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RESEARCH ARTICLE

Staphylococcus aureus Preferentially Liberates Inorganic Phosphate from Organophosphates in Environments where This Nutrient Is Limiting

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ABSTRACT Phosphate is an essential nutrient that *Staphylococcus aureus* and other pathogens must acquire from the host during infection. While inorganic monophosphate (P_i) is the preferred source of this nutrient, bacteria can also obtain it from phosphate-containing organic molecules. The P_i-responsive regulator PhoPR is necessary for *S. aureus* to cause infection, suggesting that P_i is not freely available during infection and that this nutrient must be obtained from other sources. However, the organophosphates from which *S. aureus* can obtain phosphate are unknown. We evaluated the ability of 58 phosphorus-containing molecules to serve as phosphate sources for *S. aureus*. Forty-six of these compounds, including phosphorylated amino acids, sugars, and nucleotides, supported growth. Among the organophosphate sources was glycerol-3-phosphate (G3P), which is commonly found in the mammalian host. Differing from the model organism *Escherichia coli*, *S. aureus* does not import G3P intact to obtain P_i. Instead, *S. aureus* relies on the phosphatase PhoB to release P_i from G3P, which is subsequently imported by P_i transporters. To determine if this strategy is used by *S. aureus* to extract phosphate from other phosphate sources, we assessed the ability of PhoB- and P_i transporter-deficient strains to grow on the same library of phosphorus-containing molecules. Sixty percent of the substrates (28/46) relied on the PhoB/P_i transporter pathway, and an additional 10/46 (22%) were PhoB independent but still required P_i transport through the P_i transporters. Cumulatively, these results suggest that in P_i-limited environments, *S. aureus* preferentially generates P_i from organophosphates and then relies on P_i transporters to import this nutrient.

IMPORTANCE For bacteria, the preferred form of the essential nutrient phosphate is inorganic monophosphate (P_i), but phosphate can also be extracted from a variety of phosphocompounds. Pathogens, including *Staphylococcus aureus*, experience P_i limitation within the host, suggesting that the use of alternative phosphate sources is important during infection. However, the alternative phosphate sources that can be used by *S. aureus* and others remain largely unexplored. We screened a library of phosphorus-containing compounds for the ability to support growth as a phosphate source. *S. aureus* could use a variety of phosphocompounds, including nucleotides, phosphosugars, and phosphoamino acids. Subsequent genetic analysis determined that a majority of these alternative phosphate sources are first processed extracellularly to liberate P_i, which is then imported through P_i transporters.

KEYWORDS *Staphylococcus aureus*, phosphate acquisition, organophosphate, glycerol-3-phosphate, alkaline phosphatase

Phosphate is an essential nutrient that pathogens must acquire from the host during infection. As such, phosphate acquisition and regulatory systems are critical for the virulence of a variety of bacterial pathogens (1–8). The preferred source of phosphate

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for bacteria is inorganic monophosphate (P_i). Phosphate can also be obtained from a variety of phosphate-containing molecules (9, 10). However, outside of model organisms and closely related microbes, how pathogens cope with the absence of P_i is unknown (9, 11). Capable of infecting a wide variety of tissues, *Staphylococcus aureus* is a significant threat to human health, and public health organizations have called for new strategies to combat *S. aureus* (12–15). Understanding the aspects of staphylococcal physiology that are important during infection and how pathogens obtain essential nutrients such as phosphate has the potential to inform the development of antimicrobials.

Both P_i importers and a phosphate-responsive signal transduction system are necessary for *S. aureus* to cause infection, and altering these processes has been associated with changes in antibiotic sensitivity (16–20). *S. aureus* possesses three P_i transporters that enable it to acquire P_i in distinct environments (16). Two of these transporters, PitA and NptA, are expressed regardless of P_i availability but function optimally at opposite pHs: PitA functions best at acidic pHs, and NptA functions best at alkaline pHs. The third transporter, PstSCAB, is an ABC-type transporter that is expressed only during P_i limitation, where it is also optimal for P_i acquisition (16). The loss of NptA and either PitA or PstSCAB significantly attenuates the virulence of *S. aureus*, underscoring the importance of P_i transporters for infection (16). Yet despite its three P_i transporters, *S. aureus* additionally requires regulatory systems that respond to P_i limitation for infection, indicating that P_i is not freely available within the host (17). This observation suggests that *S. aureus* must use alternative phosphate sources during infection. However, the organophosphates from which *S. aureus* can obtain P_i are largely unknown, as are the pathways through which they are acquired.

To maintain P_i homeostasis, many bacteria, including *S. aureus*, contain a P_i-responsive two-component system named PhoPR (PhoBR in *Escherichia coli*) that is important for growth during P_i starvation and infection (9, 17, 21, 22). Upon P_i limitation, PhoPR is activated and induces the expression of a set of genes responsible for scavenging phosphate, collectively known as the Pho regulon (23–25). In *E. coli* and others, the Pho regulon includes the high-affinity PstSCAB system, a secreted, nonspecific alkaline phosphatase, and transporters for alternative organic sources of phosphate, such as glycerol-3-phosphate (G3P) (9, 23–26). Although the Pho regulon has not been comprehensively elucidated in *S. aureus*, the expression of NptA and PstSCAB is PhoPR dependent (17). Notably, in some tissues, the requirement of PhoPR for staphylococcal infection extends beyond its regulation of NptA and PstSCAB, indicating that P_i transporter-independent Pho regulon members are important for virulence (17).

G3P can be a source of both phosphate and carbon for *S. aureus* (27, 28). In the context of the host, G3P is found in various host tissues and fluids as well as the cell wall of cocolonizing bacteria (29, 30). *S. aureus* encodes a glycerophosphodiesterase, GlpQ, that liberates G3P from both wall teichoic acids produced by closely related staphylococci and host-derived phospholipids (27, 28). Of note, the expression of GlpQ increases upon phosphate starvation (28). Together, these data suggest that G3P may be an important source of phosphate for *S. aureus* within skin-associated bacterial communities and in the context of infection. However, how *S. aureus* utilizes G3P liberated by GlpQ as a source of phosphate is unknown. In *E. coli*, two PhoBR-regulated pathways promote the use of G3P as a phosphate source. First, G3P is a substrate for the conserved, periplasmic phosphate starvation-inducible alkaline phosphatase PhoA, which cleaves G3P into glycerol and P_i, releasing P_i for uptake through a P_i importer (9, 10). The homolog of this enzyme in *S. aureus* is PhoB, which is located on the surface of the bacterium (31). Second, *E. coli* possesses UgpBAEC, an ABC-type transporter that imports G3P (9, 10, 32, 33). Thus, *E. coli* can use G3P as a phosphate source in both a P_i transporter-dependent and -independent manner.

Given the evidence that insufficient P_i is available for *S. aureus* during infection, we set out to identify other phosphocompounds that can serve as phosphate sources for *S. aureus* and the mechanisms by which they are used. A library of 58 phosphorus-containing compounds was screened for their ability to promote staphylococcal

growth in P_i-depleted medium. Forty-six of the compounds supported staphylococcal growth, including a variety of inorganic and organophosphate molecules, among them G3P. Next, the pathway by which *S. aureus* acquires phosphate from G3P was assessed. These studies revealed that, differing from *E. coli*, *S. aureus* does not contain a Ugp system for G3P import. Instead, *S. aureus* depends solely on the phosphatase PhoB to liberate P_i, which is subsequently imported by P_i transporters. Further analysis revealed that the majority of the alternative phosphate sources are used by *S. aureus* in a PhoB- and/or P_i transporter-dependent manner. Cumulatively, these findings suggest that *S. aureus* can obtain P_i from a variety of phosphate sources but that their utilization is dependent on the extracellular release of P_i and its subsequent import by P_i transporters.

