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MOLECULAR PATHOGENESIS

Acquisition of the Phosphate Transporter NptA Enhances *Staphylococcus aureus* Pathogenesis by Improving Phosphate Uptake in Divergent Environments

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ABSTRACT During infection, pathogens must obtain all inorganic nutrients, such as phosphate, from the host. Despite the essentiality of phosphate for all forms of life, how *Staphylococcus aureus* obtains this nutrient during infection is unknown. Differing from *Escherichia coli*, the paradigm for bacterial phosphate acquisition, which has two inorganic phosphate (P_i) importers, genomic analysis suggested that *S. aureus* possesses three distinct P_i transporters: PstSCAB, PitA, and NptA. While *pitA* and *nptA* are expressed in phosphate-replete media, expression of all three transporters is induced by phosphate limitation. The loss of a single transporter did not affect *S. aureus*. However, disruption of any two systems significantly reduced P_i accumulation and growth in divergent environments. These findings indicate that PstSCAB, PitA, and NptA have overlapping but nonredundant functions, thus expanding the environments in which *S. aureus* can successfully obtain P_i. Consistent with this idea, in a systemic mouse model of disease, loss of any one transporter did not decrease staphylococcal virulence. However, loss of NptA in conjunction with either PstSCAB or PitA significantly reduced the ability of *S. aureus* to cause infection. These observations suggest that P_i acquisition via NptA is particularly important for the pathogenesis of *S. aureus*. While our analysis suggests that NptA homologs are widely distributed among bacteria, closely related less pathogenic staphylococcal species do not possess this importer. Altogether, these observations indicate that P_i uptake by *S. aureus* differs from established models and that acquisition of a third transporter enhances the ability of the bacterium to cause infection.

KEYWORDS *Staphylococcus aureus*, phosphate metabolism, PstSCAB, PitA, NptA, transporter, Gram positive, infection

Staphylococcus aureus, carried asymptomatically by approximately one-third of the population, is capable of establishing infection in virtually every host tissue (1–3). The threat of staphylococcal infections is amplified by prolific resistance to a variety of antibiotics among health care-associated isolates and the spread of antibiotic resistance to community-associated strains (4, 5). These factors have led organizations such as the Centers for Disease Control and Prevention and the World Health Organization to designate *S. aureus* as a serious threat to human health and to call for the development of new strategies to battle *S. aureus* (6, 7). During infection, pathogens must obtain their nutrients from the host. Understanding how pathogens such as *S. aureus* obtain vital nutrients during infection has the potential to lead to the identification of novel targets for therapeutic intervention.

Phosphate is an essential nutrient for all organisms due to its critical role in signaling, metabolism, and macromolecular structure. Because it is an inorganic nutrient, invading microbes must acquire phosphate from the host. The importance of phosphate acquisition is emphasized by the observation that disruption of phosphate

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transporters in pathogenic *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and other *Enterobacteriaceae*, as well as other species, including *Vibrio cholerae*, *Mycobacterium tuberculosis*, and *Streptococcus pneumoniae*, compromises their ability to cause infection (8–15). However, the repertoire of phosphate importers expressed by pathogens outside the *Enterobacteriaceae* and their contribution to the ability of pathogens such as *S. aureus* to cause infection are largely unknown.

The preferred source of phosphate for most bacteria is inorganic phosphate (P_i). Bacteria are known to possess three distinct classes of P_i importers: the PstSCAB (phosphate-specific transport) system, PitA (phosphate inorganic transport), and NptA (Na-dependent phosphate transport) (16–20). Significant insight into the molecular features of the Pst and Pit systems has been gained by studying their contribution to P_i uptake in laboratory isolates of *E. coli* and *Bacillus subtilis* (16–18, 21–25). PstSCAB is an ABC family P_i transporter in which PstS is the solute-binding protein, PstA and PstC comprise the transmembrane channel, and PstB is the ATPase that energizes translocation. The Pst system is a high-affinity (K_m of ~0.4 μM) importer that transports P_i ions with high specificity (16, 18, 22). PstSCAB is the most highly upregulated target of the phosphate-responsive two-component regulatory system PhoBR and is important for P_i transport when this nutrient is scarce (16, 18, 21, 26). In *E. coli*, mutation of the *pstSCAB* genes results in dysregulated, constitutive expression of the P_i starvation-induced Pho regulon due to activation of PhoBR (26). In contrast, when P_i is in excess, it is predominantly transported by the Pit system. PitA of *E. coli* has a lower affinity than Pst (K_m of ~38 μM) and is constitutively expressed (16, 18, 21). Pit transporters are proton motive force (PMF) driven and translocate P_i complexed with a divalent cation, such as Mg^{2+}, as a neutral metal-phosphate complex ($MeHPO_4$) (25). Analysis of NptA in bacteria has largely been limited to *Vibrio cholerae* and *Streptococcus pneumoniae* (19, 20). NptA belongs to the NaPi-2 family of eukaryotic sodium-phosphate cotransporters, which have been more extensively studied in eukaryotic organisms (27). Differing from the Pit and Pst systems, NptA uses sodium to move P_i into the cell at a stoichiometry of 3 Na^+ atoms to 1 P_i molecule. Transport via NaPi-2 transporters is augmented by increased Na^+ concentration and increased pH (19, 28). Analysis of the *nptA* gene of *V. cholerae* expressed in *E. coli* cells revealed that NptA has a considerably lower affinity (K_m of ~300 μM) for P_i than either the Pit or Pst system of *E. coli* (19). This observation led to the suggestion that NptA is important during the establishment of infection when the need for nutrients is especially great (19). However, the contribution of NptA to bacterial pathogenesis has not been directly evaluated.

Given the importance of phosphate, we set out to identify the P_i uptake systems expressed by *S. aureus* and elucidate their respective contributions to pathogenesis. Our analysis revealed that *S. aureus* encodes three distinct P_i transporters, PstSCAB, PitA, and NptA. Expression of all three systems increases in response to P_i limitation. Analysis of a panel of single and double transporter mutants revealed that each importer is attuned to import P_i optimally in a discrete environment. While no single transporter is necessary for virulence, only NptA is sufficient to mediate full pathogenesis of *S. aureus*. Notably, this indicates that *S. aureus* differs significantly from many *Enterobacteriaceae*, including *E. coli*, which currently serves as the paradigm for bacterial P_i transport. These findings also illuminate the important contribution of NptA, a widely distributed but little-studied transporter, to bacterial pathogenesis.

