Identification of two phosphate starvation-induced wall teichoic acid hydrolases provides first insights into the degradative pathway of a key bacterial cell wall component

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ABSTRACT
The cell wall of most Gram-positive bacteria contains equal amounts of peptidoglycan and the phosphate-rich glycopolymer wall teichoic acid (WTA). During phosphate-limited growth of the Gram-positive model organism Bacillus subtilis 168, WTA is lost from the cell wall in a response mediated by the PhoPR two component system, which regulates genes involved in phosphate conservation and acquisition. It has been thought that WTA provides a phosphate source to sustain growth during starvation conditions, however, WTA degradative pathways have not been described for this, or any condition of bacterial growth. Here, we uncover roles for the Bacillus subtilis PhoP regulon genes glpQ and phoD as encoding secreted phosphodiesterases that function in WTA metabolism during phosphate starvation. Unlike the parent 168 strain, ΔglpQ or ΔphoD mutants retained WTA and ceased growth upon phosphate limitation. Characterization of GlpQ and PhoD enzymatic activities, in addition to X-ray crystal structures of GlpQ, revealed distinct mechanisms of WTA depolymerisation for the two enzymes – GlpQ catalyzes exolytic cleavage of individual monomer units, while PhoD catalyzes endo-hydrolysis at non-specific sites throughout the polymer. The combination of these activities appears requisite for the utilization of WTA as a phosphate reserve. Phenotypic characterization of the ΔglpQ and ΔphoD mutants revealed altered cell morphologies, and effects on autolytic activity and antibiotic susceptibilities that, unexpectedly, also occurred in phosphate-replete conditions. Our findings offer novel insight into the B. subtilis phosphate starvation response and implicate WTA hydrolase activity as a determinant of functional properties of the Gram-positive cell envelope.

INTRODUCTION
A key distinguishing feature of the Gram-positive cell envelope is the presence of wall teichoic acids (WTAs) covalently attached to peptidoglycan (PG) in the cell wall. These anionic polymers typically feature repeating polyol-phosphate units and extend from PG to beyond the cell surface. WTAs are important for multiple cellular processes including PG synthesis, morphogenesis and the regulation of autolysins (1). WTA is also critical for the expression of virulence (2) and drug resistance in S. aureus (3), spurring recent interest in the development of WTA biosynthesis inhibitors as lead antimicrobial compounds (3, 4).

Biosynthetic pathways for canonical poly(glycerol-phosphate) WTA in B. subtilis, and poly(ribitol-phosphate) WTA in S. aureus have been intensively studied. In B. subtilis, synthesis is accomplished by sequential actions of the tag gene products (TagO, TagA, TagB, TagF), producing WTA of 30 – 50 glycerol phosphate (GroP) units that is modified with α-glucose (TagE) and D-alanine, then transferred (TagT, TagU and TagV are implicated) to the 6'-hydroxyl on muramic acid in the glycan strands of PG (reviewed in (1)). WTAs contribute roughly half the cell wall mass, including the entire wall phosphate content, yet are dispensable for viability in B. subtilis or S. aureus, though cells lacking WTA synthesis are phenotypically typified by aberrant growth and morphology (5, 6).

Throughout normal growth, bacteria turnover up to half the cell wall material per generation (7). Gram-negative organisms such as E. coli use complex pathways to recover and recycle these turnover products, and while it is uncertain whether the same occurs in Gram-positive bacteria (8), it is perhaps implicit that WTA degradation occurs along with PG-lytic activities. Little, however, is known on this process. Early studies in B. subtilis and S. aureus found virtually identical turnover rates for WTA and PG (9), but it was likely that this WTA was in polymeric form still attached to PG fragments produced by autolysis. Direct evidence of WTA turnover was recorded over three decades ago with the discovery and partial characterization of so-called teichoicase activity in cell extracts from sporulating B. subtilis (10, 11), but the responsible enzymes and corresponding genes remain unidentified. More recently, B. subtilis impaired in the transfer of WTA to PG, contrary to S. aureus likewise impaired (12), was shown not release WTA into the extracellular medium or accumulate WTA intermediates in the cell envelope (13), possible signs of WTA turnover and recovery. Nevertheless, the only enzymes shown to catalyze WTA degradation are of
While the relevance of WTA turnover during nutrient-sufficient bacterial growth is unknown, WTA turnover and metabolism are thought to be important aspects of the phosphate starvation response in *B. subtilis* (16). Following exhaustion of inorganic phosphate (Pi) from growth media, WTA is replaced with teichuronic acid (TUA), an alternative anionic polymer devoid of phosphate (17). The switch is transcriptionally regulated by the PhoPR two component system, which responds to phosphate stress by repressing expression from WTA biosynthetic operons, while activating expression of the tuaABCDEFGH operon for TUA biosynthesis (18). Expression is also induced from genes annotated to function in phosphate scavenging, including genes encoding PhoA and PhoB that account for the majority of secreted alkaline phosphatase (APase) activity during phosphate limitation (19), the pst operon encoding a Pi transporter complex (20), and phosphodiesterase genes *glpQ* and *phoD* (21). That GlpQ and PhoD are also secreted proteins (22) has prompted notions of possible functions in WTA degradation, but this has not been examined to date.

The catalytic activity of PhoD has been studied to some degree (23, 24). *B. subtilis* GlpQ, on the other hand, has been poorly characterized, however, studies on homologous proteins such as *E. coli* GlpQ and UgpQ (25, 26) and protein D from *Haemophilus influenzae* (27) allude to possible catalytic activity and biological function. These enzymes demonstrate a strict requirement for glycerophosphodiester substrates, and there is evidence of roles for *E. coli* GlpQ in the utilization of GroP as a carbon source, and UgpQ in phosphate acquisition, with deacylated glycerophospholipids as the presumed biological substrates in either case (26).

Here, we investigate roles for *glpQ* and *phoD* in WTA metabolism during phosphate starvation of *B. subtilis* 168. Deletion of either gene suppressed post-exponential growth after depletion of Pi from culture media, and the phosphate content in the cell wall of these mutants was significantly higher than in the parent strain. We confirmed teichoicase activity by GlpQ and PhoD *in vitro* using WTA isolated from *B. subtilis* 168, as well as synthetically derived analogs of WTA intermediates and WTA fragments. Both enzymes showed a preference for undecorated WTA, but effected WTA depolymerisation in distinct manners. Processivity studies, along with structural data provide insight into the divergent catalytic activities. Finally, we present evidence of an involvement of *glpQ* and *phoD*, and thus WTA turnover, in cell envelope integrity. Our findings define functional roles for WTA degradation and recycling during the *B. subtilis* phosphate starvation response and offer novel insight on the relationship of WTA turnover to cell wall properties.

**RESULTS**

*glpQ* and *phoD* are involved in WTA metabolism during phosphate starvation of *B. subtilis* 168.

Cultures of *B. subtilis* strains disrupted in the phoPR locus become static upon depletion of Pi from growth media (28). With *glpQ* and *phoD* among the most highly activated PhoP regulon genes (29), we sought to determine their involvement in the *B. subtilis* 168 phosphate-starvation response, first by examining growth of single gene deletion mutants and a double deletion mutant, under phosphate-limited conditions. We were careful to ensure at the start of the experiment cells were not in a phosphate-limited state by using inocula from phosphate-replete cultures undergoing exponential growth, unlike slow, post-exponential growth of the wild-type strain following phosphate depletion, cultures of the mutant strains remained static (Fig. 1A), remarkably mirroring the effect from complete absence of PhoPR activity. Phosphate-limited growth of the deletion mutants was not stimulated by exogenous *B. subtilis* WTA whereas that of wild-type was (Fig. 1B), and mutant strains underwent quicker transition from vegetative growth to sporulation (Fig. 1C).

