR products and the plasmid pMUTinHis (**Table EV1**) (Ishikawa et al., 2006) were digested by *EcoRI* and *XhoI*, ligated together and transformed into *E. coli* DH5 α . Each insertion '*lytE*', '*ltaS*' and '*yfnI*' was verified by PCR and plasmid sequencing using primers oAG368-369.

To construct pAM-21, plasmid pET28a (+) (Novagen) and *lytE* (from the 76th bp, removing the coding sequence for the signal peptide) were amplified by PCRs using primer pairs oAA01-02 and oAA03-04, respectively. DNA fragments generated were assembled using NEBuilder HiFi DNA assembly kit (NEB). The resulting plasmid was verified by sequencing using primers oAA05-06.

Bacillus directed mutagenesis

Steps leading to construction of new mutants are indicated in the last column of **Table EV2** with additional details in this section. New mutants were often obtained by transformation of competent cells with a clean genomic DNA extracted from another existing *B. subtilis* mutants. Construction of some of the markerless class A PBPs mutants were previously described (Emami et al., 2017). Additional mutants described in this study were obtained using the plasmids pG+host9::*ΔponA*, pG+host10::*ΔpbpD*, pG+host10::*ΔpbpF* (**Table EV1**) (Emami et al., 2017). PCR with primers oAG124 to oAG129 were consistently performed to check markerless deletions were not lost (Emami et al., 2017).

All transformations of the background strain with, and/or leading to, deletion of *ponA*, *mreB*, *mbl*, *gtab*, *ugtP*, *ltaS* were selected for on NA medium supplemented with MgSO₄ (20-25 mM) with the appropriate antibiotic.

To construct strain AG181 (*ΔmprF*), DNA fragments upstream and downstream the *mprF* coding sequence were amplified by PCR using primers oAG103-104 (2390 bp) and oAG105-

106 (2414 bp), respectively and were digested by *EcoRI* and *XbaI*, respectively. The phleomycin resistance antibiotic cassette was amplified by PCR with primers PhleoXbaIfor and PhleoEcoRIrev from plasmid pIC22 and digested by both *EcoRI* and *XbaI*. The 3 digested PCR-fragments were ligated to generate a linear DNA, transformed into 168CA with selection for phleomycin resistance. The deletion of *mprF* was confirmed by PCR using primers oAG107-108.

Strain AG304 (*amyE::P_{hyspank}mprF, spc*) was obtained by transformation of 168CA with pDR111-*P_{hyspank}-mprF* plasmid (Table EV1). Correct recombination was confirmed by the absence of amylase activity when grown on NA with 0.2% starch and by PCR (oAG300-301 primers). The same methods were used after transformation of other pDR111-derivatives.

We constructed our own *lytE* deletion mutant (Δ *lytE::cat*). For this, regions of genomic DNA upstream and downstream of *lytE* were amplified by PCRs with oAG350-351 (2048 bp) and oAG352-353 (2500 bp) primers pairs, respectively. These products were then digested by *XbaI* or *BamHI* (Table EV3). The chloramphenicol resistance cassette from pCotC-GFP plasmid (Table EV1) was amplified with primers oAG197-198. The resulting PCR products (850 bp) was then digested by *XbaI* and *BamHI* and the three DNA fragments were ligated and transformed into *B. subtilis* 168CA. Strain AG547 was obtained from this transformation and the deletion of *lytE* confirmed by PCR using primers oAG340-341 (Table EV3).

Strains AG565 and AG586 were obtained by integration of pMUTin-*lytE-his* (erythromycin selection) in 168CA and Δ *cwI*O (AG474 strain) background, respectively. The plasmid integration (single cross-over) in *B. subtilis* resulted in expression of recombinant proteins with LEMRGSH₁₂ tag in C-terminus, under the control of its native promoter. Since the integration of this plasmid into the Δ *cwI*O background did not result in any phenotypic change it indicated that the LytE-His₁₂ was functional, as a double mutant *lytE cwI*O is lethal (Hashimoto et al., 2012). Thereafter, genomic DNA of AG565 strain was used to transform all the strains needed

for Western blot analysis (**Table EV2**). Of note, AG1541 strain (ΔcwI) obtained by genomic DNA recombination has the same genotype as AG586 strain obtained by plasmid integration.

Random transposition mutageneses

Random transposition mutageneses were performed as previously described (Claessen et al., 2008, Surdova et al., 2013) using the pMarB plasmid that carries the transposon TnYLB-1 encoding kanamycin resistance. pMarB was introduced into *B. subtilis* strains carrying pLOSS-*P_{spac}-ponA* (Emami et al., 2017) (**Table EV1**), a plasmid with an unstable origin of replication and *lacZ* which helps to monitor the plasmid stability by observing formation of blue or white colonies. After screening the random library of mutants, genomic DNA extracted from positive clones were backcrossed into the parental strain used for the transposon screen. Transposon insertion sites were identified by inverse PCR using gDNA extracted from the backcrossed positive clones (Surdova et al., 2013) and sequencing the resulting PCR products. Further verification of the transposon's site of insertion was obtained using specific primers.

For the suppressor screen in *ponA pbpD pbpF* mutant background, we constructed strain AG141 (**Table EV2**) which carries deletions of the three vegetative class A PBPs, *lacA* gene (Surdova et al., 2013) and pLOSS-*P_{spac}-ponA* (Emami et al., 2017). The transposon mutant library was screened on PAB agar supplemented with MgSO₄ (10 mM), X-Gal and IPTG (1 mM) and white colonies selected. The strain SWA11a (**Table EV2**) had a transposon in *mprF* coding sequence which was verified using specific primers oAG81-82 (**Table EV3**).

A conditional lethal screen was also carried out in a *ponA pbpD pbpF mprF lacA* pLOSS-*P_{spac}-ponA* strain (AG200, **Table EV2**). Here the transposon mutant library was screened on NA and PAB supplemented with X-Gal and IPTG (1mM). Blue colonies indicating plasmid stability were selected. One mutant was isolated and its genomic DNA was backcrossed into AG200

background. The resulting blue forming AG200BK#42 strain (**Table EV2**) was found to carry a transposon in *gtab*'s coding sequence using primers oAG165-166 (**Table EV3**).

Spot growth assays

Strains were grown in NB with 20 mM MgSO₄ at 30°C overnight. The cultures were then diluted 1:100 into fresh NB 10 mM MgSO₄ (or when applicable 20 mM) and grown up to mid exponential phase at 37°C. Cells were harvested, washed in NB, and diluted to an OD_{600nm} ≈ 0.3 in NB. Each strain sample was then serially diluted 1:10 using a 96-multiwell plate and NB. Using a multichannel pipette, 5 µl of each dilution were transferred to various agar plates. As a control all the serial dilutions presented in the figures were also spotted on media supplemented with 10 mM or 20 mM MgSO₄ to ensure that growth occurred for all spots (our unpublished observations). Plates were incubated at 37°C overnight and scanned at different time points. Images were edited in Photoshop.

Microscopic imaging

Cells were grown to exponential growth phase at 37 °C in NB medium. Before the cells were mounted onto microscopic slides, the slides were covered with a thin layer of NA medium supplemented with the FM5-95 membrane dye at 140 µg/ml (Invitrogen). Fluorescence microscopy was carried out with a Zeiss Axiovert 200M microscope attached to a Sony Cool-Snap HQ cooled CCD camera with Nikon APO x100/1.40 oil Ph3 objective lens. Images were acquired using Metamorph 6 imaging software (Molecular Devices, Inc). Images were analysed with ImageJ (<http://rsb.info.nih.gov/ij/>) and assembled with Photoshop CS software. Images stained with the FM5-95 membrane dye were processed using the plugin ObjectJ in ImageJ (<http://rsb.info.nih.gov/ij/>) to measure cell diameter and cell length of about 400 cells

per strain. Measurements were performed only once as cell diameter change was obvious under the microscope and on the images of **Fig 2D** (details in **Appendix Figure S5**).

Transmission electron microscopy

Strains were grown in NB medium (with IPTG when applicable) until reaching an OD_{600nm} of about 0.4. The cell pellets were fixed in 2% glutaraldehyde in sodium cacodylate buffer at 4°C for at least 24 h. Samples were spun and molten 4% agarose was added to the sample and respun. Sample were allowed to cool in the fridge for 30 min. The agarose block was removed from each tube and cut into 1 mm³ pieces. The samples were then dehydrated by microwave processing using the Pelco Biowave® Pro+ incorporating the Pelco Coldspot® Pro. In the vacuum chamber, samples were rinsed 3 times in buffer (150 watts for 40s), then treated in 1% osmium, pulsed (100 watts for 8 min) and rinsed 3 times in distilled water (150 watts for 40s). They were then taken out of the vacuum chamber and a graded series of dried acetone was applied to the samples (25%, 50%, 75%, and 3 times in 100%, at 150 watts for 40 s for each step). This was followed by impregnation with a series of increasing concentrations of epoxy resin (Taab Medium resin) in acetone (25%, 50%, 75%, 3 times in 100% at 300 watts for 3 min. for each step). Samples were embedded in fresh 100% resin and polymerised at 60°C for 24 h in a conventional oven. Once polymerised the resin block were cut in semi-thin survey sections (0.5 µm) and stained with 1% toluidine blue in 1% borax. Ultrathin sections (70 nm approx.) were then cut using a diamond knife on a Leica EM UC7 ultramicrotome. The sections were stretched with chloroform to eliminate compression and mounted on Pioloform-filmed copper grids. Grids were stained with uranyl acetate (2%) and Lead citrate and viewed on a Hitachi HT7800 transmission electron microscope using an Emsis Xarosa camera.

Samples preparation for LTA polymer detection

B. subtilis strains were grown overnight at 30°C with shaking in NB supplemented with 5 mM MgSO₄ (or 10 mM for *mbi* mutants). The cells were then diluted (1:60-1:80 depending on the strain) to an approximate OD_{600nm} of 0.02 in prewarmed NB and NB supplemented with 0.2% glucose. Strains were then incubated at 37°C shaking and grown for about 4.5 h (late exponential phase). At the sampling time the OD_{600nm} of the cultures were measured before harvesting 15-25 ml of culture at 3273 g for 10 min, using a swing out centrifuge at room temperature. Pellets were washed with 200 µl of solution A (with 10 mL of Tris-HCl at 100 mM pH 7.4 and one cOmplete Mini EDTA-free tablet of protease inhibitor cocktail from Roche) and pelleted with benchtop centrifuge. Cells pellets were then frozen in liquid nitrogen and stored at -20°C until processing. Samples were resuspended in a volume of solution B (an equal volume of solution A and LDS sample buffer, Life Technologies) ranging from 70 to 200 µl in proportion to their OD_{600nm}. Samples were boiled 30 min at 100°C, cooled down on ice for 3 min and incubated each with 100 U of benzonase (E1014, Sigma) for 30 min at 37°C. Samples were pelleted at 4°C, supernatants containing the extracted LTA were collected. Supernatants were frozen in liquid nitrogen and kept at -25°C. For the LTA Western blots, the quantity of sample loaded was normalized using the Bio-Rad Protein assays such that the final OD_{595nm} was approximately 0.35. Sample sets to be compared were grown, extracted, measured and normalized together for each experiment.

S. aureus strains were grown overnight at 37°C with shaking in LB. The cells were then diluted (about 300-fold) to an approximate OD_{600nm} of about 0.02 and grown in LB at 37°C until reaching late exponential and an OD_{600nm} of about 1. 10 ml of cultures were harvested, and samples were prepared as for *B. subtilis* with the following changes: 60 µl of solution B were used and about 18-20 µl of samples were loaded on SDS-PAGE with samples normalised to their OD_{600nm}.

