WhyD tailors surface polymers to prevent bacteriolysis and direct cell elongation in Streptococcus pneumoniae

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SUMMARY

Penicillin and related antibiotics disrupt cell wall synthesis in bacteria and induce lysis by misactivating cell wall hydrolases called autolysins. Despite the clinical importance of this phenomenon, little is known about the factors that control autolysins and how penicillins subvert this regulation to kill cells. In the pathogen Streptococcus pneumoniae (Sp), LytA is the major autolysin responsible for penicillin-induced bacteriolysis. We recently discovered that penicillin treatment of Sp causes a dramatic shift in surface polymer biogenesis in which cell wall-anchored teichoic acids (WTAs) increase in abundance at the expense of lipid-linked lipoteichoic acids. Because LytA binds to these polymers, this change recruits the enzyme to its substrate where it cleaves the cell wall and elicits lysis. In this report, we identify WhyD (SPD_0880) as a new factor that controls the level of WTAs in Sp cells to prevent LytA misactivation and lysis. We show that WhyD is a WTA hydrolase that restricts the WTA content of the wall to areas adjacent to active PG synthesis. Our results support a model in which the WTA tailoring activity of WhyD directs PG remodeling activity required for proper cell elongation in addition to preventing autolysis by LytA.
INTRODUCTION

Most bacterial cells are surrounded by a cell wall matrix composed of peptidoglycan (PG). This exoskeletal layer fortifies the cell membrane against internal osmotic pressure and is essential for cell integrity. The PG consists of glycan polymers with a repeating disaccharide unit of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). Attached to the MurNAc sugar is a short peptide that is used to form crosslinks between adjacent glycan polymers, generating an interconnected PG matrix. Synthesis of the PG heteropolymer is mediated by the penicillin-binding proteins (PBPs), some of which possess both glycosyltransferase and transpeptidase activity needed to polymerize and crosslink the glycan strands of PG, respectively (Goffin & Ghuysen, 1998; Sauvage et al., 2008). The glycans are also polymerized by SEDS-family proteins that work in unison with monofunctional PBPs possessing crosslinking activity (Cho et al., 2016; Emami et al., 2017; Meeske et al., 2016; Rohs et al., 2018; Sjodt et al., 2018; Taguchi et al., 2019). Because PBPs are the targets of penicillin and related β-lactam drugs (Cho et al., 2014; Strominger & Tipper, 1965; Tipper & Strominger, 1965), much of the research focus in the field of cell wall biology has been on the regulation of cell wall synthesis by these enzymes and their inhibition by cell wall targeting antibiotics.

Since the PG layer is a continuous matrix, cell wall biogenesis also requires the activity of enzymes that cut bonds in the network called PG hydrolases or autolysins. These space making factors are important for breaking bonds in the matrix to allow for its expansion and the insertion of new material into the preexisting meshwork (Bisicchia et al., 2007; Carballido-López et al., 2006; Dohi et al., 2019; Dörr et al., 2013; Meisner et al., 2013; Singh et al., 2012; Sycuro et al., 2010; Vollmer et al., 2008). They also play important roles in cleaving
shared cell wall material connecting daughter cells during cytokinesis (Vollmer et al., 2008).

Given their potential to induce cell wall damage, it has long been appreciated that bacteria must employ robust mechanisms to prevent aberrant PG cleavage and lysis by autolysins. Notably, β-lactams and related antibiotics have long been known to kill bacteria by corrupting the activity of PG hydrolases to damage the cell wall and cause catastrophic lysis (Cho et al., 2014; Salamaga et al., 2021; A Tomasz & Waks, 1975; Alexander Tomasz et al., 1970).

Despite the relevance of this phenomenon for antibiotic development, surprisingly little is known about the regulatory mechanisms governing when and where autolysins are activated during normal growth and how antibiotics disrupt these processes to induce lysis. Identifying the factors involved would address an outstanding question in microbiology and reveal attractive new vulnerabilities in bacterial cells to target for the discovery of novel lysis-inducing drugs.

One of the main challenges in elucidating mechanisms controlling PG hydrolase activity has been the genetic redundancy of these enzymes. Mutants defective for one or even several PG hydrolases rarely display a phenotype that can be exploited for genetic analysis of their regulation. To circumvent this difficulty, we used the gram-positive pathogen *Streptococcus pneumoniae* (Sp) because it only requires a single autolysin, LytA, to trigger its characteristic growth-phase-dependent and antibiotic-induced lysis phenotypes (A Tomasz & Waks, 1975; Alexander Tomasz et al., 1970). This reliance on a single enzyme for lysis induction allowed us to design a transposon sequencing (Tn-Seq) screen for factors that control LytA activity based on the identification of essential genes that become dispensable when LytA is inactivated. The screen revealed that LytA regulation is intimately linked to the biogenesis of surface glycopolymers called teichoic acids (TAs) (Flores-Kim et al., 2019).
TAs are major constituents of the gram-positive cell envelope and include both lipoteichoic acids (LTAs), which are membrane anchored, and wall teichoic acids (WTAs), which are covalently attached to the PG (Brown et al., 2013). In Sp cells, both types of TAs are made from a common precursor linked to the lipid carrier undecaprenyl phosphate (Und-P) (Brown et al., 2013; Denapaite et al., 2012; Fischer et al., 1993; Gisch et al., 2013; Heß et al., 2017). They are also decorated with choline moieties, which serve as binding sites for LytA and other Sp enzymes with choline-binding domains (CBDs) (Brown et al., 2013; Denapaite et al., 2012; Fischer et al., 1993; Gisch et al., 2013; Heß et al., 2017). We previously found that during exponential growth, LTAs predominate and sequester LytA at the membrane thereby preventing cell wall damage (Flores-Kim et al., 2019). However, upon antibiotic treatment or upon prolonged periods in stationary phase, the membrane protease FtsH degrades the LTA synthase TacL, causing a dramatic decrease in LTA levels, and an increase in WTAs. This switch in TA polymer abundance leads to the re-localization of LytA from the membrane to the cell wall where its cleavage activity compromises wall integrity inducing lysis (Flores-Kim et al., 2019).

In this report, we characterize another essential Sp gene, spd_0880, that becomes dispensable in cells lacking LytA. We have renamed this gene whyD (WTA hydrolase) based on our results showing that it encodes a membrane-anchored enzyme that removes WTAs from the Sp cell wall. Cells inactivated for WhyD accumulate high levels of WTAs in their cell wall during growth when these polymers are normally low in abundance relative to LTAs. Thus, LytA is constitutively recruited to the wall in whyD mutant cells where its PG cleavage activity elicits lysis. Notably, we found that WhyD is not only needed to control the abundance of
WTAs but is also required to restrict their localization in the wall to areas of cell elongation. We further show that the activity of choline-binding PG hydrolases that associate with WTAs are important for the normal elongation of Sp cells. Taken together, our results support a model in which the tailoring of WTAs by WhyD helps direct the activity of space maker PG hydrolases to locations of cell wall expansion in addition to preventing autolysis by LytA.

RESULTS

Identification of WhyD

To identify regulators of autolysis, we previously performed a Tn-Seq screen for essential Sp genes that become dispensable in a ΔlytA mutant (Flores-Kim et al., 2019). In addition to tacL described in our original report, we found that the whyD (spd_0880) gene also displayed a pattern of essentiality/non-essentiality expected for a LytA regulator (Figure 1A). In wild-type cells, relatively few transposon insertions were mapped in whyD, consistent with previous genomic studies that reported it to be an essential gene (Liu et al., 2017; Opijnen et al., 2009; Opijnen & Camilli, 2012). By contrast, in cells lacking LytA, insertions in whyD were readily detected (Figure 1A). To validate the Tn-seq results, we constructed a LytA-depletion strain in which the sole copy of lytA was under the control of a zinc-regulated promoter (Eberhardt et al., 2009). When LytA was absent (no inducer), cells were viable in the presence or absence of whyD (Figure 1B). However, when LytA was expressed (+Zn), viability was severely compromised only in cells deleted for whyD (Figure 1B). Furthermore, LytA production in cells lacking WhyD during growth in liquid medium caused premature lysis in late exponential phase (Figure 1C). Finally, the sensitivity of the ΔwhyD strain to LytA activity could be recapitulated by the addition of purified recombinant LytA (rLytA) to cells (Figure 1D). Addition of rLytA to a ΔlytA mutant during exponential growth restored the stationary phase autolysis phenotype.
exhibited by wild-type cells whereas its addition to a $\Delta lytA \Delta whyD$ double mutant resulted in lysis almost immediately after exposure (Figure 1D). Thus, $whyD$ has the properties expected for a gene encoding a factor that restrains LytA activity at the cell surface.

**Cells lacking WhyD contain high levels of WTAs**

Given our previous findings that LytA activity in *Sp* is controlled by the balance of LTAs versus WTAs (Flores-Kim et al., 2019), we tested the effect of WhyD inactivation on the levels of these surface polymers in exponentially growing cells (Figure 2). LTAs were detected in membrane preparations by immunoblotting with commercial antibodies specific for the phosphocholine (PCho) modifications whereas WTAs were detected in a parallel set of samples by alcian blue-silver staining of polymers released from purified cell wall sacculi. As a control, we analyzed LTA and WTA levels in mutants inactivated for the LTA synthase TacL. As expected, LTAs were undetectable in these cells and WTA levels dramatically increased (Figure 2). In mutants defective for WhyD, a similarly dramatic increase in WTAs was observed. However, in this case, LTA levels were unaffected (Figure 2). Expression of $whyD$ from an ectopic locus restored wild-type levels of WTAs, indicating that the phenotype was due to the absence of the WhyD protein rather than an effect of the deletion on the expression of a nearby gene (Figure 2).

We previously showed that in cells treated with penicillin or those grown for an extended period in stationary phase, TacL is degraded, leading to a decrease in LTAs and an increase in WTAs (Flores-Kim et al., 2019). However, unlike TacL, WhyD protein levels remained unchanged during exponential growth and under autolytic conditions (Figure 2 - Figure supplement 1), indicating that the rise in WTA abundance in stationary phase or following
penicillin treatment does not involve the degradation of WhyD. Additionally, the change in WTA/LTA abundance under autolytic conditions was found to be unaffected by WhyD inactivation (Figure 2 - Figure supplement 2). Although further work will be required to determine whether WhyD activity is inhibited during the induction of autolysis, the results thus far clearly indicate that WhyD is required to prevent LytA-induced autolysis during normal exponential growth by limiting the accumulation of WTAs in the cell wall.

**WhyD is a WTA hydrolase**

The *whyD* gene encodes a protein with seven predicted N-terminal transmembrane segments followed by an extracellular GlpQ phosphodiesterase domain (Figure 3A). Proteins with this domain from *Bacillus subtilis* and *Staphylococcus aureus* have recently been shown to hydrolyze WTAs (Jorge et al., 2018; Myers et al., 2016; Walter et al., 2020). Together with the findings presented above, we hypothesized that WhyD hydrolyzes and releases WTAs from the cell wall during exponential growth to prevent LytA recruitment to the wall and the subsequent destruction of the PG layer (Figure 3A). To test this possibility, we purified the C-terminal GlpQ domain of WhyD (WhyD<sup>CT</sup>) and monitored its ability to release WTAs from purified sacculi (Figure 3B). Sacculi were incubated with or without purified WhyD<sup>CT</sup>, and free WTAs in the supernatant were analyzed after pelleting. WTA polymers that remained associated with the PG sacculi in the pellet fraction were also measured following alkaline hydrolysis. As anticipated, WhyD<sup>CT</sup>, but not buffer alone, released WTAs from sacculi into the supernatant (Figure 3B). Orthologues of WhyD<sup>CT</sup> (GlpQ) in *B. subtilis* and *S. aureus* were previously shown to require Ca<sup>2+</sup> ions for activity (Jorge et al., 2018; Myers et al., 2016; Walter et al., 2020). Similarly, we found that WTA release by WhyD<sup>CT</sup> was inhibited by the addition of the chelator EDTA. Given that the polymeric chemical units of WTAs and LTAs are identical
Sp cells, we tested whether purified WhyD<sub>CT</sub> was active against LTAs in membrane preparations (Figure 3C). Consistent with our in vivo data showing that inactivation of WhyD has no effect on the abundance of LTAs (Figure 2), WhyD<sub>CT</sub> was unable to release LTAs from purified membranes (Figure 3C). Altogether, these results indicate that WhyD functions as a WTA hydrolase in Sp cells.