Growth in phosphate-depleted cultures of the mutants resumed upon addition of KH₂PO₄ or GroP (Fig. 1D), indicating that phosphate starvation-induced APase activity and Pi transport are intact in these mutants. After
addition of GroP, the amplitude of growth for the ΔphoD strain appeared higher than with wild-type, however, the growth rates for these strains were largely similar. Notably, corresponding cultures for the ΔglpQ and double deletion mutants were markedly reduced both in the amplitude and rate of growth, indicating that recovery from phosphate-starvation in the presence of GroP was attenuated in these strains compared to ΔphoD or wild-type cells. In addition, all strains exhibited similar phosphate-replete growth, verifying that the deletions did not hamper growth in the absence of phosphate stress (Figs. 1B, 1D). Together, these findings support roles for glpQ and phoD in phosphate acquisition from WTA.

We hypothesized that an inability to turnover WTA during phosphate starvation would lead to an increase in the cell wall phosphate content, and measured cell wall phosphate in wild-type and mutant strains after phosphate-replete and phosphate-deplete growth. Consistent with previous reports (17, 30), wall phosphate decreased significantly (~62%) in the wild-type strain after phosphate starvation (Figs. 2A, 2B). Nevertheless, wall phosphate in the ΔphoD mutant and the double mutant was unchanged while that of the ΔglpQ strain decreased by ~36% (Figs. 2A, 2B). Notably, the ΔglpQ mutant and the double mutant had higher wall phosphate than the ΔphoD mutant or the wild-type strain, after phosphate-replete growth (Fig. 2A). Overall, these results showed that deletion mutants retained significant amounts of WTA after phosphate starvation. Moreover, phosphate-starved deletion mutants contained comparable amounts of cell wall uronic acid as the wild-type strain (Fig. 2C), and exhibited similar secreted APase activity (Fig. 2D), both indicative of functional PhoPR activity. We conclude from these findings that glpQ and phoD play a major role in the loss of WTA in phosphate-starved B. subtilis that is separate from genetic repression of WTA synthesis.

GlPQ and PhoD catalyze degradation of WTA

The above results strongly suggested that glpQ and phoD encode WTA-active proteins. To test for teichoicase activity, we incubated purified recombinant GlpQ and PhoD with WTA isolated from B. subtilis 168 and examined reaction products by PAGE. Fig. 3A clearly shows PhoD was active on wild-type (glycosylated) WTA, while there appeared to be a slight decrease in apparent median polymer length after incubation with GlpQ, based on densitometric analysis. GlpQ and PhoD were next tested for activity on non-glycosylated WTA isolated from a ΔtagE mutant of B. subtilis 168 (31), and a substantial disappearance of polymeric material was observed in the presence of either enzyme (Fig. 3A), suggesting that modification by glucose reduces the susceptibility of WTA to degradation.

If GlpQ and PhoD catalyzed phosphodiester bond cleavage in WTA, Pi could be released from phosphomonoester products by the enzymes themselves, or with exogenous APase. Thus, we assayed WTA degradation reactions for Pi production. While Pi output from PhoD reactions increased proportionately over time and with increasing enzyme concentration (Fig. 3B), Pi was not detected above background levels in parallel reactions with GlpQ (not shown). Indeed, a dependence of Pi production on time and enzyme concentration in GlpQ reactions was evident only on addition of APase (Fig. 3B). Apparent turnover (phosphodiester bonds cleaved per unit time) in these reaction conditions indicated strong preference for non-glycosylated WTA by either enzyme, with faster kinetics for GlpQ (2.4 ± 0.1 min⁻¹ and 1.9 ± 0.01 min⁻¹ with glycosylated WTA; 110 ± 9.3 min⁻¹ and 52.7 ± 0.9 min⁻¹ with non-glycosylated WTA, for GlpQ and PhoD, respectively). The latter seemed to contradict PAGE findings, but it should be noted that polymeric species less than ~20 units are not well detected by PAGE (32). Also, turnover was likely underestimated as it was not feasible to assess degradation kinetics with higher WTA concentrations due to increased background interference, particularly with non-glycosylated WTA. Nonetheless, we infer from these data that PhoD catalyzes phosphodiester cleavage of WTA and acts on the phosphomonester products generated, while GlpQ strictly catalyzes phosphodiesterase activity. Pi was also released on incubating of GlpQ or PhoD with B. subtilis cell wall, demonstrating activity on WTA covalently linked to PG (Fig. 3C).
We endeavored to complement the phosphate starvation growth phenotype in mutant strains by introducing a copy of *glpQ* or *phoD* at the *amyE* locus. To do this we used a xylose inducible promoter (33), but these attempts were frustrated by leakiness in the induction system that seemed to mask the effect of complementing genes during phosphate-limited growth. More compelling results were achieved with experiments that showed exogenous WTA stimulated phosphate-starved growth in complemented strains (supplemental Fig. S1). Therefore, for further proof of the involvement of GlpQ and PhoD in WTA metabolism, we asked whether phosphate-starved growth in the mutants could be rescued by a partial hydrolysate produced by simultaneous treatment of WTA with both enzymes. As shown in Fig. 4, the extent of growth stimulation between mutants was comparable, but less than that in the parent strain, indicating that the mutants were unable to utilize polymeric WTA in the hydrolysate, and growth was stimulated only by the WTA degradation products generated *in vitro*. Collectively, these results suggest that the activities catalyzed by GlpQ and PhoD enable the utilization of WTA as a phosphate reserve under limiting conditions.

**GlpQ and PhoD catalyze distinct modes of WTA degradation**

Recognizing that the heterogeneity of WTA isolated from cells hindered enzyme kinetic studies and characterization of degradation products, chemically defined synthetic analogs of WTA intermediates were employed as substrates. We prepared tridecane-containing analogs of the TagF biosynthetic product (lipid φ.40 analog) with ^14^C incorporated into the GroP polymer and monitored product formation after reactions with GlpQ or PhoD using high performance anion exchange chromatography (HPAEX) (15). GlpQ reaction products comprised GroP and species similar in apparent size to the starting substrate (Fig. 5A), consistent with exo-degradation that releases monomer units from the polymer terminus. The absence of Gro corroborated exclusive phosphodiesterase activity by this enzyme. Conversely, turnover of lipid φ.40 analog by PhoD was accompanied by the generation of multiple products varying in size (Fig. 5B), suggesting endo-degradation. Weak signal intensity for individual product species was attributed to the dispersal of radioactivity originating in the starting substrate across numerous products, which were more clearly visualized using higher PhoD concentrations (supplemental Fig. S2). It was also evident that many PhoD-generated products would be undetectable by PAGE based on apparent sizes less than 20 polymer units (15).

We took advantage of robust product characterization afforded from the use of lipid φ.40 analog to investigate enzyme turnover using a 6-fold higher starting concentration of phosphodiesteres than in reactions with *B. subtilis* WTA. Under these conditions GlpQ turnover was 8-fold faster (881 ± 25.6 min\(^{-1}\); Fig. 5C) than was apparent with the biological substrate, while PhoD turnover increased slightly (62.4 ± 1.8 min\(^{-1}\); Fig. 5D). Worth noting is that GlpQ turnover was based on GroP production, whereas PhoD turnover reflected the disappearance of starting material, thus we have greater confidence in the rates for the former. In that context, the rate GlpQ activity was linear with enzyme concentration over the range tested while it was non-linear for PhoD. Notably, neither enzyme produced measurable activity with glycosylated lipid φ.40 analog in the same reaction conditions (not shown).