RESULTS

***Staphylococcus aureus* encodes three putative P_i transporters.** As an initial step to identify the staphylococcal P_i transporters, the *S. aureus* Newman genome was analyzed via BLAST for known P_i importers. This analysis identified three potential P_i transporters: *pstSCAB*, *pitA*, and *nptA* (see Fig. S1A in the supplemental material). In addition to the putative P_i transport genes, each locus encoded a PhoU homolog (*pstSCAB* and *pitA* loci) or domain (*nptA* locus). The presence at each locus of *phoU*, which contributes to regulating the P_i starvation response in *E. coli* and other organisms (26), strengthens the presumption that the staphylococcal PstSCAB, PitA, and NptA

TABLE 1 Distribution of *nptA* among bacteria

Taxonomic group	No. of genomes[a]	No. of *nptA* copies[b]
Actinobacteria	224	0.10
Bacteroidetes	109	0.34
Chlamydiae	13	0.00
Firmicutes	331	0.82
Bacilli	193	0.68
Staphylococcaceae	15	0.40
Clostridia	127	1.03
Others	11	0.73
Fusobacteria	6	1.17
Proteobacteria	749	0.57
Alphaproteobacteria	196	0.56
Betaproteobacteria	131	0.46
Deltaproteobacteria	56	0.61
Epsilonproteobacteria	38	0.61
Gammaproteobacteria	328	0.61
Enterobacteriaceae	105	0.76
Spirochaetes	39	0.64
Tenericutes	59	0.08

[a]Number of individual species' genomes in the KEGG database.
[b]Average gene copy per genome.

homologs are P_i transporters. A comprehensive analysis of ~9,000 staphylococcal genomes, representing 38 species available from the NCBI database, revealed that *pstSCAB* and *pitA* are almost universally conserved (*pstSCAB* is missing from only a single species, *Staphylococcus microti*) (Table S1). In contrast, *nptA* had a much more heterogeneous distribution, with only 60% (24/38) of species, including *S. aureus*, encoding an NptA ortholog (Table S1). Comparative genome alignments of the *nptA*-containing regions in *S. aureus* and the other staphylococcal species indicated that *nptA* is located in distinct genomic locations (Fig. S1B). Intriguingly, our analysis of the *nptA* loci suggests that following an ancestral loss event, *S. aureus* reacquired *nptA* from a staphylococcal donor (Fig. S1C and D). An expanded investigation of the distribution of NptA homologs in a variety of bacteria revealed that this P_i transporter was widely distributed among bacterial phyla (Table 1). NptA homologs were found to be particularly common among the *Firmicutes*, with an average copy number of 0.82 per genome, as well as in the family *Enterobacteriaceae*, with an average of 0.76 copy per genome (Table 1).

Expression of three putative P_i transporters in *S. aureus* increases upon P_i limitation. To facilitate studying phosphate acquisition in *S. aureus*, a P_i-limiting, defined medium (PFM9), based on M9 salts and a previously described defined staphylococcal growth medium (29), was created so that P_i starvation could be imposed in culture. In this medium, *S. aureus* growth is dependent on the addition of P_i, with maximal optical density observed in the presence of 1.58 mM P_i or higher (Fig. 1A). The reduced growth rate of *S. aureus* throughout the growth assay in medium containing 50 and 158 μM P_i suggested that it could be utilized to impose P_i starvation. To confirm this assumption, intracellular phosphate levels were assessed during exponential growth in PFM9 supplemented with various concentrations of P_i. Concentrations of P_i that limited the staphylococcal growth rate also resulted in reduced accumulation of intracellular phosphate (Fig. 1B). At the lowest concentration tested, *S. aureus* accumulated ~7-fold less phosphate than when bacteria were grown in P_i-replete medium (5 mM P_i). In total, these results demonstrate that PFM9 can be used to impose phosphate limitation on *S. aureus*.

If PstSCAB, PitA, and NptA are P_i importers, their expression would be expected to increase in response to reduced P_i availability. Using transcriptional reporter fusions, we found that while *pitA* and *nptA* were expressed under P_i-replete conditions, expression

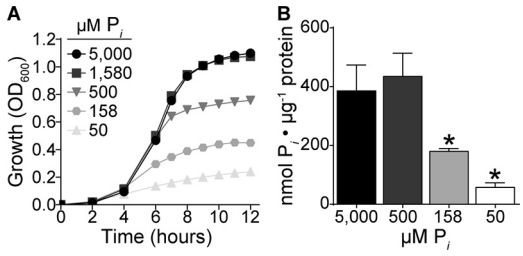

FIG 1 Intracellular P_i concentrations in *S. aureus* decrease during P_i limitation. (A) Growth of wild-type *S. aureus* in PFM9, pH 7.4, supplemented with various concentrations of P_i, measured by OD_{600}. $n = 3$; error bars indicate SEMs and are frequently smaller than the symbols. (B) Intracellular P_i levels normalized to protein concentration in wild-type *S. aureus* grown in PFM9 supplemented with the indicated concentrations of P_i. $n \geq 2$; error bars indicate SEMs. *, $P < 0.05$ via unpaired *t* test.

of both systems increased significantly in P_i-limiting medium (Fig. 2A and B). While we observed negligible expression of *pstSCAB* in P_i-replete medium, the system was induced ~6,000-fold when P_i was limiting (Fig. 2C). In total, these results support the hypothesis that PstSCAB, PitA, and NptA are P_i importers.

PstSCAB, PitA, and NptA promote growth of *S. aureus* in divergent environments. To elucidate under which conditions the putative transporters support growth of *S. aureus*, Δ*pstSCAB*, Δ*pitA*, and Δ*nptA* mutants were grown in medium supplemented with high and low concentrations of P_i. No growth defects were observed with any of the single mutants (Fig. S2), indicating that none of the transporters are essential under these conditions. As *S. aureus* encodes multiple putative P_i transporters, we reasoned that the systems might be able to compensate for one another *in vitro*. To evaluate this possibility, a series of double mutants (Δ*pstSCAB* Δ*pitA*, Δ*pstSCAB* Δ*nptA*, and Δ*nptA* Δ*pitA* mutants) was constructed. Multiple attempts to construct a Δ*pstSCAB* Δ*nptA* Δ*pitA* triple mutant were unsuccessful, suggesting that PstSCAB, PitA, and NptA are the only P_i transporters expressed by *S. aureus* under standard laboratory conditions.