RESULTS

***S. aureus* can utilize a variety of organophosphates as alternative phosphate sources.** While it is highly likely that *S. aureus* can use organophosphates as a source of phosphate, the diversity of compounds that it can use is unknown. To address this gap in knowledge, a library of 58 phosphorus-containing compounds was screened for the ability to support the growth of *S. aureus* in a P_i-limited growth medium (phosphate-free M9-based medium [PFM9]). The library consisted of organic sources of phosphate, including phosphorylated sugars, amino acids, and nucleotides, as well as inorganic sources, such as thiophosphate. The ability of *S. aureus* to grow on these compounds was compared to that with growth medium supplemented with P_i or no phosphate source (Fig. 1). Twelve (21%) of the compounds did not support the growth of *S. aureus* as a phosphate source (optical density at 600 nm [OD_{600}] of <0.1), including several cyclic nucleotides and three phosphonate (carbon-phosphorus bond-containing) molecules (Fig. 1, substrates below the red dotted line; Table 1; see Fig. S1 in the supplemental material). Six of the remaining 46 compounds (13%) resulted in intermediate growth of *S. aureus*, defined as a final growth yield less than 75% of the growth on P_i (Fig. 1, substrates between the red and green dotted lines). The other 40 out of 46 phosphorylated compounds (87%) promoted the growth of *S. aureus* to within 25% of the yield obtained with P_i, suggesting that these are strong alternative phosphate sources (Fig. 1, substrates above the green dotted line). Cumulatively, these results indicate that *S. aureus* is capable of using a wide variety of phosphate-containing molecules, including phosphorylated amino acids, nucleotides, and sugars, as phosphate sources.

The use of G3P as a phosphate source induces the expression of alkaline phosphatase. Among the organophosphates on which *S. aureus* grew robustly was G3P, a phospholipid and bacterial cell wall component which has previously been shown to act as a phosphate source for *S. aureus* (27). As G3P might represent a significant source of phosphate for *S. aureus* both during infection and in polymicrobial communities, how phosphate is obtained from this molecule was investigated in more detail. *S. aureus* contains three genes that may be involved in the use of G3P as a phosphate source: an alkaline phosphatase gene, *phoB* (*NWMN_2526*); a G3P/P_i antiporter gene, *glpT* (*NWMN_0329*); and a putative high-affinity G3P importer subunit gene, *ugpC* (*NWMN_0151*). Of note, while NWMN_0151 is annotated as the ATP-binding subunit UgpC of a Ugp system, it is encoded within a four-gene operon (*NWMN_0151* to *NWMN_0154*) that is predicted to encode an ABC-type maltose importer (34). Initially, the expression of these three loci was assessed using *yfp* promoter fusions following growth with both excess (5 mM) and limiting (0.05 mM) P_i. The expression of *phoB*, but not *glpT* or the *ugpC* locus, was strongly induced in phosphate-limited medium (Fig. 2). These results indicate that only *phoB* responds strictly to phosphate availability. However, it was conceivable that *glpT* or the *ugpC* locus is induced only when P_i is limiting and that its substrate is present. When 0.05 mM G3P was provided as a phosphate source, *phoB*, but not *glpT* or the *ugpC* locus, was strongly induced relative to when either 5 mM P_i or G3P was provided (Fig. 2). Notably, when a high concentration (5 mM) of G3P was provided, *phoB* was expressed at a higher level than when a similar

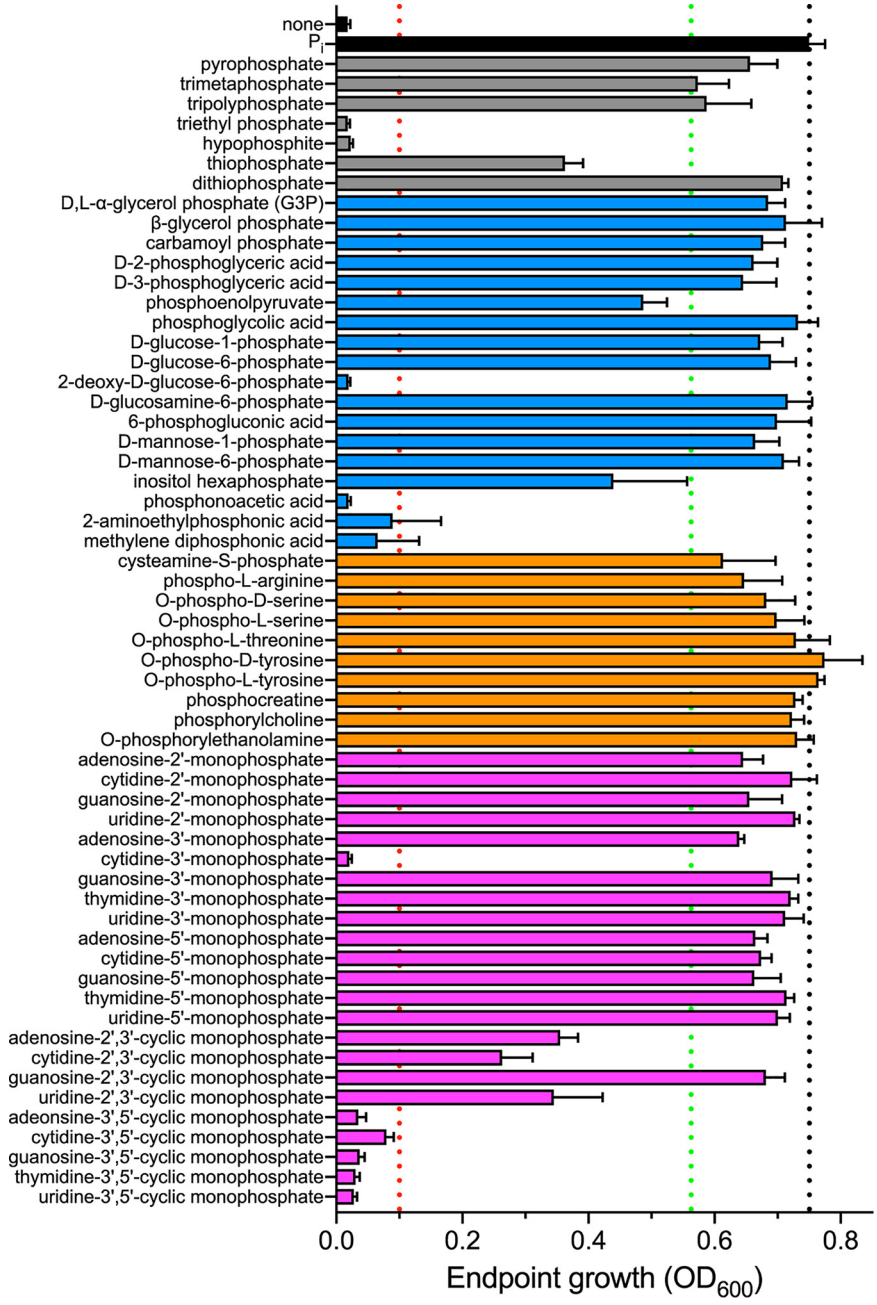

FIG 1 *S. aureus* can use a variety of phosphorylated molecules as phosphate sources. A library of 58 phosphorus-containing compounds, including inorganic molecules (gray), carbohydrates (blue), amino acids (orange), and nucleotides (purple), was screened for the growth of *S. aureus* in defined, phosphate-depleted medium buffered to pH 7.4. Bacteria were precultured overnight in a P_i-limiting medium. Growth was monitored by assessing optical density at 600 nm (OD_{600}), and endpoint growth after 10 h is reported. The screen was performed in biological triplicate; error bars indicate standard deviations. An OD_{600} of $>$0.1 (red dotted line) was used as the threshold to define phosphate sources that support growth. The black dotted line delineates growth on the positive control, P_i. The green dotted line denotes 75% of the growth on the positive control, used as the threshold to define good phosphate sources.