To directly compare catalytic activities of GlpQ and PhoD, we measured enzyme kinetic parameters on simple phosphate ester-containing substrates. As previously observed (24), PhoD was active on pNPP, b-pNPP, pNPC and on GroP (Table 1). GlpQ lacked measurable activity on these compounds, thus catalytic activity was assessed on WTA oligomer mimics glycerophosphoglycerol (GPG) and bis-glycerophosphoglycerol (bGPG). Compared to PhoD, GlpQ catalyzed faster turnover, with greater specificity for both substrates, with 30- and 16-fold greater *k_*\(_{cat}\) and *k_*\(_{cat}/K_m\) for GPG, and 16- and 3-fold greater *k_*\(_{cat}\) and *k_*\(_{cat}/K_m\) for bGPG (Table 2). Altogether, the findings portray PhoD as a promiscuous phosphodiesterase/phosphomonoesterase and GlpQ as highly selective for the poly(GroP) WTA backbone.
Processivity of exolytic degradation by GlpQ

Although the product distribution resulting from the action of GlpQ on lipid $\phi.40$ analog was consistent with processive polymer degradation, a distributive mechanism could not be excluded. To resolve this ambiguity, we performed a substrate trapping experiment where degradation was initiated on $^{14}$C-lipid $\phi.40$ analog, then an excess amount of unlabeled competing substrate was added to the reaction to trap dissociated enzyme. For the latter, we used a poly(GroP) polymer prepared with CDP-Gro as the acceptor, denoted CMP-poly(GroP) (15). As shown in Fig. 6A, turnover of $^{14}$C-lipid $\phi.40$ analog ceased upon addition of the trapping substrate, indicating that GlpQ has a distributive mechanism, dissociating from the polymer between catalytic events.

Structure of GlpQ

To gain molecular insight into GlpQ catalysis, we solved crystal structures of the enzyme in the presence and absence of GroP, and refined the models to 1.48-1.62 Å resolution (Table 3). A bicine molecule, supplied by the crystallization condition, bound GlpQ in the absence of GroP, and soaking of GlpQ-bicine crystals with GPG yielded complexes GlpQ-G3P-1 from a 5-minute soak, and GlpQ-G3P-2 from a 1-hour soak. Crystallized GlpQ hydrolyzed GPG, and thus our models represent the catalytically active enzyme.

GlpQ was found possessing a triose phosphate isomerase (TIM) barrel domain (34) and a glycerophosphodiester phosphodiesterase insert (GDPD-I) domain (35) (Fig. 6B). The active site is centrally located in the TIM barrel and includes a residue from the GDPD-I domain (Fig. 6D). E70, D72 and E152 form the metal binding site where Ca$^{2+}$ adopts a pentagonal bipyramidal coordination. In the GlpQ-G3P-1 structure (Fig. 6E), Gro hydroxyl groups are coordinated to Ca$^{2+}$ and the phosphate is positioned by H-bonds to H43 NE2, R44 NH2 and H85 NE2, of which highly conserved H43 and H85 are implicated in acid-base catalysis (36, 37). H43 NE2 also formed a H-bond to the 2-hydroxyl of Gro, which is buried inside a pocket composed of Q188, F279, Y259 and L210. The GlpQ-G3P-2 complex (Fig. 6F) featured a salt bridge between K154 and the phosphate, altering the orientation of GroP and loop 11 relative to the GlpQ-G3P-1 complex. This movement decreased the distance between Ca$^{2+}$ and the phosphorus in GroP from 4.8 Å to 3.6 Å, and between the phosphorus and 2-hydroxyl in Gro from 3.5 Å to 3.0 Å. As well, H-bonding between H43 NE2 and 2-hydroxyl in Gro was lost, while a new H-bond formed between E70 OE2 and the 1-hydroxyl.

The orientation of bound GroP, with the Gro moiety buried adjacent to the active site, suggests GlpQ is restricted from accessing internal regions of polymeric substrates, which supports the discerned exolytic mechanism of degradation. We predict that poly(GroP) would protrude to the exterior surface of loop 11 and encounter the negatively charged region adjacent to the GDPD-I (Fig. 6G), providing an efficient means for product release.

GlpQ and PhoD appear wholly responsible for degradation of poly(GroP) WTA during phosphate starvation

Given the unique product profiles resulting from lipid $\phi.40$ analog degradation by GlpQ or PhoD, we tested supernatants from phosphate-limited cultures for matching activity (Fig. 7). Incubation of $^{14}$C-lipid $\phi.40$ analog with culture supernatant from the wild-type strain yielded products consistent with actions by both enzymes. GroP was the main product generated after incubation of lipid $\phi.40$ analog with culture supernatant from the ΔphoD strain, the Gro present presumably due to activity by PhoA and/or PhoB. PhoD activity was not evident in culture supernatant from the wild-type strain until after extended phosphate-starvation. Importantly, culture supernatant from the double mutant was devoid of teichoicase activity, suggesting that GlpQ and PhoD are necessary for the depolymerization of poly(GroP) WTA.

ΔglpQ and ΔphoD mutants exhibit phenotypes related to the cell envelope

Considering the importance of WTA to cell wall biogenesis, we wondered whether impaired WTA turnover would be manifest in effects on the cell envelope. Indeed, a rod to sphere transition is characteristic of B. subtilis cells depleted for WTA biosynthetic enzymes (5, 13). Interestingly, over the course of phosphate-
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limitation, we noted that wild-type cells became shorter and wider, progressively losing their rod shape (Fig. 8). Both the ΔphoD and ΔglpQ strains showed a similar progression, but this was markedly delayed. Suppression of this rod to sphere transition under phosphate limitation was particularly striking in the double mutant (ΔglpQΔphoD), where cells maintained rod-like shape throughout phosphate-limited growth. In light of these observations, we predicted the integrity and cell wall properties in deletion mutants would be impacted during phosphate starvation. Key to this integrity is the balance between PG synthesis and PG lysis, both processes influenced by WTA. Since WTA synthesis was functional in the mutants, we suspected the latter would be dysregulated during phosphate starvation when PG synthesis slows significantly (38). In support of this hypothesis, phosphate-starved deletion mutants were found to exhibit accelerated rates of triton-induced autolysis compared to the wild-type (Fig. 9A). Surprisingly, this was also evident with phosphate-replete growth (Fig. 9A), which may reflect low, constitutive transcription of genes within the phoPR operon that occurs independent of the PhoPR-mediated response (i.e. in the absence of phosphate stress) (39). As well, phosphate limitation was less inhibitory to cell lysis in the deletion mutants. Similar trends were noted for lysis in the absence of triton (Fig. 9B) and together, these findings implicate glpQ and phoD in PG metabolism via WTA turnover-dependent modulation of autolytic activity.

To further investigate cell envelope effects from deficient WTA turnover, we determined the minimum inhibitory concentrations (MICs) of antibiotics against wild-type and mutant strains, focusing on aminoglycosides and β-lactams as these drug classes are known to be affected by teichoic acids (3, 40). Most notable was the dramatic desensitization of the double mutant to highly charged aminoglycosides neomycin and paromomycin in both phosphate-replete and deplete conditions (Fig. 9C, 9D). For β-lactams, the ΔglpQ and the double deletion mutants were sensitized to cefuroxime and ceftizoxime under phosphate-replete conditions (Fig. 9C). This effect was reduced during phosphate-deplete growth, however all mutant strains were in addition sensitized to ampicillin and methicillin (Fig. 9D). MICs were unaffected for spectinomycin, gentamycin (net charge < 5), or phosphomycin, vancomycin (non-β-lactam cell wall-targeting drugs).