Changes in WTA levels affect cell elongation

Mutants defective for WTA biogenesis have been studied in several different gram-positive bacteria, and their phenotypes have implicated these polymers in many physiological processes, including cell shape determination, cell division, virulence, and phage infection (Boylan et al., 1972; Brown et al., 2012, 2013; Heß et al., 2017; Johnsborg & Håvarstein, 2009; Pollack & Neuhaus, 1994; Xia et al., 2010, 2011; Ye et al., 2018). Additionally, in some gram-positive organisms like B. subtilis, WTAs can account for up to 30-50% of the dry weight of the cell wall (Brown et al., 2013; Ellwood, 1970). By contrast, in Sp cells, we find that WTAs are kept at low levels during exponential phase via the activities of TacL and WhyD (Flores-Kim et al., 2019). Despite this low abundance and unlike most well-studied gram-positive bacteria, WTAs are essential in Sp (Johnsborg & Håvarstein, 2009; Ye et al., 2018). We therefore reasoned that although WTA levels are maintained at low levels in these cells, they must be contributing to a vital part of the cell growth process.

To gain insights into the role of WTAs during growth, we modulated the levels of these polymers in Sp cells by inactivating or overproducing WhyD and monitoring the effects of these changes on cell morphology (Figure 4). Cells lacking WhyD were found to be longer, wider, and overall larger (Figure 4A and 4B). This assay was performed in a strain lacking LytA to...
prevent the autolysis of cells inactivated for WhyD. We note that lytA inactivation had a small impact on cell size in comparison to wildtype, as observed previously (Barendt et al., 2011; Rivas et al., 2002; Sanchez-Puelles et al., 1986) (Figure 4 – figure supplement 1).

Conversely, overexpression of whyD (whyD++), which lowers WTA abundance to nearly undetectable levels (Figure 4 – figure supplement 2), reduced cell length and overall cell size (Figure 4C-E). These results argue that WhyD and WTAs are important for normal cell elongation.

We hypothesized that the effect of WTAs on the process of cell elongation might be related to the activity of PG hydrolases that function as space makers for the expansion of the PG layer during growth. In this case, the low levels of WTAs maintained in exponentially growing cells would ensure that PG cleavage by LytA and other PG hydrolases with CBDs occurs at levels that promote cell elongation without causing autolysis. To test this possibility, we examined the morphology of mutants containing normal WTA levels (Figure 5C) but lacking LytA, LytB, and LytC (ΔlytABC), the three main WTA-binding hydrolases expressed during exponential growth (Figure 5) (Kausmally et al., 2005; Maestro & Sanz, 2016). Consistent with other reports and the function of LytB as the main Sp cell separase, ΔlytABC cells displayed a cell separation defect (Figure 5A) (Rivas et al., 2002; Zucchini et al., 2018). However, like cells with reduced WTA levels, the cells within the ΔlytABC chains were also shorter and overall smaller than wild-type, indicative of an elongation defect (Figure 5B). We therefore infer that Sp cells likely employ WhyD to maintain a level of WTAs in the wall that are low enough to prevent autolysis but sufficient to allow WTA-binding hydrolases to promote PG expansion and cytokinesis.
**WhyD is enriched at sites of cell wall synthesis at midcell**

To investigate whether WhyD activity is localized to specific areas within cells, we constructed several different fluorescent protein fusions (**Figure 6**). Most fusions were not functional or resulted in unstable proteins that displayed no fluorescence. However, a fusion of GFP to the N-terminus of WhyD (GFP-WhyD) without a linker between the two proteins was fluorescent and stable enough to complement the Δ*whyD* mutant phenotype (**Figure 6 – figure supplement 1A**). To investigate WhyD localization, *gfp-whyD* was expressed from a zinc-regulated promoter (Pzn-*gfp-whyD*) as the sole copy of *whyD*. Cells were grown in the presence of Zn\(^{2+}\) and the fluorescent D-amino acid (FDAA) HADA to monitor both GFP-WhyD localization and active sites of PG synthesis, respectively (Boersma et al., 2015). Exponentially growing cells displayed a significant cytoplasmic GFP signal that was likely caused by some cleavage of the GFP-WhyD fusion (**Figure 6A and Figure 6 – figure supplement 1B**). Nevertheless, an enrichment of GFP-WhyD at midcell was observable that co-localized with the HADA signal (**Figure 6A and 6B**). These data suggest that WhyD is recruited to areas of nascent PG synthesis at midcell.

**WTAs are most abundant in areas of zonal PG synthesis**

We next wanted to determine whether the low steady-state level of WTAs that accumulate in *Sp* cells localizes to specific subcellular regions (**Figure 7**). To do so, we used an assay that takes advantage of LytA’s ability to bind to the PCho moieties that decorate *Sp* teichoic acids (Fernández-Tornero et al., 2001; Li et al., 2015; Mellroth et al., 2012, 2014). Recombinant LytA (rLytA) and a catalytically inactive variant (rLytA\(^*\)) were fluorescently labeled with Alexa-Fluor594 (**Figure 7 – figure supplement 1A**) (Flores-Kim et al., 2019; Mellroth et al., 2012).
Importantly, rLytA-Alexa triggered growth-phase dependent autolysis at rates indistinguishable from unlabeled rLytA (Figure 7 – figure supplement 1B) indicating that labeling did not affect LytA activity. As expected, rLytA*-Alexa did not induce lysis and was used for all imaging experiments to avoid complications of PG cleavage (Figure 7 – figure supplement 1B). Since WTA and LTA are identical polymers with the same PCho moieties, we next investigated whether rLytA*-Alexa labels both polymers or exclusively labels WTAs. To do so, we used the Pzn-whyD strain that over-expresses WhyD and reduces WTA levels (Figure 7 - figure supplement 2). Surface labeling by rLytA*-Alexa was readily detectable on wild-type Sp and cells harboring Pzn-whyD without exogenous Zn\(^{2+}\). However, rLytA*-Alexa was undetectable when WhyD was over-expressed (+Zn) (Figure 7 - figure supplement 2). Furthermore, we confirmed that rLytA*-Alexa exclusively labels WTAs from Sp (Figure 7 - figure supplement 1C) and purified Sp sacculi, provided that WTAs had not been removed (Figure 7 - figure supplement 3). Altogether, these results indicate that rLytA*-Alexa specifically binds WTAs when added to intact cells.

Having established that rLytA*-Alexa labeling can be used as a proxy for the in vivo localization of WTAs, we monitored the subcellular positions of WTAs relative to newly synthesized PG in exponentially growing cells. To follow nascent PG and recently synthesized wall material that had moved away from midcell during cell elongation, we first pulse-labeled cells with HADA and 5 minutes later added the compatibly labeled FDAA sBADA. We then washed the cells with medium containing 1% choline to remove native choline binding proteins from the WTAs to ensure that the choline moieties were fully accessible to rLytA*-Alexa. Cells were then incubated with rLytA*-Alexa for 30 seconds, washed to remove unbound probe and imaged (Figure 7A). Elongating cells displayed a weak rLytA*-Alexa signal at midcell that co-
localized with sBADA. The sBADA signal at midcell was flanked by two prominent fluorescent bands of rLytA*-Alexa that co-localized with HADA-labeled peripheral PG (Figure 7B and 7C). Consistent with WhyD hydrolyzing WTAs at midcell, co-localization analysis of GFP-WhyD and WTAs showed an anti-correlation between GFP-WhyD enrichment at midcell and WTA localization (Figure 7 – figure supplement 4). In cells nearing the completion of cell division, the pattern changed with rLytA*-Alexa and HADA co-localizing within the deep cell constrictions (Figures 7B and Figure 7 – figure supplements 5 and 6). Structured illumination microscopy (SIM) and image deconvolution analyses revealed similar localization patterns with rLytA*-Alexa localizing in regions offset from midcell in elongating cells and at deep constrictions in cells that have nearly completed division (Figure 7 – figure supplement 5C). In support of the idea that WhyD is responsible for promoting the observed WTA localization, rLytA*-Alexa was present throughout the PG matrix in cells lacking WhyD (Figure 7C). Altogether, these results support a model (Figure 8) in which WhyD removes most, but not all, WTAs from nascent PG at midcell, resulting in low levels of WTAs being incorporated in the peripheral PG that brackets the septum. This localization of WTAs likely allows the recruitment of PG hydrolases to the peripheral region to promote the zonal expansion of the cell wall for elongation. Similarly, the change in WTA localization to deep constrictions in late-divisional cells is consistent with the role for the polymers in recruitment of LytB and other choline-binding PG hydrolases to catalyze the last stage of cell separation. Thus, localized pruning of WTAs from nascent PG by WhyD provides a mechanism by which Sp can direct the activity of its PG hydrolases to facilitate growth and division of the PG matrix (see Discussion).
WTAs are required for normal cell growth and division in many gram-positive bacteria, and in several cases, these polymers have been implicated in controlling the localization of PG hydrolases as part of their morphogenic function (Brown et al., 2013; Kasahara et al., 2016; Schlag et al., 2010; Zamakhaeva et al., 2021). However, the molecular mechanism(s) by which WTAs are localized and how they might participate in the spatiotemporal regulation of PG hydrolase activity have remained unclear for some time. Here, we show that WTA tailoring by the WhyD hydrolase plays an important role in this control process by promoting the localized accumulation of WTA polymers at sites adjacent to active areas of PG synthesis in Sp cells. This pruning of WTAs prevents the excessive recruitment of the LytA PG hydrolase to the cell wall to avoid autolysis during exponential growth. Additionally, our results suggest that the localization of WTAs promoted by WhyD also functions to guide the activity of WTA-binding PG hydrolases to specific subcellular sites where they can promote the remodeling of the wall necessary for proper cell elongation and division.

**WTA turnover and localization in Sp cells**

In our previous study, we found that inactivation of the LTA synthase TacL resulted in the dramatic accumulation of WTAs in the cell wall of Sp cells (Flores-Kim et al., 2019). Because LTAs and WTAs are made from the same precursor (Brown et al., 2013; Denapaite et al., 2012; Fischer et al., 1993; Gisch et al., 2013; Heß et al., 2017), this observation suggested that LTAs predominate in the envelope in exponentially growing cells due to TacL outcompeting the WTA ligases (LCP proteins) for their common substrate. However, the discovery that WhyD inactivation also causes a dramatic increase in WTA accumulation in exponentially growing cells without affecting LTA accumulation (**Figure 2**) indicates that
instead of substrate competition, it is likely that the continuous degradation of WTAs maintains their low levels in the cell wall of actively growing cells.

