Staphylococcus aureus counters phosphate limitation by scavenging wall teichoic acids from other staphylococci via the teichoicase GlpQ

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Staphylococcus aureus **is part of the human nasal and skin microbiomes along with other bacterial commensals and opportunistic pathogens. Nutrients are scarce in these habitats, demanding effective nutrient acquisition and competition strategies. How *S. aureus* copes with phosphate limitation is still unknown. Wall teichoic acid (WTA), a polyol-phosphate polymer, could serve as a phosphate source, but whether *S. aureus* can utilize it during phosphate starvation remains unknown. *S. aureus* secretes a glycerophosphodiesterase, GlpQ, that cleaves a broad variety of glycerol-3-phosphate (GroP) headgroups of deacylated phospholipids, providing this bacterium with GroP as a carbon and phosphate source. Here we demonstrate that GlpQ can also use glycerophosphoglycerol derived from GroP WTA from coagulase-negative *Staphylococcus lugdunensis*, *Staphylococcus capitis*, and *Staphylococcus epidermidis*, which share the nasal and skin habitats with *S. aureus*. Therefore, *S. aureus* GlpQ is the first reported WTA-hydrolyzing enzyme, or teichoicase, from *Staphylococcus*. Activity assays revealed that unmodified WTA is the preferred GlpQ substrate, and the results from MS analysis suggested that GlpQ uses an exolytic cleavage mechanism. Importantly, GlpQ did not hydrolyze the ribitol-5-phosphate WTA polymers of *S. aureus*, underscoring its role in interspecies competition rather than in *S. aureus* cell wall homeostasis or WTA recycling. *glpQ* expression was strongly up-regulated under phosphate limitation, and GlpQ allowed *S. aureus* to grow in the presence of GroP WTA as the sole phosphate source. Our study reveals a novel and unprecedented strategy of *S. aureus* for acquiring phosphate from bacterial competitors under the phosphate-limiting conditions in the nasal or skin environments.**

Staphylococcus aureus colonizes the nares of approximately 30% of the human population, which represents a major risk factor for invasive infections (1). In addition, *S. aureus* is a transient or permanent member of the skin microbiome in healthy persons or atopic dermatitis patients (2). Competition with other bacteria, in particular with coagulase-negative staphylococci (CoNS)[4] of the nasal or skin microbiome, is thought to enable or prevent *S. aureus* carriage, depending on the success of individual microbiome members, but the mechanism of *S. aureus* interaction with nasal commensals remain superficially understood (3, 4). The nose and skin are known to be very nutrient-poor environments, suggesting that bacteria compete for scant nutrients (5–9). Along this line, we have recently shown that *S. aureus* utilizes glycerophosphodiesters released from host cell lipids using the secreted enzyme GlpQ, which is not produced by most CoNS (10). Our findings have revealed that GlpQ from *S. aureus* is able to cleave a broad variety of glycerol-3-phosphate (GroP) headgroups from deacylated phospholipids, such as glycerophosphocholine, present in different body fluids, into GroP and the corresponding headgroup and plays an important role under nutrient limitation (10). Additionally, GlpQ is a major *S. aureus* antigen in infection, indicating that it may have an important role in invasive infections (11, 12).

The bacterial cell wall polymer wall teichoic acid (WTA) could be a rich store for alditols and phosphate under conditions of nutrient limitation, but it is not known whether staphylococci can recycle WTA from their own or from competitors' cell walls. WTAs are attached to the peptidoglycan layer via a highly conserved linkage unit followed by a poly-alditol-phosphate repeat unit, composed of 40 to 60 monomers, building up the actual WTA chain (13). In most *Staphylococcus* species,

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This article contains Figs. S1–S3, Tables S1–S3, and references.

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[4] The abbreviations used are: CoNS, coagulase-negative staphylococci; GroP, glycerol-3-P_i; WTA, wall teichoic acid; RboP, ribitol-5-P_i; MBP, maltose-binding protein; LB, Luria broth; MHB, Müller–Hinton broth; BM, basic medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative PCR; TSA, tryptic soy broth agar.

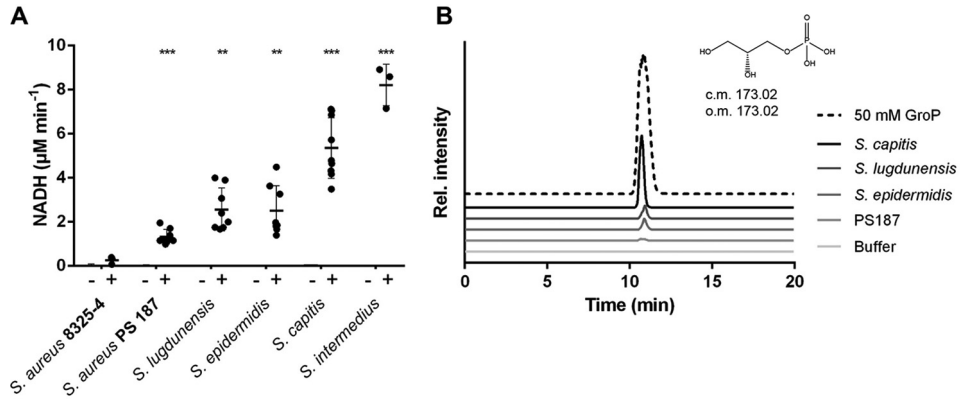

Figure 1. *S. aureus* GlpQ cleaves glycerol-type WTA derived from *S. aureus* PS187, *S. lugdunensis*, *S. epidermidis*, *S. capitis*, and *S. intermedius*. *A*, glycerophosphodiesterase activity was measured at pH 9, with 2.5 mM isolated WTA from different staphylococcal species in the presence of 5 mM $CaCl_2$ and 5 μg of MBP-GlpQ (*black bars*, +) or MBP alone as a negative control (*gray bars*, −). *Error bars* represent standard deviations of at least three independent assays done in triplicate. Significant differences between *S. aureus* 8325-4– and GroP WTA–type strains were calculated with Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$. *B*, extracted ion chromatograms of WTAs before (*Buffer*) and after treatment with MBP-GlpQ; the GroP standard was analyzed by LC/MS. Diagrams show calculated (*c.m.*) and observed masses (*o.m.*) of GroP. The chromatograms of all WTA samples without incubation with MBP-GlpQ (*Buffer*) overlap.

these monomers are either ribitol-5-phosphate (RboP), as for *S. aureus*, or GroP, as in most CoNS. The monomers are linked via phosphodiester bonds and are often substituted with a variety of (amino) sugars and D-alanine (14). Although WTA biosynthesis is well-characterized in *S. aureus* and *Bacillus subtilis*, little is known about WTA degradation (13).