DISCUSSION

Constituting half the cell wall mass, WTA embodies a valuable resource in B. subtilis faced with phosphate deficiency. In a biological setting, GlpQ and PhoD ostensibly encounter glycosylated, D-alanylated WTA, yet these enzymes showed a preference for undecorated WTA in vitro. The PhoD catalytic mechanism is proposed to involve attack of phosphorus by a Fe³⁺-bound hydroxide, followed by attack of the resulting phosphomonoester by the hydroxide bridging two active site metals coordinated by the phosphate (Fig. 10A) (24). This allows for endolytic degradation as observed here, but implies that hydroxyl groups from glucose attached to Gro moieties flanking a phosphate may perturb coordination of active site metals. Based on our structural investigation, we propose a distinct mechanism for GlpQ that is similar to Thermoanaerobacter tengcongensis GlpQ (36) (Fig. 10B). Deprotonation of the 2-hydroxyl of a terminal GroP by H43 enables nucleophilic attack of the phosphorus, aided by K154-mediated substrate reorientation. H85, stabilized by H-bonding with D86, protonates the leaving group oxygen, leading to a cyclic phosphate intermediate and release of the alcohol leaving group; the WTA polymer with one less GroP unit. This explains the lack of activity with fully glycosylated lipid φ.40 analog, where all 2-hydroxyl groups in Gro moieties are occupied. Roles for the catalytic histidine residues are reversed to enable GroP release. Considering incomplete glycosylation of WTA and turnover of labile D-alanine esters in vivo (41), combined degradation by GlpQ and PhoD as shown here is sufficiently robust to be biologically relevant during phosphate-limited growth when doubling time exceeds 150 mins (28). In the simplest scenario, WTA decoration may serve to control the rate of depolymerization, averting rapid losses that could be costly to the cell. However, our findings draw attention to the possibility of
phosphate starvation-induced genes for as yet unspecified glycoside hydrolases or esterases that render the polymer more susceptible to degradation by GlpQ and PhoD.

Despite efficient WTA turnover by GlpQ and PhoD in vitro, corresponding single gene deletion mutants did not exhibit intermediate phenotypes in phosphate-limited growth. The discovery of different modes of depolymerization catalyzed by GlpQ and PhoD therefore implies that these enzymes act in complementary fashion, with PhoD converting WTA into fragments, thereby elevating concentrations of substrates more efficiently turned over by GlpQ. Restriction of this activity to WTA attached to PG at the cell wall periphery, however, would leave a significant portion of WTA embedded within the PG matrix (41) and inaccessible. It is noteworthy that GlpQ is more highly secreted than PhoD during phosphate limitation (42), which coincides with our observations of low secreted teichoicase activity in the phosphate-starved ΔglpQ strain, and the reported association of PhoD with the cell wall prior to slow processing and extracellular transport (43). Taking all into account, the most parsimonious explanation of the requirement for composite teichoicase activity entails action of PhoD at the cell wall, and to a lesser extent in the extracellular milieu, followed by extracellular GlpQ action on the WTA fragments generated.

Much of the current understanding on the correlation between WTA synthesis and PG assembly has come about though the study of bacterial strains impaired in WTA synthesis (3, 5, 6, 13, 44-47). These studies highlighted the sensitivity of PG synthesis to the abundance of the shared biosynthetic intermediate, undecaprenyl diphosphate, and to its perturbation by blocks in WTA synthesis. Further, those efforts have described β-lactam sensitivity in S. aureus strains where WTA biosynthesis is prevented. Interestingly, the work reported here shows that such phenotypes – cell shape and β-lactam sensitivity – are also dependent on machinery dedicated to WTA depolymerization. Significantly, this influence is exerted with WTA synthetic machinery intact, and so the observations reported are likely not due to impacts on PG synthesis that arise from perturbations in WTA synthesis. Moreover, we found an impact of WTA turnover on autolytic activity. WTAs regulate such activity by directing the localization of autolysins (48, 49), and by promoting a low cell wall pH that is often inhibitory to their action (50). In B. subtilis however, cell wall binding of the major secreted autolysin, N-acetylmuramoyl-l-alanine amidase is highly dependent on interactions with WTA (45, 51, 52). Accordingly, a net increase in WTA negative charge caused by an absence of D-alanine has been correlated with enhanced autolysis in B. subtilis (53). Our findings therefore support the view that an increased presence of WTA provides additional sites for interaction of this major B. subtilis autolysin, consequently causing an imbalance between slowed PG synthesis during phosphate starvation and PG lysis. Underscoring the effect from this altered PG landscape was the sensitization to β-lactams, which appear to be potentiated by the increased autolytic activity. Conversely, neomycin and paromomycin were antagonized, seemingly because these positively charged molecules become sequestered at a more negatively charged cell wall.

Herein, we provide substantial evidence that PhoP regulon genes glpQ and phoD encode teichoicases active during phosphate starvation in B. subtilis 168. While teichoic acid hydrolytic activity was first described several decades ago, this is first genetic assignment of teichoicase activity in bacteria. Our findings illustrate a requirement of composite activity by mechanistically divergent teichoicases for WTA metabolism that extends vegetative growth after environmental phosphate becomes limiting. The widespread occurrence of glpQ homologs in the Bacillaceae and Staphylococcaceae families, and phoD homologs in Bacillaceae (Bacillus) and Streptomyces family, may signify a conserved function in WTA turnover for these genes. In addition, we show the significance of teichoicase activity to cell shape, autolytic susceptibility and β-lactam sensitivity. Remarkably, phenotypes in glpQ or phoD deletion strains were present to lesser degrees during phosphate-replete growth, implying some relevance in the absence of phosphate stress. It thus conceivable that teichoicase activity in alternate contexts is of consequence to cell wall structure and
properties. Continued discovery and characterization of such enzymes will provide unique perspectives for understanding the pathways and significance of cell wall turnover in Gram-positive bacteria, and presents possibilities for the manipulation of WTA degradation to probe drug susceptibility and virulence in WTA-synthesizing pathogens.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The strains, plasmids and oligonucleotides used in this study are listed in supplemental Table S1. Genetic manipulation and propagation of strains was performed in LB medium with appropriate addition of antibiotics. Defined media for phosphate limitation studies was that of Grant (16); phosphate-replete and phosphate-deplete media contained 2.5 mM KH₂PO₄ and 0.0625 mM KH₂PO₄, respectively. To assess phosphate-limited growth, strains were streaked on phosphate-replete agar plates and single colonies emerging after overnight growth at 37°C were used to inoculate 5 ml cultures in phosphate-replete liquid media. At the late log phase, cultures were used to inoculate (1/100) 50 ml of phosphate-deplete media, and growth monitored at 37°C, shaking at 250 rpm. Growth supplementation experiments were carried out in 200 µl in a 96-well microplate; phosphate-deplete media was inoculated (1/50) with late-log phosphate-replete cells, and 190 µl of this cell suspension was mixed with 10 µl of 10 mg/ml WTA, 50 mM KH₂PO₄, or sterile ddH₂O. The OD at 600 nm was monitored with a Sunrise microplate reader (Tecan), at 37°C with aeration.