597 ***Total protein samples preparation***

598 For LytE or LtaS Western blots, cells were grown, harvested and processed in the same way
599 as the LTA-samples, with the exception that to detect the his tagged LytE, the strains were
600 grown in presence of erythromycin. An 8 ml culture sample was resuspended in 180-200 µl of
601 a solution A and sonicated until a clear lysate was obtained. Samples were centrifuged at 16,000
602 g for 10 min at 4°C and a volume of sample supernatant loaded on the SDS-gel, normalized
603 based on the OD_{600nm} of the cells culture collected.

604 ***SDS-PAGE, LTA and proteins Western blots, detection blots***

605 For SDS-PAGE, samples were diluted in solution B with the addition of reducing agent (1.5 µl
606 per sample) in final volume of 20 µl, heated at 70°C for 10 min, loaded on the gel (NuPAGE
607 4-12% Bis-Tris gradient Midi Gel, Life Technologies) and separated by electrophoresis. For
608 the LTA-PBP2B experiment, MES buffer, Novex Sharp prestained protein Ladder (Life
609 Technologies) and 0.2 µm Amersham Hybond sequencing PVDF membrane (GE Healthcare)
610 were used. Whereas for LytE (37 kDa) or LtaS (74 kDa) Western blots, MOPS buffer, Abcam
611 prestained mid-range protein ladder (Ab116027) and 0.45 µM Amersham Hybond PVDF
612 membrane (GE Healthcare) were used. The SDS-PAGE gels were transferred to membranes
613 using the Trans-Blot Turbo transfer system (Bio-Rad) using an in-house transfer buffer (tris
614 600 mM, glycine 600 mM, tricine 280 mM, EDTA 2.5 mM and SDS 0.05%). After transfer,
615 the membranes were washed 3x 5 min in PBS using a rocking shaker, afterwards the
616 membranes were cut where required to allow different detection methods.

617 For the LTA Western blot, membrane was cut at the 60 kDa ladder position. The bottom part
618 of the membrane was used for the LTA-blot whereas the top part was used to detect the
619 membrane protein PBP2B (79 kDa) as a loading/extraction control. All LTA Western blot steps

were performed at room temperature in a rolling shaker and using 50 mL falcon tubes. The LTA-membranes were incubated 1h15 in PBS buffer with 3% BSA (A7030, Sigma), then 1h30 in fresh PBS-3% BSA with a dilution 1:1000 of Gram-positive LTA monoclonal antibody (MA1-7402, Thermofischer). The membrane was then washed briefly 2 times, followed by 4x 8 min in PBS and then incubated in PBS-5% dried semi-skimmed milk with 1:10,000 anti-mouse HRP-linked secondary antibody (A9044, Sigma) for 1 h. This was then followed by 2 brief and 6x 8 min washes steps prior to detection.

For proteins Western blots to detect LytE-His₁₂ or LtaS and PBP2B, all the following steps were performed at room temperature using a rocking shaker. The membranes were washed after transfer in PBS-Tween20 at 0.1% (here after noted PBS-T) and blocked in PBS-T with 5% milk for 1h15 (i.e PBS-T-milk) at room temperature. Thereafter the LytE-His₁₂/PBP2B membrane blot was cut at about 53 kDa ladder position. Membranes were placed in fresh buffer PBS-T-milk for 1h (or 1h30 when performing the LTAs western blot in parallel) with the polyclonal rabbit anti-PBP2B antibody (at 1:5000, lab. stock or available at Merck ABS2199), the mouse monoclonal Penta-His antibody (Qiagen n°34660 stock at 200 µg/ml, used at dilution 1:2000) or polyclonal rabbit anti-LtaS antibody (at 1:1000, lab. stock). Two brief washing steps were performed in PBS-T followed by 3 washes of 10 min. Membranes were incubated in PBS-T-milk for 1 h using 1:10000 dilutions of anti-rabbit or anti-mouse HRP-linked secondary antibodies (A0545 or A9044 Sigma antibodies, respectively) as required. Membranes were then washed as described at the previous step.

Pierce ECL Plus Western Blotting Substrate (32132, Thermo Scientific) was used for detection as recommended by manufacturer. Chemiluminescence was detected using ImageQuant LAS 4000 mini digital imaging system (GE Healthcare) and membranes were exposed with 1 min increment for 20 min. Membranes were also imaged by Epi-illumination to detect the pre-stained proteins ladder on the membrane. In our conditions, quantities of LTAs samples loaded

were scaled down to avoid burn out of signals observed in some of the tested strains. Occasionally for the LTAs blot, strains (in NB-only) can display a very weak signal above the 30 kDa ladder position. This phenomenon was observed by others performing *Bacillus* LTA Western blots and was assumed not to be related to LTA but possibly a cross-reactivity of the antibody to WTAs that also contains glycerolphosphate (Wormann et al., 2011). Images were edited in ImageJ (Fiji), the same contrast enhancement was applied for the membranes of the same set experiment. Three independent sets of experimental samples were generated by Western blots. Contrast was sometimes added to the images, in particular to observe LytE signal in low-expressing strains.

LytE purification and raising antiserum

Plasmid pAM-21 was transferred into *E. coli* BL21 (DE3) strain. Newly obtained strain grew at 37°C in LB kanamycin, when cell reached an approximative OD_{600nm} of 0.5 *lytE* expression was induced with 1 mM IPTG for 3 h. 50 ml culture was collected by centrifugation at 9,000 g for 5 min and resuspended in 15 ml of a solution of PBS at 4 µg/ml of lysosyme. After incubation for 20 min at room temperature (RT), cells were sonicated, and once clear lysate was obtained cells were harvested at 3,000 g for 4 min. The cell pellet was resuspended in PBS buffer at 8 M Urea and incubated for 30 min at RT on a rolling shaker. The cells were then centrifuged at 9,000 g for 10 min and supernatant was collected and mixed gently with 200 µl of Ni Sepharose High Performance (GE Healthcare) and incubated for 1 h in same condition as above. Then the sample was washed 3 to 4 times in buffer A (50 mM Tris (pH 8) and 300 mM NaCl), and the last wash was done in buffer B (50 mM Tris, 300 mM NaCl, 5 mM Imidazole). Sample was eluted five times in buffer C (PBS at 8 M Urea and 100mM imidazole) and elution fractions were analysed by SDS-PAGE. Ice-cold acetone was added at a 4:1 volume ratio to the desired eluted sample and incubated overnight at -20°C. Sample was centrifuged at 4 °C for 10 min at 15,000 g and the pellet was left to dry at RT. The pellet was suspended in

200 µl of PBS and 100 µg aliquot was sent to raise antiserum (Eurogentec). The LytE antiserum was used in Western blot at 1:5000 dilution and with the anti-rabbit secondary antibody (1:10000).

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Author contributions

Conceptualization: AG; Funding acquisition, RAD; Methodology: AG; Investigation: AG and AA; Methodology: AG; Project administration: RAD; Supervision: RAD; Validation: AG; Visualization: AG; Writing—original draft: AG; Writing—review & editing: AG and RAD.

Conflict of interest

The authors declare that they have no conflict of interest.

Supporting information

Expanded View Figures, Expanded View Tables and Appendix are available for this manuscript. This study includes no data deposited in external repositories.

References

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. 1961. Requirements for Transformation in *Bacillus subtilis*. *J Bacteriol*, 81, 741-6.
- ATILA, M., KATSELIS, G., CHUMALA, P. & LUO, Y. 2016. Characterization of N-Succinylation of L-Lysylphosphatidylglycerol in *Bacillus subtilis* Using Tandem Mass Spectrometry. *J Am Soc Mass Spectrom*, 27, 1606-13.
- BERTSCHE, U., YANG, S. J., KUEHNER, D., WANNER, S., MISHRA, N. N., ROTH, T., NEGA, M., SCHNEIDER, A., MAYER, C., GRAU, T., BAYER, A. S. & WEIDENMAIER, C. 2013. Increased cell wall teichoic acid production and D-alanylation are common phenotypes among daptomycin-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates. *PLoS One*, 8, e67398.
- BRANDA, S. S., GONZALEZ-PASTOR, J. E., DERVYN, E., EHRLICH, S. D., LOSICK, R. & KOLTER, R. 2004. Genes involved in formation of structured multicellular communities by *Bacillus subtilis*. *J Bacteriol*, 186, 3970-9.
- CLAESSEN, D., EMMINS, R., HAMOEN, L. W., DANIEL, R. A., ERRINGTON, J. & EDWARDS, D. H. 2008. Control of the cell elongation-division cycle by shuttling of PBP1 protein in *Bacillus subtilis*. *Mol Microbiol*, 68, 1029-46.
- COE, K. A., LEE, W., STONE, M. C., KOMAZIN-MEREDITH, G., MEREDITH, T. C., GRAD, Y. H. & WALKER, S. 2019. Multi-strain Tn-Seq reveals common daptomycin resistance determinants in *Staphylococcus aureus*. *PLoS Pathog*, 15, e1007862.
- DO, T., PAGE, J. E. & WALKER, S. 2020. Uncovering the activities, biological roles, and regulation of bacterial cell wall hydrolases and tailoring enzymes. *J Biol Chem*, 295, 3347-3361.
- DOBIHAL, G. S., BRUNET, Y. R., FLORES-KIM, J. & RUDNER, D. Z. 2019. Homeostatic control of cell wall hydrolysis by the WalRK two-component signaling pathway in *Bacillus subtilis*. *Elife*, 8.
- DOMINGUEZ-CUEVAS, P., PORCELLI, I., DANIEL, R. A. & ERRINGTON, J. 2013. Differentiated roles for MreB-actin isologues and autolytic enzymes in *Bacillus subtilis* morphogenesis. *Mol Microbiol*, 89, 1084-98.
- EMAMI, K., GUYET, A., KAWAI, Y., DEVI, J., WU, L. J., ALLENBY, N., DANIEL, R. A. & ERRINGTON, J. 2017. RodA as the missing glycosyltransferase in *Bacillus subtilis* and antibiotic discovery for the peptidoglycan polymerase pathway. *Nat Microbiol*, 2, 16253.
- ERICKSON, H. P. 2021. How Teichoic Acids Could Support a Periplasm in Gram-Positive Bacteria, and Let Cell Division Cheat Turgor Pressure. *Front Microbiol*, 12, 664704.
- ERNST, C. M. & PESCHEL, A. 2019. MprF-mediated daptomycin resistance. *Int J Med Microbiol*, 309, 359-363.
- ERNST, C. M., SLAVETINSKY, C. J., KUHN, S., HAUSER, J. N., NEGA, M., MISHRA, N. N., GEKELER, C., BAYER, A. S. & PESCHEL, A. 2018. Gain-of-Function Mutations in the Phospholipid Flippase MprF Confer Specific Daptomycin Resistance. *mBio*, 9.