In addition to reducing the total WTA content attached to the PG matrix (Figures 2 and 3), the WTA cleavage activity of WhyD also results in the localized accumulation of these polymers at sites adjacent to areas of active wall growth (Figure 7). Determining how this localization is achieved will require further investigation, but this phenomenon is likely to arise from the enrichment of WhyD at midcell where most (Figure 6), if not all, of the nascent PG synthesis takes place in Sp cells (Boersma et al., 2015; Briggs et al., 2021; Perez et al., 2021; Trouve et al., 2021). Biochemical studies suggest that WTAs are most efficiently attached to nascent PG before the newly synthesized glycans are crosslinked into the preexisting meshwork (Rausch et al., 2019; Schaefer et al., 2017). Therefore, the balance between WTA addition and cleavage at midcell could explain the observed pattern of WTA localization. In this scenario, the enrichment of WhyD in the septal region is likely to result in the removal of most but not all WTAs added to nascent PG. Zonal PG synthesis would then be expected to push the WTA-decorated PG material away from the cell center (Figure 8). If processing of WTAs from this older material were less efficient due to the lower concentration of WhyD outside midcell and/or the reduced accessibility of WTAs attached to more mature PG, the expected result would be a gradient of WTA accumulation centered at positions adjacent to midcell, as observed (Figure 8). Re-localization of WhyD to the future daughter cell septa to prepare for the next cell cycle could then be responsible for the midcell accumulation of WTAs displayed by cells in the final stages of division (Figure 8).

Possible role of WTAs in directing the activity of space-making PG hydrolases
However the observed WTA localization is achieved, PG hydrolases and other proteins with choline-binding domains are expected to be concentrated where the choline-containing polymers accumulate. As observed in other gram-positive bacteria, including *Streptococcus mutans*, the observed concentration of WTAs in late-stage septa is likely to promote the midcell recruitment of hydrolases to facilitate daughter cell separation (Zamakhaeva et al., 2021). The finding that ΔlytABC cells lacking three major WTA-binding PG hydrolases have a short cell phenotype that resembles that of cells overproducing WhyD (Figures 4 and 5), which reduces their WTA content, suggests an additional role for WTA-guided PG hydrolases during cell elongation. In many bacteria, such space-making PG cleavage activity is essential for growth (Bisicchia et al., 2007; Carballido-López et al., 2006; Dobihal et al., 2019; Dörr et al., 2013; Meisner et al., 2013; Singh et al., 2012; Sycuro et al., 2010; Vollmer et al., 2008).

Although WTA biogenesis is essential in *Sp* cells in accordance with such a role in cell elongation, the ΔlytABC mutant lacking all the known WTA-binding PG hydrolases produced during normal exponential growth is viable. Thus, either there are other WTA-binding PG hydrolases with a space-making function yet to be identified in *Sp* cells or WTAs have an essential function other than promoting cell wall expansion. In either case, the viability of the ΔlytABC mutant suggests that there are additional space-making PG hydrolases in *Sp* cells. Whether they are also directed by WTAs or are membrane-associated factors regulated by potential parallel pathways like PcsB and/or MltG (MpgA) remains to be determined (Briggs et al., 2021; Perez et al., 2021; Sham et al., 2011; Taguchi et al., 2021;Trouve et al., 2021).

**WTA cleavage activity of WhyD**

WhyD has seven predicted N-terminal transmembrane segments in addition to a C-terminal GlpQ-like domain (WhyD<sub>CT</sub>; Figure 3A). GlpQ-containing proteins from other gram-positive
bacteria like *B. subtilis* and *S. aureus* have been shown to function as WTA hydrolases (Jorge et al., 2018; Myers et al., 2016; Walter et al., 2020). Unlike WhyD, which is membrane-anchored and essential for normal growth, the previously characterized GlpQ-containing proteins are secreted and function to promote growth during phosphate-limitation (Jorge et al., 2018; Myers et al., 2016; Walter et al., 2020). In *B. subtilis* (strain 168), LTAs and WTAs are both glycerolphosphate polymers, and *B. subtilis* GlpQ (*BsglpQ*) has been shown to act as an exolytic enzyme that specifically degrades WTAs from their unlinked termini based on their distinct enantiomeric configuration relative to LTAs (Brown et al., 2013; Jorge et al., 2018; Myers et al., 2016; Walter et al., 2020). Our biochemical results indicate that WhyD is also specific for WTAs (Figures 2 and 3). However, because WTAs and LTAs in *Sp* cells are built from a common undecaprenyl-linked precursor and have an identical polymeric structure (Denapaite et al., 2012), WhyD is unlikely to cleave within the polymer itself. Consistent with this idea, in contrast to *BsglpQ*, which completely hydrolyzes its WTA substrate (Myers et al., 2016; Walter et al., 2020), WhyD releases polymers from the wall with a length distribution that mirrors that of the LTAs (Figure 8 – figure supplement 1). Given that the only major difference between WTAs in *Sp* cells relative to the precursor or the LTAs is its linkage to the PG (Brown et al., 2013; Denapaite et al., 2012; Fischer et al., 1993; Gisch et al., 2013; Heß et al., 2017), we favor a model in which WhyD works as an endoenzyme, specifically cleaving the phosphate linkage between the WTA polymer and PG. In support of this, WhyD<sup>CT</sup> is unable to release TA polymers from membranes (Figure 3), indicating that it is unable to hydrolyze LTAs or the Und-P linked TA precursor.

*Are WTAs recycled by WhyD?*
A mechanism for controlling the WTA content of the wall and its localization by cleaving a significant portion of the polymers that are made seems wasteful and inefficient. However, such a scenario is not that different from the synthesis of the cell wall itself, which involves the turnover of up to 50% of the PG layer per generation (Borisova et al., 2016; Johnson et al., 2013). It is therefore possible that WTAs may be recycled in Sp cells in a manner similar to how PG turnover products are recycled in many different bacterial species. A WTA recycling activity seems especially important for Sp given that it is auxotrophic for the choline moieties that decorate its teichoic acids. Notably, the N-terminal multi-pass transmembrane domain of WhyD shares remote homology with a family of plasma-membrane choline transporters (PF04515), raising the intriguing possibility that this domain might function to import the WTA polymers cleaved by WhyD\textsuperscript{CT} to recycle choline and other components of the polymers.

**WhyD activity and autolysis**

We discovered WhyD based on its essential function in preventing LytA-induced autolysis of Sp cells (Figures 1, 2, and Figure 2 – figure supplement 2). Like mutants lacking the LTA synthase TacL, cells inactivated for WhyD accumulate high levels of WTAs in their walls during normal exponential growth (Figures 1, 2, and Figure 2 – figure supplement 2) (Flores-Kim et al., 2019). These levels of WTAs are comparable to those observed in cells treated with penicillin where the excess recruitment of LytA to the PG layer results in damage to the wall and lysis (Figures 1, 2, and Figure 2 – figure supplement 2). Our previous work found that the accumulation of WTAs in penicillin-treated cells requires the FtsH-mediated degradation of TacL (Flores-Kim et al., 2019). By contrast, WhyD is stable under autolytic conditions (Figure 2 – figure supplement 1). Whether its activity must also be inhibited for autolytic induction remains unknown and requires further investigation. Nevertheless, our results clearly show
that inhibition of WhyD during exponential phase has the potential to trigger cell lysis (Figure 2 – figure supplement 1). Notably, *B. subtilis* mutants inactivated for GlpQ are hypersensitive to several beta-lactam antibiotics (Myers et al., 2016), suggesting that WTA remodeling is generally important for controlling autolysis in gram-positive bacteria. Thus, WhyD and other related GlpQ family members represent attractive targets for the development of new classes of lysis-inducing antibiotics and/or potentiators of existing beta-lactam drugs.

MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.**

All *S. pneumoniae* strains were derived from the unencapsulated strain (D39 Δ*cps*) (Lanie et al., 2007). Cells were grown in Todd Hewitt (Beckton Dickinson) medium supplemented with 0.5% yeast extract (THY) at 37 °C in an atmosphere containing 5% CO₂ or on pre-poured tryptic soy agar 5% sheep blood plates (TSAII 5% sheep blood, Beckton Dickinson) with a 5 ml overlay of 1% nutrient broth (NB) agar containing the required additives. When required, TSA agar plates containing 5% defibrinated sheep blood (Northeast laboratory) were used. *E. coli* strains were grown on Luria-Bertani (LB) broth or LB agar. Wild-type *Bacillus subtilis* strain PY79 (Youngman et al., 1983) was grown in LB broth or LB agar as described previously (Fenton et al., 2016, 2018; Flores-Kim et al., 2019). For both *S. pneumoniae* and *E. coli*, antibiotics were used as previously described. A list of strains, plasmids, and oligonucleotides used in this study can be found in the Key Resources Table in Supplementary files 1, 2, and 3, respectively.

**Transformation of *S. pneumoniae*.**
Transformations were performed as described (Fenton et al., 2016, 2018; Flores-Kim et al., 2019). Briefly, cells in early exponential phase were back-diluted to an optical density at 600 nm (OD\textsubscript{600}) of 0.03 and competence was induced with 500 pg/ml competence stimulating peptide 1 (CSP-1), 0.2% BSA, and 1 mM CaCl\textsubscript{2}. Cells were transformed with 100 ng chromosomal or plasmid DNA and selected on TSAII overlay plates containing the appropriate additives.

**Growth curves.**

To monitor growth kinetics and autolysis, \textit{S}p cells in early exponential phase were diluted to an OD\textsubscript{600} of 0.025 and grown to mid exponential phase in THY medium containing the appropriate additives at 37 °C in an atmosphere containing 5% CO\textsubscript{2}. Cells were diluted to OD\textsubscript{600} of 0.025 in THY with the indicated additives and growth was monitored by OD\textsubscript{600} every 30 min. The figures that report growth curves are representative of experiments that were performed on at least two independent samples.

**Library generation and transposon insertion sequencing (Tn-seq).**

Tn-seq was performed as described previously (Flores-Kim et al., 2019). Two independently generated libraries in wild-type and ΔlytA were used and reanalyzed in this study. Briefly, genomic DNA mutagenized with the Magellan6 transposon was transformed into competent \textit{S}p. Approximately 302,000 (wt) and 305,000 (ΔlytA) transformants were recovered for each library. Genomic DNA was then isolated and digested with Mmel, followed by adapter ligation. Transposon–chromosome junctions were amplified and sequenced on the Illumina HiSeq 2500 platform using TruSeq Small RNA reagents (Tufts University Core Facility Genomics). Reads were de-multiplexed, trimmed, and transposon insertion sites mapped onto the D39.
genome. After normalization, a Mann Whitney U test was used to identify genomic regions with significant differences in transposon insertions. Transposon insertion profiles were visualized using the Artemis genome browser (v10.2).

Isolation and analysis of pneumococcal LTAs.

Sp strains were grown in THY medium with required additives at 37 °C in 5% CO2 to the indicated growth phase and normalized to an OD$_{600}$ of 0.5. 20 ml of the normalized culture were collected by centrifugation at 5000 xg for 5 min and the cell pellet was washed twice with 2 ml SMM (0.5 M sucrose, 20 mM maleic acid pH 6.5, 20 MgCl$_2$) and then re-suspended in 2 ml SMM. Protoplasts were generated by addition of lysozyme (1mg/ml final concentration) and 100 units mutanolysin (Sigma) and incubation at 37 °C for 30 minutes. Complete protoplasting was monitored by light microscopy. Protoplasts were pelleted by centrifugation at 5000 xg for 5 min and resuspended in 2 ml cold hypotonic buffer (20 mM HEPES (Na$^+$) pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM MgCl$_2$, 1 mM CaCl$_2$, 2X complete protease inhibitors (Roche), 6 µg/ml RNAse A, 6 µg/ml DNAse. Unbroken protoplasts were removed by centrifugation at 20,000 xg for 10 min, and the lysate was then subjected to ultracentrifugation at 100,000 xg for 1 hr at 4 °C. Membrane pellets were resuspended in 1ml Tris-tricine sample buffer (200 mM Tris-HCl pH 6.8, 40% glycerol, 2% SDS, 0.04% Coomassie Blue G-250), boiled for 10min, and analyzed by Tris-tricine PAGE followed by immunoblotting. The results in figures analyzing LTA levels are representative of experiments that were performed on at least two independently collected samples.
Isolation and analysis of pneumococcal WTAs.