Recently it has been shown that WTA can be recycled in *B. subtilis* when phosphate becomes limiting, a process that requires two secreted phosphodiesterases, the exolytic GlpQ and the endolytic PhoD enzymes (15). It has been suggested that the *B. subtilis* GlpQ acts on WTA fragments released by the PhoD phosphodiesterase and that the two enzymes have a cooperative role in the recycling of phosphate from the GroP WTA. Although *S. aureus* does not secrete a PhoD homolog, we recently showed that its GlpQ enzyme, which is 50% identical to the *B. subtilis* GlpQ, has only glycerophosphodiesterase activity but no activity against *S. aureus*' own RboP WTA or own lipoteichoic acid (10). The GlpQ enzymes belong to the family of glycerophosphodiester phosphodiesterases (EC 3.1.4.46) (16).

We explored here the substrate spectrum of GlpQ toward phosphodiester bonds of purified GroP WTA derived from *Staphylococcus lugdunensis*, *Staphylococcus capitis*, and *Staphylococcus epidermidis*. We demonstrate that GlpQ can be classified as a teichoicase, a WTA-hydrolyzing enzyme. Teichoicase activity was first described in a soil isolate of *Acinetobacter* spp. (17). Such activities have also been reported before in *Bacillus* (18) and in some bacteriophages (19–21), but the enzymes have hardly been isolated and never before described in staphylococci. Thus, *S. aureus* GlpQ and the recently described homologous enzyme from *B. subtilis* are the first identified bacterial teichoicases important for phosphate supply. GlpQ was induced by phosphate limitation and enabled *S. aureus* to thrive in medium with limited phosphate content by mobilizing GroP from WTA polymers of CoNS.

Results

GlpQ cleaves GroP-type but not RboP-type WTA

We showed previously that *S. aureus* GlpQ has glycerophosphodiesterase activity and cleaves phospholipid headgroups to produce GroP, which can be utilized by *S. aureus*. Although the homologous enzyme of *B. subtilis* can also cleave its own GroP WTA polymer, such an activity was not observed for the *S. aureus* RboP WTA. To analyze whether the *S. aureus* GlpQ may be able to hydrolyze the GroP WTA polymers of co-colonizing CoNS, GroP WTA was isolated from *S. lugdunensis*, *S. epidermidis*, *S. capitis*, *Staphylococcus intermedius,* and the unusual *S. aureus* PS187 strain producing GroP WTA (22). GroP WTA was then incubated with purified recombinant GlpQ (expressed in *Escherichia coli* without signal peptide and fused to the C terminus of a maltose-binding protein (MBP) (10). The release of GroP was monitored via a coupled NADH detection assay, and the identity of reaction products was confirmed by MS analysis (Fig. 1). GlpQ was indeed active toward WTA from all tested GroP WTA types, confirming that it has a broad substrate range for glycerophosphodiesters, including GroP WTA. *S. intermedius* and *S. capitis* WTAs were the best substrates, whereas WTA from *S. aureus* PS187, *S. lugdunensis*, and *S. epidermidis* were less efficiently degraded. In contrast, RboP WTA from *S. aureus* 8325-4 was not hydrolyzed (Fig. 1), which is in agreement with our previous data (10) and demonstrates that GlpQ cannot degrade ribitolphosphodiesters.

MS analysis showed that monomeric GroP is the only product of the reaction, as no GroP dimers, trimers, or other polymers were found (Tables S1 and S2). Moreover, no GroP with D-alanine or sugar substitutions was detected, suggesting that GlpQ preferentially hydrolyzes unsubstituted WTA repeating units. Quantification of the released GroP by MS supported the results from the NADH activity assay and confirmed that *S. capitis* WTA is more readily degraded by GlpQ than *S. lugdunensis* or *S. epidermidis* WTA (Table 1).

GlpQ releases GroP from cell wall–bound WTA

To investigate whether WTA that is still attached to the bacterial cell wall can be directly degraded by GlpQ, cell wall samples were extracted from GroP-WTA–producing bacteria without exposure to harsh acidic conditions to keep WTA attached to peptidoglycan. The insoluble cell walls were incubated with recombinant GlpQ linked to MBP, and the release of soluble organic phosphate to the supernatant was determined

Table 1
MS quantification of GroP released from WTA or from cell walls, extracted from different *Staphylococcus* species after incubation with GlpQ

GlpQ substrate	*S. lugdunensis* N920243	*S. epidermidis* RP62A	*S. capitis* ATCC 27840	*S. aureus* PS187	*S. aureus* PS187 GN1
WTA (mM GroP)	0.80	0.64	6.82	0.60	19.55
Cell wall (nmol GroP/ mg cell wall)	87.52	99.80	6573.07	60.82	12631.02

and compared with the negative control using MBP protein alone or buffer (Fig. 2). MBP-GlpQ, but not MBP, released phosphate from the cell walls of *S. lugdunensis*, *S. epidermidis*, and *S. capitis*, demonstrating that GlpQ is not only active against released but also against cell wall–attached GroP WTA polymers. The fact that *S. capitis* WTA did not lead to a pronounced release of phosphate, as observed in the NADH-coupled activity assay where GroP release is measured, might be related to a much lower amount of phosphate in the cell wall of *S. capitis*, which has been described previously (23).