When growth of the double transporter mutants was assessed in PFM9 medium supplemented with high and low concentrations of P_i (buffered to pH 7.4), the Δ*pstSCAB* Δ*pitA* and Δ*pstSCAB* Δ*nptA* mutants grew similarly to the wild type in both P_i-replete and -deplete medium, suggesting that NptA and PitA, respectively, are sufficient for growth under these conditions (Fig. 3A and B). While the Δ*nptA* Δ*pitA* mutant, which presumptively relies on PstSCAB for P_i uptake, also grew similarly to the wild type in PFM9 (Fig. S3A), we observed that it formed smaller colonies on tryptic soy agar (TSA) plates (Fig. S3B). Additionally, when growth of the Δ*nptA* Δ*pitA* mutant was assessed in P_i-replete rich medium (tryptic soy broth [TSB]), this strain grew more slowly than wild-type *S. aureus* or the other single and double mutants (Table 2). Ectopic expression of either *nptA* or *pitA* reversed the growth rate defect of the Δ*nptA* Δ*pitA*

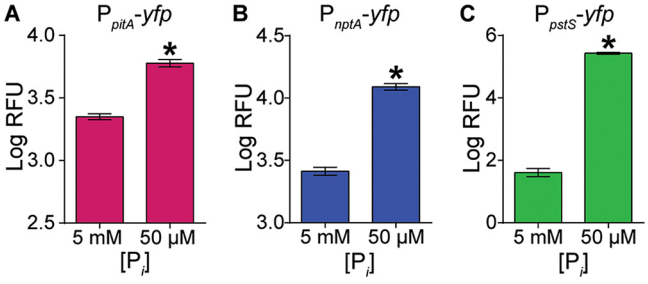

FIG 2 Expression of the three P_i transporters in *S. aureus* increases upon P_i limitation. Shown is expression of *pitA* (A), *nptA* (B), and *pstSCAB* (C) in wild-type *S. aureus* after 9 h of growth in PFM9, pH 7.4, supplemented with 5 mM (excess) or 50 μM (limiting) P_i. Expression was assessed using the reporter plasmids P_{pitA}-*yfp*, P_{nptA}-*yfp*, and P_{pstS}-*yfp*. *, $P < 0.05$ compared to result with 5 mM P_i via unpaired *t* test. $n = 3$; error bars indicate SEMs. A similar pattern of expression was observed at other time points (data not shown).

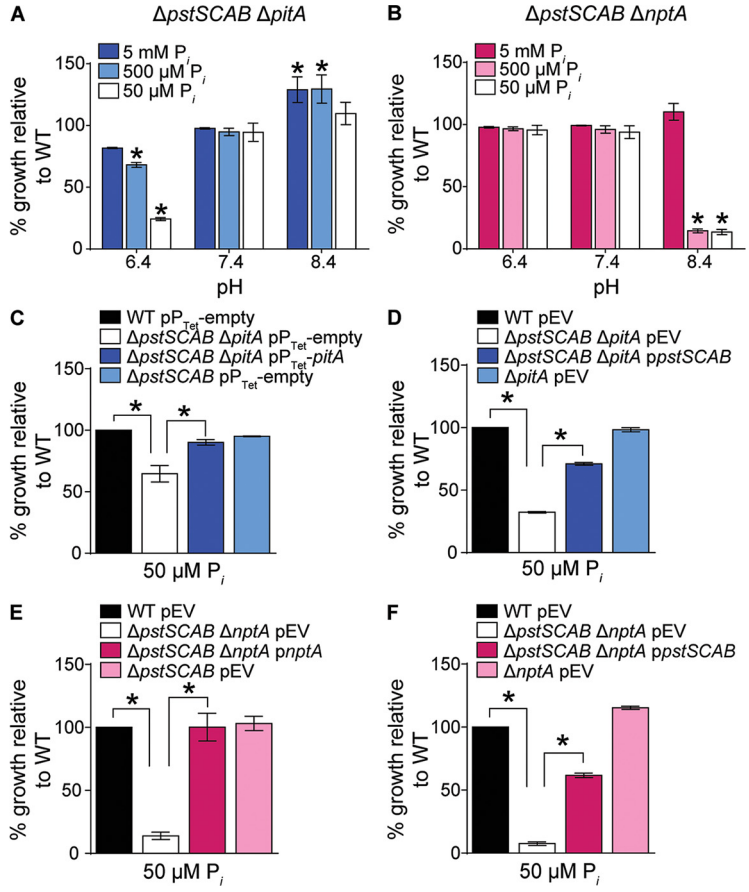

FIG 3 NptA and PitA promote growth of *S. aureus* in divergent environments. (A and B) Growth of Δ*pstSCAB* Δ*pitA* (A) and Δ*pstSCAB* Δ*nptA* (B) mutants in PFM9 adjusted to pH 6.4, 7.4, or 8.4 with different supplemental P_i concentrations. Growth was measured by assessing OD_{600} at 12 h. *, $P < 0.05$ compared to result for the wild type (WT) via two-way analysis of variance (ANOVA) with Dunnett's posttest. $n = 3$; error bars indicate SEMs. Similar results were obtained when growth was compared at earlier time points (data not shown). (C to F) Growth of the indicated strains measured by OD_{600} at 12 h and normalized to the wild type in PFM9, pH 6.4 (C and D) or 8.4 (E and F), with 50 μM supplemental P_i. pEV, empty vector. *, $P < 0.05$ for the indicated comparisons via one-way ANOVA with Sidak's posttest. $n = 3$; error bars indicate SEMs.

mutant in TSB (Table 2). Together, these data suggest that P_i acquisition via the Pst system is insufficient to support robust growth of *S. aureus* in rich medium. When combined with the observation that *pstSCAB* is expressed in low- but not high-P_i medium, these data suggest that the Pst system largely contributes to P_i uptake when the availability of this nutrient is limiting.