- FLORES-KIM, J., DOBIHAL, G. S., FENTON, A., RUDNER, D. Z. & BERNHARDT, T. G. 2019. A switch in surface polymer biogenesis triggers growth-phase-dependent and antibiotic-induced bacteriolysis. *Elife*, 8.
- FORMSTONE, A. & ERRINGTON, J. 2005. A magnesium-dependent *mreB* null mutant: implications for the role of *mreB* in *Bacillus subtilis*. *Mol Microbiol*, 55, 1646-57.
- FRIEDMAN, L., ALDER, J. D. & SILVERMAN, J. A. 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrob Agents Chemother*, 50, 2137-45.
- GRAY, D. A. & WENZEL, M. 2020. More Than a Pore: A Current Perspective on the In Vivo Mode of Action of the Lipopeptide Antibiotic Daptomycin. *Antibiotics (Basel)*, 9.
- GREIN, F., MULLER, A., SCHERER, K. M., LIU, X., LUDWIG, K. C., KLOCKNER, A., STRACH, M., SAHL, H. G., KUBITSCHKE, U. & SCHNEIDER, T. 2020. Ca²⁺-Daptomycin targets cell wall biosynthesis by forming a tripartite complex with undecaprenyl-coupled intermediates and membrane lipids. *Nat Commun*, 11, 1455.
- GRUNDLING, A. & SCHNEEWIND, O. 2007a. Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. *J Bacteriol*, 189, 2521-30.
- GRUNDLING, A. & SCHNEEWIND, O. 2007b. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A*, 104, 8478-83.
- HACHMANN, A. B., ANGERT, E. R. & HELMANN, J. D. 2009. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. *Antimicrob Agents Chemother*, 53, 1598-609.
- HACHMANN, A. B., SEVIM, E., GABALLA, A., POPHAM, D. L., ANTELMANN, H. & HELMANN, J. D. 2011. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. *Antimicrob Agents Chemother*, 55, 4326-37.
- HAMOEN, L. W., SMITS, W. K., DE JONG, A., HOLSAPPEL, S. & KUIPERS, O. P. 2002. Improving the predictive value of the competence transcription factor (ComK) binding site in *Bacillus subtilis* using a genomic approach. *Nucleic Acids Res*, 30, 5517-28.
- HANSON, B. R. & NEELY, M. N. 2012. Coordinate regulation of Gram-positive cell surface components. *Curr Opin Microbiol*, 15, 204-10.
- HASHIMOTO, M., OOIWA, S. & SEKIGUCHI, J. 2012. Synthetic lethality of the *lytE* *cwlO* genotype in *Bacillus subtilis* is caused by lack of D,L-endopeptidase activity at the lateral cell wall. *J Bacteriol*, 194, 796-803.
- HASHIMOTO, M., SEKI, T., MATSUOKA, S., HARA, H., ASAI, K., SADAIE, Y. & MATSUMOTO, K. 2013. Induction of extracytoplasmic function sigma factors in *Bacillus subtilis* cells with defects in lipoteichoic acid synthesis. *Microbiology*, 159, 23-35.
- HESSER, A. R., MATANO, L. M., VICKERY, C. R., WOOD, B. M., SANTIAGO, A. G., MORRIS, H. G., DO, T., LOSICK, R. & WALKER, S. 2020a. The length of lipoteichoic acid polymers controls *Staphylococcus aureus* cell size and envelope integrity. *J Bacteriol*.

- HESSER, A. R., SCHAEFER, K., LEE, W. & WALKER, S. 2020b. Lipoteichoic acid polymer length is determined by competition between free starter units. *Proc Natl Acad Sci U S A*, 117, 29669-29676.
- ISHIKAWA, S., KAWAI, Y., HIRAMATSU, K., KUWANO, M. & OGASAWARA, N. 2006. A new FtsZ-interacting protein, YlmF, complements the activity of FtsA during progression of cell division in *Bacillus subtilis*. *Mol Microbiol*, 60, 1364-80.
- JOHNSON, C. M. & GROSSMAN, A. D. 2016. The Composition of the Cell Envelope Affects Conjugation in *Bacillus subtilis*. *J Bacteriol*, 198, 1241-9.
- JORASCH, P., WOLTER, F. P., ZAHNINGER, U. & HEINZ, E. 1998. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. *Mol Microbiol*, 29, 419-30.
- JOYCE, L. R., MANZER, H. S., DA C. MENDONÇA, J., VILLARREAL, R., DORAN, K. S., PALMER, K. L. & GUAN, Z. 2021. *Streptococcus agalactiae* MprF synthesizes a novel cationic glycolipid and plays a role in brain entry and meningitis. *bioRxiv*, 2020.12.01.406702.
- KASAHARA, J., KIRIYAMA, Y., MIYASHITA, M., KONDO, T., YAMADA, T., YAZAWA, K., YOSHIKAWA, R. & YAMAMOTO, H. 2016. Teichoic Acid Polymers Affect Expression and Localization of dl-Endopeptidase LytE Required for Lateral Cell Wall Hydrolysis in *Bacillus subtilis*. *J Bacteriol*, 198, 1585-1594.
- KAWAI, Y., ASAI, K. & ERRINGTON, J. 2009a. Partial functional redundancy of MreB isoforms, MreB, Mbl and MreBH, in cell morphogenesis of *Bacillus subtilis*. *Mol Microbiol*, 73, 719-31.
- KAWAI, Y., DANIEL, R. A. & ERRINGTON, J. 2009b. Regulation of cell wall morphogenesis in *Bacillus subtilis* by recruitment of PBP1 to the MreB helix. *Mol Microbiol*, 71, 1131-44.
- KOO, B. M., KRITIKOS, G., FARELLI, J. D., TODOR, H., TONG, K., KIMSEY, H., WAPINSKI, I., GALARDINI, M., CABAL, A., PETERS, J. M., HACHMANN, A. B., RUDNER, D. Z., ALLEN, K. N., TYPAS, A. & GROSS, C. A. 2017. Construction and Analysis of Two Genome-Scale Deletion Libraries for *Bacillus subtilis*. *Cell Syst*, 4, 291-305 e7.
- LAZAREVIC, V., SOLDI, B., MEDICO, N., POOLEY, H., BRON, S. & KARAMATA, D. 2005. *Bacillus subtilis* α -phosphoglucomutase is required for normal cell morphology and biofilm formation. *Appl Environ Microbiol*, 71, 39-45.
- LE BRETON, Y., MOHAPATRA, N. P. & HALDENWANG, W. G. 2006. *In vivo* random mutagenesis of *Bacillus subtilis* by use of TnYLB-1, a mariner-based transposon. *Appl Environ Microbiol*, 72, 327-33.
- MAMOU, G., FIYAKSEL, O., SINAI, L. & BEN-YEHUDA, S. 2017. Deficiency in Lipoteichoic Acid Synthesis Causes a Failure in Executing the Colony Developmental Program in *Bacillus subtilis*. *Front Microbiol*, 8, 1991.
- MATSUOKA, S., HASHIMOTO, M., KAMIYA, Y., MIYAZAWA, T., ISHIKAWA, K., HARA, H. & MATSUMOTO, K. 2011. The *Bacillus subtilis* essential gene *dgbB* is dispensable in mutants with defective lipoteichoic acid synthesis. *Genes Genet Syst*, 86, 365-76.

- 822 MCPHERSON, D. C., DRIKS, A. & POPHAM, D. L. 2001. Two class A high-molecular-
823 weight penicillin-binding proteins of *Bacillus subtilis* play redundant roles in
824 sporulation. *J Bacteriol*, 183, 6046-53.
- 825 MCPHERSON, D. C. & POPHAM, D. L. 2003. Peptidoglycan synthesis in the absence of
826 class A penicillin-binding proteins in *Bacillus subtilis*. *J Bacteriol*, 185, 1423-31.
- 827 MEESKE, A. J., RILEY, E. P., ROBINS, W. P., UEHARA, T., MEKALANOS, J. J.,
828 KAHNE, D., WALKER, S., KRUSE, A. C., BERNHARDT, T. G. & RUDNER, D.
829 Z. 2016. SEDS proteins are a widespread family of bacterial cell wall polymerases.
830 *Nature*, 537, 634-638.
- 831 MISHRA, N. N., BAYER, A. S., WEIDENMAIER, C., GRAU, T., WANNER, S.,
832 STEFANI, S., CAFISO, V., BERTUCCIO, T., YEAMAN, M. R., NAST, C. C. &
833 YANG, S. J. 2014. Phenotypic and genotypic characterization of daptomycin-resistant
834 methicillin-resistant *Staphylococcus aureus* strains: relative roles of *mprF* and *dlt*
835 operons. *PLoS One*, 9, e107426.
- 836 MULLER, A., WENZEL, M., STRAHL, H., GREIN, F., SAAKI, T. N. V., KOHL, B.,
837 SIERSMA, T., BANDOW, J. E., SAHL, H. G., SCHNEIDER, T. & HAMOEN, L.
838 W. 2016. Daptomycin inhibits cell envelope synthesis by interfering with fluid
839 membrane microdomains. *Proc Natl Acad Sci U S A*, 113, E7077-E7086.
- 840 MURRAY, T., POPHAM, D. L. & SETLOW, P. 1998. *Bacillus subtilis* cells lacking
841 penicillin-binding protein 1 require increased levels of divalent cations for growth. *J*
842 *Bacteriol*, 180, 4555-63.
- 843 NEUHAUS, F. C. & BADDILEY, J. 2003. A continuum of anionic charge: structures and
844 functions of D-alanyl-teichoic acids in Gram-positive bacteria. *Microbiol Mol Biol*
845 *Rev*, 67, 686-723.
- 846 NICOLAS, P., MADER, U., DERVYN, E., ROCHAT, T., LEDUC, A., PIGEONNEAU, N.,
847 BIDNENKO, E., MARCHADIER, E., HOEBEKE, M., AYMERICH, S., BECHER,
848 D., BISICCHIA, P., BOTELLA, E., DELUMEAU, O., DOHERTY, G., DENHAM,
849 E. L., FOGG, M. J., FROMION, V., GOELZER, A., HANSEN, A., HARTIG, E.,
850 HARWOOD, C. R., HOMUTH, G., JARMER, H., JULES, M., KLIPP, E., LE
851 CHAT, L., LECOINTE, F., LEWIS, P., LIEBERMEISTER, W., MARCH, A.,
852 MARS, R. A., NANNAPANENI, P., NOONE, D., POHL, S., RINN, B.,
853 RUGHEIMER, F., SAPP, P. K., SAMSON, F., SCHAFFER, M., SCHWIKOWSKI,
854 B., STEIL, L., STULKE, J., WIEGERT, T., DEVINE, K. M., WILKINSON, A. J.,
855 VAN DIJL, J. M., HECKER, M., VOLKER, U., BESSIERES, P. & NOIROT, P.
856 2012. Condition-dependent transcriptome reveals high-level regulatory architecture in
857 *Bacillus subtilis*. *Science*, 335, 1103-6.
- 858 NOWAK, J., VISNOVSKY, S. B., CRUZ, C. D., FLETCHER, G. C., VAN VLIET, A. H.
859 M., HEDDERLEY, D., BUTLER, R., FLINT, S., PALMER, J. & PITMAN, A. R.
860 2021. Inactivation of the gene encoding the cationic antimicrobial peptide resistance
861 factor MprF increases biofilm formation but reduces invasiveness of *Listeria*
862 *monocytogenes*. *J Appl Microbiol*, 130, 464-477.
- 863 OKU, Y., KUROKAWA, K., MATSUO, M., YAMADA, S., LEE, B. L. & SEKIMIZU, K.
864 2009. Pleiotropic roles of polyglycerolphosphate synthase of lipoteichoic acid in
865 growth of *Staphylococcus aureus* cells. *J Bacteriol*, 191, 141-51.