Cell wall– derived soluble products were also analyzed by MS (Table 1) and, similar to what was observed for the degradation of released WTA, only monomeric GroP but no dimers, polymers, or modified GroP units could be identified. Quantification of the amount of GroP revealed a higher release of GroP from cell walls of *S. capitis* compared with *S. lugdunensis* and *S. epidermidis*, which is in accordance to what was observed for released soluble WTA molecules (Table 1).

GlpQ prefers nonglycosylated WTA as substrate

Because only unsubstituted GroP residues were detected after incubation of GlpQ with GroP WTA, we hypothesized that the different GlpQ activities observed for the tested WTA samples may be correlated to the degree of substitution of the WTA backbones with sugar residues. WTAs are usually modified with a variety of hexoses, such as GlcNAc, GalNAc, or glucose, in a species- and strain-specific manner (14, 23, 24). Therefore, differences in glycosylation could explain the different activities of GlpQ for the different CoNS WTA types (Table 2). To verify whether sugar modification indeed affects GlpQ activity, we compared the degradation of GroP WTA from the model strain *S. aureus* PS187, which is substituted with GalNAc, and from the isogenic mutant GN1 lacking glycosylation (22). Remarkably, the GlpQ activity for purified WTA isolated from strain GN1 showed a drastic increase in the NADH-based (Fig. 3*A*) and MS-based enzyme assays compared with WTA from the WT (Fig. 3*B* and Table 1). Similarly, the cell wall from strain GN1 could be more effectively degraded by GlpQ and released more GroP than cell walls of the parental strain PS187 (Table 1). Hence, our data indicate that WTA glycosylation has a strong inhibitory influence on GlpQ activity in degrading GroP-WTA.

To further analyze to which extent WTA is degraded by GlpQ, we analyzed the end products of WTA-GlpQ reactions by PAGE. Degradation was visible only for the nonglycosylated GN1 strain, where the amount of WTA was clearly reduced (Fig. 4), corroborating the previous findings that GlpQ activity is higher for nonglycosylated WTA, whereas the presence of GalNAc modifications in PS187 might inhibit cleavage of the full chain, allowing only the release of probably unmodified GroP from the distal ends.

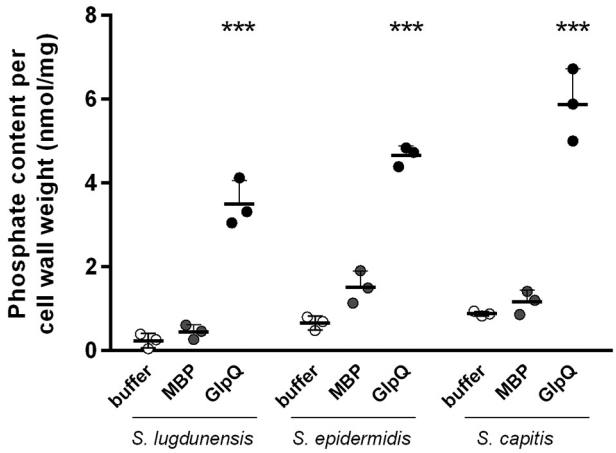

Figure 2. GlpQ is active on cell wall–attached WTA. Shown is phosphate released to the supernatant after incubation of cell wall extracts of *S. lugdunensis*, *S. epidermidis*, and *S. capitis* with recombinant MBP-GlpQ, negative control MBP, or only buffer. *Error bars* represent standard deviations of at least three independent assays done in triplicate. Significant differences between MBP control and GlpQ treated samples were calculated with Student's *t* test. ***, $p < 0.001$.

To ascertain that lack of GlpQ activity toward RboP-type WTA was not due to the glycosylation of WTA, we used *S. aureus* strains lacking the enzymes TarM and TarS, responsible for GlcNAc modification in this strain (25, 26). Importantly, unglycosylated RboP WTA from the *S. aureus* RN4220Δ*tarM*Δ *tarS* double mutant strain did not serve as a substrate for GlpQ (Fig. S1), confirming that RboP WTA is not a substrate for GlpQ.

S. aureus profits from WTA as phosphate source under phosphate limitation

Although *B. subtilis* GlpQ is known to recycle WTA phosphate from the producer's own cell wall, we hypothesized that *S. aureus* GlpQ might also help to cope with phosphate limitation by using the GroP WTA of competing CoNS as a substrate instead of its own RboP WTA. We tested this hypothesis by analyzing the growth of a *glpQ* mutant strain (*S. aureus* USA300 Δ*glpQ*) in phosphate-deficient synthetic medium (RPMI-P_i). We observed no growth either with or without GlpQ, as expected. However, when nonglycosylated GroP-WTA from strain PS187 GN1 was added, it restored growth in the presence of GlpQ (Fig. 5*A*). Heat-inactivated GlpQ was not able to improve growth under such conditions (Fig. 5*A*), confirming that GlpQ activity is responsible for the observed growth improvement when GroP WTA is the only source of phosphate. The slight growth observed in the absence of GlpQ could be justified by the presence of other teichoicases. However, we did not detect any additional putative enzymes with our activity assays.