concentration of P_i was provided (Fig. 2A). This observation suggests that PhoB is potentially important for the use of G3P as a phosphate source in the absence of P_i. In contrast, the expression of neither *glpT* nor *ugpC* increased significantly when 5 mM G3P was the sole phosphate source instead of P_i (Fig. 2B and C). To further evaluate whether these genes are potentially involved in the use of G3P as an alternative

TABLE 1 PhoB and P_i transporter dependencies of 58 phosphorus-containing compounds screened for growth of *S. aureus*[a]

Source
Nonphosphate sources
Triethyl phosphate
Hypophosphite
Adenosine-3′,5′-cyclic monophosphate
Guanosine-3′,5′-cyclic monophosphate
2-Deoxy-D-glucose-6-phosphate
Cytidine-3′-monophosphate
Cytidine-3′,5′-cyclic monophosphate
Uridine-3′,5′-cyclic monophosphate
Phosphonoacetic acid
2-Aminoethyl phosphonic acid
Methylene diphosphonic acid
Thymidine-3′,5′-cyclic monophosphate
P_i transporter-dependent, PhoB-independent phosphate sources
AMP
Guanosine-2′-monophosphate
GMP
Guanosine-2′,3′-cyclic monophosphate
D-Glucose-6-phosphate
CMP
Uridine-3′-monophosphate
UMP
Thymidine-3′-monophosphate
dTMP
PhoB- and P_i transporter-independent phosphate sources
Thiophosphate
Dithiophosphate
Carbamoyl phosphate
Guanosine-3′-monophosphate
Phosphoglycolic acid
Cysteamine *S*-phosphate
Phospho-L-arginine
O-Phospho-D-tyrosine
PhoB- and P_i transporter-dependent phosphate sources
Pyrophosphate
Trimetaphosphate
Tripolyphosphate
Adenosine-2′-monophosphate
Adenosine-3′-monophosphate
Adenosine-2′,3′-cyclic monophosphate
D,L-α-Glycerol phosphate (G3P)
β-Glycerol phosphate
D-2-Phosphoglyceric acid
D-3-Phosphoclyceric acid
Phosphoenolpyruvate
D-Glucose-1-phosphate
D-Glucosamine-6-phosphate
6-Phosphogluconic acid
Cytidine-2′-monophosphate
Cytidine-2′,3′-cyclic monophosphate
D-Mannose-1-phosphate
D-Mannose-6-phosphate
O-Phospho-D-serine
O-Phospho-L-serine
O-Phospho-L-threonine
Uridine-2′-monophosphate
Uridine-2′,3′-cyclic monophosphate
O-Phospho-L-tyrosine
Phosphocreatine
Phosphorylcholine
O-Phosphorylethanolamine
Inositol hexaphosphate

[a]Categorization of phosphate sources was based on the endpoint growth of *S. aureus* PhoB- and/or P_i transporter-deficient strains normalized to wild-type growth on P_i. Nonphosphate sources were those on which wild-type *S. aureus* did not reach a terminal OD_{600} of >0.1. PhoB- and P_i transporter-dependent phosphate sources were those in which Δ*phoB* mutants reached a terminal OD_{600} of <70% of that of the wild type at either the permissive or nonpermissive pH. P_i transporter-dependent, PhoB-independent substrates were classified as those on which the growth of the Δ*pstSCAB* Δ*pitA* mutant was <70% of that of the wild type at the nonpermissive pH compared to growth at the permissive pH. PhoB- and P_i transporter-independent phosphate sources were those in which all mutants grew to ≥70% of the growth of the wild type at both pHs.

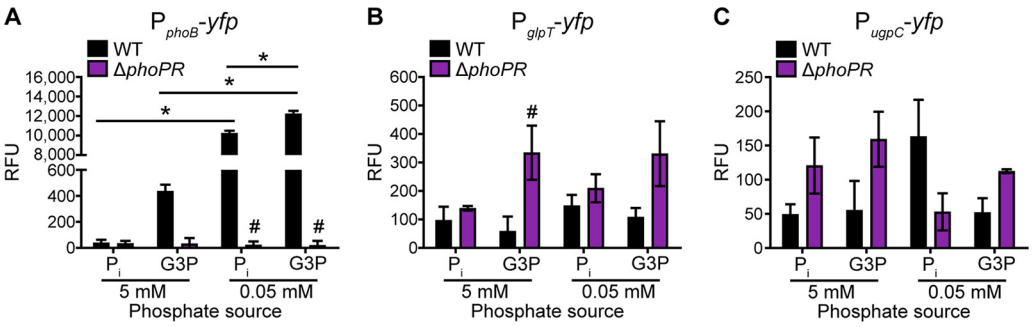

FIG 2 Expression of *phoB* responds to phosphate starvation and is PhoPR dependent. Shown are data for the expression of *phoB* (A), *glpT* (B), and *ugpC* (C) in wild-type and Δ*phoPR S. aureus* after 10 h of growth in PFM9 with 0.5% glucose supplemented with excess (5 mM) or limiting (0.05 mM) P_i or G3P as the sole phosphate source. Expression was assessed by measuring fluorescence using the P_{phoB}-*yfp*, P_{glpT}-*yfp*, and P_{ugpC}-*yfp* reporter plasmids. Strains were precultured overnight in TSB. *, $P < 0.05$ for the indicated comparison by two-way analysis of variance (ANOVA) with Sidak's multiple-comparison test; #, $P < 0.05$ for the Δ*phoPR* mutant compared to the wild type in the same growth medium by two-way ANOVA with Tukey's multiple-comparison test ($n = 3$). Error bars indicate standard errors of the means (SEM).

phosphate source, their expression was also measured in a Δ*phoPR* mutant (17). The expression of *phoB* was entirely ablated in the Δ*phoPR* mutant, suggesting that PhoB is a member of the PhoPR-controlled phosphate starvation regulon (Fig. 2A). In contrast, the expression of *glpT* and *ugpC* did not decrease in the Δ*phoPR* mutant compared to the wild type (WT) (Fig. 2B and C). Cumulatively, these results suggest that PhoB, but not GlpT or the Ugp system, is responsive to phosphate availability.

***glpT* and *ugpC* are induced by the absence of glucose.** As *glpT* and *ugpC* expression was not phosphate responsive, whether they are regulated by carbon source availability was investigated. Their expression was assessed in P_i-replete (5 mM P_i) medium in which glycerol or G3P was provided as the carbon source. Differing from glucose-containing growth medium, when glycerol and G3P were provided as the sole carbon source, *glpT* and the *ugpC* locus were expressed at appreciable levels (Fig. 3A and B). The expression levels of *glpT* and the *ugpC* locus were ~3- and 4-fold higher, respectively, in the presence of G3P than in the presence of glycerol (Fig. 3A and B). The expression of *phoB* was not substantially induced when glycerol or G3P was provided as the sole carbon source (Fig. 3C). As G3P did not induce the expression of *glpT* or the *ugpC* locus when glucose was present (Fig. 2B and C), this suggests that their expression is subject to carbon catabolite repression. These results suggest roles for GlpT and the UgpC locus in carbon metabolism rather than phosphate assimilation.