In several bacteria, the efficacy of P_i transporters is differentially affected by environmental conditions. For example, the transport rate of NptA from *V. cholerae* is augmented under alkaline conditions and PitA from *E. coli* optimally imports P_i in acidic environments (19, 30). This raises the possibility that the expanded repertoire of importers expressed by *S. aureus* may expand the environmental niches that it can occupy. To evaluate if the *S. aureus* transporters enhance growth in divergent environments, PFM9 was adjusted from physiological to acidic or alkaline pH. As before, no growth defects were observed with any of the single mutants under any of the conditions tested (Fig. S2). However, the presumably NptA-dependent Δ*pstSCAB* Δ*pitA* mutant had a severe growth defect in acidic P_i-limiting medium (Fig. 3A). Conversely, the presumptively PitA-dependent Δ*pstSCAB* Δ*nptA* mutant was unable to grow in alkaline P_i-limiting medium (Fig. 3B). The growth defects of the Δ*pstSCAB* Δ*pitA* and Δ*pstSCAB* Δ*nptA* mutants could be complemented by ectopic expression of either of the deleted transporters (Fig. 3C to F; see also Fig. S4). Both *pitA* and *nptA* were

TABLE 2 Doubling times of P_i transporter mutants in tryptic soy broth[a]

Strain description	Doubling time, mean ± SD (min)
WT	33.0 ± 1.7
Δ*pstSCAB*	31.8 ± 0.6
Δ*pitA*	32.8 ± 1.0
Δ*nptA*	30.3 ± 0.6*
Δ*pstSCAB* Δ*pitA*	32.0 ± 0.3
Δ*pstSCAB* Δ*nptA*	31.8 ± 1.0
Δ*nptA* Δ*pitA*	37.5 ± 1.5*
WT pEV	32.8 ± 0.6
Δ*pitA* pEV	32.5 ± 0.6
Δ*nptA* Δ*pitA* pEV	38.2 ± 1.1*
Δ*nptA* Δ*pitA* p*nptA*	32.2 ± 0.6
WT pP_{Tet}-empty	39.5 ± 0.7
Δ*nptA* pP_{Tet}-empty	42.7 ± 2.8
Δ*nptA* Δ*pitA* pP_{Tet}-empty	48.3 ± 2.0*
Δ*nptA* Δ*pitA* pP_{Tet}-*pitA*	40.8 ± 1.5

[a]*, $P < 0.05$ compared to wild type via one-way ANOVA with Sidak's posttest. WT, wild type; pEV, empty vector.

expressed in P_i-limited medium regardless of pH, indicating that the growth defect is due to reduced activity of the transporter rather than reduced expression of *pitA* and *nptA* (Fig. S5). Importantly, the growth defects of the mutants were also reversed by the addition of P_i, indicating that the phenotypes are not due to a generalized growth defect in either acidic or alkaline environments (Fig. 3A and B). Cumulatively, these observations suggest that P_i transport by PitA and NptA is suboptimal in basic and acidic pHs, respectively.

To further interrogate the importance of each transporter in different environments, the expression of the transporters was assessed in Δ*pstSCAB*, Δ*pitA*, and Δ*nptA* backgrounds as a function of pH and P_i level. We reasoned that in a given environment, loss of a preferred P_i importer would result in compensatory increases in expression of the other transporters due to suboptimal P_i acquisition. At neutral pH in P_i-replete medium, *nptA* and *pst* expression increased significantly in the Δ*pitA* strain, suggesting that PitA may be the optimal P_i transporter under these conditions (Fig. 4A). At this pH, loss of NptA resulted in increased expression of *pitA* but only in P_i-limited medium. In neutral, P_i-deplete medium, a change in expression was only observed with loss of NptA, which resulted in increased expression of *pitA*. At acidic pH in P_i-replete medium, expression of all three transporters significantly increased in the Δ*pitA* strain but not the Δ*pstSCAB* or Δ*nptA* strain (Fig. 4B). This suggests that PitA is the predominant P_i transporter utilized by *S. aureus* under acidic conditions. In acidic, P_i-deplete medium, no increased expression of *pitA*, *nptA*, or *pstSCAB* was observed in any of the strains compared to that in the wild type, suggesting that all three transporters were maximally expressed. At alkaline pH in P_i-replete medium, loss of NptA resulted in significantly increased expression of *pitA*, *pstSCAB*, and *nptA* (Fig. 4C). Under these conditions, loss of PitA resulted in a modest increase of *nptA*, but not *pstSCAB*, expression. In alkaline, P_i-deplete medium, only the loss of NptA resulted in any increase in expression, and then only for *pitA*. Cumulatively, these results suggest that NptA is the preferred P_i importer under alkaline conditions. Interestingly, the fact that no significant increase in the expression of the transporters was observed in the Δ*pstSCAB* mutant in any media (Fig. 4) suggests that *S. aureus* does not primarily rely on Pst for growth under the conditions tested. Together, these data indicate that *S. aureus* utilizes its three P_i transporters preferentially in different environments.

P_i acquisition via PstSCAB, PitA, and NptA is optimal under different conditions. To evaluate whether loss of the putative transporters decreases P_i acquisition by *S. aureus*, the ability of each double mutant to accumulate P_i was assessed in replete and limiting P_i at neutral, acidic, and basic pHs. The PitA- and NptA-dependent mutants