- PATEL, Y., ZHAO, H. & HELMANN, J. D. 2020. A regulatory pathway that selectively up-regulates elongasome function in the absence of class A PBPs. *Elife*, 9.
- PAZOS, M. & VOLLMER, W. 2021. Regulation and function of class A Penicillin-binding proteins. *Curr Opin Microbiol*, 60, 80-87.
- PERCY, M. G. & GRUNDLING, A. 2014. Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. *Annu Rev Microbiol*.
- PEREGO, M., GLASER, P., MINUTELLO, A., STRAUCH, M. A., LEOPOLD, K. & FISCHER, W. 1995. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation. *J Biol Chem*, 270, 15598-606.
- PESCHEL, A., JACK, R. W., OTTO, M., COLLINS, L. V., STAUBITZ, P., NICHOLSON, G., KALBACHER, H., NIEUWENHUIZEN, W. F., JUNG, G., TARKOWSKI, A., VAN KESSEL, K. P. & VAN STRIJP, J. A. 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J Exp Med*, 193, 1067-76.
- RAJAGOPAL, M. & WALKER, S. 2017. Envelope Structures of Gram-Positive Bacteria. *Curr Top Microbiol Immunol*, 404, 1-44.
- SALZBERG, L. I. & HELMANN, J. D. 2008. Phenotypic and transcriptomic characterization of *Bacillus subtilis* mutants with grossly altered membrane composition. *J Bacteriol*, 190, 7797-807.
- SALZBERG, L. I., POWELL, L., HOKAMP, K., BOTELLA, E., NOONE, D. & DEVINE, K. M. 2013. The WalRK (YycFG) and s¹ RsgI regulators cooperate to control CwlO and LytE expression in exponentially growing and stressed *Bacillus subtilis* cells. *Mol Microbiol*, 87, 180-95.
- SCHIRNER, K. & ERRINGTON, J. 2009. The cell wall regulator s¹ specifically suppresses the lethal phenotype of *mbl* mutants in *Bacillus subtilis*. *J Bacteriol*, 191, 1404-13.
- SCHIRNER, K., MARLES-WRIGHT, J., LEWIS, R. J. & ERRINGTON, J. 2009. Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in *Bacillus subtilis*. *EMBO J*, 28, 830-42.
- SCHNEEWIND, O. & MISSIAKAS, D. 2014. Lipoteichoic acids, phosphate-containing polymers in the envelope of Gram-positive bacteria. *J Bacteriol*, 196, 1133-42.
- SCHNEEWIND, O. & MISSIAKAS, D. 2016. Lipoteichoic acid synthesis and function in gram-positive bacteria. *Biogenesis of Fatty Acids, Lipids and Membranes*, 1-18.
- SLAVETINSKY, C., KUHN, S. & PESCHEL, A. 2017. Bacterial aminoacyl phospholipids - Biosynthesis and role in basic cellular processes and pathogenicity. *Biochim Biophys Acta Mol Cell Biol Lipids*, 1862, 1310-1318.
- SOLDO, B., LAZAREVIC, V. & KARAMATA, D. 2002. *tagO* is involved in the synthesis of all anionic cell-wall polymers in *Bacillus subtilis* 168. *Microbiology (Reading)*, 148, 2079-2087.
- SONG, D., JIAO, H. & LIU, Z. 2021. Phospholipid translocation captured in a bifunctional membrane protein MprF. *Nat Commun*, 12, 2927.

- 908 STAUBITZ, P. & PESCHEL, A. 2002. MprF-mediated lysinylation of phospholipids in
909 *Bacillus subtilis* - protection against bacteriocins in terrestrial habitats? *Microbiology*,
910 148, 3331-2.
- 911 STEINMETZ, M. & RICHTER, R. 1994. Plasmids designed to alter the antibiotic resistance
912 expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination.
913 *Gene*, 142, 79-83.
- 914 STRAUME, D., PIECHOWIAK, K. W., KJOS, M. & HAVARSTEIN, L. S. 2021. Class A
915 PBPs: It is time to rethink traditional paradigms. *Mol Microbiol*.
- 916 SURDOVA, K., GAMBA, P., CLAESSEN, D., SIERSMA, T., JONKER, M. J.,
917 ERRINGTON, J. & HAMOEN, L. W. 2013. The conserved DNA-binding protein
918 WhiA is involved in cell division in *Bacillus subtilis*. *J Bacteriol*, 195, 5450-60.
- 919 THITIANANPAKORN, K., AIBA, Y., TAN, X. E., WATANABE, S., KIGA, K., SATO'O,
920 Y., BOONSIRI, T., LI, F. Y., SASAHARA, T., TAKI, Y., AZAM, A. H., ZHANG,
921 Y. & CUI, L. 2020. Association of *mprF* mutations with cross-resistance to
922 daptomycin and vancomycin in methicillin-resistant *Staphylococcus aureus* (MRSA).
923 *Sci Rep*, 10, 16107.
- 924 VEENING, J. W., KUIPERS, O. P., BRUL, S., HELLINGWERF, K. J. & KORT, R. 2006.
925 Effects of phosphorelay perturbations on architecture, sporulation, and spore
926 resistance in biofilms of *Bacillus subtilis*. *J Bacteriol*, 188, 3099-109.
- 927 WILSON, S. & GARNER, E. 2021. An Exhaustive Multiple Knockout Approach to
928 Understanding Cell Wall Hydrolase Function in *Bacillus subtilis*. *bioRxiv*,
929 2021.02.18.431929.
- 930 WOOD, B. M., SANTA MARIA, J. P., JR., MATANO, L. M., VICKERY, C. R. &
931 WALKER, S. 2018. A partial reconstitution implicates DltD in catalyzing lipoteichoic
932 acid d-alanylation. *J Biol Chem*, 293, 17985-17996.
- 933 WORMANN, M. E., CORRIGAN, R. M., SIMPSON, P. J., MATTHEWS, S. J. &
934 GRUNDLING, A. 2011. Enzymatic activities and functional interdependencies of
935 *Bacillus subtilis* lipoteichoic acid synthesis enzymes. *Mol Microbiol*, 79, 566-83.
- 936 YANG, S. J., NAST, C. C., MISHRA, N. N., YEAMAN, M. R., FEY, P. D. & BAYER, A.
937 S. 2010. Cell wall thickening is not a universal accompaniment of the daptomycin
938 nonsusceptibility phenotype in *Staphylococcus aureus*: evidence for multiple
939 resistance mechanisms. *Antimicrob Agents Chemother*, 54, 3079-85.
- 940 ZHU, B. & STULKE, J. 2018. SubtiWiki in 2018: from genes and proteins to functional
941 network annotation of the model organism *Bacillus subtilis*. *Nucleic Acids Res*, 46,
942 D743-D748.

943 Expanded View figures

944 Figure EV1. Comparison of $\Delta 4$ and $\Delta ponA$ mutants in absence MprF or LtaS

945 Strains were streaked from glycerol stocks onto NA plates (supplemented with antibiotic and/or
946 magnesium, where required). After incubation at 37°C overnight, a single colony for each strain
947 was then streak across the set of plates shown above (with glucose 0.5%, MgSO₄ 10mM) and
948 scanned after 16 h incubation at 37°C. The following strains were inoculated: AG157 ($\Delta 4$),
949 AG223 ($\Delta 4 \Delta mprF$), AG342 ($\Delta 4 \Delta ltaS$), RE101 ($\Delta ponA$), AG193 ($\Delta ponA \Delta mprF$), AG383
950 ($\Delta ponA \Delta ltaS$), AG181 ($\Delta mprF$), 4285CA ($\Delta ltaS$), AG347 ($\Delta mprF \Delta ltaS$).

Figure EV2. Accumulation of LtaS-His and YfnI-His in Δ ponA cells.

Strains were grown in NB and NB supplemented with glucose 0.2% until late exponential growth at 37°C. Tested once in condition similar to that of the proteins Western blot presented in this study, following a preliminary test. * Strains used here express the following his tagged protein under the control of their native promoter: either LtaS-His₁₂ in a wild type-like background (AG569) or in Δ ponA (AG588) background; or YfnI-His₁₂ in a wild type-like (WT) (AG570 strain) or in Δ ponA (AG589) background. (A) The membrane was detected for His tagged proteins with a monoclonal Penta-His and an HRP-linked anti-mouse antibodies. (B) The membrane was reused to detected PBP2B (sample loading control) with a polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies. Experiment tested on one biological set of samples.

Figure EV3. Accumulation of LtaS/YfnI and LytE in LTA-synthase mutants.

The following strains were grown (NB $\pm$ Glc 0.2%) in condition similar to our previous assays: *B. subtilis* 168CA, 4285CA (Δ ltaS), 4289CA (Δ yfnI), 4292CA (Δ yqgS), AG595 (Δ yqgS Δ yfnI), AG593 (Δ ltaS Δ yqgS), AG594 (Δ ltaS Δ yfnI), AG600 (Δ ltaS Δ yfnI Δ yqgS). In this figure, the Western blot experiments were carried out in same condition as other assays (polyclonal anti-LtaS antibody cross reacts with YfnI, unpublished Errington's lab) (A), except for the use of our newly produced polyclonal anti-LytE antibody (B). Experiment tested on one biological set of samples.

Figure EV4. Results of three independent experiments supporting Fig 5.

A Samples of methicillin-sensitive *Staphylococcus aureus* strains SA113 and SA113 Δ mpf (Peschel et al., 2001) were probed for LTA by Western Blot. Three independent set of cultures grown in LB at 37°C were tested simultaneously.

B-D It has been shown that a modified allele of *pgsA*(A64V) was the major determinant of daptomycin resistance of *B. subtilis* Dap^R1 and Dap^R20 strains selected by serial daptomycin antibiotic passages (Hachmann et al., 2011). The strains 168 1A1 (wild type, WT), Dap^R1, Dap^R20, HB15516 (Dap^R1 *pgsA*^{WT}) and HB15507 (Dap^R20 *pgsA*^{WT}) were grown in NB overnight at 30°C and at late exponential growth phase (37°C, using theses overnight cultures). For each set of samples, LTA was detected along with PBP2B productions by Western Blots. Bottom panel shows the cellular abundance of PB2B and the LTA-synthase detected with anti-LtaS antibody (also detect YfnI, unpublished Errington's lab). Samples used in the bottom panel where those of the LTA/PBP2B experiment loaded onto a new SDS-gel, after minor samples normalization based on PBP2B signal shown on the upper panel.

Expanded View Tables

Table EV1: Plasmids used in this study (by alphabetic order)

Plasmids	Relevant features ¹	Source or reference
pAM-21	<i>kan P_{T7} LytE^(26-334AA)-6His lacI</i>	This study
pCotC-GFP	used as matrix to amplify <i>cat</i>	(Veening et al., 2006)
pDR111	<i>bla amyE::(lacI P_{hyspank} spc)</i>	Rudner, D., unpublished
pDR111- <i>P_{hyspank}-ltaS</i>	<i>bla amyE::(lacI P_{hyspank}-ltaS, spc)</i>	This study
pDR111- <i>P_{hyspank}-lytE-mprF</i>	<i>bla amyE::(lacI P_{hyspank}-lytE-mprF, spc)</i>	This study
pDR111- <i>P_{hyspank}-mprF</i>	<i>bla amyE::(lacI P_{hyspank}-ATGmprF, spc)</i>	This study
pDR111- <i>P_{hyspank}-mprF-lytE</i>	<i>bla amyE::(lacI P_{hyspank}-ATGmprF-lytE, spc)</i>	This study
pDR111- <i>P_{hyspank}-yfnI</i>	<i>bla amyE::(lacI P_{hyspank}-yfnI, spc)</i>	This study
pDR111- <i>P_{hyspank}-yqgS</i>	<i>bla amyE::(lacI P_{hyspank}-yqgS, spc)</i>	This study
pET28a (+)	<i>kan P_{T7} his lacI</i>	Novagen
pG ⁺ host9:: <i>ΔponA</i>	<i>erm</i> , carry flanking DNA regions of <i>ponA</i>	(Emami et al., 2017)
pG ⁺ host10:: <i>ΔpbpD</i>	<i>bla</i> , carry flanking DNA regions of <i>pbpD</i>	(Emami et al., 2017)
pG ⁺ host10:: <i>ΔpbpF</i>	<i>bla</i> , carry flanking DNA regions of <i>pbpF</i>	(Emami et al., 2017)
pIC22	used as matrix to amplify <i>phleo</i>	(Steinmetz and Richter, 1994)
pLOSS*	<i>bla spc P_{spac}-mcs P_{divIVA}-lacZ lacI rep_{pLS20}(GA→CC)</i>	(Claessen et al., 2008)
pLOSS- <i>P_{spac}-lytE</i>	<i>bla spc P_{spac}-lytE P_{divIVA}-lacZ lacI rep_{pLS20}(GA→CC)</i>	This study
pLOSS- <i>P_{spac}-ponA</i>	<i>bla spc P_{spac}-ponA P_{divIVA}-lacZ lacI rep_{pLS20}(GA→CC)</i>	(Emami et al., 2017)
pMarB	<i>bla erm TnYLB-1::kan HimarI</i> transposase	(Le Breton et al., 2006)
pMUTinHis	<i>bla erm P_{spac} mcs 12xhis lacZ lacI</i>	(Ishikawa et al., 2006)
pMUTin- <i>lytE-his</i>	<i>bla erm P_{spac} mcs LytE-6AA-12His lacZ lacI</i>	This study
pMUTin- <i>ltaS-his</i>	<i>bla erm P_{spac} mcs LtaS-6AA-12His lacZ lacI</i>	This study
pMUTin- <i>yfnI-his</i>	<i>bla erm P_{spac} mcs YfnI-6AA-12His lacZ lacI</i>	This study

¹ *cat*, chloramphenicol resistance; *bla*, ampicillin resistance; *spc*, spectinomycin resistance; *erm*, erythromycin resistance; *phleo*, phleomycin resistance; *kan*, kanamycin resistance; *lacI*, encodes lactose repressor; *mcs*, multiple cloning site; *lacZ*, encodes β-galactosidase; AA, amino acids; *P_{hyspank}* and *P_{spac}* are IPTG-inducible promoters with *P_{hyspank}* known to be stronger; *rep_{pLS20}(GA→CC)*, unstable replication in *Bacillus*.