Table 2
Reported WTA architecture of the investigated strains

Species	Strain	Polyol backbone	Reported sugar modifications and anomeric configuration[a]	References	GlpQ activity[b]
S. lugdunensis	N920243	GroP	NA	–	++
S. epidermidis	RP62A	GroP	α-Glc, α-GlcNAc, D-Ala and α-Glc6Ala	36	++
S. intermedius	CCM5739	GroP	GlcNAc	24	+++
S. capitis	ATCC 27840	GroP	α-GlcNAc	24	+++
S. aureus	PS187	GroP	α-GalNAc	29	+
S. aureus	PS187 GN1	GroP	NA	22	++++
S. aureus	8325-4	RboP	GlcNAc	37	−
S. aureus	RN4220	RboP	α, ß-GlcNAc	25, 38	−
S. aureus	RN4220 Δ*tarM*Δ*tarS*	RboP	NA	25, 29	−

[a] Anomeric configurations are shown only for available data; NA, data not available.
[b] GlpQ activity refers to the initial kinetic experiment and the formation of NADH in μM min^{-1}. −, <0.8; +, >1; ++, >5; +++, >7.5; ++++, >10 μM min^{-1}.

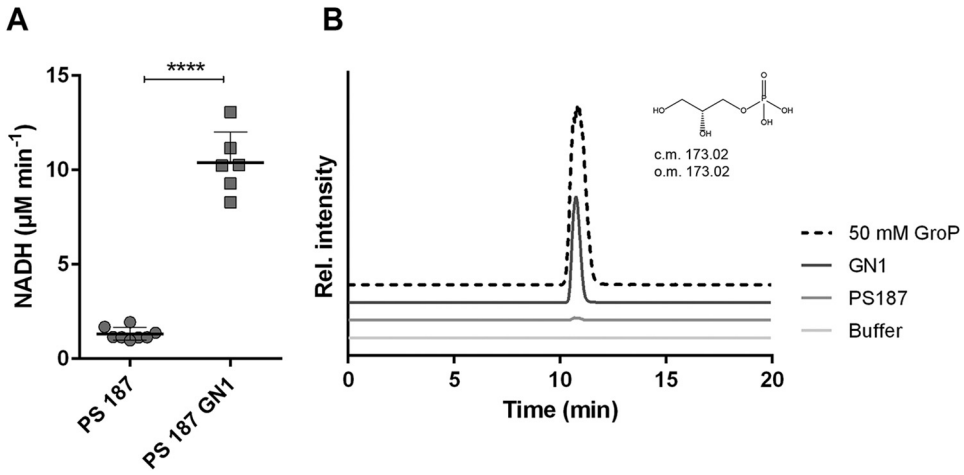

Figure 3. Influence of WTA sugar modifications on GlpQ activity. *A*, 2.5 mM WTA was incubated with 5 μg of recombinant MBP-GlpQ in the presence of 5 mM $CaCl_2$. Means and standard deviations from at least three independent experiments done in triplicates are shown. Significant differences between PS187 WT and PS187 GN1 WTA were calculated with Student's *t* test. ****, $p < 0.0001$. *B*, extracted ion chromatograms of WTA from *S. aureus* PS187 and nonglycosylated mutant GN1 before (*Buffer*, *light gray*) and after treatment with MBP-GlpQ, analyzed by LC/MS. The diagrams show calculated (*c.m.*) and observed masses (*o.m.*) of GroP. Data were plotted relative to the intensity of the GroP peak of *S. aureus* GN1 to allow comparison.

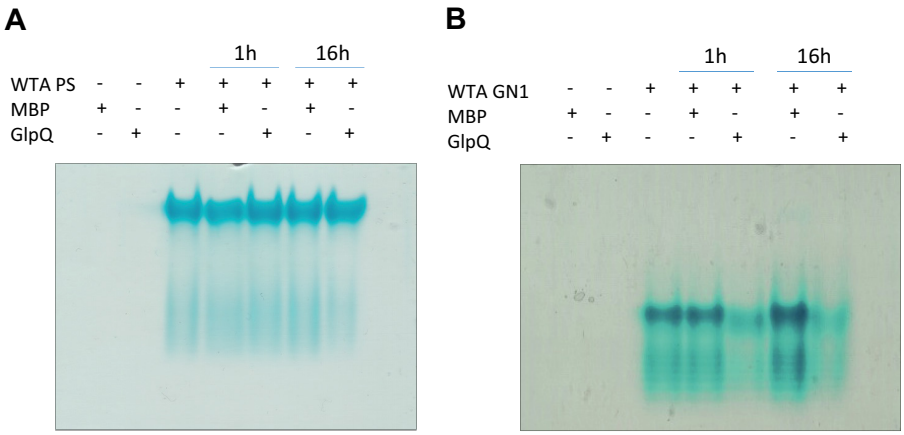

Figure 4. Activity of GlpQ visualized by WTA-PAGE. *A* and *B*, 1 mM WTA from parental *S. aureus* PS187 WT (*PS*, *A*) or nonglycosylated GN1 mutant strain (*B*) was incubated for 1 h or 16 h at room temperature with purified GlpQ or with MBP as a negative control in the presence of 5 mM $CaCl_2$.

GlpQ expression is induced under phosphate-limiting conditions

The observation that growth under phosphate-limiting conditions could be rescued by WTA and that GlpQ permitted the utilization of WTA suggests that GlpQ could be a component of the phosphate limitation stress response of *S. aureus*. How *S. aureus* copes with phosphate limitation has hardly been studied in the past. To determine whether *glpQ* expression was induced by phosphate-limiting conditions, we quantified the amount of *glpQ* transcript by RT-PCR from *S. aureus* USA300 cultures grown in RPMI-P_i supplemented with different phosphate concentrations. *glpQ* expression levels were substantially higher at low (0.025 mM) phosphate than for high (1 mM) P_i, indicating that GlpQ is a component of the P_i stress response of *S. aureus* (Fig. 5*B*). In a different approach, a 30-min incubation in P_i-free medium was enough to strongly induce the expression of *glpQ* (Fig. 5*C*).