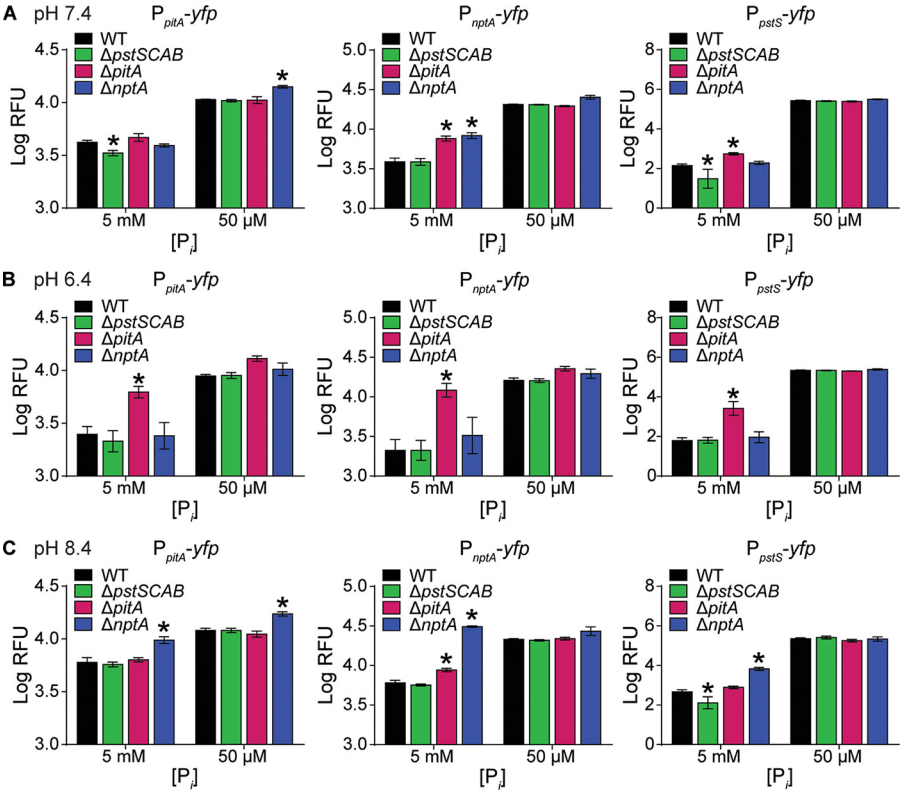

FIG 4 *S. aureus* differentially compensates for loss of P_i transporters depending on the environmental conditions. Shown is expression of *pitA*, *nptA*, and *pstSCAB* in the wild type and Δ*pstSCAB*, Δ*pitA*, and Δ*nptA* mutants following growth in PFM9 supplemented with 5 mM (excess) or 50 μM (limiting) P_i adjusted to pH 7.4 (A), 6.4 (B), or 8.4 (C). Expression was assessed using the reporter plasmids P_{pitA}-*yfp*, P_{nptA}-*yfp*, and P_{pstS}-*yfp*. *, $P < 0.05$ compared to the value for the wild type via two-way ANOVA with Dunnett's posttest. $n = 3$; error bars indicate SEMs.

(the Δ*pstSCAB* Δ*nptA* and Δ*pstSCAB* Δ*pitA* mutants, respectively) accumulated as much P_i as wild-type bacteria at neutral pH (Fig. 5A). In contrast, the Pst-dependent Δ*nptA* Δ*pitA* mutant accumulated approximately 25% less P_i than did the wild type under both P_i-replete and -limiting conditions at neutral and acidic pHs (Fig. 5A and B). Coupled with the diminished growth rate of the Δ*nptA* Δ*pitA* mutant, this observation suggests that the Pst system is unable to satiate a rapidly growing cell's need for phosphate. In acidic P_i-limiting medium, the NptA-dependent Δ*pstSCAB* Δ*pitA* mutant contained less P_i than the wild type, suggesting that its growth defect is due to a reduced ability to

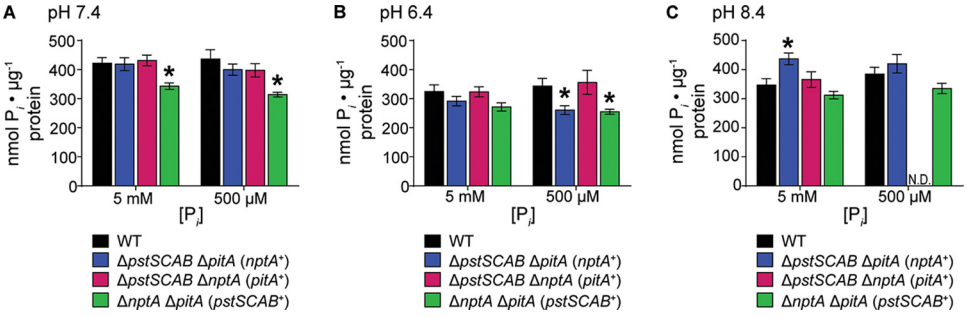

FIG 5 Loss of P_i transporters diminishes the ability of *S. aureus* to accumulate P_i. (A to C) The wild type and Δ*pstSCAB* Δ*pitA*, Δ*pstSCAB* Δ*nptA*, and Δ*nptA* Δ*pitA* mutants were grown in PFM9 in various concentrations of P_i adjusted to pH 7.4 (A), 6.4 (B), or 8.4 (C), and intracellular P_i was measured. *, $P < 0.05$ compared to the value for the wild type via two-way ANOVA with Dunnett's posttest. $n = 5$; error bars indicate SEMs. N.D., not determined.

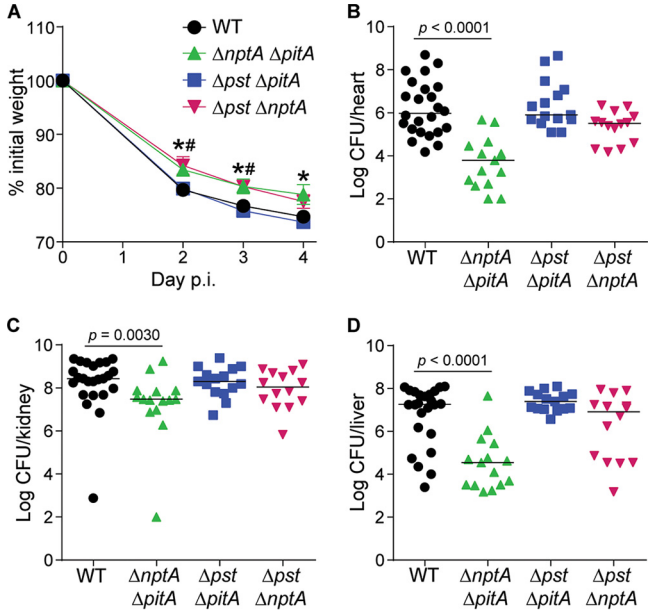

FIG 6 NptA but not PitA or Pst is sufficient to mediate virulence of *S. aureus*. Wild-type C57BL/6J mice were infected with the *S. aureus* wild type and Δ*pstSCAB* Δ*pitA*, Δ*pstSCAB* Δ*nptA*, and Δ*nptA* Δ*pitA* mutants. Weight loss was monitored (A) and bacterial burdens in the heart (B), kidneys (C), and liver (D) were enumerated 4 days postinfection. (A) #, $P < 0.05$ for the Δ*pstSCAB* Δ*nptA* mutant compared to the wild type; *, $P < 0.05$ for the Δ*nptA* Δ*pitA* mutant compared to the wild type via two-way ANOVA with Dunnett's posttest. Error bars indicate SEMs. (B to D) *P* values were determined by Mann-Whitney test; only significant *P* values are shown. The lines indicate medians. The data are results from three independent experiments. $n \geq 14$ for each group. Δ*pst*, Δ*pstSCAB*.