Sp strains were grown and harvested as above. The pellets were resuspended in 2 ml of buffer 1 (50 mM 2-(N-morpholino ethanesulfonic acid (MES)) pH 6.5) and centrifuged at 7000 xg for 5 min. The resulting pellets were resuspended in 2 ml buffer 2 (50 mM MES pH 6.5, 4% (w/v) SDS) and incubated in boiling water for 1 hr. The sample was then centrifuged at 7,000 xg for 5 min and the pellet was washed with 2 ml buffer 2. The sample was transferred into a clean microfuge tube and centrifuged at 16,000 xg for 5 min. The pellet was then washed with 2 ml buffer 2, followed by successive washes with 2 ml buffer 3 (50 mM MES pH 6.5, 2% (w/v) NaCl) and 2 ml buffer 1. The samples were then centrifuged at 16,000 x g for 5 min, resuspended in 2 ml of buffer 4 (20 mM Tris-HCl pH 8.0, 0.5% (w/v) SDS) supplemented with 2 µl proteinase K (20 mg/ml), and incubated at 50 °C for 4 hr with shaking (1000 rpm). The pellet was then collected by centrifugation and washed with 2 ml buffer 3 followed by 3 washes with distilled water. The pellet was collected by centrifugation and subjected to alkaline hydrolysis in 0.5 ml of 0.1 N NaOH and incubation at 25 °C for 16 h with shaking (1000 rpm). The samples were then pelleted by centrifugation and the supernatants containing the extractable WTA were collected and resuspended in 0.5 ml native sample buffer (62.5 mM Tris-HCl pH 6.8, 40% glycerol, 0.01% bromophenol blue). Samples were analyzed by native PAGE followed by alcian blue-silver staining. The results in figures analyzing LTA levels are representative of experiments that were performed on at least two independently collected samples.

Purification of LytA (rLytA) and LytA* (rLytA) and labeling with Alexa Fluor594.

rLytA or rLytA* were over-expressed in E. coli BL21(DE3) ΔfhuA2 (New England Biolabs) containing the pET21amp-lytA or pET21amp-lytA* expression vectors. Cells were grown in LB
supplemented with 100 μg/mL ampicillin at 37 °C and expression was induced at an OD$_{600}$ of 0.5 with 1 mM IPTG for 2 h at 37 °C. Cells were collected by centrifugation and stored overnight at -20 °C. The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 200 μg/ml DNase, and 2X complete protease inhibitors (Roche)) and lysed by two passages through a cell disruptor (Constant systems Ltd.) at 25,000 psi. Unbroken cells were discarded by centrifugation. The supernatant was then passed over a DEAE cellulose column (Sigma). After washing with 20 column volumes of wash buffer (20mM NaPO$_4$ pH 7, 1.5 M NaCl), LytA was eluted with 2 column volumes of wash buffer supplemented with 140 mM choline chloride. Protein-containing fractions were pooled and dialyzed against 20 mM NaPO$_4$ pH 7.5, 150 mM NaCl, 10% glycerol, and 5 mM choline chloride. Purified rLytA and rLytA* were labeled with the Alexa Fluor594 protein labeling kit according to manufacturer instructions (Thermo Fisher Scientific).

**Purification of WhyD$^{CT}$ and antibody production.**

The C-terminal domain of WhyD (WhyD$^{CT}$) was expressed in *E. coli* BL21(DE3) ΔfhuA using the P$_{T7}$-His$_6$-SUMO-whyd$^{CT}$ expression vector (pTD68-whyD). Cells were grown in LB supplemented with 100 μg/mL ampicillin at 37 °C to an OD$_{600}$ of 0.5. Cultures were allowed to equilibrate at room temperature for 30 min and then transferred to 30 °C. his6-sumo-whyD$^{CT}$ expression was induced with 0.5 mM IPTG for 3 hr. Cells were collected by centrifugation, resuspended in 50 ml Buffer A (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM Imidazole, and 2X complete protease inhibitor tablets (Roche)), and stored at −80 °C. The cell suspension was thawed on ice and lysed by two passes through a cell disruptor at 25,000 psi. The lysate was clarified by ultracentrifugation at 35 Krpm for 30 min at 4 °C. The supernatant was added to 1 mL Ni$^{2+}$-NTA resin (Qiagen) and incubated for 1 hr at 4 °C. The suspension was loaded
into a 10 ml column (BioRad), washed twice with 4 ml Buffer A, and eluted with 2.5 ml Buffer B
(100 mM Tris-HCl pH 8.0, 500 mM NaCl, 300 mM Imidazole). 10 µL of purified His<sub>6</sub>-Ulp1 (1.25 mg/ml) was added to the eluate, and the mixture was dialyzed into 100mM Tris-HCl pH 8, 100 mM NaCl, 10% glycerol overnight at 4 °C. The next morning 10 µL more His<sub>6</sub>-Ulp1 was added to the dialysate and incubated for 1 hr at 30 °C. The dialysate was mixed with 1 mL of Ni<sup>2+</sup>-NTA resin for 1 hr at 4 °C and then loaded onto a column and the WhyD<sub>CT</sub>-containing flow-through was collected, dialyzed into 100mM Tris-HCl pH 8, 100 mM NaCl, 1mM CaCl<sub>2</sub>, 10% glycerol overnight at 4 °C and stored at −80°C. The purified protein was used for in vitro assays and to generate rabbit polyclonal antibodies (Covance).

**In vitro WTA and LTA release assays using WhyD<sub>CT</sub>.**

For the WTA release assays, the activity of WhyD<sub>CT</sub> was assayed using purified sacculi (from ΔlytAΔwhyD cells to obtain larger quantities of WTAs attached to sacculi) prepared as described above without the alkaline hydrolysis step to retain WTA. The release assays were conducted with 0.1 mg sacculi and 10 µg/ml WhyD<sub>CT</sub>, 10 µg/ml WhyD<sub>CT</sub> + 1 mM EDTA, or no WhyD<sub>CT</sub> in 1 ml reaction buffer (0.1 M Tris-HCl pH 8, 1 mm CaCl<sub>2</sub>) incubated at room temperature with gentle shaking. Released WTAs were collected by centrifugation. To recover WTAs that were not released, the sacculi pellets were then treated with 0.1 M NaOH overnight at room temperature with gentle shaking. Alkaline-released WTAs were collected by centrifugation and analyzed alongside the WhyD-released WTAs by SDS-PAGE followed by alcian blue-silver staining.

LTA assays were performed in reaction buffer with 0.1 mg homogenized membrane extracts (from ΔlytAΔwhyD cells) prepared as described above. 0.1 mg of the homogenized
membranes were incubated with 10 µg/ml WhyD<sup>CT</sup>, 10 µg/ml WhyD<sup>CT</sup> + 1 mM EDTA, or no WhyD<sup>CT</sup> in 1 ml reaction buffer (0.1 M Tris-HCl pH 8, 1 mm CaCl<sub>2</sub>), and incubated at room temperature with gentle shaking. After incubation, the reactions were quenched with 1 mM EDTA. Released and membrane-associated LTAs were then analyzed by 16% Tris-tricine SDS-PAGE and probed with a monoclonal antibody specific for phosphocholine. These assays are representative of experiments that were performed on at least two independently collected samples.

**In vivo WTA labeling experiments using LytA<sup>*</sup>-Alexa in Sp and B. subtilis.**

Strains were grown to mid exponential phase and labeled for 5 min with HADA. After labeling, the cells were washed with fresh medium and labeled for 5 min with sBADA. The equivalent of 1 mL of cells at OD<sub>600</sub> of 0.5 was washed with fresh medium containing 1% choline and then was incubated with 1 µg/ml rLytA<sup>*</sup>-Alexa for 30s with gentle shaking. Cells were washed twice with 1X phosphate buffer saline (PBS) and analyzed by fluorescence microscopy. These assays are representative of experiments that were performed on at least three independently collected samples.

**S. pneumoniae sacculi preparation and labeling.**

Cells were grown to mid exponential phase, labeled with sBADA for 5 min, and sacculi with or without WTAs were prepared as described above. Sacculi from the equivalent of 1ml of cells at OD<sub>600</sub> of 0.5 were labeled with 1 µg/ml rLytA<sup>*</sup>-Alexa as described above the samples were imaged by fluorescence microscopy. These assays are representative of experiments that were performed on at least three independently collected samples.

**Fluorescence microscopy.**
Cells were harvested and concentrated by centrifugation at 6800 x g for 1.5 min, re-suspended in 1/10th volume growth medium, and then immobilized on 2% (wt/vol) agarose pads containing 1XPBS. Fluorescence microscopy was performed on a Nikon Ti inverted microscope equipped with a Plan Apo 100x/1.4 Oil Ph3 DM phase contrast objective, an Andor Zyla 4.2 Plus sCMOS camera, and Lumencore SpectraX LED Illumination. Images were acquired using Nikon Elements 4.3 acquisition software. HADA was visualized using a Chroma ET filter cube for DAPI (49000); sBADA and GFP were visualized using a Chroma ET filter cube for GFP (49002); LytA*-AlexaFluor594 was visualized using a Chroma ET filter cube for mCherry (49008). Image processing was performed using Metamorph software (version 7.7.0.0) and Oufti was used for quantitative image analysis (Paintdakhi et al., 2016).

**Structured illumination microscopy (SIM).**

Acquisitions were performed on an Elyra 7 system with SIM², with dual PCO Edge 4.2 sCMOS cameras, on an inverted microscope via a motorized Duolink camera adapter (Carl Zeiss Microscopy, Jena Germany, and PCO, Kelheim, Germany). Channels were set up as two line-switched imaging tracks with 405 and 561 acquired simultaneously in one track, and 488 on a separate line-switched track. Samples were imaged with a Plan Apochromat 63x/1.4 oil objective with immersion medium Immersol 518 F (30 °C) with a 1.6x optovar in the light-path providing 60 nm acquisition pixel spacing. Emission windows were set via a combination of a long-pass 560 nm dichroic in the light-path, and dual-pass emission filters (420-480+495-550 nm; and 570-620+LP 655 nm) for the two cameras. A single grid spacing for both tracks was automatically selected by the Zen 3.0 software based on the wavelength and objective combination. Nine SIM phases were acquired for each planar step per channel, while Z-stacks were acquired with a spacing of 270 nm in the “Leap Mode” over a range of 4-5 µm.
Laser powers were set up to achieve ~3000 gray values in the 16-bit raw image per channel.

SIM² processing was performed based on theoretical PSF’s using the built-in unified SIM² processing function in Zen, with 25 iterations of the Constrained Iterative method at a regularization weight of 0.015, with processing pixel-sampling scale-up resulting in 16 nm pixel sizes in x-y, and 90 nm step-size in z.

**Quantification and Statistical Analyses.**

Cell size was calculated using meshes generated by Oufti (Paintdakhi et al., 2016) and the Matlab script getCellDimensions. Cell fluorescence, normalized to cell area, was calculated using meshes generated by Oufti (Paintdakhi et al., 2016) and the Matlab script getSignal1.