Generation of glpQ and phoD deletion mutants

Gene targeting DNA fragments were generated by joining PCR of three fragments; an antibiotic resistance cassette and 1kb 5' and 3' flanking sequences of target gene. For preparation of antibiotic resistance cassettes, PAGE-purified primer pairs Ab-F and Ab-R (0.5 µM final each) were mixed with 10 ng of purified template plasmid (pDR240a for KanR and pDR242a for ErmR) and amplified by PCR under standard conditions, using Phusion hot-start DNA polymerase (New England Biolabs). Amplified KanR or ErmR fragments were purified by gel extraction. For preparation of flanking sequences of target genes, targeting gene specific primer pairs, 5pL, 5pR and 3pL, 3pR (0.5 µM final each) were mixed with 20 ng of purified B. subtilis 168 genomic DNA, and amplified by PCR under standard conditions. Amplified flanking DNA fragments were purified using the Agencourt AMPure XP (Beckman Coulter) magnetic beads. Antibiotic resistance cassette and 5' and 3' flanking DNA fragments (approximately 15 ng of each DNA) were mixed and subjected to the joining PCR in the presence of 5pL and 3pR (0.5 µM final each) under following conditions: 1 min at 98°C; (10 s at 98°C, 20 s at 55°C, 80 s at 72°C) for 30 cycles; 5 min at 72°C using detergent-free HF buffer and Phusion hot-start DNA polymerase. The joined PCR products were directly used for transformation. Competent cells were prepared by following protocol; wild-type B. subtilis 168 cells were inoculated into 3 ml of MC medium (10.7 mg/ml K₂HPO₄, 5.2 mg/ml KH₂PO₄, 20 mg/ml glucose, 0.88 mg/ml trisodium citrate dihydrate, 0.022 mg/ml ferric ammonium citrate, 1 mg/ml casamino acids, 2.2 mg/ml potassium glutamate monohydrate, 20 mM MgSO₄, 300 mM MnCl₂, 20 mg/l L-tryptophan) and incubated at 37 °C overnight with aeration. The overnight culture was diluted to an OD₆₀₀ of 0.1 in 30 mL competence medium (10.7 mg/ml K₂HPO₄, 5.2 mg/ml KH₂PO₄, 20 mg/ml glucose, 0.88 mg/ml sodium citrate dihydrate, 0.022 mg/ml ferric ammonium citrate, 2.5 mg/ml potassium aspartate, 10 mM MgSO₄, 150 mM MnCl₂, 40 mg/l L-tryptophan, 0.05% (w/v) yeast extract), then grown in a 125 ml flask at 37 °C with shaking (250 rpm) until cells reached an OD at 600 nm of ~1.5. 120 µl of culture was then mixed with 10 µl of gene targeting PCR fragments arrayed in a deep 96-well plate, covered with a breathable film, and incubated at 37 °C with shaking (900 rpm). After 2 hours of incubation, cells were plated on LB agar containing selective antibiotics (7.5 µg/ml kanamycin or 1 µg/ml erythromycin, and 12.5 µg/ml lincomycin [by activity]). After overnight incubation, single colonies from each plate were purified by re-streaking on new selection plate. Deletion of target genes was confirmed by PCR carried out using different combination of
primers; 3pR and antibiotic resistance cassette-specific primers. Gene deletions were confirmed by ~1.2 kb PCR product from the reaction.

To construct a double knockout in glpQ and phoD, genomic DNA isolated from the phoD::KanR strain and used to transform competent cells from the glpQ::ErmR strain according to established procedures (54). Successful transformants were identified by double selection on LB-agar containing kanamycin and erythromycin.

Genetic Complementation

Bacteria were grown in LB (supplemented with 0.2% (w/v) xylose when required) at 37°C. Cultures were supplemented with chloramphenicol (10 µg/ml) or erythromycin (5 µg/ml), as required. DNA manipulations were performed according to established procedures (55). T4DNA ligase, Antarctic phosphatase and restriction endonucleases were from New England Biolabs. Transformation of E. coli Novablue was performed by electroporation. PCR was performed with Phusion DNA polymerase (Thermo Fisher Scientific) using B. subtilis 168 genomic DNA as a template.

The primers listed in Table S1 were used to place a consensus ribosome binding site (56) upstream of the gene and PCR reactions were optimized for each primer pair. Resulting PCR products were purified, double-digested with PacI and BamHI and ligated into pSWEET-bgaB digested with the same restriction enzymes. The ligation mixture was electroporated into E. coli Novablue as previously described. The correct integration of sequences encoding phoD or glpQ into pSWEET was confirmed by PCR and the sequence verified by DNA sequencing (MOBIX Lab, McMaster University). Competent cells of the individual gene knockout strains (B. subtilis 168 phoD::erm or glpQ::erm) were prepared as previously described (54, 57) with modifications. Mutant strains were inoculated into 5 mL of pre-transformation medium (8.968 ml 1x T-Base, 1% (w/v) Casamino acids, 1.2% (w/v) MgSO4, 25% (w/v) glucose, 10% (w/v) yeast extract, 0.1 M EGTA and deionized water to 20 ml) and mixed with 1 µg PstI-linearized pSWEET-phoD or pSWEET-glpQ, in 1 ml aliquots, and incubated for 90 minutes at 37°C with shaking (250 rpm). Chloramphenicol was added to a final concentration of 1 µg/ml and incubation continued for 30 minutes. Cells were plated undiluted or after 10-fold dilution onto LB-agar supplemented with chloramphenicol. Chromosomal integration (via double recombination) downstream of the xylose promoter into the amyE locus was verified by PCR, and confirmed by plating on LB-agar containing 1% (w/v) starch and incubation at 37°C overnight after which amylase activity was detected by adding Lugol’s iodine solution. Lack of amylase activity indicated the disruption of amyE gene and chromosomal integration of the gene of interest.

Expression and purification of GlpQ and PhoD

Synthetic DNA sequences codon optimized for expression in E. coli (ThermoFisher Scientific) and encoding mature GlpQ (residues 27 – 293) or PhoD (residues 57 – 583) were cloned into the expression vector pDEST17 (ThermoFisher Scientific) with a TEV protease cleavable N-terminal hexa-histidine tag. Plasmid constructs were transformed into E. coli BL21 DE.3 PlysS and cells cultured in LB broth supplemented with ampicillin (50 µg/ml) and chloroamphenicol (34 µg/ml) at 37°C, until an OD at 600 nm of 0.6 – 0.8. Cultures were cooled to 16°C, and protein expression induced by addition of IPTG to a final concentration of 0.5 mM. Expression was continued for 18 hours at 16°C, and the cells harvested by centrifugation. Cell pellets were washed in 0.9% (w/v) saline, then resuspended in buffer A (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 25 mM imidazole). DNase and RNase were added, each at final concentrations of 10 µg/ml, as well as complete protease inhibitor (Roche). Cells were lysed by disruption at 30,
000 p.s.i. and the lysates clarified by centrifugation (48,000 g) prior to loading onto a 5 ml HisTrapHP column (GE Healthcare) pre-equilibrated with buffer A. His-tagged proteins were eluted over a linear gradient of 25 – 500 mM imidazole in buffer A, and fractions containing pure protein, as confirmed by SDS-PAGE, were pooled and the buffer exchanged for 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$ using a 5ml HiTrap desalting column (GE Healthcare). Proteins were divided into aliquots and stored at -80°C.

**Crystallography and structural solution GlpQ**

GlpQ (residues 27-293; 5 mg/ml) was crystallized at room temperature by sitting-drop vapour diffusion using 1 µl of protein solution (5 mg/ml of purified protein in 50 mM Tris-HCl pH 7.4, 200 mM NaCl and 1 mM CaCl$_2$) mixed with 1 µl of reservoir solution (100 mM bicine pH 8.5 and 25% (w/v) PEG6000). X-ray diffraction data for the GlpQ-bicine complex were collected on a single crystal flash-frozen in liquid N$_2$, and structures of unique GlpQ-GroP complexes acquired after soaking crystals in 100 mM HEPES (pH 8.4) containing 25% (w/v) PEG6000 and 25 mM GPG. All diffraction data were processed using Xia2 (58). Structures were solved by molecular replacement using the BALBES webserver (59), selecting the *Bacillus anthracis* GlpQ structure (PDB ID: 4R70) as the starting model. Model building and refinement were performed with Phenix and Coot (60, 61). Coordinates and structure factors were deposited in the Protein Data Bank with accession codes 5T91 (GlpQ-bicine), 5T9B (GlpQ-G3P-1) and 5T9C (GlpQ-G3P-2).

**Isolation and PAGE of WTA**

Cell wall was isolated from late-log cells by SDS extraction (30). WTA was released from PG by incubation in 0.1 M NaOH at room temperature for 16 hours. The pH was adjusted to ~7.0 with acetic acid and insoluble material removed by centrifugation (21,000 x g). The supernatant containing WTA was desalted over a PD MidiTrap G10 gel filtration column (GE Healthcare), eluting with ddH$_2$O, and lyophilized.