PhoB is important for the use of G3P as a phosphate source. As *phoB* is induced when G3P is present as a phosphate source, we next assessed whether PhoB is needed for growth when G3P is present as the sole phosphate source. The Δ*phoB* mutant grew similarly to the wild type when P_i was the phosphate source, regardless of the concentration (Fig. 4A and B). However, when excess (5 mM) G3P was the phosphate

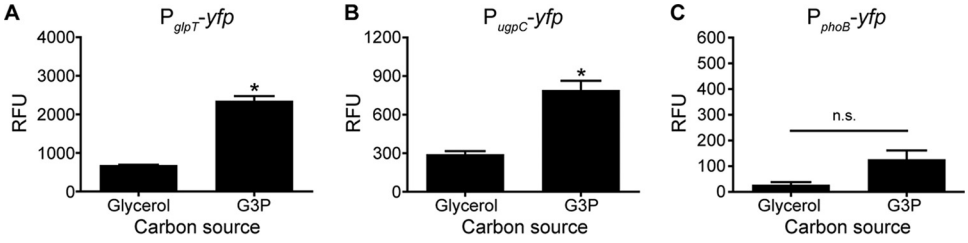

FIG 3 Expression of *glpT* and *ugpC* responds to carbon availability. Shown are data for the expression of *glpT* (A), *ugpC* (B), and *phoB* (C) in wild-type *S. aureus* after 10 h of growth in PFM9 with 5 mM P_i and 5 mM either glycerol or G3P as the sole carbon source. Expression was assessed by measuring fluorescence using the P_{glpT}-*yfp*, P_{ugpC}-*yfp*, and P_{phoB}-*yfp* reporter plasmids. Strains were precultured overnight in TSB. *, $P < 0.05$ for G3P compared to glycerol by an unpaired *t* test ($n = 3$); n.s., not significant. Error bars indicate SEM.

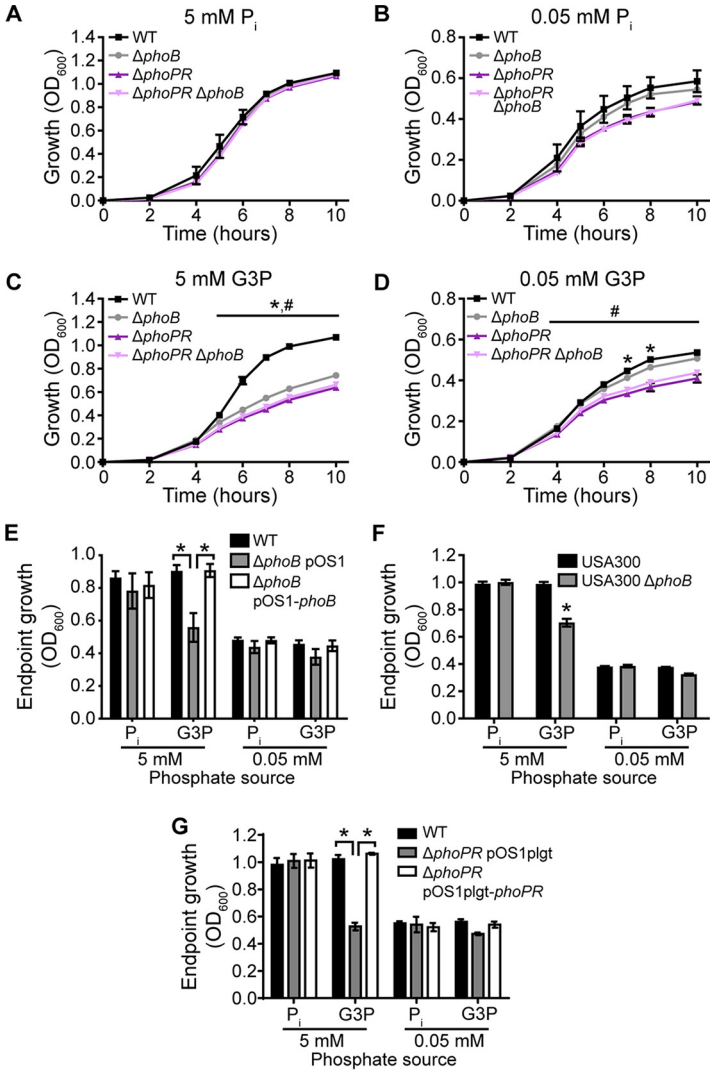

FIG 4 PhoB is important for the use of G3P as a phosphate source. (A to D) Growth of wild-type *S. aureus* (Newman) and the indicated mutants in PFM9 with 0.5% glucose supplemented with the phosphate source P_i (A and B) or G3P (C and D) in excess (A and C) or limiting (B and D) amounts. *, $P < 0.05$ for the Δ*phoB* mutant compared to the wild type; #, $P < 0.05$ for the Δ*phoPR* mutant compared to the Δ*phoB* mutant (by two-way ANOVA with Tukey's multiple-comparison test) ($n = 4$). (E) Endpoint growth after 10 h of wild-type *S. aureus* with an empty vector (pOS1), the Δ*phoB* mutant with an empty vector, or the Δ*phoB* mutant with a vector containing *phoB* in PFM9 with 0.5% glucose and the indicated concentrations of P_i or G3P as the phosphate source. *, $P < 0.05$ for the indicated comparison by two-way ANOVA with Tukey's multiple-comparison test ($n = 3$). (F) Endpoint growth after 10 h of wild-type *S. aureus* USA300 (JE2) or the Δ*phoB* mutant in PFM9 with 0.5% glucose supplemented with the indicated concentrations of P_i or G3P as the phosphate source. *, $P < 0.05$ for the *phoB*::*erm* strain compared to the wild type by two-way ANOVA with Sidak's multiple-comparison test ($n = 3$). (G) Endpoint growth after 10 h of wild-type *S. aureus* with an empty vector (pOS1), the Δ*phoPR* mutant with an empty vector, or the Δ*phoPR* mutant with a vector containing *phoPR* in PFM9 with 0.5% glucose and the indicated concentrations of P_i or G3P as the phosphate source. *, $P < 0.05$ for the indicated comparison by two-way ANOVA with Tukey's multiple-comparison test ($n = 3$). For panels A to G, strains were precultured overnight in TSB. Error bars indicate SEM.

source, the Δ*phoB* mutant had a strong growth defect (Fig. 4C), suggesting that PhoB is important for utilizing G3P as a phosphate source. With a limiting amount (0.05 mM) of G3P in the medium, the Δ*phoB* mutant grew similarly to the wild type, presumably because the amount of phosphate that PhoB could liberate from G3P is relatively negligible compared to background growth under these conditions (Fig. 4D). The growth phenotype of the Δ*phoB* mutant in 5 mM G3P could be complemented by the ectopic expression of *phoB* (Fig. 4E). The loss of *phoB* in the community-acquired

methicillin-resistant *S. aureus* (MRSA) strain USA300 JE2 also resulted in a growth defect when the strain was grown on G3P as a phosphate source (Fig. 4F). Cumulatively, these results suggest that PhoB is critical for the ability of *S. aureus* to use G3P as a P_i source.