Demographics were generated using Oufti’s built-in demograph feature (Paintdakhi et al., 2016). Fluorescence intensity profiles along the cell length were generated using meshes generated by Oufti (Paintdakhi et al., 2016) and the Matlab script signal1_alonglength. For statistical comparisons of cell size between strains, unpaired t-tests with Welch’s correction were performed. For all tests, at least 300 cells from each sample were analyzed. Scripts, output data, and calculated p-values can be found in the source data files associated with each figure.

**Strain Construction.**

**S. pneumoniae** deletion strains

All *Sp* deletion strains were generated using PCR fragments as described previously and are listed in Table S1. Briefly, two products representing the regions (~1 kb each) flanking the target gene were amplified, and an antibiotic resistance cassette ligated between them using...
Gibson assembly. Assembled PCR products were transformed directly into Sp as described above. In all cases, deletion primers were given the name: “gene name”\_5FLANK\_F/R for 5’ regions and “gene name”\_3FLANK\_F/R for 3’ regions. Antibiotic markers were amplified from ∆bgaA::antibiotic cassette (bgaA gene disrupted with an antibiotic cassette) strains using the AB_Marker\_F/R primers. A full list of primer sequences can be found in the Table S3. Extracted gDNA from deletion strains was confirmed by PCR using the AntibioticMarker\_R primer in conjunction with a primer binding ~200 bp 5’ of the disrupted gene; these primers were given the name: “gene name”\_Seq\_F. Confirmed gDNAs of single gene deletions were used for the construction of multiple knockout strains. For strains containing multiple deletions and construct integrations, transformants were verified by re-streaking on media containing the relevant antibiotics. When needed, each construct was confirmed by diagnostic PCR and/or sequencing.

**Pzn-lytA**

The lytA ORF, with its native RBS, was amplified using primers lytA\_F\_nativeRBS\_XhoI and lytA\_R\_BamHI. The primers introduced XhoI and BamHI sites used for cloning into pLEM023 (Fenton et al., 2016) cut with the same enzymes, resulting in plasmid pJFK004. The plasmid was sequenced and used to transform strain D39 ∆cps ∆bgaA::kan lytA::erm. Integration into the bga locus was confirmed by antibiotic marker replacement and PCR using the bgaA\_FLANK\_F and bgaA\_FLANK\_R primers. gDNA from the resulting strain was prepared and then used to transform the appropriate Sp strains.

**Pzn-whyD**

The whyD ORF, with its native RBS, was amplified using primers whyD_F_optRBS_XhoI and whyD_R_BamHI. The primers introduced XhoI and BamHI sites used for cloning into pLEM023 cut with the same enzymes, resulting in plasmid pJFK003. The plasmid was sequenced and used to transform strain D39 Δcps Δbga::kan. Integration into the bga locus was confirmed by antibiotic marker replacement and PCR using the bgaA_FLANK_F and bgaA_FLANK_R primers. gDNA from the resulting strain was prepared and then used to transform the appropriate Sp strains.

Pzn-gfp-whyD (pGD147)

The plasmid was generated in a 3-piece isothermal assembly reaction with (1) a PCR product containing gfp with an optimized RBS (oligonucleotide primers oGD267/268), (2) a PCR product containing whyD (oligonucleotide primers oGD369/270), and (3) pLEM023 digested with XhoI and BamHI. The resulting construct was sequence-confirmed and used to transform strain D39 Δcps Δbga::kan. Integration into the bga locus was confirmed by antibiotic marker replacement and PCR using the bgaA_FLANK_F and bgaA_FLANK_R primers. gDNA from the resulting strain was used to transform the appropriate Sp strains.

pET24-lytA^{H26A} (pET24-lytA*)

The lytA ORF was amplified using primers lytA_F_purification_Ndel and lytA_R_purification_HindIII from the chromosome of D39 Δcps lytA-H26A (JFK_Spn004). The primers introduced Ndel and HindIII sites used for cloning into pET24a cut with the same enzymes, resulting in plasmid pJFK004. The plasmid was confirmed by sequencing.

pTD68-whyD^{CT}
The whyD<sup>CT</sup> ORF was amplified using primers whyD<sub>CTERM BamHI F</sub>_pTD68 and whyD<sub>CTERM XhoI R</sub>_pTD68 from the chromosome of D39 Δcps, and cloned into pTD68 (Uehara et al., 2010) cut with the same enzymes to generate pJFK005. The plasmid was confirmed by sequencing.

**pER111**

The plasmid was generated using a 2-piece ligation reaction containing spd1526'-1527' from pER87 (unpublished) and the kanamycin cassette digested from pBB283 (Wang et al., 2014) using BglII/SalI. The resulting construct was sequence-confirmed.

**pER111-Pf6-gfp**

pGD160 was generated using a 3-piece isothermal assembly reaction containing (1) a PCR product containing the PF6 promoter and an optimized RBS (oligonucleotide primers oGD391/392 from pPEPY-PF6-lacl(Liu et al., 2017), (2) a PCR product containing gfp (oligonucleotide primers oGD67/193), and (3) pER111 digested with Xhol and BamHI. The resulting construct was sequence-confirmed. The plasmid was sequenced and used to transform strain D39 Δcps Integration into spd1526-1527 locus was confirmed by PCR using the primers oSp95 and oSp98. gDNA from the resulting strain was prepared and then used to transform the appropriate Sp strains.

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COMPETING INTERESTS

The other authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS

JFK, GSD, DZR, and TGB designed research; JFK and GSD performed research; JFK, GSD, DZR, and TGB analyzed data; JFK, GSD, DZR, and TGB wrote the paper.

FIGURE LEGENDS

Figure 1. whyD essentiality is lytA-dependent.

(A) Transposon insertion profiles in wild-type (WT) and the ΔlytA mutant. Mariner transposon libraries were generated in WT and ΔlytA mutant strains and insertions sites and their abundance were mapped to the S. pneumoniae genome. A region including the whyD locus is ...
shown. Each line indicates an insertion site and its height reflects the number of sequencing reads. Transposon insertions in whyD were underrepresented in WT compared to ΔlytA. (B) Serial dilutions of the indicated strains in the presence (+Zn) and absence of inducer. The indicated strains were grown to exponential phase, normalized and 10-fold serially diluted. Aliquots (5 μl) of each dilution were spotted onto TSAII 5% SB plates in the presence or absence of 200 μM ZnCl₂. Plates were incubated at 37 °C in 5% CO₂ and then imaged. (C) Expression of lytA in cells lacking whyD results in growth arrest and lysis in exponential phase. Strains containing a zinc-inducible lytA allele (Pzn-lytA) were grown in THY to mid-exponential phase. Cultures were diluted into fresh THY to an OD₆₀₀ of 0.025 in the presence or absence of 200 μM ZnCl₂ and grown at 37 °C in 5% CO₂. Growth was monitored by OD₆₀₀ measurements approximately every 30 min for 10 h. (D) Cells lacking WhyD are sensitive to exogenous LytA. Growth curves of the indicated strains before and after the addition of 1 mg/ml recombinant LytA (rLytA) at an OD₆₀₀ of ~0.2. Growth was monitored by OD₆₀₀ approximately every 30 min for 15 h. The ΔwhyD mutant rapidly lyse after rLytA addition. By contrast and as reported previously, the ΔlytA strain lysed in stationary phase in a manner similar to LytA⁺ cells. Right: Coomassie-stained gel of rLytA purified from *E. coli*. Molecular weight markers (M) are shown.

**Figure 2. Cells lacking WhyD have elevated levels of WTAs.**

Analysis of WTA and LTA levels in strains lacking WhyD or TacL. The indicated strains were grown to exponential phase and harvested. WTAs were released from purified cell wall sacculi and separated by SDS-PAGE followed by alcian blue-silver staining. Membrane-associated LTAs were resolved by 16% Tris-tricine SDS-PAGE, transferred to nitrocellulose and probed with an anti-phosphocholine monoclonal antibody. A region of the nitrocellulose membrane
was stained with Ponceau S to control for loading (LC). Strains with complementing alleles of *whyD* or *tacL* under control of the Zn-inducible promoter were grown in the presence of 100 µM ZnCl₂.

**Figure 2 – figure supplement 1. WhyD levels remain constant throughout growth and under autolytic conditions.**

(A) Immunoblot analysis of WhyD in the indicated strains. Cells were harvested in exponential phase (E), stationary phase (S), and at a time point when *lytA*+ cells undergo autolysis (A). Samples were normalized to an OD<sub>600</sub> of 0.5, lysed, and immediately analyzed by SDS-PAGE followed by immunoblotting with anti-WhyD antibodies. (B) TacL-FLAG levels were analyzed by immunoblot under the same conditions. ∆*lytA* ∆*whyD* and ∆*lytA* lacking TacL-FLAG served as specificity controls for the anti-WhyD and anti-FLAG antibodies, respectively. A wild-type (*lytA*+) strain was grown in parallel to determine the timing of autolysis. A nonspecific protein recognized by the anti-FLAG antibody is indicated with an asterisk. LC, loading control.

**Figure 2 – figure supplement 2. Cells lacking WhyD do not accumulate LTAs under autolytic conditions.**

(A) Growth curves of the indicated strains and the time points that were analyzed. Samples from the ∆*lytA*, and ∆*lytA* ∆*whyD* strains were collected in exponential phase (E), stationary phase (S), and autolysis (A) and normalized to an OD<sub>600</sub> of 0.5. The wild-type strain (WT) was used as reference for the timing of autolysis. (B) Analysis of WTA and LTA levels. Samples from (A) were processed as described in Materials and Methods to detect WTAs released from purified sacculi and LTAs from membrane preparations. A region of the nitrocellulose membrane used for anti-phosphocholine immunoblot was stained with Ponceau S (LC) to
control for loading. (C) Growth curves of the indicated strains and the time points that were analyzed. At an OD$_{600}$ of $\sim$0.5 the cultures were treated with penicillin G (PenG) (0.5 $\mu$g/ml final). Samples from the $\Delta$lytA and $\Delta$lytA $\Delta$whyD strains were collected before PenG addition (B), the plateau after addition (P); and during lysis (L), and normalized to an OD$_{600}$ of 0.5. A wild-type strain (WT) treated with PenG was used as a reference for the timing of lysis. (D) Analysis of WTA and LTA levels. Harvested cells from (C) were processed as described in Materials and Methods to detect LTAs and WTAs.

**Figure 3.** WhyD releases WTAs from the pneumococcal cell wall.

(A) Schematic diagram of WhyD-dependent release of WTAs from the cell wall of *S. pneumoniae*. WTAs are attached to the cell wall peptidoglycan (PG) (grey) and LTAs are anchored in the lipid bilayer. TA polymers have the same chemical structure but differ in their linkages to the PG or glycolipid anchor. WhyD is a polytopic membrane protein with a predicted C-terminal WTA-hydrolase domain (CT). (B) The CT domain of WhyD releases WTAs from purified sacculi. Coomassie-stained gel of the recombinant CT domain of WhyD (WhyD$^{CT}$) purified from *E. coli*. M, Molecular weight markers. Alcian blue-silver stained gel of WTAs released from purified sacculi (top) and those that remain associated with the PG (bottom) after incubation with 10 $\mu$g/ml WhyD$^{CT}$, 10 $\mu$g/ml WhyD$^{CT}$ + 1 mM EDTA, or no WhyD$^{CT}$. The reactions were incubated overnight at room temperature and then quenched with 1 mM EDTA. To release WTAs that remained associated with the sacculi, the reactions were further treated with 0.1 M NaOH overnight at room temperature. The alkaline-hydrolyzed WTAs were then collected from the supernatant. (C) Immunoblot analysis of membrane preparations treated with WhyD$^{CT}$ to assess its ability to release LTAs. Membranes from $\Delta$lytA $\Delta$whyD cells were treated with 10 $\mu$g/ml WhyD$^{CT}$, 10 $\mu$g/ml WhyD$^{CT}$ + 1 mM EDTA, or no
WhyD\textsuperscript{CT}. The reactions were incubated overnight at room temperature. The samples were then resolved by 16% Tris-tricine SDS-PAGE, transferred to nitrocellulose and probed with an anti-phosphocholine monoclonal antibody.