acquire P_i under these conditions (Fig. 5B). The PitA-dependent Δ*pstSCAB* Δ*nptA* strain was unable to be tested in basic medium with limiting P_i because it could not grow under this condition; notably, however, the ability of this strain to grow and acquire P_i could be complemented by the addition of P_i (Fig. 5C). In total, the ability of each double mutant to acquire P_i under at least one tested growth condition highlights a role for all three systems as P_i importers. In sum, these data indicate that PstSCAB, PitA, and NptA are P_i transporters that optimally promote P_i acquisition in discrete environments.

NptA is sufficient for systemic *S. aureus* infection. To evaluate the contribution of the staphylococcal P_i importers to virulence, C57BL/6 mice were infected with wild-type *S. aureus* and the transporter single mutants (the Δ*pstSCAB*, Δ*pitA*, and Δ*nptA* mutants). Similar to the growth assays, none of the single mutants had a virulence defect (Fig. S6). To determine if any transporter is sufficient for *S. aureus* pathogenesis, mice were infected with the transporter double mutants (the Δ*pstSCAB* Δ*pitA*, Δ*pstSCAB* Δ*nptA*, and Δ*nptA* Δ*pitA* mutants). Mice infected with the Δ*pstSCAB* Δ*nptA* and Δ*nptA* Δ*pitA* mutants lost less weight compared to mice infected with wild-type *S. aureus* (Fig. 6A). Significantly reduced bacterial burdens were recovered from the hearts, kidneys, and livers of mice infected with the Δ*nptA* Δ*pitA* mutant relative to those infected with wild-type *S. aureus* (Fig. 6B to D). Remarkably, the Δ*pstSCAB* Δ*pitA* mutant was as virulent as wild-type bacteria, indicating that P_i transport by NptA is sufficient to mediate *S. aureus* infection. Cumulatively, these observations highlight an important role for NptA in staphylococcal infection.

DISCUSSION

S. aureus is a versatile pathogen that can thrive in a multitude of environments, enabling it to infect virtually all host tissues. Despite the essential role of P_i in many cellular processes, the contribution of P_i transport to virulence remains unstudied for *S. aureus* and many other pathogens. In this work, we found that *S. aureus* expresses three

P_i transporters, PstSCAB, PitA, and NptA, which optimally facilitate growth and P_i acquisition in distinct environments. While none of the transporters are essential for systemic infection, NptA, a member of a conserved but sparsely studied family of bacterial P_i transporters, is the only transporter sufficient to mediate wild-type levels of staphylococcal disease. This is particularly surprising, as PstSCAB is necessary for the pathogenesis of *E. coli* and other *Enterobacteriaceae* (8). While NptA homologs are widely distributed in bacteria, within the staphylococci, NptA is present in only ~60% of staphylococcal species, including *S. aureus*. Of note, our analysis indicates that a lineage of human-associated *Staphylococcus* species that are closely related to but less pathogenic than *S. aureus* (namely, *S. epidermidis*, *S. lugdunensis*, and *S. haemolyticus*) underwent a shared loss of *nptA* but has not reacquired this gene (Fig. S1D) (31). In total, the current observations suggest that acquisition of NptA enhances the ability of *S. aureus* to obtain P_i and cause infection and may contribute to the heightened pathogenicity of this species.

The maintenance of proper cellular levels of phosphate via acquisition and regulation has been extensively characterized for *E. coli*. This has led *E. coli* to become the paradigm for bacterial phosphate homeostasis, a position borne out by virulence studies with many enterobacterial pathogens (8–12). However, P_i acquisition and homeostasis in *S. aureus* do not follow the rules of *E. coli*, as is the case for several other organisms, including *B. subtilis*, *S. pneumoniae*, *M. tuberculosis*, and *V. cholerae* (14, 17, 19, 20, 32). The most notable difference is that in addition to PitA and PstSCAB, *S. aureus* possesses a third functional P_i importer, NptA. *E. coli* strains lacking PitA and PstSCAB are incapable of growth on P_i as a phosphate source (33). Beyond this, our results suggest that *S. aureus* takes a profoundly different approach to coping with P_i limitation. Intracellular P_i concentrations of *E. coli* are thought to be quite stable, with *E. coli* experiencing only a maximal 4-fold reduction in intracellular P_i when the nutrient is limiting (21, 34). Additionally, when grown in P_i-limiting media, *E. coli* transiently accumulates high levels of polyphosphate, a storage form of P_i, during logarithmic growth (35, 36). In *S. aureus*, however, there was an approximately 7-fold reduction in the total cellular P_i concentration between *S. aureus* grown in P_i-replete versus -limiting media even after treatment with a polyphosphatase. This observation indicates that the dynamic range of intracellular P_i concentrations in *S. aureus* is larger than that of *E. coli*. Overall, these findings indicate that P_i acquisition and homeostasis in *S. aureus* differ substantially from the paradigm established by *E. coli*. This idea is further supported by the observation that disruption of the Pst structural genes in *S. aureus* does not result in constitutive expression of the P_i importers as observed in *E. coli* and other bacteria, including *M. tuberculosis* (22, 37). The presence of a *phoU* gene at the *pst* locus in *S. aureus* suggests that staphylococcal phosphate homeostasis is also distinct from that of the more closely related *B. subtilis*, which does not possess a PhoU homolog (38). Intriguingly, the presence of *phoU* genes/domains in addition to the *pst*-associated *phoU* further suggests that regulation of *S. aureus* phosphate homeostasis differs from established models. While the presence of multiple copies of *phoU* is not unique to *S. aureus*, how the presence of multiple PhoU proteins, particularly those associated with *pit* and *nptA* loci, impacts control of phosphate homeostasis is largely unknown.