PhoPR is necessary to utilize G3P as a phosphate source. The finding that *phoB* is induced in a PhoPR-dependent manner when G3P is the sole phosphate source raises the possibility that other components of the Pho regulon could be necessary for the use of G3P as a phosphate source. To test this idea, the growth of a Δ*phoPR* mutant was compared to that of wild-type *S. aureus* and a Δ*phoB* mutant in excess (5 mM)- and limiting (0.05 mM)-phosphate media with either P_i or G3P provided as the sole phosphate source. Consistent with our previous findings (17), the Δ*phoPR* mutant grew similarly to the wild type in P_i-replete medium (Fig. 4A) but had a growth defect in P_i-limiting medium (Fig. 4B). When 5 mM G3P was provided as a phosphate source, the Δ*phoPR* mutant had a pronounced growth defect relative to the wild type (Fig. 4C). This defect was reversed by expressing *phoPR* from a plasmid (Fig. 4G). Notably, the growth defect of the Δ*phoPR* mutant was slightly but statistically significantly more pronounced than that of the Δ*phoB* mutant. The growth of the Δ*phoPR* mutant in the presence of 0.05 mM G3P was similar to that in 0.05 mM P_i but again was slightly worse than that of the Δ*phoB* mutant (Fig. 4B and D). A Δ*phoPR* Δ*phoB* double mutant grew similarly to the Δ*phoPR* mutant under all conditions tested (Fig. 4A to D). Cumulatively, these results suggest that the induction of PhoB by PhoPR substantially contributes to the growth of *S. aureus* on G3P but that other components of the Pho regulon, such as those involved in the phosphate-sparing response (17), may also be important.

The use of G3P by *S. aureus* as a phosphate source is P_i transporter dependent. The observation that PhoB is necessary for the use of G3P as a phosphate source suggests that P_i would be liberated extracellularly, necessitating the subsequent use of P_i transporters. To test this model, a phenotypically P_i transport-deficient strain was used to determine if *S. aureus* requires both PhoB and P_i transporters in order to use G3P as a phosphate source. A Δ*pstSCAB* Δ*pitA* mutant grown at an acidic pH becomes phenotypically a triple P_i transporter mutant, as the remaining P_i transporter, NptA, functions poorly under this condition (16). We assessed the growth of the wild type and the Δ*phoB*, Δ*pstSCAB* Δ*pitA*, and Δ*pstSCAB* Δ*pitA* Δ*phoB* mutants at permissive and nonpermissive pHs (pH 7.4 and 6.4, respectively) with either P_i or G3P provided as the phosphate source. Consistent with previous results (16), the Δ*pstSCAB* Δ*pitA* double mutant grew similarly to the wild type on P_i at pH 7.4 but reached a lower terminal optical density at pH 6.4 (Fig. 5A and B). Similar to the observations in Fig. 4, the Δ*phoB* mutant grew as well as wild-type bacteria when P_i was provided as a phosphate source but had a severe growth defect when G3P was provided (Fig. 5A and C). The Δ*pstSCAB* Δ*pitA* mutant also had a growth defect relative to the wild type at the nonpermissive pH when G3P was provided as the phosphate source (Fig. 5D). The expression of *phoB* from a plasmid reversed the growth defect of the Δ*phoB* mutant when G3P was provided as the sole phosphate source in acidic medium (Fig. 5E). Similarly, the ectopic expression of *pitA* reversed the growth defect of the Δ*pstSCAB* Δ*pitA* mutant (Fig. 5F). To preserve the native regulation of *pitA*, the complementation construct also included the upstream gene *pitR*. These results indicate that P_i transporters are necessary for *S. aureus* to use G3P as a phosphate source. As expected, the Δ*pstSCAB* Δ*pitA* Δ*phoB* triple mutant grew worse than the wild type when G3P was the phosphate source or when P_i was provided at the nonpermissive pH (Fig. 5B to D). Unexpectedly, the Δ*phoB* mutant grew to a higher optical density when G3P was the phosphate source in medium at pH 6.4 than in medium at pH 7.4 (Fig. 5C and D). However, this pathway is P_i transporter dependent, as the increased growth of the Δ*phoB* mutant is abolished in the Δ*pstSCAB* Δ*pitA* Δ*phoB* strain (Fig. 5D). This observation suggests an increase in the presence of P_i produced independently of PhoB in acidic environments; regardless, P_i transporters would still be necessary to take up the liberated P_i. Cumulatively, these observations establish that PhoB and P_i transporters are necessary for *S. aureus* to use G3P as a phosphate source.

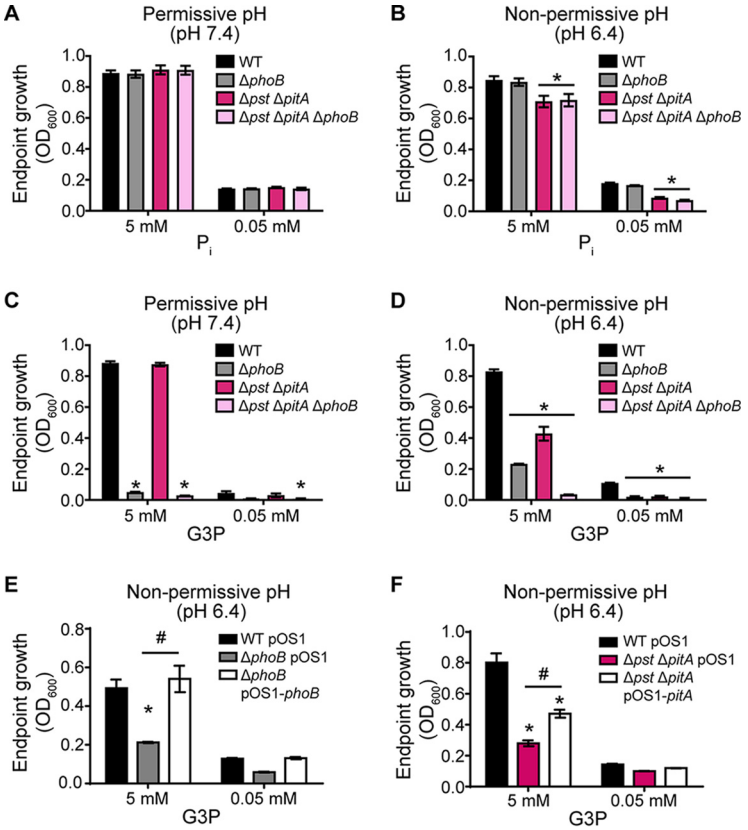

FIG 5 Use of G3P as a phosphate source by *S. aureus* is PhoB and P_i transporter dependent. Shown are data for endpoint growth after 10 h of wild-type *S. aureus* and the indicated mutants (Δ*pst* indicates Δ*pstSCAB*) in PFM9 with 0.5% glucose supplemented with the indicated concentrations of P_i (A and B) or G3P (C to F) as the phosphate source and buffered to pH 7.4 (A and C) or pH 6.4 (B, D, and F). Strains were precultured overnight in a P_i-limiting medium at the permissive pH so that the cells were equivalently phosphate limited at the start of the growth assay. *, $P < 0.05$ compared to the wild type by two-way ANOVA with Tukey's multiple-comparison test; #, $P < 0.05$ for the indicated comparison by two-way ANOVA with Tukey's multiple-comparison test ($n \geq 3$). Error bars indicate SEM.

***S. aureus* relies on PhoB and P_i transporters to obtain phosphate from most alternative phosphate sources.** Given the finding that *S. aureus* relies on PhoB and P_i transporters to use G3P, we investigated if this was a general strategy employed by *S. aureus* or specific to G3P. To address this question, we evaluated the ability of Δ*phoB*, Δ*pstSCAB* Δ*pitA*, and Δ*pstSCAB* Δ*pitA* Δ*phoB* mutants to grow on the same library of phosphorus-containing compounds that was previously screened with wild-type *S. aureus*. In addition to screening the library at a pH of 7.4, it was also screened at a pH of 6.4, where the Δ*pstSCAB* Δ*pitA* and Δ*pstSCAB* Δ*pitA* Δ*phoB* mutants would have a reduced ability to utilize P_i liberated by PhoB. Changing the pH of the growth medium did not change which phosphorous-containing compounds wild-type *S. aureus* could use as a phosphate source (Fig. S2).