**Figure 4. WhyD and WTAs are important for cell elongation.**

(A) Cells lacking WhyD with high levels of WTAs are longer and larger than cells with normal levels of WTAs. Representative phase-contrast and fluorescent images of Δ\textit{lytA} and Δ\textit{lytA} Δ\textit{whyD} strains grown in THY at 37 °C in 5% CO\textsubscript{2} to mid-exponential phase. Cells were labeled with sBADA for 5 min prior to imaging. Scale bar, 3 \textmu m. (B) Quantitative analysis of cell length, width, and area for the strains shown in (A). The violin plots indicate the median (bold lines) and quartiles. P-values were obtained using a Welch’s t-test. P<0.01, **; P<0.0001, ****. (C) Cells over-expressing WhyD with low levels of WTAs are shorter and smaller than cells with WT levels of WTAs. Representative phase-contrast and fluorescent images of wild-type (WT) and cells with a second copy of \textit{whyD} under zinc-inducible control (\textit{whyD}\textsuperscript{++}). Strains were grown in THY to mid-exponential phase, diluted into fresh THY at an OD\textsubscript{600} of 0.025 in the presence of 200 \textmu M ZnCl\textsubscript{2} and incubated at 37 °C with 5% CO\textsubscript{2} for 2 h. The cells were then labeled with HADA for 5 min prior to imaging. Both strains also contain a cytoplasmic fluorescent marker (GFP). Scale bar, 3 \textmu m. (D) Quantitative analysis of cell length, width, and area as in (B). (E) Immunoblot analysis to assess the levels of WhyD. Samples from (C) were collected and normalized to an OD\textsubscript{600} of 0.5 and analyzed by SDS-PAGE and anti-WhyD immunoblotting. LC: control for loading. WTA levels in these strains are shown in Figure 4 - figure supplement 1.

**Figure 4 – figure supplement 1. Cells lacking LytA are similar in size to wild-type.**
Quantitative analysis of cell length, width, and area of wild-type *Sp* (WT) and cells lacking LytA ($\Delta$lytA). Violin plots indicate the median (bold lines) and quartiles. P-values were obtained using a Welch’s t-test. ns, not significant.

**Figure 4 – figure supplement 2. Analysis of WTA, LTA, and WhyD levels in wild-type and cells overexpressing WhyD.**

The indicated strains were grown to mid-exponential phase in the presence or absence of 200 $\mu$M ZnCl$_2$ and WhyD, WTA, and LTA levels were analyzed as described in Materials and Methods. Top: anti-WhyD immunoblot. Middle: alcian blue-silver stained gel of WTAs released from purified sacculi and separated by SDS-PAGE. Bottom: Immunoblot analysis of membrane-associated LTAs resolved by 16% Tris-tricine SDS-PAGE and probed with an anti-phosphocholine monoclonal antibody.

**Figure 5. Mutants lacking WTA-bound cell wall hydrolases have a short cell phenotype.**

(A) Cells lacking the cell wall hydrolases that bind WTAs are shorter and smaller than wild-type. Representative phase-contrast and fluorescent images of wild-type (WT) and cells lacking LytA, LytB, and LytC ($\Delta$lytABC). Strains were grown in THY to mid-exponential phase and labeled with HADA for 5 min prior to imaging. Both strains also contain a cytoplasmic fluorescent marker (GFP). Scale bar, 3 $\mu$m. (B) Quantitative analysis of cell length, width, and area for the strains shown in (A). The violin plots indicate the median (bold lines) and quartiles. P-values were obtained using a Welch’s t-test. P<0.01, **; P<0.0001, ****. (C) Analysis of WTA and LTA levels in the $\Delta$lytABC mutant. The indicated strains were grown to mid-exponential phase and their WTA and LTA levels were analyzed as described in Materials and Methods.
**Figure 6.** GFP-WhyD is enriched at mid-cell.

(A) Representative fluorescent and phase-contrast images of cells harboring a GFP-WhyD fusion. The fusion was expressed from a zinc-inducible promoter in a Δ*whyD* background. Strains were grown in THY medium in the presence of 200 µM ZnCl$_2$ at 37 °C in 5% CO$_2$ to an OD$_{600}$ of 0.5. Cells were labeled with HADA for 5 min prior to imaging. Scale bar, 3 µm. (B) Demographs showing GFP-WhyD and HADA localization in a population of cells. >450 cells were quantified and the resulting heat map of fluorescence intensity for each cell were then arranged according to cell length and stacked to generate the demograph. Demographs were constructed using the open-source software package Oufti.

**Figure 6 – figure supplement 1.** The GFP-WhyD fusion is functional in vivo.

(A). Images of spot-dilutions of the indicated strains on agar plates in the presence or absence of 200 µM ZnCl$_2$. The indicated strains harboring zinc-inducible promoter fusions to *whyD* or *gfp-whyD* as the sole source of the protein with and without *lytA* were analyzed. Cells were grown in the presence of 200 µM ZnCl$_2$ to exponential phase, normalized for OD$_{600}$ and serially diluted. Aliquots (5 µl) of each dilution were spotted onto TSAII 5% SB plates with or without 200 µM ZnCl$_2$. Plates were incubated at 37 °C in 5% CO$_2$ and imaged. (B) Immunoblot analysis of WhyD and GFP-WhyD. Samples from Figure 3A were collected and normalized to an OD$_{600}$ of 0.5 and resolved by SDS-PAGE followed by anti-WhyD or anti-GFP immunoblotting. A region of the nitrocellulose membrane used for immunoblot analysis was stained with Ponceau S to control for loading (LC). A degradation product of the GFP-WhyD fusion protein detected with the anti-WhyD antisera is indicated with an asterisk.

**Figure 7.** WTAs accumulate adjacent to active zones of PG synthesis.
(A) Flow diagram of experiment. The ΔlytA mutant was grown in THY medium to mid-exponential phase, labeled with HADA for 5 min, washed with fresh THY, and then labeled with sBADA for 5 min. The sample was then collected, normalized to an OD600 of 0.5, washed with fresh medium containing 1% choline and incubated with 1 µg/ml recombinant LytA(H26A) coupled to Alexa Fluor 594 (rLytA*-Alexa) for 30 sec with gentle shaking to label WTAs. Cells were then washed twice with 1X PBS and analyzed by fluorescence microscopy. (B) Representative phase-contrast and fluorescent images of rLytA*-Alexa (WTAs) and recently synthesized (HADA) and nascent (sBADA) PG. Carets indicate nascent PG (green), recently synthesized PG (white), and the final stage of cell separation (pink) (C) Cartoon depicting WTA localization relative to PG synthesis observed in (B). (D) Representative images of WTA localization in ΔlytA and ΔlytA ΔwhyD strains. Exponentially growing cells were collected and incubated with rLytA*-Alexa as described above to label WTAs. The fluorescence intensity of the rLytA*-Alexa labeled cells was re-scaled in the right panels to visualize of WTA localization in the cells lacking WhyD.

Figure 7 – figure supplement 1. Validation of the WTA-labeling assay.

(A) Coomassie-stained gels of purified recombinant LytA (rLytA), a catalytically inactive mutant (rLytA*), and the mutant labeled with Alexa594 (rLytA*-Alexa). Note that the panel showing rLytA was also used in Figure 1D. M, Molecular weight markers. (B) rLytA-Alexa is functional. Growth curves of the indicated strains before and after the addition of 1 mg/ml rLytA, rLytA-Alexa, or rLytA*-Alexa at an OD600 of ~0.2. The ΔlytA strain incubated with rLytA or rLytA-Alexa lysed in stationary phase in a manner similar to LytA+ cells. (C) LytA*-Alexa specifically labels S. pneumoniae cells. Wild-type Bacillus subtilis and S. pneumoniae cells were incubated
with rLytA*-Alexa as described in the Materials and Methods. Cells were labeled with sBADA for 5 min prior to imaging. Scale bar, 3 μm.

**Figure 7 – figure supplement 2. rLytA*-Alexa specifically labels WTAs.**

**(A)** Overexpression of WhyD decreases rLytA*-Alexa labeling. Representative phase-contrast and fluorescent images of the indicated strains grown in THY medium in the presence or absence of 200 μM ZnCl₂ to mid-exponential phase and labeled with rLytA*-Alexa as described in the Material and Methods. Cells were imaged on 2% agarose pads. **(B)** Quantitative analysis of rLytA*-Alexa fluorescence intensity in the indicated strains using images from the experiment in (A). Bar graphs show the standard deviation in each sample. AU., arbitrary units.

**Figure 7 – figure supplement 3. rLytA*-Alexa specifically labels sacculi that contain WTAs.**

**(A)** Representative phase-contrast and fluorescent images of purified sacculi derived from cells lacking LytA that were labeled with rLytA*-Alexa. The ΔlytA mutant was grown in THY to mid-exponential phase and labeled with sBADA for 5m prior to harvest. Sacculi were purified with their WTAs intact (+WTAs) or with their WTAs removed (-WTAs) as described in the Materials and Methods. Sacculi from the equivalent of 1 ml of cells at OD₆₀₀ of 0.5 were labeled with rLytA*-Alexa as described in the Materials and Methods and imaged on 2% agarose pads. **(B)** Alcian blue-silver stained gel of WTAs present in the samples in (A).

**Figure 7 – figure supplement 4. Localization of WhyD and WTAs.**
Representative phase-contrast and fluorescent images of cells expressing GFP-WhyD and labeled with LytA*-Alexa (WTA) and HADA (PG). The ΔlytA mutant was grown in THY medium to mid-exponential phase and labeled with sBADA for 5 min. A sample was then collected normalized to an OD600 of 0.5, washed with fresh medium containing 1% choline and then incubated with 1 μg/ml rLytA*-Alexa for 30 sec with gentle shaking to label WTAs. Cells were then washed twice with 1X PBS and analyzed by fluorescence microscopy. Yellow carets, mid-cell localization of nascent PG and GFP-WhyD; pink carets, LytA*-Alexa (WTA) enrichment.

**Figure 7 – figure supplement 5. WTAs are most abundant at the edge of zonal PG synthesis.**

**(A)** Representative phase-contrast and fluorescent images of cells labeled with LytA*-Alexa (WTAs) and sBADA (nascent PG). The ΔlytA mutant was grown in THY medium to mid-exponential phase, labeled with sBADA for 5 min. The sample was collected and normalized to an OD600 of 0.5 before incubation with rLytA*-Alexa to label WTAs as described in the Material and Methods. The cells were then imaged on 2% agarose pads. **(B)** Representative deconvolved image of the ΔlytA mutant labeled with rLytA*-Alexa and sBADA as described in (A). Z-stack images were taken every 100 nm from 1.5 μm above and below the focused image plane. Deconvolution was performed using Hyugens Widefield Deconvolution Software. **(C)** Representative Structured illumination microscopy image of a ΔlytA mutant grown in THY medium to mid-exponential phase, labeled with HADA for 5 min, washed with fresh THY, and then labeled with sBADA for 5 min. The sample was then collected, normalized to an OD600 of 0.5, washed with fresh medium containing 1% choline and incubated with 1 μg/ml recombinant LytA(H26A) coupled to Alexa Fluor 594 (rLytA*-Alexa) for 30 sec with gentle shaking to label WTAs. Cells were then washed twice with 1X PBS and analyzed by fluorescence microscopy.
Cells were imaged using an Elyra 7 system with SIM² as described in Materials and Methods. Carets indicate nascent PG (green), recently synthesized PG (white), and the final stage of cell separation (pink).