Table EV2: Strains used in this study.

Strains	Relevant genotype ¹	Source or reference ²
<i>E. coli</i>		
DH5α	F ⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d/ <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169, hsdR17(r _K ⁻ m _K ⁺), λ ⁻	Lab. stock
BL21(DE3)	B F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻)</i> λ(DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>]) [<i>malB</i> ⁺] _{K-12} (λ ^S)	Novagen
<i>B. subtilis</i>		
168CA	<i>trpC2</i>	R. Daniel Lab. stock
168 1A1	<i>trpC2</i> (BGSC n° 1A1)	(Hachmann et al., 2011)
3725CA	<i>trpC2</i> Ω _{neo} Δ <i>mreB</i>	3725 (Formstone and Errington, 2005) → 168CA cells
4261CA	<i>trpC2</i> Δ <i>aml::cat</i>	4261 (Schirner and Errington, 2009) → 168CA cells
4283CA	<i>trpC2</i> Δ <i>ltaS::neo</i>	(Schirner and Errington, 2009) → 168CA cells
4285CA	<i>trpC2</i> Δ <i>ltaS::cat</i>	(Schirner and Errington, 2009) → 168CA cells
4287CA	<i>trpC2</i> Δ <i>yfnI::spc</i>	(Schirner and Errington, 2009) → 168CA cells
4289CA	<i>trpC2</i> Δ <i>yfnI::erm</i>	(Schirner and Errington, 2009) → 168CA cells
4292CA	<i>trpC2</i> Δ <i>yqgS::spc</i>	(Schirner and Errington, 2009) → 168CA cells
4293CA	<i>trpC2</i> Δ <i>yqgS::cat</i>	(Schirner and Errington, 2009) → 168CA cells
4294CA	<i>trpC2</i> Δ <i>yqgS::erm</i>	(Schirner and Errington, 2009) → 168CA cells
4295CA	<i>trpC2</i> Δ <i>yvgJ::spc</i>	(Schirner and Errington, 2009) → 168CA cells
4620	<i>trpC2</i> Δ <i>ltaS::neo</i> Δ <i>yfnI::cat</i> Δ <i>yqgS::spc</i> Δ <i>yvgJ::erm</i>	(Schirner and Errington, 2009)
AG105	<i>trpC2</i> Δ <i>pbpD</i> Δ <i>pbpF</i>	pGhost10::Δ <i>pbpF</i> (Emami et al., 2017) → RE103 cells
AG108	<i>trpC2</i> Δ <i>ponA</i> Δ <i>pbpD</i> Δ <i>pbpF</i>	pG ⁺ host10::Δ <i>pbpF</i> → RE104 cells
AG123	<i>trpC2</i> Δ <i>ponA</i> Δ <i>pbpD</i> Δ <i>pbpF</i> pLOSS- <i>P_{spac}-ponA</i>	pLOSS- <i>P_{spac}-ponA</i> → AG108 cells
AG141	<i>trpC2</i> Δ <i>ponA</i> Δ <i>pbpD</i> Δ <i>pbpF</i> pLOSS- <i>P_{spac}-ponA lacA::cat</i>	K532 → AG123 cells

AG157	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan</i>	(Emami et al., 2017)
AG181	<i>trpC2 ΔmprF::phleo</i>	This study
AG185	<i>trpC2 ΔmprF::phleo Ωneo ΔmreB</i>	3725CA → AG181 cells
AG189	<i>trpC2 ΔmprF::phleo Δmbl::cat</i>	4261CA → AG181 cell
AG193	<i>trpC2 ΔponA ΔmprF::phleo</i>	AG181 → RE101 cells
AG200	<i>trpC2 ΔponA ΔpbpD ΔpbpF pLOSS-P_{spac}-ponA ΔmprF::phleo lacA::cat</i>	AG183 → AG141 cells
AG200BK#42	<i>trpC2 ΔponA ΔpbpD ΔpbpF pLOSS-P_{spac}-ponA ΔmprF::phleo lacA::cat gtaB::TnYLB-1(kan)</i>	This study
AG210	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo lacA::cat pLOSS-P_{spac}-ponA</i>	DPVB45CA → AG200 cells
AG211	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan pLOSS-P_{spac}-ponA</i>	DPVB45CA → AG123 cells
AG223	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo</i>	AG181 → AG157 cells
AG290	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan pLOSS-P_{spac}-ponA ΔgtaB::erm</i>	SM08 → AG211 cells
AG290NopLoss	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔgtaB::erm</i>	AG290 lost plasmid on 20 mM MgSO ₄ with kan
AG292	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan pLOSS-P_{spac}-ponA Ωerm ΔtagE</i>	SM50 → AG211 cells
AG293	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ggaAB::spc</i>	SM31 → AG229 cells
AG296	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan pLOSS-P_{spac}-ponA pgcA::tet</i>	SSB122 → AG211 cells
AG304	<i>trpC2 amyE::(P_{hyspank}-mprF, spc)</i>	pDR111-P _{hyspank} -mprF → 168CA cells
AG311	<i>trpC2 ΔponA amyE::(P_{hyspank}-mprF, spc)</i>	AG304 → RE101 cells
AG317	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan amyE::(P_{hyspank}-mprF, spc)</i>	AG304 → AG157 cells
AG322	<i>trpC2 amyE::(P_{hyspank}-mprF, spc) Δmbl::cat</i>	4261CA → AG304 cells
AG327	<i>trpC2 amyE::(P_{hyspank}-mprF, spc) Ωneo ΔmreB</i>	3725CA → AG304 cells
AG341	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan dltAB::cat</i>	DLT71 (Perego et al., 1995) → AG157 cells
AG342	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔltaS::cat</i>	4285CA → AG157 cells
AG343	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔyfnI::erm</i>	4289CA → AG157 cells
AG344	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔyqgS::erm</i>	4294CA → AG157 cells

AG347	<i>trpC2 ΔmprF::phleo ΔltaS::cat</i>	4285CA → AG181 cells
AG353	<i>trpC2 amyE::(P_{hyspank}-mprF, spc) ΔyfnI::erm</i>	4289CA → AG304 cells
AG370	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔltaS::cat ΔyfnI::erm</i>	4289CA → AG342 cells
AG372	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔltaS::cat ΔyqgS::erm</i>	4294CA → AG342 cells
AG377	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔyqgS::erm ΔyfnI::spc</i>	4287CA → AG344 cells
AG380	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔltaS::cat ΔyqgS::erm ΔyfnI::spc</i>	4287CA → AG372 cells
AG383	<i>trpC2 ΔponA ΔltaS::cat</i>	4285CA → RE101 cells
AG384	<i>trpC2 ΔponA ΔyfnI::erm</i>	4289CA → RE101 cells
AG385	<i>trpC2 ΔponA ΔyqgS::spc</i>	4292CA → RE101 cells
AG389	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo ΔltaS::cat ΔyqgS::erm</i>	AG223 → AG372 cells
AG390	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo ΔyqgS::erm ΔyfnI::spc</i>	AG223 → AG377 cells
AG393	<i>trpC2 ΔponA ΔltaS::cat ΔyfnI::erm</i>	4289CA → AG383 cells
AG394	<i>trpC2 ΔponA ΔltaS::cat ΔyqgS::spc</i>	4292CA → AG383 cells
AG395	<i>trpC2 ΔponA ΔyfnI::spc ΔyqgS::spc</i>	4292CA → AG384 cells
AG399	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo ΔltaS::cat ΔyfnI::erm</i>	AG223 → AG370 cells
AG400	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo ΔltaS::cat ΔyqgS::erm ΔyfnI::spc</i>	AG223 → AG380 cells
AG403	<i>trpC2 ΔponA ΔltaS::cat ΔyfnI::erm ΔyqgS::spc</i>	4292CA → AG393 cells
AG417	<i>trpC2 ΔpbpD ΔpbpF ΔpbpG::kan</i>	DPVB45CA → AG105 cells
AG421	<i>trpC2 ΔpbpD ΔpbpF ΔpbpG::kan amyE::(P_{hyspank}-mprF, spc)</i>	AG304 → AG417 cells
AG441	<i>trpC2 ΔponA ΔltaS::cat ΔyfnI::erm ΔugtP::spc</i>	PG253 → AG393 cells
AG442	<i>trpC2 ΔponA ΔltaS::cat ΔyfnI::erm gtaB::TnYLB-1(kan)</i>	AG200BK#42 → 168CA → AG393 cells
AG443	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔugtP::spc</i>	PG253 → AG157 cells
AG444	<i>trpC2 ΔponA ΔugtP::spc</i>	PG253 → RE101 cells
AG474	<i>trpC2 ΔcwlO::spc</i>	PDC463 (Dominguez-Cuevas et al., 2013) → 168CA cells
AG475	<i>trpC2 amyE::(P_{hyspank}-lytE, spc)</i>	Dominguez-Cuevas, unpublished → 168CA cells
AG484	<i>trpC2 ΔponA amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → RE101 cells