The Δ*phoB*, Δ*pstSCAB* Δ*pitA*, and Δ*pstSCAB* Δ*pitA* Δ*phoB* mutants were unable to grow on the 12 compounds in the library that were previously found to be unable to support the growth of wild-type *S. aureus* (Fig. S1 and S3). Based on the endpoint growth yield of the mutants relative to the wild type on the 46 phosphocompounds that can serve as phosphate sources for *S. aureus*, three classes of compounds were identified: (i) PhoB- and P_i transporter-dependent phosphate sources, (ii) PhoB- and P_i transporter-independent phosphate sources, and (iii) PhoB-independent but P_i transporter-dependent phosphate sources (Fig. 6; Table 1). Consistent with a role in liberating P_i from organophosphates, all PhoB-dependent compounds were also P_i transporter dependent. More than 60% (28/46) of the compounds that *S. aureus* can use

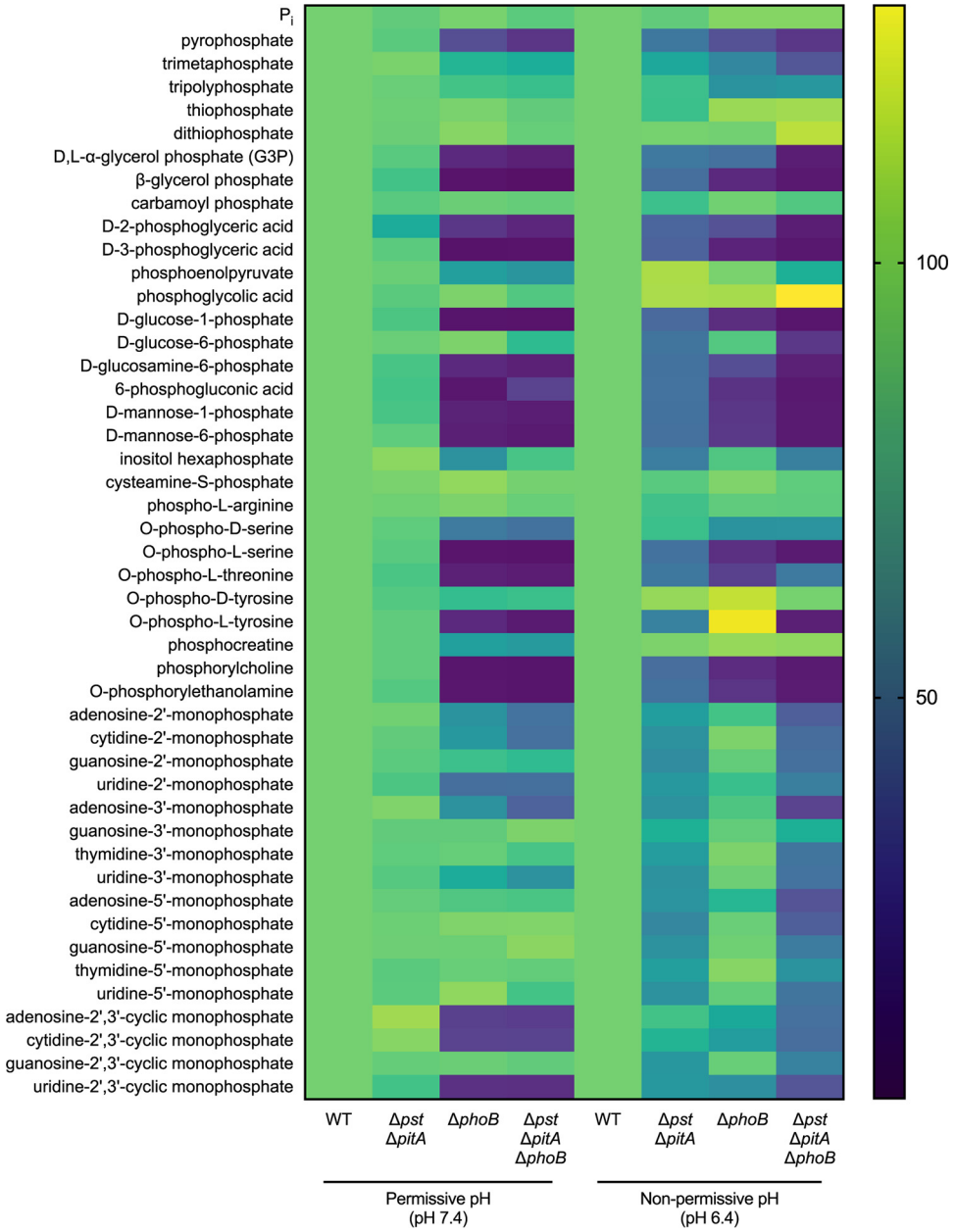

FIG 6 *S. aureus* requires its alkaline phosphatase PhoB and P_i transporters to acquire phosphate from most alternative phosphate sources. Forty-six phosphorus-containing compounds were screened for their ability to support the growth of *S. aureus* wild-type, Δ*phoB*, Δ*pstSCAB* Δ*pitA*, and Δ*pstSCAB* Δ*pitA* Δ*phoB* strains at pH 7.4 and pH 6.4 (Δ*pst* indicates Δ*pstSCAB*). Strains were precultured overnight in a P_i-limiting medium at the permissive pH so that the cells were equivalently phosphate limited at the start of the growth assay. Growth was measured by assessing the OD_{600} for 10 h. For each phosphate source, endpoint growth values were then normalized to the growth of the wild type (WT) within each pH. Percent growth relative to the WT is represented as a heat map. The screen was performed in biological triplicate.

as a phosphate source were PhoB and P_i transporter dependent (Fig. 6; Table 1). Validating the screen, G3P was identified as one of the PhoB- and P_i transporter-dependent substrates (Fig. 6; Table 1). Other substrates in this group included a range of phosphorylated sugars, nucleotides, and amino acids, suggesting that *S. aureus* relies on its inducible alkaline phosphatase and P_i importers to acquire phosphate from a wide variety of sources. Approximately 17% (8/46) of the compounds could be used independently of PhoB and P_i transporters, suggesting that *S. aureus* may express dedicated transporters to import these molecules intact. These eight substrates in-