**Figure 7 – figure supplement 6. WTA localization at different growth stages.**

(A) Representative phase-contrast and fluorescence images of wild-type cells labeled with LytA*-Alexa (WTAs) and HADA (nascent PG) and sBADA (recently synthesized PG). The ΔlytA mutant was grown in THY medium to mid-exponential phase, labeled with HADA for 5 min, washed with fresh THY medium, and then labeled with sBADA for 5 min. A sample was collected and normalized to an OD₆₀₀ of 0.5 prior to incubation with LytA*-Alexa to label WTAs as described in the Materials and Methods. The images are not normalized and were adjusted to best highlight the LytA*-Alexa distribution along the cell envelope. Each image is representative of a distinct stage in the pneumococcal cell elongation cycle: (i) early division/elongation, (ii) mid-division/elongation, (iii) early constriction, (iv) late constriction/separation. Scale bars indicate 1 μm. (B) Quantitative analysis of LytA*-Alexa, HADA, and sBADA fluorescence intensities in the indicated cells shown in (A). The graphs depict fluorescence intensities as a function of position along the cell length.

**Figure 8. WhyD tailors WTAs to direct cell elongation.**

Schematic model of WhyD function. WhyD releases the majority WTAs attached to the cell wall during nascent PG synthesis at midcell. A subset of the WTAs remain intact and as the cell elongates these polymers recruit PG hydrolases with choline binding domains (yellow, orange, red Pac-Men) to the zone of peripheral PG synthesis, promoting expansion of the cell wall meshwork and cell elongation. At a late stage of cell constriction, WTAs accumulate at...
midcell where they recruit PG hydrolases that promote cell separation (yellow). At this stage, WhyD might not be localized at midcell or its activity could be inhibited. Upon entry into stationary phase or exposure to cell wall targeting antibiotics (autolysis), WhyD is unable to keep pace with the increase in WTA synthesis and/or is actively inhibited, leading to an increase in WTAs throughout the sacculus. Recruitment of LytA and other PG hydrolases leads to cell wall cleavage and lysis.

**Figure 8 – figure supplement 1.** WhyD releases TA polymers from the cell wall with a length distribution that mirrors that of LTAs.

Immunoblot analysis of TAs released from purified sacculi by WhyD and membrane-associated LTAs. TA polymers were resolved by 16% Tris-tricine SDS-PAGE, transferred to nitrocellulose and probed with an anti-phosphocholine monoclonal antibody.

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Lipoteichoic acid deficiency permits normal growth but impairs virulence of *Streptococcus pneumoniae*. *Nature Communications*, 8(1), 2093. https://doi.org/10.1038/s41467-017-01720-z

the teichoicase GlpQ. *Journal of Biological Chemistry*, 293(38), 14916–14924. https://doi.org/10.1074/jbc.ra118.004584


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### Table S2. Oligonucleotides used in this study

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**SUPPLEMENTARY REFERENCES**


Uehara, T., Parzych, K. R., Dinh, T., & Bernhardt, T. G. (2010). Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. The EMBO Journal, 29(8), 1412–1422. doi: 10.1038/emboj.2010.36


Figure 1. whyD essentiality is lytA-dependent.

(A) Transposon insertion profiles in wild-type (WT) and the ΔlytA mutant. Mariner transposon libraries were generated in WT and ΔlytA mutant strains and insertions sites and their abundance were mapped to the S. pneumoniae genome. A region including the whyD locus is shown. Each line indicates an insertion site and its height reflects the number of sequencing reads. Transposon insertions in whyD were underrepresented in WT compared to ΔlytA. (B) Serial dilutions of the indicated strains in the presence (+Zn) and absence of inducer. The indicated strains were grown to exponential phase, normalized and 10-fold serially diluted. Aliquots (5 μl) of each dilution were spotted onto TSAII 5% SB plates in the presence or absence of 200 μM ZnCl₂. Plates were incubated at 37 °C in 5% CO₂ and then imaged. (C) Expression of lytA in cells lacking whyD results in growth arrest and lysis in exponential phase. Strains containing a zinc-inducible lytA allele (Pzn-lytA) were grown in THY to mid-exponential phase. Cultures were diluted into fresh THY to an OD₆₀₀ of 0.025 in the presence or absence of 200 μM ZnCl₂ and grown at 37 °C in 5% CO₂. Growth was monitored by OD₆₀₀ measurements approximately every 30 min for 10 h. (D) Cells lacking WhyD are sensitive to exogenous LytA. Growth curves of the indicated strains before and after the addition of 1 mg/ml recombinant LytA (rLytA) at an OD₆₀₀ of ~0.2. Growth was monitored by OD₆₀₀ approximately every 30 min for 15 h. The ΔwhyD mutant rapidly lyse after rLytA addition. By contrast and as reported previously, the ΔlytA strain lysed in stationary phase in a manner similar to LytA⁺ cells. Right: Coomassie-stained gel of rLytA purified from E. coli. molecular weight markers (M) are shown.
Figure 2. Cells lacking WhyD have elevated levels of WTAs.

Analysis of WTA and LTA levels in strains lacking WhyD or TacL. The indicated strains were grown to exponential phase and harvested. WTAs were released from purified cell wall sacculi and separated by SDS-PAGE followed by alcian blue-silver staining. Membrane-associated LTAs were resolved by 16% Tris-tricine SDS-PAGE, transferred to nitrocellulose and probed with an anti-phosphocholine monoclonal antibody. A region of the nitrocellulose membrane was stained with Ponceau S to control for loading (LC). Strains with complementing alleles of whyD or tacL under control of the Zn-inducible promoter were grown in the presence of 100 µM ZnCl₂.
Figure 2 – figure supplement 1. WhyD levels remain constant throughout growth and under autolytic conditions.

(A) Immunoblot analysis of WhyD in the indicated strains. Cells were harvested in exponential phase (E), stationary phase (S), and at a time point when lytA+ cells undergo autolysis (A). Samples were normalized to an OD$_{600}$ of 0.5, lysed, and immediately analyzed by SDS-PAGE followed by immunoblotting with anti-WhyD antibodies. (B) TacL-FLAG levels were analyzed by immunoblot under the same conditions. ΔlytA ΔwhyD and ΔlytA lacking TacL-FLAG served as specificity controls for the anti-WhyD and anti-FLAG antibodies, respectively. A wild-type (lytA+) strain was grown in parallel to determine the timing of autolysis. A nonspecific protein recognized by the anti-FLAG antibody is indicated with an asterisk. LC, loading control.
Figure 2 – figure supplement 2. Cells lacking WhyD do not accumulate LTAs under autolytic conditions.

(A) Growth curves of the indicated strains and the time points that were analyzed. Samples from the ΔlytA, and ΔlytA ΔwhyD strains were collected in exponential phase (E), stationary phase (S), and autolysis (A) and normalized to an OD₆₀₀ of 0.5. The wild-type strain (WT) was used as reference for the timing of autolysis. (B) Analysis of WTA and LTA levels. Samples from (A) were processed as described in Materials and Methods to detect WTAs released from purified sacculi and LTAs from membrane preparations. A region of the nitrocellulose membrane used for anti-phosphocholine immunoblot was stained with Ponceau S (LC) to control for loading. (C) Growth curves of the indicated strains and the time points that were analyzed. At an OD₆₀₀ of ~0.5 the cultures were treated with penicillin G (PenG) (0.5 µg/ml final). Samples from the ΔlytA and ΔlytA ΔwhyD strains were collected before PenG addition (B), the plateau after addition (P), and during lysis (L), and normalized to an OD₆₀₀ of 0.5. A wild-type strain (WT) treated with PenG was used as a reference for the timing of lysis. (D) Analysis of WTA and LTA levels. Harvested cells from (C) were processed as described in Materials and Methods to detect LTAs and WTAs.
**Figure 3. WhyD releases WTAs from the pneumococcal cell wall.**

**(A)** Schematic diagram of WhyD-dependent release of WTAs from the cell wall of *S. pneumoniae*. WTAs are attached to the cell wall peptidoglycan (PG) (grey) and LTAs are anchored in the lipid bilayer. TA polymers have the same chemical structure but differ in their linkages to the PG or glycolipid anchor. WhyD is a polytopic membrane protein with a predicted C-terminal WTA-hydrolase domain (CT). **(B)** The CT domain of WhyD releases WTAs from purified sacculi. Coomassie-stained gel of the recombinant CT domain of WhyD (WhyD\textsuperscript{CT}) purified from *E. coli*. M, Molecular weight markers. Alcian blue-silver stained gel of WTAs released from purified sacculi (top) and those that remain associated with the PG (bottom) after incubation with 10 µg/ml WhyD\textsuperscript{CT}, 10 µg/ml WhyD\textsuperscript{CT} + 1 mM EDTA, or no WhyD\textsuperscript{CT}. The reactions were incubated overnight at room temperature and then quenched with 1 mM EDTA. To release WTAs that remained associated with the sacculi, the reactions were further treated with 0.1 M NaOH overnight at room temperature. The alkaline-hydrolyzed WTAs were then collected from the supernatant. **(C)** Immunoblot analysis of membrane preparations treated with WhyD\textsuperscript{CT} to assess its ability to release LTAs. Membranes from ΔlytA ΔwhyD cells were treated with 10 µg/ml WhyD\textsuperscript{CT}, 10 µg/ml WhyD\textsuperscript{CT} + 1 mM EDTA, or no WhyD\textsuperscript{CT}. The reactions were incubated overnight at room temperature. The samples were then resolved by 16% Tris-tricine SDS-PAGE, transferred to nitrocellulose and probed with an anti-phosphoscholine monoclonal antibody.
**Figure 4.** WhyD and WTAs are important for cell elongation.

(A) Cells lacking WhyD with high levels of WTAs are longer and larger than cells with normal levels of WTAs. Representative phase-contrast and fluorescent images of ΔlytA and ΔlytA ΔwhyD strains grown in THY at 37 °C in 5% CO₂ to mid-exponential phase. Cells were labeled with sBADA for 5 min prior to imaging. Scale bar, 3 µm. (B) Quantitative analysis of cell length, width, and area for the strains shown in (A). The violin plots indicate the median (bold lines) and quartiles. P-values were obtained using a Welch's t-test. P<0.01, **; P<0.0001, ****. (C) Cells over-expressing WhyD with low levels of WTAs are shorter and smaller than cells with WT levels of WTAs. Representative phase-contrast and fluorescent images of wild-type (WT) and cells with a second copy of whyD under zinc-inducible control (whyD++). Strains were grown in THY to mid-exponential phase, diluted into fresh THY at an OD₆₀₀ of 0.025 in the presence of 200 µM ZnCl₂ and incubated at 37 °C with 5% CO₂ for 2 h. The cells were then labeled with HADA for 5 min prior to imaging. Both strains also contain a cytoplasmic fluorescent marker (GFP). Scale bar, 3 µm. (D) Quantitative analysis of cell length, width, and area as in (B). (E) Immunoblot analysis to assess the levels of WhyD. Samples from (C) were collected and normalized to an OD₆₀₀ of 0.5 and analyzed by SDS-PAGE and anti-WhyD immunoblotting. LC: control for loading. WTA levels in these strains are shown in Figure 4 - figure supplement 1.
Figure 4 – figure supplement 1. Cells lacking LytA are similar in size to wild-type.
Quantitative analysis of cell length, width, and area of wild-type Sp (WT) and cells lacking LytA (ΔlytA). Violin plots indicate the median (bold lines) and quartiles. P-values were obtained using a Welch’s t-test. ns, not significant.
Figure 4 – figure supplement 2. Analysis of WTA, LTA, and WhyD levels in wild-type and cells overexpressing WhyD.