AG501	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG157 cells
AG502	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG223 cells
AG506	<i>trpC2 ΔponA ΔgtaB::erm</i>	SM08 → RE101 cells
AG547	<i>trpC2 ΔlytE::cat</i>	This study
AG549	<i>trpC2 ΔlytE::cat pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG547 cells
AG551	<i>trpC2 pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → 168CA cells
AG557	<i>trpC2 ΔponA pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → RE101 cells
AG565	<i>trpC2 ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	pMUTinHis-lytE → 168CA cells
AG569	<i>trpC2 ΩltaS-his₁₂::(pMUTIN-ltaS, erm)</i>	pMUTinHis-ltaS → 168CA cells
AG570	<i>trpC2 ΩyfnI-his₁₂::(pMUTIN-yfnI, erm)</i>	pMUTinHis-yfnI → 168CA cells
AG586	<i>trpC2 ΔcwlO::spc ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	pMUTinHis-lytE → AG474 cells
AG587	<i>trpC2 ΔponA ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → RE101 cells
AG588	<i>trpC2 ΔponA ΩltaS-his₁₂::(pMUTIN-ltaS, erm)</i>	AG569 → RE101 cells
AG589	<i>trpC2 ΔponA ΩyfnI-his₁₂::(pMUTIN-yfnI, erm)</i>	AG570 → RE101 cells
AG593	<i>trpC2 ΔyqgS::spc ΔltaS::cat</i>	4292CA → 4285CA cells
AG594	<i>trpC2 ΔyfnI::erm ΔltaS::cat</i>	4289CA → 4285CA cells
AG595	<i>trpC2 ΔyfnI::erm ΔyqgS::spc</i>	4292CA → 4289CA cells
AG600	<i>trpC2 ΔltaS::cat ΔyfnI::erm ΔyqgS::spc</i>	4285CA → AG595 cells
AG614	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo pLOSS-P_{spac}-ponA ΔgtaB::erm</i>	SM08 → AG210 cells
AG632	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo ΔugtP::spc</i>	PG253 → AG223 cells
AG636	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔmprF::phleo ΔgtaB::erm</i>	AG614 plasmid lost on NA MgSO ₄ 20 mM with erm
AG1460	<i>trpC2 ΔpbpD ΔpbpF ΔpbpG::kan amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG417 cells
AG1461	<i>trpC2 ΔlytE::cat amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG547 cells
AG1462	<i>trpC2 ΔltaS::cat amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → 4285CA cells
AG1463	<i>trpC2 ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → 4289CA cells

AG1465	<i>trpC2 ΔponA ΔpbpD ΔpbpF ΔpbpG::kan ΔltaS::cat amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG342 cells
AG1466	<i>trpC2 ΔltaS::cat pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → 4285CA cells
AG1467	<i>trpC2 ΔyfnI::erm pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → 4289CA cells
AG1469	<i>trpC2 ΔltaS::cat ΔyfnI::erm pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG594 cells
AG1478	<i>trpC2 ΔmprF::phleo amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG181 cells
AG1479	<i>trpC2 ΔponA ΔmprF::phleo amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG193 cells
AG1480	<i>trpC2 ΔponA ΔltaS::cat amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG383 cells
AG1481	<i>trpC2 ΔmprF::phleo pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG181 cells
AG1482	<i>trpC2 ΔponA ΔmprF::phleo pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG193 cells
AG1483	<i>trpC2 ΔponA ΔltaS::cat pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG383 cells
AG1486	<i>trpC2 ΔmprF::phleo ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → AG181 cells
AG1487	<i>trpC2 ΔponA ΔmprF::phleo ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → AG193 cells
AG1488	<i>trpC2 ΔponA ΔltaS::cat ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → AG383 cells
AG1489	<i>trpC2 ΔltaS::cat ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → 4285CA cells
AG1490	<i>trpC2 ΔltaS::cat ΔyqgS::erm</i>	4294CA → 4285CA cells
AG1491	<i>trpC2 ΔyqgS::cat ΔyfnI::erm</i>	4293CA → 4289CA cells
AG1492	<i>trpC2 ΔyqgS::cat amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → 4293CA cells
AG1493	<i>trpC2 ΔyqgS::cat pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → 4293CA cells
AG1497	<i>trpC2 ΔltaS::cat ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG594 cells
AG1498	<i>trpC2 ΔltaS::cat ΔyqgS::erm amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG1490 cells
AG1499	<i>trpC2 ΔyqgS::cat ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc)</i>	AG475 → AG1491 cells
AG1500	<i>trpC2 ΔltaS::cat ΔyqgS::erm pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG1490 cells
AG1502	<i>trpC2 ΔyqgS::cat ΔyfnI::erm pLOSS-P_{spac}-lytE</i>	pLOSS-P _{spac} -lytE → AG1491 cells

AG1535	<i>trpC2 ΔugtP::spc ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → PG253 cells
AG1541	<i>trpC2 ΔcwlO::spc ΩlytE-his₁₂::(pMUTin-lytE, erm)</i>	AG565 → AG474 cells
AG1581	<i>trpC2 Δmbl::zeo amyE::(P_{hyspank}-mprF, spc)</i>	AK045B → AG304 cells
AG1593	<i>trpC2 ΔmreBH::kan</i>	BGSC strain* → 168CA cells
AG1594	<i>trpC2 ΔugtP::kan</i>	BGSC strain* → 168CA cells
AG1604	<i>trpC2 ΔponA Ωneo ΔmreB</i>	KS36 → RE101 cells
AG1605	<i>trpC2 ΔponA Δmbl::zeo</i>	AK045B → RE101 cells
AG1643	<i>trpC2 ΔugtP::kan amyE::(P_{hyspank}-lytE, spc)</i>	AG1594 → AG475 cells
AG1663	<i>trpC2 ΔmprF::kan</i>	BGSC strain* → 168CA cells
AG1667	<i>trpC2 ΔltaS::cat ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc) ΔmprF::kan</i>	AG1663 → AG1497 cells
AG1668	<i>trpC2 ΔltaS::cat ΔyqgS::erm amyE::(P_{hyspank}-lytE, spc) ΔmprF::kan</i>	AG1663 → AG1498 cells
AG1669	<i>trpC2 ΔyqgS::cat ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc) ΔmprF::kan</i>	AG1663 → AG1499 cells
AG1670	<i>trpC2 ΔltaS::cat ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc) ΔugtP::kan</i>	AG1594 → AG1497 cells
AG1671	<i>trpC2 ΔltaS::cat ΔyqgS::erm amyE::(P_{hyspank}-lytE, spc) ΔugtP::kan</i>	AG1594 → AG1498 cells
AG1672	<i>trpC2 ΔyqgS::cat ΔyfnI::erm amyE::(P_{hyspank}-lytE, spc) ΔugtP::kan</i>	AG1594 → AG1499 cells
AG1676	<i>trpC2 amyE::(P_{hyspank} mprF, spc) aprE::(P_{spac} lytE, erm)</i>	PCR on gDNA PDC620 → AG304 cells
AG1677	<i>trpC2 amyE::(P_{hyspank} mprF, spc) aprE::(P_{xyl} lytE, erm)</i>	PCR on gDNA PDC702 → AG304 cells
AG1684	<i>trpC2 ΔponA ΩlytE-his₁₂::(pMUTin-lytE, erm) amyE::(P_{hyspank}-mprF, spc)</i>	AG304 → AG587 cells
AG1685	<i>trpC2 ΔponA ΩlytE-his₁₂::(pMUTin-lytE, erm) ΔugtP::kan</i>	AG1594 → AG587 cells
AG1707	<i>trpC2 amyE::(P_{hyspank} -mprF-lytE)</i>	pDR111 <i>P_{hyspank} mprF-lytE</i> → 168CA cells
AG1709	<i>trpC2 amyE::(P_{hyspank} -lytE-mprF)</i>	pDR111 <i>P_{hyspank} lytE-mprF</i> → 168CA cells
AG1711	<i>trpC2 ΔponA amyE::(P_{hyspank} -mprF-lytE)</i>	AG1707 → RE101 cells
AG1713	<i>trpC2 ΔponA amyE::(P_{hyspank} -lytE-mprF)</i>	AG1709 → RE101 cells
AG1715	<i>trpC2 ΔltaS::cat amyE::(P_{hyspank} -mprF-lytE)</i>	AG1707 → 4285CA cells
AG1717	<i>trpC2 ΔltaS::cat amyE::(P_{hyspank} -lytE-mprF)</i>	AG1709 → 4285CA cells
AK045B	<i>trpC2 Δmbl::zeo</i> (in 168CA background)	H. Strahl's lab, A. Koh
Dap ^R 1	DAP-resistant isolate derived from 168 1A1	(Hachmann et al., 2011)

Dap ^R 20	DAP-resistant isolate derived from 168 1A1	(Hachmann et al., 2011)
DLT71CA	<i>trpC2 dltAB::cat</i>	DLT71 (Perego et al., 1995) → 168CA cells
DPVB45CA	<i>trpC2 ΔpbpG::kan</i>	DPVB45 (McPherson et al., 2001) → 168CA
HB15507	Dap ^R 20 <i>pgsA</i> ^{WT}	(Hachmann et al., 2011)
HB15516	Dap ^R 1 <i>pgsA</i> ^{WT}	(Hachmann et al., 2011)
K532	<i>trpC2 lacA::cat</i>	D. Claessen, unpublished
KS36	<i>trpC2 Ωneo ΔmreB</i> (in 168CA background)	H. Strahl's lab, K. Seistrup
PDC620	<i>trpC2 aprE::(P_{spac}-lytE, erm)</i>	(Dominguez-Cuevas et al., 2013)
PDC702	<i>trpC2 ΔlytE::cat::spc aprE::(P_{xyt}-lytE erm)</i>	(Dominguez-Cuevas et al., 2013)
PG253	<i>trpC2 ΔugtP::spc</i>	P. Gamba, unpublished
RE101	<i>trpC2 ΔponA</i>	(Emami et al., 2017)
RE102	<i>trpC2 ΔpbpF</i>	pG ⁺ host10::Δ <i>pbpF</i> (Emami et al., 2017) → 168CA cells
RE103	<i>trpC2 ΔpbpD</i>	pG ⁺ host10::Δ <i>pbpD</i> (Emami et al., 2017) → 168CA cells
RE104	<i>trpC2 ΔponA ΔpbpD</i>	pG ⁺ host9::Δ <i>ponA</i> (Emami et al., 2017) → RE103 cells
SM08	<i>trpC2 ΔgtaB::erm</i>	S. Moore, unpublished
SM31	<i>trpC2 ggaAB::spc</i>	S. Moore, unpublished
SM50	<i>trpC2 Ωerm ΔtagE</i>	S. Moore, unpublished
SSB122	<i>trpC2 pgcA (yhxB)::tet</i>	(Branda et al., 2004)
SWA11a	<i>trpC2 ΔponA ΔpbpD ΔpbpF lacA::cat mprF::TnYLB-1(kan)</i>	This study
<i>S. aureus</i>		
SA113	SA113 (wild type, methicillin-sensitive)	T. Palmer, lab collection
SA113 Δ <i>mprF</i>	SA113 Δ <i>mprF::erm</i>	(Peschel et al., 2001)

¹ Antibiotic resistance to *spc*, spectinomycin; *kan*, kanamycin; *erm*, erythromycin; *neo*, neomycin; *cat*, chloramphenicol; *tet*, tetracycline; *zeo*, zeocin and *phleo*: phleomycin. *TnYLB-1(kan)* transposon that originates from pMarB (Table EV1). *his*₁₂ indicates a coding sequence that corresponds to a 12 His tag at the C terminus. *P_{hyspank}* and *P_{spac}* are IPTG-inducible promoters with *P_{hyspank}* known to be stronger. Other abbreviations: Δ, deletion; Ω, insertion.

² Arrows indicate how the strain was constructed by transformation using either a plasmid DNA, a chromosomal DNA (strain code is then indicated) or a PCR on chromosomal DNA. BGSC* refers to the deletion mutant collection of the Bacillus Genetic Stock Center (Koo et al., 2017).