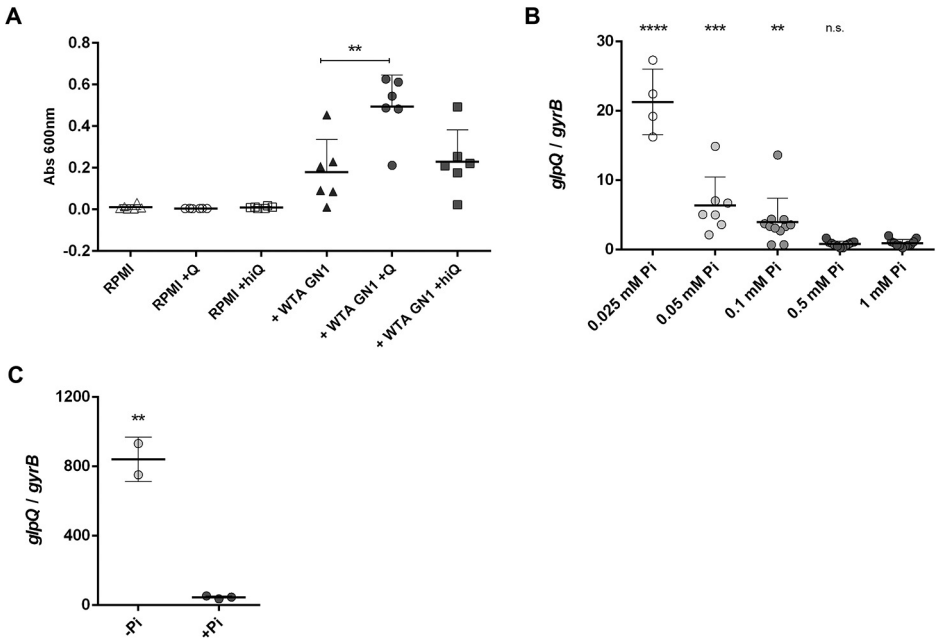

Figure 5. *A*, nonglycosylated purified GroP WTA (from strain GN1) restores growth of *S. aureus* USA300 Δ*glpQ* under phosphate-limiting conditions. Purified, active GlpQ (+*Q*), but not heat-inactivated GlpQ (*hiQ*), improves growth when WTA is the only phosphate source in RPMI-P_i medium. Means represent at least three independent assays done in triplicate. Growth after 6 h is shown. *B* and *C*, GlpQ expression is induced under phosphate-limiting conditions. Relative amounts of the *glpQ* transcript were obtained from RNA extracted from cultures grown for 20 h in RPMI-P_i supplemented with different phosphate concentrations (*B*) or after a 30-min shock without phosphate (*C*). Values are given as means and standard deviations ($n \geq 3$). Statistically significant differences, calculated using an unpaired two-tailed Student's *t* test, are indicated as follows: *n.s.*, not significant, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Discussion

In this study, we characterized the capacity of the secreted GlpQ enzyme from *S. aureus* to release GroP from GroP polymers in addition to glycerophospholipid headgroups. We show that GlpQ hydrolyzes GroP WTA from a variety of staphylococcal strains that co-inhabit the human nares with *S. aureus*, including the commensal *S. capitis* and the opportunistic pathogens *S. lugdunensis*, *S. epidermidis*, and *S. intermedius* (Table 2). Although all tested WTA molecules from different CoNS species were found to be substrates of GlpQ, the amounts of GroP monomers released from WTA varied between the sources of WTA. Enzymatic activity assays revealed unmodified WTA as the most potent substrate for the enzyme, suggesting that differences in the degree and pattern of WTA glycosylation between the various CoNS species may lead to different susceptibility to GlpQ degradation.

MS analysis shed light on the cleavage mechanism of GlpQ by identifying GroP as the only reaction product, whereas oligomers of GroP or substituted monomers could not be detected as products of GlpQ activity on WTA. Consequently, the enzyme most likely follows an exolytic cleavage mechanism that may be inhibited by modified GroP monomers, similar to the *B. subtilis* GlpQ homolog (15). Importantly, GlpQ could also act directly on crude cell wall extracts, which mimic natural substrates, demonstrating the ability of GlpQ to directly cleave WTA attached to the peptidoglycan layer. This finding emphasizes the classification of GlpQ as a GroP teichoicase.

B. subtilis uses the closely related GlpQ enzyme during P_i starvation to recycle its phosphate-containing GroP WTA, which is at the same time replaced by the phosphate-free polymer teichuronic acid (27). Staphylococci lack teichuronic acid biosynthetic genes and cannot switch to production of a phosphate-free polymer as a phosphate starvation response. Instead, *S. aureus* appears to use GlpQ to "steal" phosphate from GroP WTA of CoNS colonizing the same habitats. This idea is supported by our observation that GroP WTA could restore growth of *S. aureus* when phosphate was limiting and that *glpQ* expression levels were induced by phosphate-limiting conditions. Indeed, the concentrations of phosphate vary strongly in different parts of the human body, being as low as 0.057 ± 0.03 mM in sweat (28), 0.38 ± 0.03 mM in human blood (6), 28 ± 36 mM in saliva (7), or between 0.75 and 7 mM in nasal secretions (8, 9). In certain environments *S. aureus* may depend on GlpQ to ensure sufficient availability of phosphate for robust growth. We suggest here that *S. aureus* secretes GlpQ to thrive in phosphate-poor habitats encountered on the host, where it can profit from the GroP WTA co-habitating bacteria.

The fact that GlpQ seems to be unable to completely degrade sugar-modified WTA suggests that GlpQ may only trim CoNS WTA without causing complete WTA loss or impairment of cell wall integrity. This idea is supported by the fact that CoNS growth or phage absorption was never found to be affected by treatment of CoNS cells with *S. aureus* GlpQ or by the heterologous overexpression of GlpQ in CoNS (Fig. 6). Along this line, *S. aureus* PS187 from the rare clonal complex CC395 with GroP WTA also secretes active GlpQ without notable inhibitory effects on growth or phage susceptibility (Fig. S2).