In P_i-replete environments, PitA is the primary system utilized by *E. coli* to obtain this critical nutrient, with the PstSCAB system expressed in P_i-limiting environments (21, 22). While there are differences in how the two organisms handle P_i limitation, *S. aureus* utilizes a similar regulatory logic to control the expression of its P_i importers. Similar to the case with *E. coli*, in *S. aureus*, PitA appears to be the primary transporter expressed under neutral, P_i-replete conditions. In response to loss of PitA in acidic environments (Fig. 4B) or environments where the ability of PitA to transport P_i is compromised, such as in alkaline media (Fig. S5B), *S. aureus* induces the expression of *nptA* and *pstSCAB*. Interestingly, under both of these conditions, *nptA* expression reaches a value closer to its maximal induction under P_i-limiting

conditions than *pstSCAB* expression. This suggests that when PitA is unable to supply the cell with sufficient P_i, *S. aureus* may selectively induce NptA before the Pst system. How *S. aureus* would achieve this differential regulation in response to presumptively the same stimulus is unclear. An intriguing possibility is that the expanded repertoire of PhoU-like molecules enables a more nuanced approach to controlling the expression of the *S. aureus* importers.

Differing from most essential nutrients, the biologically available form of P_i is strongly influenced by pH, as well as the identity and abundance of monovalent and divalent cations. Prior biochemical analysis of PitA and NptA homologs from *E. coli*, *V. cholerae*, and other bacteria revealed that these importers optimally transport P_i in distinct environments (19, 25, 30). Our analysis demonstrates that this difference in preferred substrates expands the environments in which *S. aureus* can satiate its need for P_i. Throughout the host, the abundance of potential counterions can change; for example, kidneys are rich in monovalent ions like Na^+ and K^+. Intriguingly, Na^+ increases P_i transport rates of NptA from *V. cholerae* and rat (19, 39). As the importance of PitA and NptA is revealed in the absence of PstSCAB, the question remains, do these systems provide an advantage? In culture, when forced to rely on PstSCAB, *S. aureus* grows more slowly, and accumulates less P_i, in nutrient- and P_i-replete environments than wild-type bacteria. This suggests that relying on PstSCAB can be suboptimal. The virulence defect of the Δ*nptA* Δ*pitA* mutant suggests that a similar situation also occurs during systemic infection. In conjunction with the virulence defect of a strain forced to rely on PitA to obtain P_i, these results suggest that acquisition of NptA enhances the ability of *S. aureus* to cause infection. At the same time, the recovery of a *pitA* mutant from a screen for factors that contribute to the development of osteomyelitis indicates that in some host environments PitA is the preferred P_i importer (40). While the current studies do not provide a definitive rationale for the retention of the PstSCAB system, it seems unlikely that *S. aureus* would maintain three distinct transporters unless the bacterium encounters conditions in which each is necessary within the host. As PstSCAB is a high-affinity importer, it is tempting to speculate that it is most important for growth in environments where phosphate availability is limited, due to either reduced absolute abundance or competition with other microbes. In any case, as demonstrated by the ability of the Δ*pitA* and Δ*nptA* mutants to grow and infect mice as well as the wild type, it is clear that PstSCAB can sufficiently augment P_i uptake when either PitA or NptA is not functioning optimally. Our results suggest that the acquisition of NptA and the expression of three distinct P_i importers enhance *S. aureus* fitness by enabling optimal P_i acquisition in divergent environments.

In *S. aureus* and *S. pneumoniae*, NptA functions as a P_i importer. While PstSCAB and PitA are the only P_i importers expressed by *E. coli*, it does possess an NptA homolog, YjbB. However, overexpression studies suggest that in *E. coli* YjbB functions as a P_i exporter (41). While the physiological role of YjbB remains unclear, this observation suggests that the NptA homologs possessed by other bacteria may function as either importers or exporters. While transporters are typically unidirectional, in *E. coli* and *Metallosphaera sedula*, Pit family transporters have been demonstrated to mediate efflux of P_i (42, 43). Intriguingly, in these cases, the PitA homologs are thought to protect the bacteria from metal toxicity due to their ability to export a neutral phosphate-metal complex. Given the widespread distribution of NptA, particularly within the *Enterobacteriaceae* and other pathogenic organisms, it seems likely that homologs will be found to contribute to P_i acquisition by other virulent species. However, due to the ability of NptA and PitA homologs to function as P_i efflux pumps, the specific role of each system to the lifestyle of any given bacterium will need to be directly evaluated.

As the efficacy of current antibiotic therapies continues to decrease, infection has once again become a significant threat to human health. As both over- and underaccumulation of phosphate are detrimental to bacteria (21, 44), phosphate homeostasis

represents a regulatory network primed for therapeutic disruption. Thus, continued investigations into this aspect of staphylococcal physiology will not only expand our understanding of how bacteria obtain phosphate and control cellular levels of this essential nutrient but also provide critical insight that may facilitate the development of new approaches for combating infection.

MATERIALS AND METHODS

Ethics statement. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (IACUC license number 15059) and performed according to NIH guidelines, the Animal Welfare Act, and U.S. federal law.

Bioinformatics analysis of distribution of inorganic phosphate transporter loci. Inorganic phosphate transporters (PstSCAB, PitA, and NptA) and associated phosphate regulatory proteins (PhoU and PhoBR) of interest were identified from the literature. An initial BLASTP search using default parameters was completed with characterized representatives against the *S. aureus* strain Newman genome sequence to identify possible homologs. A detailed analysis was carried out using genomes retrieved from GenBank for ~9,000 staphylococcal genomes, representing 38 species from NCBI and several outgroups (see Data Set S1 in the supplemental material). Predicted coding sequences were searched using HMMRSCAN (--cut_tc) with TIGRFAM, PFAM, and custom hidden Markov models for 13 universally conserved proteins in addition to the P_i transporters (Table S2) (45, 46). Representatives were manually checked to validate ortholog predictions, and tBLASTn was used to confirm the absence of NptA using default search parameters. Universally conserved proteins were individually aligned with MUSCLE, concatenated, and used to generate a phylogenetic tree with RAxML on the CIPRES website using default parameters (47, 48). The distribution and genomic context of the P_i transporters were examined and mapped to the phylogeny and aligned with MAUVE (49). Predicted gains and losses of *nptA* were inferred based on parsimony.