Table EV3: primers used in this study

Primer name	Sequence (in bold restriction enzyme site)	Used for ¹
oAA01	TTTTTCACCTTAATGCTTTGCATGGTATA TCTCCTTCTTAAAGTTAAACAAAATTATT TCTAGAGG	To amplify pET28a (+)
oAA02	ACCTCGGTGCGAAAAGATTCCACCACCA CCACCACCACT	To amplify pET28a (+)
oAA03	TAAGAAGGAGATATACCATGCAAAGCAT TAAGGTGAAAAAAGGCGAC	To amplify <i>lytE</i> from base 76 th - 1002 th
oAA04	CAGTGGTGGTGGTGGTGGTGGGAATCTTTT CGCACCGAGGTAACG	To amplify <i>lytE</i> from base 76 th - 1002 th
oAA05	ACGCCGAAACAAGCGCTC	To check pET28a- <i>lytE</i> - <i>his</i> plasmid
oAA06	GCTAGTTATTGCTCAGCG	To check pET28a- <i>lytE</i> - <i>his</i> plasmid
oAG18	GTTGACTTTATCTACAAGGT	To check insert cloned in pLOSS- <i>P_{spac}</i> - <i>lytE</i>
oAG19	GGTACCAGTAGTTCACCAC	To check pLOSS- <i>P_{spac}</i> - <i>lytE</i>
oAG81	GAATCGCCTGGCTCACATCATC	To check <i>B. subtilis</i> <i>mprF</i> ::TnYLB-1
oAG82	CGGCAAAGAACAGTCCCAAG	To check <i>B. subtilis</i> <i>mprF</i> ::TnYLB-1
oAG103	CCTGTATGTCTGGCACTCAC	To amplify upstream <i>mprF</i>
oAG104	GCTCTAGATGGTCTCTCCAATCATATTC	To amplify upstream <i>mprF</i> (<i>EcoRI</i>)
oAG105	CGGAATTCTAAGACGGAGTCTTTTTTTAT TTCG	To amplify downstream <i>mprF</i> (<i>XbaI</i>)
oAG106	CGGAGATGCGAGAGGGTTTC	To amplify downstream <i>mprF</i>
oAG107	TGCGGCATGCTCATATCCAG	To check <i>B. subtilis</i> <i>ΔmprF</i>
oAG108	TGGGCCAATCAGTGGACAAG	To check <i>B. subtilis</i> <i>ΔmprF</i>
oAG124	CCATCATCTGGTGCAGAAAGG	(Emami et al., 2017), to check <i>B. subtilis</i> <i>ΔponA</i>
oAG125	CCGCAAAGCCGATTAATTGG	
oAG126	TCTATTGGCGAGTGCTTC	
oAG127	AGCATCGACTCCGTATTG	(Emami et al., 2017), to check <i>B. subtilis</i> <i>ΔpbpF</i>
oAG128	TTATTCGGAATGGCGATGGG	(Emami et al., 2017), to check <i>B. subtilis</i> <i>ΔpbpD</i>
oAG129	CCTTAATGGCTGCAGTTGAC	
oAG165	CTAACCTGGCTGACATTCAC	To check <i>B. subtilis</i> <i>gtaB</i> ::TnYLB-1
oAG166	TGATCAGGTCTTCGCAGTTG	To check <i>B. subtilis</i> <i>gtaB</i> ::TnYLB-1
oAG197	GCTCTAGAGCACCCATTAGTTCAACAAA CG	To amplify <i>cat</i> (<i>XbaI</i>)
oAG198	GCGGATCCAGTACAGTCGGCATTATCTC	To amplify <i>cat</i> (<i>BamHI</i>)
oAG271	GGGGTTCGACAAAGGAGATTCTTAGGAT GCTGATTAAAAAGAATGCT	To construct pDR111- <i>P_{hyspank}</i> - <i>mprF</i>
oAG272	GGGGCATGCTTGATGATATTGAAACCT	To construct pDR111- <i>P_{hyspank}</i> - <i>mprF</i>
oAG292	AGGCACTCGTGAAAGGAATC	To check plasmid by sequencing
oAG294	TTCAGCGTGCGGTATTAGTG	To check plasmid by sequencing
oAG300	CAATCACGAAACAATAATTGG	To check cloned insert in pDR111

oAG301	GTTGACTTTATCTACAAGGTG	To check cloned insert in pDR111
oAG308	GGGGCTAGCAAAGGAGATTCTAGGAT GAAAACATTTATAAAGAAAGAGG	To construct pDR111- <i>P_{hysp}ank-ltaS</i> (<i>NheI</i>)
oAG309	CCGGCATGCCCCGAATGTGGAATTTGC	To construct pDR111- <i>P_{hysp}ank-ltaS</i> (<i>SphI</i>)
oAG310	GGGGCTAGCAAAGGAGATTCTAGGAT GAAGAACTTTTTTCTTACAAAC	To construct pDR111- <i>P_{hysp}ank-yfnI</i> (<i>NheI</i>)
oAG311	CCGGCATGCTGTAATGATATGAGAGAAAGC	To construct pDR111- <i>P_{hysp}ank-yfnI</i> (<i>SphI</i>)
oAG312	GGGGCTAGCAAAGGAGATTCTAGGAT GCGAAAAACGTTTTTTTCGAAG	To construct pDR111- <i>P_{hysp}ank-yqgS</i> (<i>NheI</i>)
oAG313	CCGGCATGCGAAAGCCTCCCGCTCACTTC	To construct pDR111- <i>P_{hysp}ank-yqgS</i> (<i>SphI</i>)
oAG340	GTCACATGAAGTCAAGACTATT	To check <i>ΔlytE</i>
oAG341	ACGGTTTATCAAGGAAGGACTC	To check <i>ΔlytE</i>
oAG348	GTGGCGGCCGCATGAAAAAGCAAATCA TTACAG	To construct pLOSS- <i>P_{spac}-lytE</i> (<i>NotI</i>)
oAG349	CCGGGATCCGATTGCCCTTTATGAAAATAAG	To construct pLOSS- <i>P_{spac}-lytE</i> (<i>BamHI</i>)
oAG350	GTCTGTGCTTGAGGATAAGG	To amplify upstream <i>lytE</i> for construction <i>ΔlytE</i>
oAG351	GGGTCTAGACTGCTGTCGTAGCTGTAATG	To amplify upstream <i>lytE</i> (<i>XbaI</i>) for construction <i>ΔlytE</i>
oAG352	CCGGGATCCGCGAAAAGATTCTAATTTTAG	To amplify downstream <i>lytE</i> (<i>BamHI</i>), for construction <i>ΔlytE</i>
oAG353	GATCCGTTTGCGTGTTTC	To amplify downstream <i>lytE</i> , for construction <i>ΔlytE</i>
oAG354	GGGGAATTTCGGCGGAACGACAACTTCAG	To construct pMUTin- <i>lytE-his</i> , amplify 3' terminus <i>lytE</i> (no stop codon)
oAG355	GGGCTCGAGGAATCTTTTCGCACCGAGG	To construct pMUTin- <i>lytE-his</i> , amplify 3' terminus <i>lytE</i> (no stop codon)
oAG360	GGCGAATTCGATTTATTCTCTAAAGACCACG	To construct pMUTin- <i>yfnI-his</i> , amplify 3' terminus <i>yfnI</i> (no stop codon)
oAG361	GGGCTCGAGTTTGATTCTTTCTCCTTGC CG	To construct pMUTin- <i>yfnI-his</i> , amplify 3' terminus <i>yfnI</i> (no stop codon)
oAG366	GGCGAATTCGAAACGGAGACTTTATTT CAC	To construct pMUTin- <i>ltaS-his</i> , amplify 3' terminus <i>ltaS</i> (no stop codon)
oAG367	GGGCTCGAGTTTATCTTCGTTATCCTTTG AC	To construct pMUTin- <i>ltaS-his</i> , amplify 3' terminus <i>ltaS</i> (no stop codon)
oAG368	CAAGGTGTGGCATAATGT	To check integration in pMUTinHis
oAG369	GACATCCAGAGGCACTTC	To check integration in pMUTinHis
oAG412	CACCTGGTTTTCTGCTATAGT	To check <i>B. subtilis AgtaB</i>
oAG413	GTTTAACTCTACAATCAGTG	To check <i>B. subtilis AgtaB</i>

PhleoXbaI-fw	GCTCTAGATCTTCCTTCAGGTTATGAC	To amplify <i>phleo</i> from pIC22 (<i>XbaI</i>)
PhleoEcoRI-rev	CGAATTCCGCGCCCGATTGCTGAAC	To amplify <i>phleo</i> from pIC22 (<i>EcoRI</i>)
oAG686	AAGAGAATGCGCCGGAACATC	To amplify <i>aprE</i> locus and generate strain AG1676-1677
oAG689	TGACATTTCGGCACACTCC	To amplify <i>aprE</i> locus and generate strain AG1676-1677
oAG911	TGATTGGCAAAAGCAAAAAAGACTCCGTCTAATAGTCGACAAAGGAGATTCTAGGatgaaaaagcaaatcattacagc	To construct pDR111- <i>P_{hysp}ank-mprF-lytE</i> (co-expression)
oAG912	GACCTCGTTTCCACCGAATTAGCTTGCATGCTTGATGATATTGAAACCTAttagaatcttttcgcaccgag	To construct pDR111- <i>P_{hysp}ank-mprF-lytE</i> (co-expression)
oAG913	GGTACGTACGATCTTTTCAGC	To check plasmid construct
oAG914	GGAGTGGCGAGGGAAATAC	To check plasmid construct
oAG915	ccttaatgctttgtgcagatg	To check plasmid construct
oAG916	GTTAACTCTAGAGCTGCCTG	To check plasmid construct
oAG917	GTGTGTAATTGTGAGCGGATAACAATTAAGCTTAGTCGACAAAGGAGATTCTAGGatgaaaaagcaaatcattacagc	To construct pDR111- <i>P_{hysp}ank-lytE-mprF</i> (co-expression)
oAG918	TATTGATAAAGCATTCTTTTAAATCAGCATCCTAGGAATCTCCTTTGTCGACTAttagaatcttttcgcaccgag	To construct pDR111- <i>P_{hysp}ank-lytE-mprF</i> (co-expression)
oAG919	GATGACCATGAGCGTACG	To check plasmid construct
oAG920	ccgtctgttggtgatttcg	To check plasmid construct

¹ *cat*, chloramphenicol resistance; *phleo*, phleomycin cassette; *P_{spac}* and *P_{hysp}ank* are promoter inducible with IPTG, the latter is known to be stronger; *rep_{PLS20}*(*GA*→*CC*), unstable replication in *Bacillus*, *TnYLB-1* transposon associated to kanamycin resistance.

APPENDIX

Insights into the role of lipoteichoic acids in *Bacillus subtilis*- a new function for MprF

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TABLE OF CONTENT

Appendix Figure S1. Other growth condition assays supporting Fig 1.

Appendix Figure S2. Growth of actin like mutants in 168CA background and in absence of *ponA*.

Appendix Figure S3. Additional TEM images of *B. subtilis* strains.

Appendix Figure S4. Absence of *gtab* or *ugtP* causes severe growth defect in absence of *ponA*.

Appendix Figure S5. Cell morphogenesis changes associated with deletion of LTA-synthase genes combined with class A PBPs null mutants of *B. subtilis*.

Appendix Figure S6. Effect of LTA-synthase deletions in Δ *ponA* background.

Appendix Figure S7. Results of three independent LTA Western blots supporting Fig 3A.

Appendix Figure S8. Results of three independent LTA Western blots supporting Fig 3B.

Appendix Figure S9. Results of three independent LTA Western blots supporting Fig 3C.

Appendix Figure S10. LytE overexpression is conditionally lethal in absence of PBP1 or LtaS.

Appendix Figure S11. Results of three independent experiments supporting Fig 4C.

Appendix Figure S12. Results of three independent experiments supporting Fig 4D.

Appendix Figure S13. Effect of magnesium in culture medium on the production of LytE and LtaS and accumulation of LtaS/YfnI and LytE in synthetically sick strains Δ *ponA* MprF $\uparrow$ and Δ *ugtP*.

Appendix Figure S1. Other growth condition assays supporting Fig 1.

