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BACTERIAL INFECTIONS

Disruption of Phosphate Homeostasis Sensitizes *Staphylococcus aureus* to Nutritional Immunity

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ABSTRACT To control infection, mammals actively withhold essential nutrients, including the transition metal manganese, by a process termed nutritional immunity. A critical component of this host response is the manganese-chelating protein calprotectin. While many bacterial mechanisms for overcoming nutritional immunity have been identified, the intersection between metal starvation and other essential inorganic nutrients has not been investigated. Here, we report that overexpression of an operon encoding a highly conserved inorganic phosphate importer, PstSCAB, increases the sensitivity of *Staphylococcus aureus* to calprotectin-mediated manganese sequestration. Further analysis revealed that overexpression of *pstSCAB* does not disrupt manganese acquisition or result in overaccumulation of phosphate by *S. aureus*. However, it does reduce the ability of *S. aureus* to grow in phosphate-replete defined medium. Overexpression of *pstSCAB* does not aberrantly activate the phosphate-responsive two-component system PhoPR, nor was this two-component system required for sensitivity to manganese starvation. In a mouse model of systemic staphylococcal disease, a *pstSCAB*-overexpressing strain is significantly attenuated compared to wild-type *S. aureus*. This defect is partially reversed in a calprotectin-deficient mouse, in which manganese is more readily available. Given that expression of *pstSCAB* is regulated by PhoPR, these findings suggest that overactivation of PhoPR would diminish the ability of *S. aureus* to resist nutritional immunity and cause infection. As PhoPR is also necessary for bacterial virulence, these findings imply that phosphate homeostasis represents a critical regulatory node whose activity must be precisely controlled in order for *S. aureus* and other pathogens to cause infection.

KEYWORDS *Staphylococcus aureus*, phosphate homeostasis, PstSCAB, manganese, calprotectin, nutritional immunity, infection

Staphylococcus aureus is a pervasive and versatile pathogen. It is carried asymptomatically by ~30% of the population, but it causes a wide variety of diseases once it crosses the epithelial barrier, including endocarditis, osteomyelitis, and bacteremia (1–3). *S. aureus* is adept at averting protective immune responses (4, 5), and isolates of *S. aureus* are increasingly resistant to antibiotics, complicating prevention and treatment of staphylococcal infection (6–8). For these reasons, agencies that include the Centers for Disease Control and Prevention and the World Health Organization have designated *S. aureus* a serious threat to human health and have called for new strategies to combat this pathogen (9, 10). The ability of *S. aureus* to infect any tissue in the host indicates that it can thrive in diverse and often dynamic environments.

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Understanding how this pathogen adapts to host niches and avoids immune insult provides us with the potential to identify new opportunities for therapeutic intervention.

To inhibit the growth of invading pathogens, the host withholds essential nutrients, a process called nutritional immunity (11). The best-studied example of this defense is the sequestration of transition metals, such as iron (Fe), zinc (Zn), and manganese (Mn), which are required for all forms of life (12–14). An important factor that mediates this response is the transition metal-binding immune effector calprotectin (CP) (11). One of the most abundant immune proteins present at sites of infection, CP concentrations can exceed 1 mg/ml (15, 16). Mice lacking CP have defects in Mn sequestration during staphylococcal infection and are more sensitive to infection by a variety of Gram-positive, Gram-negative, and fungal organisms, including *S. aureus* (17–22). CP is a heterodimer of S100A8 and S100A9 and binds transition metals at two binding sites. Site 1 binds Mn, Fe, or Zn with nanomolar to picomolar affinities, while site 2 binds Zn with subpicomolar affinity (18, 23–25). This prevents *S. aureus* and other pathogens from obtaining Mn, Zn, and potentially other metals during infection (17, 18, 26–28).

S. aureus and other pathogens express multiple Mn and Zn transporters in order to compete with the host for these metals (11, 20, 29–35). However, while these systems are necessary for *S. aureus* and other pathogens to cause infection, they do not prevent the host from imposing metal starvation (17, 26–30, 36). Thus, in addition to transporters, other adaptations are needed to cause infection. In *S. aureus*, this includes expression of a superoxide dismutase that can use either Mn or Fe and the rerouting of metabolism away from glucose consumption, decreasing the cellular demand for Mn (37–39). Similar strategies have also been described in other pathogens (28, 39–42). While many strategies for overcoming nutritional immunity have been reported, the intersection between metal starvation and other inorganic nutrients has not been explored.