The indicated strains were grown to mid-exponential phase in the presence or absence of 200 μM ZnCl₂ and WhyD, WTA, and LTA levels were analyzed as described in Materials and Methods. Top: anti-WhyD immunoblot. Middle: alcian blue-silver stained gel of WTAs released from purified sacculi and separated by SDS-PAGE. Bottom: Immunoblot analysis of membrane-associated LTAs resolved by 16% Tris-tricine SDS-PAGE and probed with an anti-phosphocholine monoclonal antibody.
Figure 5. Mutants lacking WTA-bound cell wall hydrolases have a short cell phenotype. (A) Cells lacking the cell wall hydrolases that bind WTAs are shorter and smaller than wild-type. Representative phase-contrast and fluorescent images of wild-type (WT) and cells lacking LytA, LytB, and LytC (ΔlytABC). Strains were grown in THY to mid-exponential phase and labeled with HADA for 5 min prior to imaging. Both strains also contain a cytoplasmic fluorescent marker (GFP). Scale bar, 3 μm. (B) Quantitative analysis of cell length, width, and area for the strains shown in (A). The violin plots indicate the median (bold lines) and quartiles. P-values were obtained using a Welch's t-test. P<0.01, **; P<0.0001, ****. (C) Analysis of WTA and LTA levels in the ΔlytABC mutant. The indicated strains were grown to mid-exponential phase and their WTA and LTA levels were analyzed as described in Materials and Methods.
Figure 6. GFP-WhyD is enriched at mid-cell. (A) Representative fluorescent and phase-contrast images of cells harboring a GFP-WhyD fusion. The fusion was expressed from a zinc-inducible promoter in a ΔwhyD background. Strains were grown in THY medium in the presence of 200 µM ZnCl₂ at 37 °C in 5% CO₂ to an OD₆₀₀ of 0.5. Cells were labeled with HADA for 5 min prior to imaging. Scale bar, 3 µm. (B) Demographs showing GFP-WhyD and HADA localization in a population of cells. >450 cells were quantified and the resulting heat map of fluorescence intensity for each cell were then arranged according to cell length and stacked to generate the demograph. Demographs were constructed using the open-source software package Oufit.
Figure 6 – figure supplement 1
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(A) Images of spot-dilutions of the indicated strains on agar plates in the presence or absence of 200 μM ZnCl₂. The indicated strains harboring zinc-inducible promoter fusions to whyD or gfp-whyD as the sole source of the protein with and without lytA were analyzed. Cells were grown in the presence of 200 μM ZnCl₂ to exponential phase, normalized for OD₆₀₀ and serially diluted. Aliquots (5 μl) of each dilution were spotted onto TSAII 5% SB plates with or without 200 μM ZnCl₂. Plates were incubated at 37 °C in 5% CO₂ and imaged. (B) Immunoblot analysis of WhyD and GFP-WhyD. Samples from Figure 3A were collected and normalized to an OD₆₀₀ of 0.5 and resolved by SDS-PAGE followed by anti-WhyD or anti-GFP immunoblotting. A region of the nitrocellulose membrane used for immunoblot analysis was stained with Ponceau S to control for loading (LC). A degradation product of the GFP-WhyD fusion protein detected with the anti-WhyD antisera is indicated with an asterisk.
Figure 7. WTAs accumulate adjacent to active zones of PG synthesis.

(A) Flow diagram of experiment. The ΔlytA mutant was grown in THY medium to mid-exponential phase, labeled with HADA for 5 min, washed with fresh THY, and then labeled with sBADA for 5 min. The sample was then collected, normalized to an OD600 of 0.5, washed with fresh medium containing 1% choline and incubated with 1 µg/ml recombinant LytA(H26A) coupled to Alexa Fluor 594 (rLytA*-Alexa) for 30 sec with gentle shaking to label WTAs. Cells were then washed twice with 1X PBS and analyzed by fluorescence microscopy. (B) Representative phase-contrast and fluorescent images of rLytA*-Alexa (WTAs) and recently synthesized (HADA) and nascent (sBADA) PG. Carets indicate nascent PG (green), recently synthesized PG (white), and the final stage of cell separation (pink). (C) Cartoon depicting WTA localization relative to PG synthesis observed in (B). (D) Representative images of WTA localization in ΔlytA and ΔlytA ΔwhyD strains. Exponentially growing cells were collected and incubated with rLytA*-Alexa as described above to label WTAs. The fluorescence intensity of the rLytA*-Alexa labeled cells was re-scaled in the right panels to visualize of WTA localization in the cells lacking WhyD.
Figure 7 – figure supplement 1. Validation of the WTA-labeling assay.
(A) Coomassie-stained gels of purified recombinant LytA (rLytA), a catalytically inactive mutant (rLytA*), and the mutant labeled with Alexa594 (rLytA*-Alexa). Note that the panel showing rLytA was also used in Figure 1D. M, Molecular weight markers. (B) rLytA-Alexa is functional. Growth curves of the indicated strains before and after the addition of 1 mg/ml rLytA, rLytA-Alexa, or rLytA*-Alexa at an OD_600~0.2. The ΔlytA strain incubated with rLytA or rLytA-Alexa lysed in stationary phase in a manner similar to LytA* cells. (C) LytA*-Alexa specifically labels S. pneumoniae cells. Wild-type Bacillus subtilis and S. pneumoniae cells were incubated with rLytA*-Alexa as described in the Materials and Methods. Cells were labeled with sBADA for 5 min prior to imaging. Scale bar, 3 μm.
Figure 7 – figure supplement 2. rLytA*-Alexa specifically labels WTAs. 

(A) Overexpression of WhyD decreases rLytA*-Alexa labeling. Representative phase-contrast and fluorescent images of the indicated strains grown in THY medium in the presence or absence of 200 µM ZnCl₂ to mid-exponential phase and labeled with rLytA*-Alexa as described in the Material and Methods. Cells were imaged on 2% agarose pads. (B) Quantitative analysis of rLytA*-Alexa fluorescence intensity in the indicated strains using images from the experiment in (A). Bar graphs show the standard deviation in each sample. AU, arbitrary units.
Figure 7 – figure supplement 3. rLytA*-Alexa specifically labels sacculi that contain WTAs. (A) Representative phase-contrast and fluorescent images of purified sacculi derived from cells lacking LytA that were labeled with rLytA*-Alexa. The ΔlytA mutant was grown in THY to mid-exponential phase and labeled with sBADA for 5m prior to harvest. Sacculi were purified with their WTAs intact (+WTAs) or with their WTAs removed (-WTAs) as described in the Materials and Methods. Sacculi from the equivalent of 1 ml of cells at OD_{600} of 0.5 were labeled with rLytA*-Alexa as described in the Materials and Methods and imaged on 2% agarose pads. (B) Alcian blue-silver stained gel of WTAs present in the samples in (A).
Figure 7 – figure supplement 4. Localization of WhyD and WTAs.
Representative phase-contrast and fluorescent images of cells expressing GFP-WhyD and labeled with LytA*-Alexa (WTA) and HADA (PG). The ΔlytA mutant was grown in THY medium to mid-exponential phase and labeled with sBADA for 5 min. A sample was then collected normalized to an OD600 of 0.5, washed with fresh medium containing 1% choline and then incubated with 1 µg/ml rLytA*-Alexa for 30 sec with gentle shaking to label WTAs. Cells were then washed twice with 1X PBS and analyzed by fluorescence microscopy. Yellow carets, mid-cell localization of nascent PG and GFP-WhyD; pink carets, LytA*-Alexa (WTA) enrichment.
Figure 7 – figure supplement 5. WTAs are most abundant at the edge of zonal PG synthesis.  
(A) Representative phase-contrast and fluorescent images of cells labeled with LytA*-Alexa (WTAs) and sBADA (nascent PG). The ΔlytA mutant was grown in THY medium to mid-exponential phase, labeled with sBADA for 5 min. The sample was collected and normalized to an OD$_{600}$ of 0.5 before incubation with rLytA*-Alexa to label WTAs as described in the Material and Methods. The cells were then imaged on 2% agarose pads. (B) Representative deconvolved image of the ΔlytA mutant labeled with rLytA*-Alexa and sBADA as described in (A). Z-stack images were taken every 100 nm from 1.5 μm above and below the focused image plane. Deconvolution was performed using Hyugens Widefield Deconvolution Software. (C) Representative Structured illumination microscopy image of a ΔlytA mutant grown in THY medium to mid-exponential phase, labeled with HADA for 5 min, washed with fresh THY, and then labeled with sBADA for 5 min. The sample was then collected, normalized to an OD$_{600}$ of 0.5, washed with fresh medium containing 1% choline and incubated with 1 μg/ml recombinant LytA(H26A) coupled to Alexa Fluor 594 (rLytA*-Alexa) for 30 sec with gentle shaking to label WTAs. Cells were then washed twice with 1X PBS and analyzed by fluorescence microscopy. Cells were imaged using an Elyra 7 system with SIM$^2$ as described in Materials and Methods. Carets indicate nascent PG (green), recently synthesized PG (white), and the final stage of cell separation (pink).
Figure 7 – figure supplement 6. WTA localization at different growth stages.

(A) Representative phase-contrast and fluorescence images of wild-type cells labeled with LytA*-Alexa (WTAs) and HADA (nascent PG) and sBADA (recently synthesized PG). The ΔlytA mutant was grown in THY medium to mid-exponential phase, labeled with HADA for 5 min, washed with fresh THY medium, and then labeled with sBADA for 5 min. A sample was collected and normalized to an OD₆₀₀ of 0.5 prior to incubation with LytA*-Alexa to label WTAs as described in the Materials and Methods. The images are not normalized and were adjusted to best highlight the LytA*-Alexa distribution along the cell envelope. Each image is representative of a distinct stage in the pneumococcal cell elongation cycle: (i) early division/elongation, (ii) mid-division/elongation, (iii) early constriction, (iv) late constriction/separation. Scale bars indicate 1 µm. (B) Quantitative analysis of LytA*-Alexa, HADA, and sBADA fluorescence intensities in the indicated cells shown in (A). The graphs depict fluorescence intensities as a function of position along the cell length.
Figure 8. WhyD tailors WTAs to direct cell elongation.
Schematic model of WhyD function. WhyD releases the majority WTAs attached to the cell wall during nascent PG synthesis at midcell. A subset of the WTAs remain intact and as the cell elongates these polymers recruit PG hydrolases with choline binding domains (yellow, orange, red Pac-Men) to the zone of peripheral PG synthesis, promoting expansion of the cell wall meshwork and cell elongation. At a late stage of cell constriction, WTAs accumulate at midcell where they recruit PG hydrolases that promote cell separation (yellow). At this stage, WhyD might not be localized at midcell or its activity could be inhibited. Upon entry into stationary phase or exposure to cell wall targeting antibiotics (autolysis), WhyD is unable to keep pace with the increase in WTA synthesis and/or is actively inhibited, leading to an increase in WTAs throughout the sacculus. Recruitment of LytA and other PG hydrolases leads to cell wall cleavage and lysis.
Figure 8 – figure supplement 1
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Figure 8 – figure supplement 1. WhyD releases TA polymers from the cell wall with a length distribution that mirrors that of LTAs. Immunoblot analysis of TAs released from purified sacculi by WhyD and membrane-associated LTAs. TA polymers were resolved by 16% Tris-tricine SDS-PAGE, transferred to nitrocellulose and probed with an anti-phosphocholine monoclonal antibody.