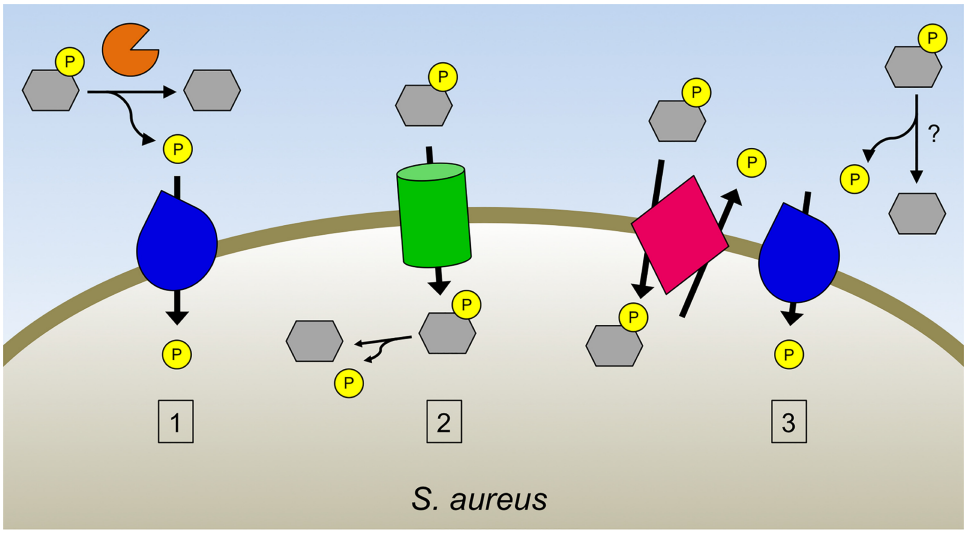

FIG 7 Model of phosphate acquisition from alternative phosphate sources by *S. aureus*. *S. aureus* can acquire phosphate from a variety of phosphosubstrates during phosphate starvation through three pathways: the alkaline phosphatase PhoB (orange) cleaves P_i (yellow) from phosphorylated compounds (gray) extracellularly, with the liberated P_i subsequently imported by P_i importers (blue) (pathway 1); phosphorylated molecules can be imported intact through phosphosubstrate importers (green), with P_i subsequently released intracellularly (pathway 2); and extracellular P_i is generated independently of PhoB, such as by phosphosubstrate/P_i antiporters (pink) or by other phosphatases, which is then imported through P_i importers (pathway 3).

cluded several phosphorylated amino acids (cysteamine *S*-phosphate, phospho-L-arginine, and *O*-phospho-D-tyrosine), one nucleotide (guanosine-3′-monophosphate), and other organophosphate (carbamoyl phosphate and phosphoglycolic acid) and inorganic phosphate (thiophosphate and dithiophosphate) compounds (Fig. 6; Table 1). The remaining 22% (10/46) of the compounds that support staphylococcal growth, nine of which were nucleotides, are PhoB independent but P_i transporter dependent (Fig. 6; Table 1). In total, 38 of the 46 compounds that *S. aureus* could use required the presence of a functional P_i importer. Cumulatively, the results of the screen suggest that the liberation of P_i and the subsequent import of P_i are the preferred mechanisms for obtaining phosphate by *S. aureus*.

DISCUSSION

Pathogens, including *S. aureus*, must obtain essential inorganic nutrients from a variety of environments within the host. One of these nutrients, phosphate, can be taken up via dedicated importers for P_i monomers or transported as part of organophosphate compounds (9, 10). While the preferred source of phosphate for bacteria is P_i, the requirement of P_i starvation-responsive two-component systems for the virulence of many pathogens indicates that P_i is not freely available in all host environments (1, 17, 22, 35–38). Given that *S. aureus* is among the pathogens that experience P_i limitation *in vivo*, we screened a library of 58 phosphorus-containing compounds and identified 46 phosphate sources for *S. aureus*, including phosphorylated sugars, nucleotides, and amino acids, as well as several inorganic phosphate sources that can be used to provide phosphate. Subsequent experiments revealed that the majority of these compounds are used as phosphate sources in a PhoB- and P_i transporter-dependent manner. These findings lead to a model in which *S. aureus* preferentially uses PhoB in combination with P_i transporters to extract phosphate from a variety of organophosphates (Fig. 7).

Among the phosphate sources for *S. aureus* identified in the screen was G3P, a compound that has been studied as an alternative phosphate source for *E. coli* (39). While G3P is known to promote the growth of *S. aureus* as a phosphate and carbon source (27), the pathways through which it is used for these purposes had not been determined. Here, we reveal that to use G3P as a phosphate source, *S. aureus* must first

process G3P extracellularly with its inducible alkaline phosphatase, PhoB, and then import P_i through P_i transporters. Differing from *E. coli*, our results indicate that *S. aureus* lacks a phosphate starvation-regulated G3P importer to transport G3P intact. Based on its expression pattern, the annotated staphylococcal *ugpC* gene does not appear to encode part of a Ugp-like transporter but instead is likely part of an operon involved in carbohydrate uptake, as previously suggested (34). The Pho regulon of *Bacillus subtilis* also lacks a Ugp system, but compared to *E. coli*, which possesses one inducible alkaline phosphatase, *B. subtilis* contains an expanded repertoire of three phosphatases (24). These observations cumulatively suggest that some organisms, including *B. subtilis* and *S. aureus*, may more strongly rely on phosphatases and subsequent P_i import to acquire phosphate. Of note, while both *E. coli* and *S. aureus* contain PstSCAB and PitA P_i transporters, *S. aureus* also possesses an additional P_i importer, NptA, which expands the environments in which it can import P_i (16). *E. coli* does have an NptA homolog; however, a *pstSCAB pitA* mutant of *E. coli* cannot grow on P_i, indicating that *E. coli* NptA does not function as a P_i importer (39, 40). Therefore, although *S. aureus* lacks a dedicated transporter for a common organophosphate source, it may compensate for this by an increased biochemical capacity for P_i import.

A screen of phosphate sources revealed that, similar to the pathway through which *S. aureus* uses G3P as a phosphate source, this pathogen relies on the enzymatic function of PhoB and then its P_i importers to acquire phosphate from most of the phosphocompounds that support growth. Given the importance of PhoB in using alternative phosphate sources, this finding suggests that PhoB may represent one of the PhoPR-regulated factors other than P_i transporters that are important for systemic staphylococcal infection (17). The screen also revealed that 8 compounds, including amino acids, a nucleotide, and other organic and inorganic phosphocompounds, can be phosphate sources independent of P_i transporters, suggesting that *S. aureus* may express dedicated importers for their uptake. The observation that nucleotides and phosphorylated amino acids can serve as phosphate sources in this manner was unexpected because aside from G3P and a few hexose-phosphates, phosphorylated compounds are not thought to be able to cross the cytoplasmic membrane (9). Continued genetic and biochemical analyses will be required to appreciate how *S. aureus* obtains phosphate from these molecules.

Interestingly, *S. aureus* can also use 10 compounds, including 9 nucleotides and hexose-6-phosphate, as phosphate sources in a PhoB-independent yet P_i transporter-dependent manner. We envision *S. aureus* having two mechanisms for using such substrates: (i) a substrate/P_i antiporter that would release P_i, subsequently allowing for the reuptake of P_i via the P_i transporters and, ultimately, net phosphate acquisition, or (ii) another phosphatase to act on the substrate. Correspondingly, one of the compounds in this category, glucose-6-phosphate, can be imported by *S. aureus* via the substrate-inducible hexose-6-phosphate/P_i antiporter UhpT (41). However, given that an additional export-and-import step must be taken for a net gain of one phosphate, we anticipate that such pathways of phosphosubstrate utilization are artificial. While nonphosphorylated nucleosides can be transported across the membrane, nucleotide transporters have not been described (42–44). Similar to *B. subtilis*, *Vibrio cholerae* encodes three inducible phosphatases, in addition to a PhoB homolog, that promote phosphate acquisition from molecules, including nucleotides (44). The observation that the *S. aureus* Δ*phoB* mutant grows better on G3P as a phosphate source at pH 6.4 than at pH 7.4 (Fig. 5B and D) suggests that there may be an additional phosphatase deployed under this condition that can act on G3P. Intriguingly, a secreted tyrosine phosphatase, PtpA, was recently found to be required for the virulence of *S. aureus* (45). While PtpA interacts with the host protein coronin-1A, presumably to modulate signaling pathways during infection, perhaps P_i liberated by PtpA and potentially other host-targeting phosphatases can also serve as a phosphate source.