A Ten-fold spot growth assays of strains *B. subtilis* wild type 168CA, AG157 ($\Delta ponA \Delta pbpD \Delta pbpF \Delta pbpG$ renamed $\Delta 4$), AG223 ($\Delta 4 \Delta mprF$), SWA11a ($\Delta ponA \Delta pbpD \Delta pbpF mprF::TnYLB-1 lacA$), AG417 ($\Delta pbpD \Delta pbpF \Delta pbpG$ renamed $\Delta 3$), RE101 ($\Delta ponA$), AG193 ($\Delta ponA \Delta mprF$) and AG181 ($\Delta mprF$). Serial dilutions were spotted onto nutrient agar (NA) with or without glucose 1% (**Fig 1B**) as well as on PAB agar (this figure). Plates were incubated at 37°C for 24 h and imaged.

B Overnight cultures of strains were diluted back in NB and grown at 37°C in presence or absence of IPTG (dashed or solid lines, respectively). Growth curves of strains carrying *P_{hyspank}-mprF* are displayed with the following code: wild-type-like MprF ↑ (black, diamond symbol, AG304 strain), $\Delta ponA$ MprF ↑ (red, round symbol, AG311 strain), $\Delta 4$ MprF ↑ (blue, triangle symbol, AG317 strain) and Δmbl MprF ↑ (green, square-cross symbol, AG322 strain).

Appendix Figure S2. Growth of actin like mutants in 168CA background and in absence of *ponA*.

Strains were streaked from the glycerol stock onto NA plates supplemented with antibiotic and/or magnesium, where required. After incubation at 37°C overnight, a single colony for each strain was then streak across the set of plates (Glc 0.5%) shown above and scanned after 24 h or 48 h incubation at 37°C. The following strains obtained in 168CA background were inoculated: (A) *ΔmreB* (KS36), *Δmbl* (AK045B), *ΔmreBH* (AG1593); (B) *ΔponA* (RE101), AG1605 (*ΔponA Δmbl*), AG1604 (*ΔponA ΔmreB*).

Appendix Figure S3. Additional TEM images of *B. subtilis* strains.

Figure shows images of cross-sections of *B. subtilis* 168CA (A), *mprF* mutant (AG181) (B) and strain overexpressing *mprF* (AG304) (C) grown in nutrient broth (with IPTG present to overexpress *mprF*). Samples were taken in mid-exponential growth. Arrows indicate abnormal cell wall structures that seem specific to the overexpression of *mprF*. All images were taken at 15,000x magnification and processed in ImageJ (Fiji) with scale bars attached.

Appendix Figure S4. Absence of *gtaB* or *ugtP* causes severe growth defect in absence of *ponA*.

Strains were taken from the glycerol stocks onto NA plate supplemented with antibiotic and/or magnesium, where required. After incubation at 37°C overnight, a single colony was then streak across a set of plates shown above. The top plan indicates the strains used and their position in the pictures taken after 24 h and 72 h incubation at 37°C.

Appendix Figure S5. Cell morphogenesis changes associated with deletion of LTA-synthase genes combined with class A PBPs null mutants of *B. subtilis*.

All the strains grew exponentially in NB with 10 mM MgSO₄, were washed and diluted in NB. Strains 168CA, AG157 ($\Delta 4$), AG342 ($\Delta 4 \Delta ltaS$), AG377 ($\Delta 4 \Delta yfnI \Delta yqgS$), AG370 ($\Delta 4 \Delta ltaS \Delta yfnI$), AG372 ($\Delta 4 \Delta ltaS \Delta yqgS$), AG380 ($\Delta 4 \Delta ltaS \Delta yfnI \Delta yqgS$) and AG223 ($\Delta 4 \Delta mprF$) (Table EV2) grown for 120 min until mid-exponential at 37°C. Cells were stained with FM5-95 dye and microscopy images were acquired. Cells were measured using plugin ObjectJ in ImageJ. About 400 cells were counted for each strain. Content of the figure is from data acquired by one experiment only. Due to the formation of twisted cell chains, the cells measurement of $\Delta 4 \Delta ltaS \Delta yfnI \Delta yqgS$ strain was done on the straight regions of the chain. Distributions of cell width (A) and cell length (B). (C) indicates the average of cell width and cell length in the cell population analysed.

Appendix Figure S6. Effect of LTA-synthase deletions in Δ ponA background.

A 10-fold serial dilutions were prepared in NB as in previous assays for strains 168CA, RE101 (Δ ponA), AG383 (Δ ponA Δ ltaS), AG384 (Δ ponA Δ yfnI), AG385 (Δ ponA Δ yqgS), AG393 (Δ ponA Δ ltaS Δ yfnI), AG394 (Δ ponA Δ ltaS Δ yqgS), AG395 (Δ ponA Δ yfnI Δ yqgS) and AG403 (Δ ponA Δ ltaS Δ yfnI Δ yqgS) (**Table EV2**). Plates were incubated at 37°C and imaged at 22 h.

B Strains described in (A) were diluted in NB and grown at 37°C for 120 min. When cells reached mid-exponential phase, cell membranes were stained with a FM5-95 dye and observed under a microscope. Representative images were assembled in photoshop. Scale bar represents 5 μ m.

Appendix Figure S7. Results of three independent LTA Western blots supporting Fig 3A.

The lower part of the membrane was probed using a monoclonal LTA antibody and an HRP-linked anti-mouse antibody. The other membrane part was probed using polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies, to provide a sample loading control. Samples were prepared from strains *B. subtilis* wild type (168CA), 4285CA ($\Delta ltaS$), 4289CA ($\Delta yfnI$), 4292CA ($\Delta yqgS$), AG595 ($\Delta yfnI \Delta yqgS$), AG593 ($\Delta ltaS \Delta yqgS$), AG594 ($\Delta ltaS \Delta yfnI$), AG600 ($\Delta ltaS \Delta yfnI \Delta yqgS$) and 4620 ($\Delta ltaS \Delta yfnI \Delta yqgS \Delta yvgJ$, noted here ΔLTA).

Appendix Figure S8. Results of three independent LTA Western blots supporting Fig 3B.

The lower part of the membrane was probed using a monoclonal LTA antibody and an HRP-linked anti-mouse antibody. The other membrane part was probed using polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies, to provide a sample loading control. Samples were prepared from strains *B. subtilis* wild type (168CA), AG417 ($\Delta pbpD \Delta pbpF \Delta pbpG$), RE101 ($\Delta ponA$), AG181 ($\Delta mprF$), AG193 ($\Delta ponA \Delta mprF$), AG304 (MprF $\uparrow$), AG311 ($\Delta ponA$ MprF $\uparrow$), 4261CA (Δmbi), AG322 (Δmbi MprF $\uparrow$).

Appendix Figure S9. Results of three independent LTA Western blots supporting Fig 3C.

The lower part of the membrane was probed using a monoclonal LTA antibody and an HRP-linked anti-mouse antibody. The other membrane part was probed using polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies, to provide a sample loading control. Samples were extracted from *B. subtilis* 168CA, 4285CA (Δ ltaS), AG383 (Δ ltaS Δ ponA), PG253 (Δ ugtP) and AG444 (Δ ugtP Δ ponA) strains.

Appendix Figure S10. LytE overexpression is synthetically lethal in absence of PBP1 or LtaS.

lytE overexpression was analysed in strains which integrated at the *amyE* locus an IPTG-inducible *P_{hyspank}-lytE*, represented here LytE ↑. The strains tested had the following relevant features: AG484 (*ΔponA* LytE ↑), AG1460 (*Δ3 ponA⁺* LytE ↑), AG501 (*Δ4* LytE ↑), AG502 (*Δ4 ΔmprF* LytE ↑), AG1465 (*Δ4 ΔltaS* LytE ↑), AG1478 (*ΔmprF* LytE ↑), AG1479 (*ΔponA ΔmprF* LytE ↑), AG1462 (*ΔltaS* LytE ↑), AG1480 (*ΔponA ΔltaS* LytE ↑), AG1497 (*ΔltaS ΔyfnI* LytE ↑), AG1498 (*ΔltaS ΔyqgS* LytE ↑) and AG1499 (*ΔyqgS ΔyfnI* LytE ↑). The following strains that did not display any obvious phenotype are not represented here: AG475 (168CA LytE ↑), AG1461 (*ΔlytE* LytE ↑), AG1463 (*ΔyfnI* LytE ↑), AG1492 (*ΔyqgS* LytE ↑). NA plates were supplemented with IPTG (0.1 mM), glucose Glc (0.5%) and MgSO₄ (10 mM). Plates were incubated at 37°C and scanned after 24 h (Fig 4A) and 48 h.

Appendix Figure S11. Results of three independent experiments supporting Fig 4C.

Relative abundance of LytE and PBP2B protein levels in mutant strains detected by Western blots. Strains expressing LytE-His₁₂ under the control of its native promoter were grown in NB and NB supplemented with glucose 0.2% until late exponential growth at 37°C. For simplification on this figure only the relevant background feature is displayed for the following strains: wild type-like AG565 (WT*), AG587 (Δ ponA), AG1486 (Δ mprF), AG1487 (Δ ponA Δ mprF), AG1489 (Δ ltaS), AG1488 (Δ ponA Δ ltaS), AG1535 (Δ ugtP) and AG1541 (Δ cw1O). In addition, the wild type 168CA (i.e. not expressing LytE-His₁₂) was grown in parallel and used here as a negative control for the LytE-blot. The bottom part of the membrane (split at ~ 53 kDa position) was incubated with a monoclonal Penta-His and an HRP-linked anti-mouse antibodies. The top membrane part was detected with a polyclonal anti-PBP2B and HRP-linked anti-rabbit antibodies. # For each section, the middle panel corresponds to the raw image of the LytE-His₁₂ blot at a detection time where one of the sample signals has already reached saturation.

Appendix Figure S12. Results of three independent experiments supporting Fig 4D.

Relative abundance of LTA-synthases LtaS, YfnI and PBP2B protein levels in mutant strains detected by Western blots. The samples used in Appendix Figure S10 were loaded on a new SDS-gel and the Western blot was carried out with polyclonal anti-LtaS (that cross reacts with YfnI, unpublished Errington's lab), anti-PBP2B antibodies, and HRP-linked anti-rabbit antibody. A weak non-specific protein band is observed between PBP2B and LtaS and is detected by the anti-PBP2B antibody. Strains expressing LytE-His₁₂ under the control of its native promoter were grown in NB and NB supplemented with glucose 0.2% until late exponential growth at 37°C. For simplification on this figure only the relevant background feature is displayed for the following strains: 168CA, wild type-like AG565 (WT*), AG587 (Δ ponA), AG1486 (Δ mprF), AG1487 (Δ ponA Δ mprF), AG1489 (Δ ltaS), AG1488 (Δ ponA Δ ltaS), AG1535 (Δ ugtP) and AG1541 (Δ cwlO).

Appendix Figure S13. Effect of magnesium in culture medium on the production of LytE and LtaS and accumulation of LtaS/YfnI and LytE in synthetically sick strains Δ ponA MprF ↑ and Δ ponA Δ ugtP.

Detection of LytE-His₁₂ and PBP2B by Western blots. Strains expressing LytE-His₁₂ under the control of its native promoter were grown in NB supplemented with glucose 0.2% with or without MgSO₄ (10 mM) at 37°C. For simplification on this figure only the relevant background feature is displayed for the following strains: wild type like AG565 (WT*), AG587 (Δ ponA), AG1487 (Δ ponA Δ mprF), AG1488 (Δ ponA Δ ltaS), AG1541 (Δ cwlO), AG1684 (Δ ponA MprF ↑) and AG1685 (Δ ponA Δ ugtP). The last two strains were also grown in parallel in NB. The top membrane part was incubated with polyclonal anti-LtaS (that cross reacts with YfnI, unpublished Errington's lab), anti-PBP2B antibodies, and HRP-linked anti-rabbit antibody. The bottom part of the membrane was incubated with a monoclonal Penta-His and an HRP-linked anti-mouse antibodies. Images were processed in ImageJ software. Results of three independent experiments are presented here.