It remains unclear why *S. aureus* evolved a specific RboP-type WTA that distinguishes it from most CoNS. Although our study suggests that it might be a mechanism of immunity against its own GlpQ, RboP WTA could also be an evolutionary strategy to govern attachment of specific transducing phages

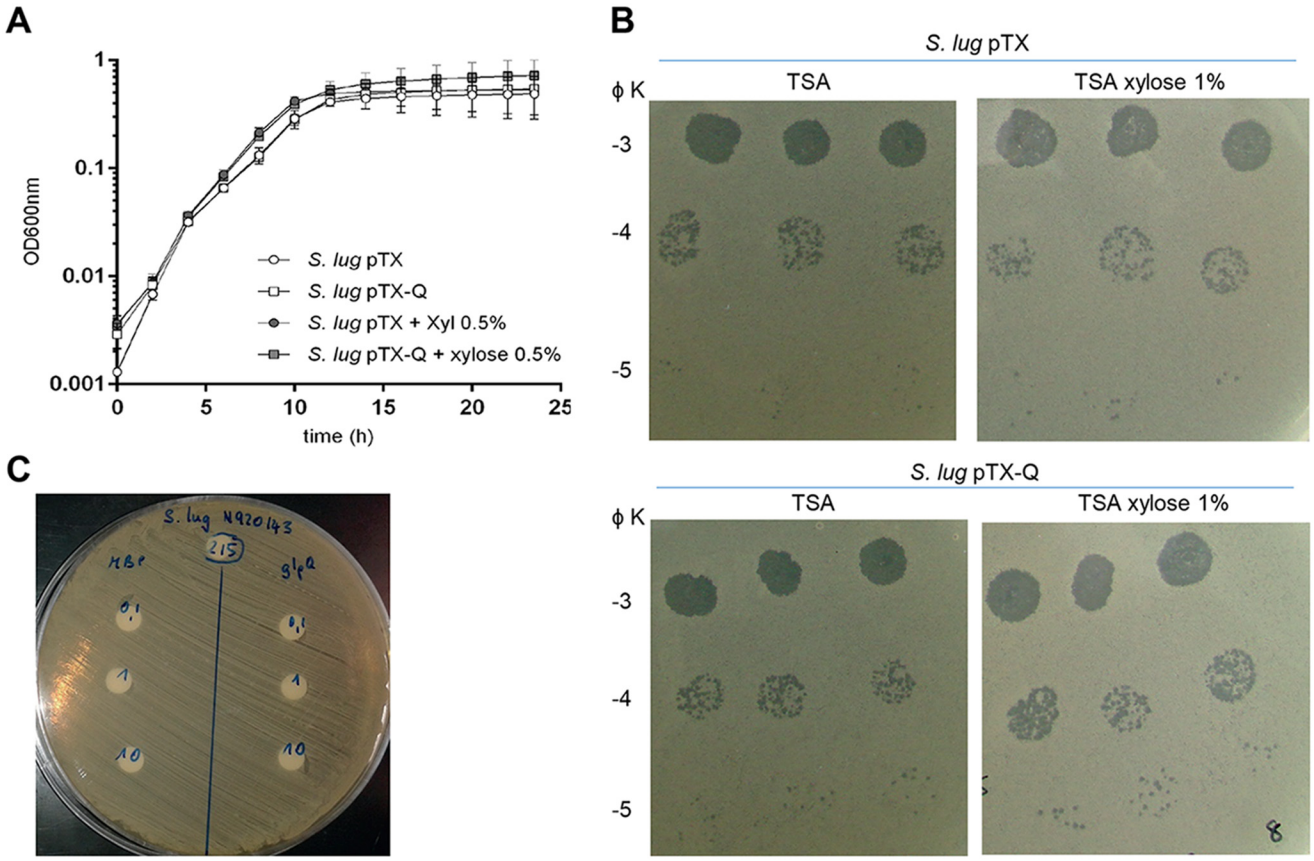

Figure 6. *A* and *B*, heterologous expression of *S. aureus* GlpQ in *S. lugdunensis* does not affect *S. lugdunensis* (*S. lug*) growth (*A*) or susceptibility to phage K (*B*). *S. lugdunensis* harboring a xylose-inducible *glpQ* expression plasmid (*pTX-Q*) or empty plasmid (*pTX*), was grown in MHB (*A*) or on TSA (*B*) with or without xylose. Serial dilutions of phage K (10^{-3} to 10^{-5}) were spotted in triplicate on *S. lugdunensis* lawns (*B*). *C*, treatment of *S. lugdunensis* with recombinant GlpQ is not growth-inhibitory. *S. lugdunensis* grown on TSA was treated with 0.1, 1, or 10 μg of recombinant purified MBP (negative control) or GlpQ.

with an advantage for horizontal gene transfer (29). Additionally, it could shape the interaction with epithelial binding partners such as the EGF-domain containing scavenger receptor SR-F1 (SREC-I) (30). The existence of teichoicases such as GlpQ has long been suggested but difficult to prove. Here we provide evidence for the existence of such an enzyme in *S. aureus* specific for GroP WTA, similar to the one found in *B. subtilis* but with a somewhat different function. Our findings that *S. aureus* GlpQ cannot cleave its own RboP WTA and that most CoNS do not secrete active GlpQ-like enzymes (10) suggests that staphylococci may have different, currently unknown WTA recycling and P_i mobilization strategies. Nevertheless, even bacteria targeted by the *S. aureus* GlpQ may profit from the release of phosphate from GroP WTA.