Overall, this study provides molecular insight into the range of phosphate sources available to *S. aureus*. Detailed analysis of the use of G3P as a phosphate source and subsequent screening of potential phosphate sources underscore the importance of

TABLE 2 Strains used in this study

Genotype or name	Description	Reference
Newman	Wild-type strain of *Staphylococcus aureus*	
Δ*phoB*	*phoB*::*erm* allele from the NTML[a] transduced into Newman	This study
Δ*phoPR*	Clean deletion of *phoPR* in Newman	17
Δ*phoPR* Δ*phoB*	*phoB*::*erm* antibiotic resistance cassette swapped to *kan* and transduced into Δ*phoPR*	This study
Δ*pstSCAB* Δ*pitA*	*pitA*::*erm* allele from the NTML transduced into the strain with a clean deletion of *pstSCAB*	16
Δ*pstSCAB* Δ*pitA* Δ*phoB*	*phoB*::*erm* antibiotic resistance cassette swapped to *kan* and transduced into the Δ*pstSCAB* Δ*pitA* mutant	This study
USA300 JE2	Wild-type strain of *Staphylococcus aureus*	46
Δ*phoB* (USA300 JE2)	*phoB*::*erm* mutant from the NTML	46

[a]NTML, Nebraska Transposon Mutant Library (46).

PhoB and the P_i acquisition systems in *S. aureus*. In addition, the work here adds to a growing body of literature that reveals key differences in phosphate acquisition and metabolism in pathogenic organisms such as *S. aureus* and established models built from the study of nonpathogenic organisms such as *E. coli* and *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and cloning. *S. aureus* strain Newman and derivatives were used for all experiments unless otherwise noted. *S. aureus* was cultivated in tryptic soy broth (TSB) and on tryptic soy agar (TSA) plates and stored in brain heart infusion (BHI) broth containing 30% glycerol at −80°C. *E. coli*, used for subcloning, was grown in LB and on LB agar plates and stored in LB containing 30% glycerol at −80°C. Both species were grown at 37°C. As needed to maintain plasmids, 100 μg/ml ampicillin or 10 μg/ml chloramphenicol was added to growth media.

The *phoB*::*erm* allele was obtained from the Nebraska Transposon Mutant Library (NTML) (46), and the antibiotic resistance cassette was swapped using a protocol provided in the NTML toolkit (47). Mutant alleles were subsequently transduced into the desired background via Phi85 phage (Table 2). For complementation studies, *phoB* and *pitA* were cloned into the pOS1 vector (48) under the control of their native promoter using the indicated primers (Tables 3 and 4). For expression studies, the promoters of *phoB*, *glpT*, and *ugpC* were cloned into the pAH5 vector using the indicated primers (Tables 3 and 4) (49). All PCR-generated constructs were verified by sequencing.

Growth medium, growth assays, and expression analysis. Phosphate-free M9-based medium (PFM9) was used for growth assays and was described previously (16). PFM9 was buffered with 70 mM either HEPES (pH 7.4) or morpholinepropanesulfonic acid (MOPS) (pH 6.4). P_i source stocks were made by mixing NaH_2PO_4 and Na_2HPO_4 and adjusting the mixture to the desired pH. G3P (D,L-α-glycerol phosphate, purchased from Sigma [catalog no. 17766]) was resuspended in water at 20 mM and filter sterilized. For phosphate limitation growth assays, bacteria were inoculated into TSB and grown overnight on a roller drum for 16 to 18 h. Where indicated, to prestarve strains for phosphate, bacteria were inoculated into 5 ml PFM9 plus 70 mM HEPES (pH 7.4) supplemented with 0.5% glucose and 500 μM P_i and grown overnight on a roller drum for 16 to 18 h. Cultures grown overnight were inoculated 1:100 into a 96-well round-bottom plate containing 100 μl/well PFM9 containing the indicated phosphate and carbon sources. Plates were incubated at 37°C with shaking at 180 rpm. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}). Expression (RFU [relative fluorescence units]) was determined by measuring fluorescence (excitation/emission wavelengths of 505/535 nm), normalizing the values to the OD_{600}, and then subtracting the RFU of empty vector controls.

Identification of phosphate sources for *S. aureus*. Phenotype MicroArray PM4A microplate phosphorus and sulfur source plates (Biolog, Inc., Haywood, CA) were screened to identify compounds that can be used as phosphate sources by *S. aureus*. For these assays, bacteria were inoculated into 5 ml PFM9 plus 0.5% glucose and 70 mM HEPES (pH 7.4) supplemented with 500 μM P_i and grown on a roller drum for 16 to 18 h. Cultures grown overnight were diluted 1:100 into PFM9 plus 0.5% glucose with either

TABLE 3 Primers used in this study

Primer name	Sequence
phoB comp F	GGCCGAATTCCTATAATCTTCTTCCTTCAATTGAATTATCC
phoB comp R	GCGCGGATTCGAACACCTTGTTATAGTGCTCG
phoB prom F	CGCGCTGCAGCTATAATCTTCTTCCTTCAATTGAATTATCC
phoB prom R	CGCGGGTACCAAGACATCCTCCTGAGTATATGAC
pitA comp F	GATTGTACTGAGAGTGCACCATAATTTTTGAAATTAATATCAGTACACTAAAATTATAC
pitA comp R	GCTAGCTTGGCTGCAGGTCGACGTTAGAAAAATAAGTTAAGTATATAGAATAGTAAAC
glpT prom F	CGCGCTGCAGACTATCCCTCCTATTAGTATAAATTTTATACCAGC
glpT prom R	CGCGGGTACCAAAATCCTCCTTAATATGTATTTATATGCATTTTGTG
ugpC prom F	CGCGCTGCAGTTATAGAAGGGTGCCCGCAGTC
ugpC prom R	CGCGGGTACCGTTATGCCTCCCATACTTTGTTTACAGTTTGATTG

TABLE 4 Plasmids used in this study

Plasmid	Description[a]	Reference
pOS1	pOS1 (empty vector)	48
pOS1-*phoB*	pOS1 containing the *phoB* CDS under the control of the native promoter	This study
pOS1-*pitA*	pOS1 containing the *pitRA* locus under the control of the native promoter	This study
pOS1 P_{lgt}	pOS1 (empty vector) with the *lgt* promoter	48
pOS1 P_{lgt}-*phoPR*	pOS1 P_{lgt} containing the *phoPR* CDS	17
pAH5::P_{empty}-*yfp*	pAH5 with no promoter driving *yfp* expression	50
pAH5::P_{phoB}-*yfp*	pAH5 with the *phoB* promoter driving *yfp* expression	This study
pAH5::P_{glpT}-*yfp*	pAH5 with the *glpT* promoter driving *yfp* expression	This study
pAH5::P_{ugpC}-*yfp*	pAH5 with the *ugpC* promoter driving *yfp* expression	This study

[a]CDS, coding DNA sequence.

70 mM HEPES (pH 7.4) or 70 mM MOPS (pH 6.4). The Biolog plates were then inoculated with 100 μl of this mixture. The plates were incubated at 37°C with shaking at 180 rpm. Bacterial growth was monitored by measuring the OD_{600} for 10 h. The screen was performed in biological triplicate (on three separate days).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.4 MB.

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J.L.K., A.J.L.M., K.M.G., J.N.R., and T.E.K.-F. performed the research and analyzed the data. J.L.K., A.J.L.M., J.N.R., and T.E.K.-F. designed the experiments and wrote the paper.

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