Subsequently, the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology assignments for each of the NptA gene family (KEGG accession numbers K03324 and K14683) were retrieved for all *Bacteria* on 17 July 2017 (50). The results were compiled for one strain of each named species and counted to provide the average number of gene copies for phyla, classes, and families of interest.

Bacterial strains and cloning. *S. aureus* strain Newman and its derivatives were used for all experiments. *S. aureus* was routinely grown in tryptic soy broth (TSB) and on tryptic soy agar (TSA) plates, while *E. coli* was routinely cultivated in Luria broth (LB) and on Luria agar plates. Both species were grown at 37°C; all strains were stored in media containing 30% glycerol at −80°C. As needed to maintain plasmids, 100 μg/ml of ampicillin or 10 μg/ml of chloramphenicol was added to the growth medium. Anhydrotetracycline was added at 200 ng/ml for gene induction where indicated.

S. aureus Δ*pstSCAB*, Δ*nptA*, and Δ*pstSCAB* Δ*nptA* mutants were generated by amplifying the 5′ and 3′ flanking regions (~1 kb up- and downstream) of *pstSCAB* or *nptA* using the indicated primers (Table S3). Fragments were cloned into the pKOR1 knockout vector (51) via site-specific recombination using the Gateway BP Clonase II enzyme mix (Thermo Fisher Scientific). The deletions were constructed via allelic exchange as previously described (51). The *S. aureus* USA300 (JE2) *pitA*::*erm* allele was obtained from the Nebraska Transposon Mutant Library (52) and was transduced via Phi85 phage into Newman, Δ*pstSCAB*, and Δ*nptA* backgrounds. All mutant strains were confirmed to be hemolytic when grown on TSA blood agar plates. For complementation studies, *pstSCAB* and *nptA* were cloned into the pOS1 vector (53) under the control of their native promoters using the indicated primers (Table S3). *pitA* was cloned under the control of an anhydrotetracycline-inducible promoter into the pRMC2 vector (54) using the indicated primers (Table S3). To create the reporter constructs, the promoters of each transporter were cloned into the pAH5 vector (55) via the indicated primers (Table S3). All PCR-generated constructs were verified by sequencing.

Growth medium, phosphate growth assays, and expression analysis. Phosphate-free M9-based medium (PFM9) was based on a chemically defined medium reported by Richardson et al. (29). PFM9 salts consisted of 104 mM NaCl, 19 mM NH_4Cl, 22 mM KCl, 12.4 mM Tris base, and a 70 mM concentration of either morpholinepropanesulfonic acid (MOPS; for pH 6.4), HEPES (for pH 7.4), or Tris (for pH 8.4). PFM9 salts were supplemented with trace amino acids (0.06 g/liter of alanine, 0.07 g/liter of arginine, 0.09 g/liter of aspartate, 0.02 g/liter of cysteine, 0.1 g/liter of glutamate, 0.05 g/liter of glycine, 0.03 g/liter of histidine, 0.03 g/liter of isoleucine, 0.09 g/liter of leucine, 0.01 g/liter of lysine, 0.07 g/liter of methionine, 0.04 g/liter of phenylalanine, 0.06 g/liter of proline, 0.03 g/liter of serine, 0.03 g/liter of threonine, 0.01 g/liter of tryptophan, 0.05 g/liter of tyrosine, and 0.08 g/liter of valine), trace vitamins (0.2 μg/liter of biotin, 0.2 mg/liter of nicotinic acid, 0.2 mg/liter of pyridoxine hydrochloride, 0.2 mg/liter of thiamine hydrochloride, 0.1 mg/liter of riboflavin, and 0.6 mg/liter of calcium pantothenate), 0.5% glucose, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 6.2 mM β-mercaptoethanol, 1 μM $FeSO_4$, 1 μM $ZnSO_4$, and 1 μM $MnCl_2$ to constitute PFM9 medium. Phosphate levels in the media were adjusted using a combination of NaH_2PO_4 and Na_2HPO_4.

For phosphate limitation growth assays, bacteria were inoculated into 5 ml of TSB for 8 h and then back-diluted 1:10 into 5 ml of PFM9 plus 70 mM MOPS plus 158 μM P_i, pH 6.40, for 12 h. Overnight cultures were normalized with PFM9 plus 70 mM MOPS plus 158 μM P_i, pH 6.40, and inoculated at 1:100 into a 96-well round-bottom plate containing 100 μl/well of PFM9. 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 was determined by measuring the fluorescence (excitation/emission wavelengths, 505/535 nm), normalizing to the OD_{600}, and then subtracting the relative fluorescence units (RFU) of empty vector controls.

Phosphate accumulation assays. Bacteria were grown as for the phosphate growth assays at the indicated pH and P_i concentration and then harvested at similar optical densities (OD_{600} = 0.2 to 0.25). Cells were washed once and then lysed in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) by mechanical disruption. Lysates were centrifuged to remove particulate matter. To digest any polyphosphates, lysates were diluted 1:5 into polyphosphatase reaction buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$) and subsequently treated with yeast exopolyphosphatase for 30 min at 37°C. P_i monomers were then measured with BIOMOL Green reagent according to the manufacturer's instructions. Protein concentration was measured in untreated lysates using the Pierce bicinchoninic acid (BCA) assay kit. Recombinant His_6-tagged *Saccharomyces cerevisiae* exopolyphosphatase was produced in *E. coli* and purified as previously described (56).

Animal infections. Mouse infections were performed essentially as previously described, with minor modifications (57, 58). Briefly, the wild type and the six single and double mutant strains of *S. aureus* were grown in TSB for 3 h on a roller drum and then washed and resuspended in phosphate-free, carbonate-buffered saline and diluted to an approximate density of 10^8 CFU/ml. Nine-week-old female C57BL/6J mice were injected retro-orbitally with 10^7 CFU in 100 μl of buffer. The infection was allowed to proceed for 96 h before the mice were sacrificed. Livers, hearts, and kidneys were removed, the organs were homogenized, and bacterial burdens were determined by plating serial dilutions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00631-17.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.6 MB.

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