The phosphate starvation response in *S. aureus* has not been analyzed in detail. Studies are limited to the knowledge that phosphate is essential that and its limitation does not lead to a starvation survival state as in glucose limitation (31). Also, so far, there are no studies evaluating the transcriptome or proteome of *S. aureus* from phosphate-starved cultures. Such studies could unravel the existence of a teichoicase involved in the utilization of its own RboP and/or of other mechanisms of phosphate mobilization. *S. aureus* might be constantly exposed to phosphate-limiting conditions in specific colonization sites, such as skin-related habitats. Furthermore, the available form of phosphate is pH-dependent. In human body fluids, phosphate is present mostly as HPO_4^{2-} (32). However, the preferred phosphate form transported by bacteria, the orthophosphate anion PO_4^{3-}, is only present in trace amounts. As a result, phosphate limitation is likely to occur frequently in natural *S. aureus* habitats where *S. aureus* may compete with other commensals for phosphate. Recently, an *S. aureus* transporter for acquisition of phosphate has been described (33). In contrast to many other staphylococcal species, *S. aureus* has three dedicated transporters that enable the provision of phosphate under a multitude of distinct conditions, such as pH changes or phosphate limitation, thereby maintaining phosphate homeostasis. Moreover, loss of the transporter NptA in conjunction with a second transporter reduces the ability of *S. aureus* to cause infection. This study together with our findings demonstrates for the first time how phosphate homeostasis is of particular importance for *S. aureus* to thrive in the competitive host-associated microbiomes. Phosphate-sensing systems have been described to be involved in virulence in other bacteria and in cross-talk with other nutrition-regulatory pathways (34). Our findings shed light on the phosphate starvation response of *S. aureus* and expand the knowledge of a barely analyzed although crucial system that might be of importance as a therapeutic target. Moreover, our study reveals a sophisticated strategy used by *S. aureus* to increase its fitness under challenging conditions at the expense of competing microbiome members.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S3. *E. coli* was grown in Luria broth (LB) (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 100 μg ml^{-1} ampicillin and with 0.2% glucose for overexpression of MBP-GlpQ. *S. aureus* was grown in Müller–Hinton broth (MHB, Carl Roth; 0.2% beef extract, 1.75% acid hydrolysate of casein, and 0.15% starch) or in basic medium (BM) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, and 0.1% glucose) at 37 °C with shaking at 160 rpm. For phosphate-limiting growth conditions, RPMI medium without phosphate (RPMI-P_i) (c-c-pro, Oberdorla, Germany) was used. For growth curves, bacteria from overnight cultures grown in MHB were centrifuged, washed four times with RPMI-P_i, reinoculated in fresh medium to an initial $A_{600\ nm}$ of 0.05 in microtiter plates, and grown in an Epoch2 BIOTEK reader with orbital shaking (180 rpm) at 37 °C with measurement of optical densities every 30 min.

Purification of MBP-GlpQ

Heterologous expression of MBP-GlpQ was accomplished as described recently (10). Briefly, *E. coli* MT56 transformed with pMAL-glpQ was grown in LB with 0.2% glucose to an $A_{600\ nm}$ of 0.5. Expression of MBP-GlpQ was induced with 0.3 mM isopropylthiogalactoside, and cells were further grown for 16 h at 30 °C. Cells were lysed with 0.4 mg/ml lysozyme and by sonication. The lysate was purified by affinity chromatography using amylose resin (Qiagen) and 20 mM Tris-HCl (pH 7.6) and 200 mM NaCl as washing buffer. MBP-GlpQ was eluted with the same buffer but using 20 mM NaCl and 10 mM maltose. The purity of the protein was confirmed by SDS-PAGE, and protein concentration was measured using the Bradford protein assay (Bio-Rad) using BSA as the standard.

Extraction of WTA and cell wall

Extraction of WTA from *S. aureus* cells was performed as described previously (22). In brief, 2 liters of overnight culture in BM were harvested and washed with 20 mM ammonium acetate buffer (pH 4.8). Lysis was performed mechanically using a cell mill (Euler). Samples were incubated with DNase and RNase overnight at 37 °C. SDS was added to 2%, and samples were treated with sonication (output 30%, cycle 50%, 15 min) and subsequent incubation at 65 °C for 1 h. SDS was removed by extensive washing with ammonium acetate buffer, and samples were incubated with 5% TCA for 4 h at 60 °C to remove WTA from peptidoglycan before finally dialyzing against bidistilled water over 2 days at 4 °C with a membrane of 3.5 kDa molecular weight cut-off (MWCO). Samples were quantified by their phosphate amount as described previously (22), concentrated to 50 mM P_i, and stored at −20 °C. Cell wall samples were spared from TCA treatment, dialyzed against water, and quantified by their dry weight.

Enzyme activity assay

Enzyme activity was performed essentially as described previously (10) by measurement of GroP formation in a spectrometric assay coupled to an NAD-dependent enzymatic reaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma). The assay mixture was performed in a 96-well plate in a final volume of 50 μl. The reaction buffer contained 0.9 M glycine in 1 M hydrazine (pH 9), 5 mM $CaCl_2$, 0.5 M NAD^+, and 10 units/ml GAPDH. 2.5 mM extracted WTA sample was incubated with 5 μg of MBP-GlpQ or MBP alone. The kinetics of the reaction were measured until oxidation of Gro-3P by GAPDH was complete, and the Gro-3P concentration was calculated from the absorbance change at 340 nm using an NADH standard curve. The kinetics data were analyzed using Prism 6 software (GraphPad).

Activity of recombinant MBP-GlpQ was evaluated on cell wall extracts washed previously with 20 mM Tris-HCl (pH 7.8) and 20 mM NaCl. An identical sample was dried in a SpeedVac to determine its dry weight. Cell wall extracts were incubated in the presence of 5 mM $CaCl_2$ and 10 μg MBP-GlpQ or MBP alone for 24 h at 37 °C and 700 rpm shaking. After centrifugation, the supernatant was analyzed for phosphate content using the Biomol GREEN kit according to the manufacturer's instructions. As a positive control, mutanolysin (Sigma-Aldrich), an *N*-acetylmuramidase that solubilizes cell walls by specifically cleaving the β-*N*-acetylmuramyl-(1→4)–GlcNAc linkage of the peptidoglycan backbone, was used to release fragments of peptidoglycan with WTA attached. The mutanolysin-treated cell wall led to a strong release of soluble phosphate (Fig. S3).