PAGE was performed on samples containing ~ 10 µg of WTA in 10 mM Tris-HCl (pH 8.0), 5% (v/v) glycerol, 1 mM EDTA, 0.05% (w/v) bromophenol blue. Samples were loaded onto pre-cast 15% TBE gels (Bio-Rad) and separated in Mini-Protean Tetra electrophoresis cell (Bio-Rad) using a constant power (100 V) and a running buffer of 89 mM Tris-HCl, 89 mM Boric acid and 2 mM EDTA, pH 8.3. Bands were visualized with Alcian Blue/ silver staining (32) using the Bio-Rad silver stain kit (Bio-Rad). Densitometric analysis was performed with ImageJ software, and image adjustments limited to brightness, contrast and background subtraction (62).

**Cell wall phosphate quantification**

Cell wall preparations were further purified as described previously (63), then resuspended in ddH$_2$O to an OD of 0.7 – 1 and incubated in 10% (w/v) TCA at 80°C for 16 hours. Pi released was detected with the BioMol green reagent (Enzo Life Sciences) and quantified by the absorbance at 630 nm and interpolation to a standard curve generated with KH$_2$PO$_4$.

**Synthesis of WTA oligomers**

Phosphatidylglycerol or cardiolipin (100 mg; Avanti Polar Lipids) was dissolved in a 2:1 mixture of chloroform and methanol. An equimolar amount of NaOH, prepared in 100% ethanol, was added and the mixture incubated at room temperature for 2 hours. Precipitate formed in the reaction was collected and dissolved in water, then extracted with chloroform/methanol (9:1). The aqueous layer containing the sodium salt of GPG or bGPG was recovered and lyophilized.

**Synthesis of WTA intermediate analogs**

($^{14}$C-GroP) lipid φ.40 analog and CMP-poly(GroP) were synthesized as previously described (15).

**WTA degradation assays**

Degradation reactions of *B. subtilis* WTA were performed at room temperature in 50 mM Tris-HCl (pH 8.0) containing 1 mM MgCl$_2$ and 1 mM CaCl$_2$. Reactions were quenched with EDTA (50 mM) and activity assessed by quantification of liberated Pi. Reactions...
containing GlpQ received 0.5 units of bovine APase (Sigma) 2 mins prior to quenching.

Reactions with ^14^C-lipid φ.40 analog were performed as above, but quenched with urea (6.6 M). Radioactive products were separated by HPAEX (15) using a DNA PAC PA200 column (Thermo Fisher Scientific), with visualization by in-line scintillation counting.

The amount of lipid φ.40 analog remaining or GroP produced was quantified by peak integration.

**Assays for hydrolysis of GPG and bGPG**

Activity of GlpQ and PhoD with GPG or bGPG was assessed as described for *B. subtilis* WTA. Initial rates of Pi production were plotted as a function of substrate concentration and the data fit by non-linear regression (Prism 6.0, GraphPad) to the following equation:

\[ v = \frac{v_{\text{max}}[S]}{k_m + [S]} \]

**Autolysis assay**

Strains were grown to late log phase (phosphate-replete media), or 6 hours past when the onset of post-exponential growth would occur in the wild-type strain (phosphate-deplete media). Cells were collected by centrifugation, washed twice in PBS, once in ice cold ddH2O, and resuspended in PBS or PBS containing 0.05% (v/v) Triton X-100, to an OD at 600 nm of ~ 1. 200 µl of cell suspension was transferred to a single well in a 96-well microplate and the decrease OD at 600 nm monitored at 37°C with shaking.

**Fluorescence microscopy**

Bacteria were imaged using the membrane dye FM 4-64, and a Nikon Ti-E inverted microscope (1000× magnification). Cell width quantification was performed with ImageJ (62). Images were converted to 8-bit grayscale and background-subtracted using a 50 pixel rolling ball radius method. The Otsu thresholding algorithm was applied, and gaps in the resulting binary images were filled before quantification. The R statistical programming language (64) was used to examine the results, using a density function to generate frequency plots from images with sample sizes typically greater than 200 cells. The density distribution of image features, in this case cell width, is preferable over a mean value given the morphological variability existing even within a single culture.

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FOOTNOTES
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†Abbreviations used are: APase, alkaline phosphatase; bGPG, bis-glycerophosphoglycerol; bPNP, bis-p-nitrophenyl phosphate; GroP, glycerol-3-phosphate; GPG, glycerophosphoglycerol; HPAEX, high performance anion exchange chromatography; LB, Luria Bertani; Pi, inorganic phosphate; pNPC, p-nitrophenylphosphorylcholine; pNPP, p-nitrophenyl phosphate; PG, peptidoglycan; WTA, wall teichoic acid.
Table 1. Kinetic parameters for hydrolysis of \( p \)-nitrophenyl phosphates and glycerol-3-phosphate by PhoD

| Substrate | \( K_m \) (mM) | \( k_{cat} \) (min\(^{-1}\)) | \( k_{cat}/K_m \) (s\(^{-1}\)M\(^{-1}\)) |
|-----------|----------------|-----------------|---------------------|
| \( p \)NPC\(^1\) | 0.7 ± 0.034 | 65.5 ± 1.6 | 0.56 × 10\(^4\) |
| \( bp \)NPP\(^1\) | 0.034 ± 0.003 | 49.3 ± 1.0 | 2.4 × 10\(^4\) |
| \( p \)NPP\(^1\) | 0.048 ± 0.004 | 79.8 ± 1.6 | 2.8 × 10\(^4\) |
| GroP\(^2\) | 1.5 ± 0.2 | 67.4 ± 3.3 | 0.076 × 10\(^4\) |

\(^1\) Assays were performed at room temperature in 50 mM Tris-HCl (pH 8.0) containing 1 mM MgCl\(_2\) and 1 mM CaCl\(_2\). Activity was measured by continuous detection of liberated \( p \)NP at 410 nm. \( p \)NP was quantified by interpolation to a standard curve. For determination of enzyme kinetic parameters, initial rates of \( p \)NP production were plotted as a function of substrate concentration and the data fit by non-linear regression (Prisim 6.0, GraphPad) to the following equation: \( v = \frac{v_{max}}{K_m + [S]} \). Concentrations of \( p \)NPP, \( bp \)NPP, \( p \)NPC were between 0.0012 mM – 1.2 mM.

\(^2\) Reactions were performed as above, but activity assayed based on quantification of liberated Pi. GroP concentrations were 0.31 – 10 mM.
**Table 2. Kinetic parameters for hydrolysis of WTA oligomers by GlpQ and PhoD**

| Substrate | GlpQ  | PhoD  |
|-----------|-------|-------|
| GPG       | 0.96 ± 0.08 | 5.3 ± 0.6 |
|           | 1275 ± 43   | 42.3 ± 2.5 |
|           | 2.2 × 10⁴   | 0.13 × 10⁴ |
| bGPG      | 1.4 ± 0.2   | 2.6 ± 0.3   |
|           | 1517 ± 123  | 96.4 ± 4.6  |
|           | 1.9 × 10⁴   | 0.63 × 10⁴  |