End point enzyme activity was analyzed via LC/MS as described below. Reaction mixtures contained 2.5 mM WTA sample in 20 mM Tris-HCl (pH 8) with 5 mM $CaCl_2$ and 5 μg MBP-GlpQ or MBP. The samples were incubated overnight at 37 °C and analyzed for GroP formation. Supernatants from GlpQ-treated cell wall extracts prepared as described above were also analyzed by LC/MS.

LC/MS analysis of GlpQ substrates

Identification of products formed in the reaction of GlpQ with WTA and cell walls was performed using an Ultimate 3000 HPLC system (Dionex) coupled to a MicrOTOF II detector (Bruker). For HPLC, a Gemini C18 column (150 × 4.6 mm, 110 Å, 5 μM, Phenomenex) was used at 37 °C with a flow rate of 0.2 ml/min. A 5-min washing step with 96% buffer A (0.1% formic acid and 0.05% ammonium formate) was applied, followed by a linear gradient of 0% to 40% acetonitrile in buffer A for 30 min. A final washing step with 40% buffer B for 5 min and a re-equilibration step (100% buffer A) for 5 min completed the method. Samples were ionized via electron spray ionization in positive ion mode. Exact masses in positive ion mode for GroP (m/z^{+1} 173.01) and RboP (m/z^{+1} 153.07) were presented as extracted ion chromatograms with Data Analysis (Bruker). Peak areas were extracted and analyzed, and GroP was quantified using serial dilutions of a GroP standard.

Quantitative real-time qPCR

1 ml of a 16-h culture of *S. aureus* USA300 grown in MHB was washed four times with 1 ml of RPMI-P_i, reinoculated in 3 to 5 ml of fresh RPMI-P_i to an initial $A_{600\ nm}$ of 0.02, and grown for 20 h at 37 °C at 160 rpm. For a 30-min phosphate stress condition, a 16-h culture of *S. aureus* USA300 grown in MHB

was reinoculated in MHB and grown at 37 °C and 160 rpm to an $A_{600\,nm}$ of 1. The cells were then washed four times with RPMI-P_i, resuspended in the initial amount of RPMI-P_i, and grown for 30 min at 37 °C and 160 rpm.

Prior to harvesting, RNAprotect bacterial reagent (twice the culture volume, Qiagen) was added to the culture, and the mixture was immediately vortexed for 10 s. The cells were harvested, and the pellet was stored at −80 °C overnight. The next day, the cells were resuspended with 1 ml of TRIzol (Ambion, Life Technologies), followed by mechanical disruption with 0.5 ml of zirconia–silica beads (Carl Roth, Karlsruhe, Germany; 0.1 mm in diameter) using a FastPrep 24 homogenizer (MP Biomedicals) (1 cycle; 20 s; speed, 6.5 m/s). RNA was extracted with 200 μl of chloroform, recovered by precipitation with isopropyl alcohol, washed with 80% ethanol, dried under a vacuum, and resuspended in 30 to 50 μl of bidistilled water. The unlikely presence of DNA was measured with a Qubit 3.0 Fluorometer (Invitrogen). RNA was quantified using a Nanodrop ND-100 spectrophotometer, and 10 μg of RNA was digested with DNase I using the TURBO DNA-free™ kit (Thermo Fisher Scientific) and stored at −80 °C.

RNA (20 ng) was transcribed into complementary DNA and used directly for real-time qPCR using the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time qPCR was performed in a QuantStudio 3 System (Thermo Fisher Scientific) using the following conditions: 30 min at 48 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Quantification of *glpQ* expression was achieved using a standard curve method for relative quantification. *gyrB* was used as the endogenous control. The primers used for each gene were as follows: glpQ_P13 (5′-gactatgactaactcttcgaaaag-3′) and glpQ_P14 (5′-gaatagcctgaggtttatttgca-3′); gyr297_F (5′-ttagtgtgggaaattgtcgataat-3′) and gyr_P1_R (5′-tccgttactttaatccagttatc-3′). The specificity of the amplified products was verified by analysis of the dissociation curves generated by the QuantStudio™ Design & Analysis software based on the specific melting temperature for each amplicon.

Phage and GlpQ susceptibility assays

The phage K susceptibility of *S. lugdunensis* or PS187 secreting *S. aureus* GlpQ was tested by transforming the bacterial strains with the pTX-Q plasmid so that expression of heterologous *S. aureus* GlpQ was controlled by a xylose-inducible promoter. The pTX-Q plasmid was constructed by inserting a PCR fragment of GlpQ (including the Shine-Dalgarno sequence region) and using primer pair 5′-cgcGGATCCgagatgaaaggataaagactatg-3′ and 5′-ctCCCGGGctacttaatgacttctttatatttatcagcg-3′ into the BamHI/SmaI restriction site of the *Staphylococcus*-specific expression vector pTX15 (35). Phage K susceptibility was tested by growing pTX-Q-transformed (or empty vector pTX15-transformed) strains overnight in BM liquid medium with 1% xylose with aeration at 37 °C. 10 μl of phage K lysate dilutions (10^{-3}, 10^{-4}, and 10^{-5}) was spotted in triplicate on tryptic soy broth agar (TSA) supplemented or not with 0.5% or 1% xylose as indicated. Plates were grown for 16 h at 37 °C.

To test the susceptibility of *S. lugdunensis* to exogenous *S. aureus* GlpQ, a stationary phase culture of *S. lugdunensis* was swabbed on TSA, and paper discs soaked with 0.1, 1, or 10 μg of recombinant purified MBP (negative control) or GlpQ were applied on top of the bacterial lawn. Plates were grown for 16 h at 37 °C.

Statistical methods

Statistical analyses were performed with the Prism 6.0 package (GraphPad Software), and the between-group differences were analyzed for significance with a two-tailed Student's *t* test. $p \leq 0.05$ was considered statistically significant.

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