Assays were performed as described in the Experimental Procedures. GPG and bGPG concentrations ranged from 0.039 mM – 2.5 mM for GlpQ reactions and 0.16 – 10 mM in PhoD reactions.
### Table 3. X-ray data collection and refinement statistics

|                     | GlpQ-bicine (5T91) | GlpQ-GPG-1 (5T9B) | GlpQ-GPG-2 (5T9C) |
|---------------------|--------------------|--------------------|--------------------|
| **Data collection** |                    |                    |                    |
| Spacegroup          | P2,2,1             | P2,2,1             | P2,2,1             |
| Cell dimensions     |                    |                    |                    |
| a, b, c (Å)         | 50.42, 60.04, 88.25| 51.46, 60.23, 88.42| 47.10, 58.24, 87.57|
| a, b, c (°)         | 90, 90, 90         | 90, 90, 90         | 90, 90, 90         |
| Resolution (Å)      | 44.12-1.53 (1.59-1.53) | 43.83-1.62 (1.68-1.62) | 48.49-1.48 (1.53-1.48) |
| CC\(_{1/2}\)        | 0.999 (0.904)      | 0.999 (0.854)      | 0.999 (0.698)      |
| R\(_{pim}\)         | 0.017 (0.417)      | 0.023 (0.607)      | 0.025 (0.449)      |
| R\(_{meas}\)        | 0.0457 (1.085)     | 0.0624 (1.580)     | 0.0549 (0.916)     |
| I/\(\sigma\)        | 21.6 (1.7)         | 19.4 (1.6)         | 15.8 (1.8)         |
| Completeness (%)    | 99.61 (96.43)      | 99.92 (99.91)      | 99.67 (98.61)      |
| Redundancy          | 7.2 (6.6)          | 6.7 (6.7)          | 4.7 (3.8)          |
| **Refinement**      |                    |                    |                    |
| Resolution (Å)      | 44.12-1.53 (1.59-1.53) | 43.83-1.62 (1.68-1.62) | 48.49-1.48 (1.53-1.48) |
| No. of reflections  | 40983 (3889)       | 34957 (3441)       | 40774 (3981)       |
| R\(_{work}/R_{free}\) | 0.175/0.211        | 0.176/0.216        | 0.168/0.197        |
| No. of atoms        |                    |                    |                    |
| Protein             | 2062               | 2071               | 2095               |
| Ligand/ion          | 13                 | 12                 | 17                 |
| Water               | 238                | 193                | 165                |
| B factors (Å\(^2\))|                    |                    |                    |
| Protein             | 39.4               | 40.6               | 29.0               |
| Ligand/ion          | 29.2               | 29.3               | 24.7               |
| Calcium ion         | 26.8 [0.95]        | 24.5 [0.95]        | 15.7 [1]           |
| Sodium ion          | 38.6 [0.71]        | 47.3 [0.83]        | 33.3 [1]           |
| Bicine              | 25.2-32.4 [0.97]   | -                  | -                  |
| G3P\(^d\)          | -                  | 22.0-35.4 [0.92]   | 13.0-20.7 [1]      |
| Phosphate ion       | -                  | -                  | 25.2-76.0 [0.85]   |
| Water               | 43.4               | 42.3               | 37.8               |
| R.m.s. deviation    |                    |                    |                    |
| Bond lengths (Å)    | 0.006              | 0.006              | 0.006              |
| Bond angles (°)     | 0.98               | 0.92               | 1.01               |
| Ramachandran        |                    |                    |                    |
| % Favored           | 99.2               | 98.4               | 98.5               |
| % Allowed           | 0.8                | 1.6                | 1.5                |
| % Outliers          | 0                  | 0                  | 0                  |
| Molprobity          |                    |                    |                    |
| Clashscore          | 0.48               | 2.64               | 2.84               |

\(^a\) Values in parentheses represent the highest-resolution shell.  
\(^b\) 5% of reflections were excluded from refinement and used to calculate R\(_{free}\).  
\(^c\) Values in brackets indicate the occupancy.  
\(^d\) G3P; Glycerol-3-phosphate
FIGURE 1. *glpQ* and *phoD* are important for phosphate-limited growth of *B. subtilis* 168. A) Phosphate-limited growth of *B. subtilis* strains. Plots show the mean OD at 600 nm, for 50 ml cultures, in phosphate-limited media. Data from 3 independent experiments are shown. B) Growth kinetics in phosphate-replete media, phosphate-deplete media and phosphate-deplete media supplemented with 0.5 mg/ml *B. subtilis* 168 WTA. Growth was carried out in 200 µl in a 96-well microplate at 37°C. Post-exponential, phosphate-starved growth in the wild-type strain is not visible under these conditions (middle panel), but is stimulated by exogenous WTA (right panel). Plots show the mean OD at 600 nm from two independent experiments. C) Sporulation of *B. subtilis* strains. Sporulation of phosphate-starved cultures for each strain was induced in Schaffer’s sporulation media according to established methods (65). Mean and SEM from 3 independent experiments are shown. D) Supplementation with KH₂PO₄ or GroP rescues phosphate-starved growth of *B. subtilis* strains. Growth was carried out in as in B) until the exhaustion of Pi, and KH₂PO₄ or GroP added to a final concentration of 2.5 mM. Plots show the mean OD at 600 nM from two independent experiments. A, B & D: ●, wt; ■, ΔphoD; ▲, ΔglpQ; ▼, ΔglpQΔphoD.
FIGURE 2

Wall phosphate content normalized for OD after A) phosphate-replete and B) phosphate-limited growth. Means and SEM from at least 2 independent measurements are shown. Differences between groups were assessed for significance by one-way ANOVA analysis. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant. C) Presence of uronic acid in the cell wall of phosphate-starved *B. subtilis* strains. Cell wall was isolated and digested as described in the Experimental Procedures. Uronic acid was detected by the absorbance at 520 nm, after treatment of cell wall digest with concentrated H$_2$SO$_4$ and Na$_2$B$_4$O$_7$ at 100°C, followed by 3-phenylphenol as described previously (66). Data shows the mean values, normalized for culture OD, from three independent experiments. Error bars are the SEM. D) APase activity in the supernatant of phosphate-limited cultures. Supernatant (25 ml) from phosphate-limited cultures of *B. subtilis* strains was filtered (0.22 µm), then concentrated with a centrifugal filter (3 kDa molecular weight cut-off). The buffer was exchanged for 50 mM Tris-HCl (pH 8.0) containing 1 mM CaCl$_2$ and 1mM MgCl$_2$, and the sample further concentrated 10-fold. Total protein concentrations, as determined by Bradford Assay, were: wt, 0.33 mg/ml; ΔphoD, 0.19 mg/ml; ΔglpQ, 0.08 mg/ml; ΔglpQΔphoD, 0.26 mg/ml. Assays were performed at room temperature, in a 96-well microplate, by mixing 10 µl of concentrated supernatant protein with 90 µl of a mixture comprising 50 mM Tris-HCl (pH 8.0), 1 mM MgCl$_2$, 1 mM CaCl$_2$ and 20 mM pNPP. Liberated pNP was detected continuously by monitoring absorbance at 410 nm. Data from 3 independent experiments are shown. Error bars, where visible, are the SEM.
FIGURE 3

A) Representative PAGE gels of WTA (0.4 mg/ml) after incubation with 1 µM GlpQ (lanes 2, 3, 7 and 8) or 0.25 µM PhoD (lanes 4, 5, 9 and 10). Reactions were quenched with EDTA after 60 mins (lanes 2, 4, 7 and 9) or 120 mins (lanes 3, 5, 8 and 10). Lanes 1 and 6: no enzyme controls. Densitometric scans plotting pixel density over distance migrated are shown below gels; median polymer size of glycosylated WTA is indicated by the red vertical line. B) Progress of GlpQ and PhoD reactions with WTA (~ 220 µM Pi/0.2 mg/ml), based on Pi output. APase was added to GlpQ reactions 2 mins prior to quenching. Pi generated in PhoD reactions was not affected by APase (not shown) and data shows the results of PhoD assays performed without added APase. Enzyme concentrations in reactions with glycosylated WTA: 0.031 µM (●), 0.063 µM (○), 0.13 µM (■), 0.25 µM (▲), 0.5 µM (△), and 1.0 µM (▲). Enzyme concentrations in reactions with non-glycosylated WTA: 0.0026 µM (●), 0.0052 µM (○), 0.010 µM (■), 0.021 µM (▲), 0.042 µM (△) and 0.083 µM (▲). Control reactions received buffer instead of enzyme, as well as APase in the case of GlpQ reactions. Plots show the mean from two independent experiments. Apparent turnover was estimated as the slope from linear regression of initial rate as a function of enzyme concentration. C) GlpQ and PhoD are active on WTA attached to PG. Cell wall from B. subtilis 168 and the ΔtagE mutant was isolated and purified as described in Experimental Procedures, then resuspended in ddH2O to an OD at 600 nm of ~1.0. Reactions (50 µl) were performed at room temperature in 50 mM Tris - HCl (pH 8.0) containing 1 mM CaCl₂, 1 mM MgCl₂, 10 µl of cell wall (resulting in a final Pi concentration of ~ 50 µM) and 1 µM GlpQ or PhoD. Reactions were quenched with EDTA at the time points indicated (GlpQ reactions received 0.5 units of APase 2 mins prior to quenching) and the insoluble PG material removed by centrifugation (21,
000 × g) before Pi detection and quantification. Data from two independent experiments are shown. ●, PhoD; ○ GlpQ.
FIGURE 4

FIGURE 4. Supplementation of phosphate-limited growth with teichoicase-treated WTA. *B. subtilis* 168 WTA was incubated with PhoD (0.5 µM) and GlpQ (1 µM) in 50 mM Tris-HCl (pH 8.0) containing 1 mM CaCl$_2$, 1 mM MgCl$_2$ for 2 hours. Enzymes were removed using a centrifugal filter (10kDa molecular weight cut-off). 10 µl of A) buffer or B) collected filtrate (WTA partial hydrolysate) were added to 190 µl of culture, prepared in phosphate-limited media, in individual wells of a 96-well microplate. Plots show the mean OD at 600 nm from 3 independent experiments. ●, wt; ■, ΔphoD; ▲, ΔglpQ; ▼, ΔglpQΔphoD.
**FIGURE 5**

**A**

Glycerol phosphate

Radioactivity

Elution Time (mins)

**B**

Glycerol

Radioactivity

Elution Time (mins)

**C**

Rate of GroP production, μM/min

[Glq] μM

**D**

Rate of lipid ϕ.40 analog degradation, μM/min

[PhoD] μM

FIGURE 5. Degradation of lipid ϕ.40 analog by GlpQ and PhoD. HPAEX chromatograms of radioactive products from reactions of \(^{14}\)C-lipid ϕ.40 analog (30 μM, ~1.2 mM Pi) with 0.04 μM of A) GlpQ or B) PhoD. Experiments were performed at least twice, with similar results. C) Dependence of the initial rate of GroP production on GlpQ concentration. Initial rates were determined from GroP produced in 5 mins. Turnover, estimated as the slope from linear regression, was 881 ± 25.6 min\(^{-1}\). D) Dependence of the initial rate of \(^{14}\)C-lipid ϕ.40 analog degradation on PhoD concentration. Initial rates were determined from the amount of lipid ϕ.40 analog converted in 5 mins. Estimated turnover was 62.4 ± 1.8 min\(^{-1}\). C & D, means and SEM from 3 independent experiments are shown.
FIGURE 6. Processivity and structure of GlpQ. A) Reactions containing equimolar concentrations (2.5 μM) of GlpQ and 14C-lipid φ.40 analog were quenched after 5 mins with urea. HPAEX chromatograms show radioactive products for: (i) No enzyme control, (ii) Reaction in the absence of CMP-poly(GroP). (iii) Reaction with 2.5 mM CMP-poly(GroP) added after 1 min and (iv) 2 mins. (v) Reaction with buffer added after 2 mins. B) Crystal structure of GlpQ-bicine. TIM barrel and GDPD-I domains are in gray and orange, respectively. Bicine is shown as yellow sticks and Ca^{2+} as a green sphere. Heteroatoms are colored by element (N, blue; O, red; P, orange). C) Bound ligands in structures of GlpQ-bicine, GlpQ-G3P-1 and GlpQ-G3P-3 are shown with the 2F_o − F_c electron density map contoured to 2σ in a gray mesh. Carbon atoms are in yellow and heteroatoms are colored as in B). Active sites of D) GlpQ-bicine, E) GlpQ-G3P-1, and F) GlpQ-G3P-2 shown with bound ligand, Ca^{2+}, and selected residues. Metal binding sites of complexes are shown adjacent, right. Carbon atoms are colored in cyan and heteroatoms are colored by element. Ligands are shown as yellow sticks and Ca^{2+} as green spheres. GDPD-I and loop 11 are orange and magenta, respectively. G) Electrostatic potential surface of GlpQ-GPG-2 and corresponding ribbon structure, colored as in D).
FIGURE 7

**FIGURE 7. Extracellular teichoicase activity after phosphate-limited growth.** Supernatants (25 ml) from phosphate-limited cultures of *B. subtilis* strains were recovered at 6 and 14 hours past when the onset of post-exponential growth would occur in the wild-type strain and concentrated as described earlier (See Fig. 2B). Teichoicase activity was tested in a reaction comprising 50 mM Tris-HCl (pH 8.0), 1 mM CaCl₂, 1mM MgCl₂, 40 µM lipid φ.40 analog and 8 µl of concentrated supernatant (8 µl of buffer for the control). Reactions were incubated for 90 mins at room temperature and quenched with urea. Chromatograms show radioactive products separated by HPAEX. Teichoicase activity was detected in the supernatants from the ΔglpQ and ΔphoD cultures by the presence of Gro and GroP and concomitant loss of starting substrate material. Incubation of lipid φ.40 analog with supernatant of the culture from ΔglpQ mutant recovered at 14 hours resulted in a noticeable reduction in the abundance of starting material along with product emergence (indicated by the thick black arrow; see inset). The experiment was performed twice, resulting in similar profiles.
FIGURE 8

FIGURE 8. Deletion of glpQ and phoD impacts cell morphology during phosphate starvation. Strains were grown in phosphate-limited media and cells imaged along a growth curve, towards phosphate starvation. Images show cell morphologies, for typical fields, of the various strains. Frequency distributions (n ≥ 200) for cell width are shown alongside micrographs; blue corresponds to 4 hours, and red to 24 hours. Differences between groups were assessed for significance by ANOVA analysis. *, p < 0.001.
FIGURE 9. Autolytic properties and antibiotic susceptibilities for glpQ and phoD deletion mutants. Autolytic profiles for *B. subtilis* strains in A) the presence and B) absence of Triton X-100. Means and SEM (too small to be visible) from 3 independent experiments are shown. A & B: ●, wt; ■, ΔphoD; ▲, ΔglpQ; ▼, ΔglpQΔphoD. C) Antibiotic susceptibilities of deletion mutants relative to the wild-type strain in phosphate-replete and D) phosphate-deplete media. Fold change refers to the MIC in the mutant strain divided by the MIC in the wild-type strain. Fold changes < 1 indicate increased sensitivity and > 1, reduced sensitivity. MIC data are provided in supplemental Table S2.
FIGURE 10

A) PhoD is proposed to employ a catalytic mechanism akin to that described for purple acid phosphatases (67). Phosphodiester hydrolysis commences with nucleophilic attack by a Fe$^{3+}$ coordinated hydroxide leading to ejection of the alcohol product, followed by processive nucleophilic attack of the phosphomonoester product by the bridging hydroxide. B) H43 and H85 act as general bases and acids in catalysis by GlpQ that involves the formation of a cyclic phosphate intermediate.
Identification of two phosphate starvation-induced wall teichoic acid hydrolases provides first insights into the degradative pathway of a key bacterial cell wall component
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