In addition to transition metals, bacteria must also obtain other inorganic nutrients, such as phosphate, from the host during infection. *S. aureus* possesses three inorganic phosphate (P_i) importers: PstSCAB, NptA, and PitA. Widely conserved among bacteria, PstSCAB has a high affinity and specificity for P_i and is a member of the ATP-binding cassette (ABC) family of transporters, which require ATP hydrolysis for function and have a high affinity and specificity for P_i (43–45). In *S. aureus*, *pstSCAB* is only expressed in P_i-limiting environments, where it contributes to optimal growth and P_i acquisition (46). NptA and PitA are proton motive force-driven P_i transporters that are constitutively expressed in P_i-replete media, but expression of both transporters also increases upon P_i limitation (44, 46, 47). Expression of the three staphylococcal P_i transporters is regulated by the broadly distributed P_i-responsive two-component system PhoPR (named PhoBR in *Escherichia coli*). PhoPR is required for expression of *pstSCAB* and *nptA* and can influence expression of *pitA* in some conditions (48). Aside from its role in controlling P_i transporter expression, PhoPR also mediates a P_i-sparing response by inducing as-yet-unidentified processes that promote *S. aureus* growth during P_i starvation (48). Similarly to other pathogens, PhoPR is critical during systemic staphylococcal infection (48–50).

The current studies reveal that overexpression of *pstSCAB* sensitizes *S. aureus* to CP-mediated Mn sequestration. This decreased ability to resist metal starvation is not due to altered total cellular Mn or phosphate levels. While the phenotype is independent of PhoPR, as *pstSCAB* expression is dependent on this two-component system, these observations suggest that disrupting phosphate homeostasis diminishes the ability of *S. aureus* to resist Mn starvation. Overexpression of *pstSCAB* attenuates staphylococcal virulence in a systemic mouse model of disease, and the attenuation is partially relieved in a CP-deficient mouse. Thus, the current investigations reveal a previously unappreciated intersection between phosphate homeostasis and resisting nutritional immunity. As loss of PhoPR also attenuates staphylococcal virulence, the current studies underscore the critical importance of maintaining proper regulation of phosphate homeostasis to infection.

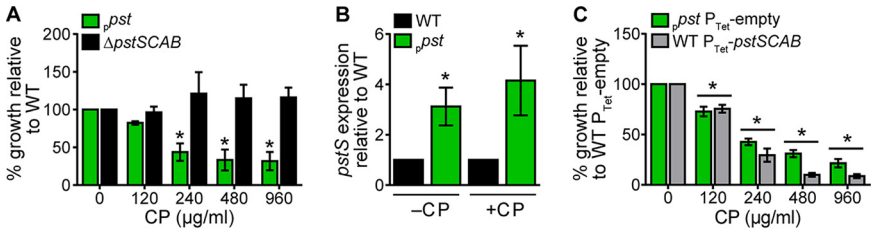

FIG 1 Overexpression of the ABC family P_i transporter PstSCAB increases the sensitivity of *S. aureus* to CP. (A) CP growth assays were performed in rich medium and growth (optical density at 600 nm [OD_{600}]) of the indicated strains after 10 h was normalized first to untreated and then to wild-type (WT) bacteria. *, $P < 0.05$ compared to WT by two-way analysis of variance (ANOVA) with Tukey's multiple-comparison test. $n = 3$; error bars indicate standard error of the mean (SEM). (B) Transcript levels of *pstSCAB* were assessed via quantitative reverse transcription-PCR (qRT-PCR) following growth of WT *S. aureus* and the $_p$*pst* mutant in the absence or presence of 120 μg/ml of CP. *, $P < 0.05$ compared to WT by *t* test. $n = 5$ biological replicates; error bars indicate SEM. (C) CP growth assays were performed in rich medium and growth (OD_{600}) of the indicated strains after 10 h was normalized first to untreated bacteria and then to WT carrying empty vector. Anhydrotetracycline (25 ng/ml) was added to induce expression of *pstSCAB*. *, $P < 0.05$ compared to WT carrying empty vector by two-way ANOVA with Tukey's multiple-comparison test. $n = 3$ biological replicates; error bars indicate SEM. See also Fig. S1 in the supplemental material. For the experiments shown in panels A and C, similar results were observed at other time points (data not shown).

RESULTS

Overexpression of a high-affinity P_i transporter increases the susceptibility of *S. aureus* to calprotectin. To identify adaptations that allow *S. aureus* to overcome host-imposed metal starvation, a nonsaturating transposon library was previously screened for mutants less resistant to CP. This analysis identified a mutant with an insertion in the promoter of the *pstSCAB* operon (see Fig. S1 in the supplemental material), subsequently referred to as the $_p$*pst* mutant. The $_p$*pst*::*erm* allele was subsequently transduced into a fresh *S. aureus* background, where it also resulted in a growth defect compared to the wild-type in the presence of CP when growth was assessed by either optical density or CFU (Fig. 1A and Fig. S1). Surprisingly, the $_p$*pst* phenotype was not recapitulated in a strain lacking *pstSCAB*, suggesting that the transposon insertion does not lead to loss of *pstSCAB* expression (Fig. 1A). While surprising, this observation is consistent with the expectation that *pstSCAB* would not be expressed in P_i-replete media (23, 48). Next, the possibility that *pstSCAB* was overexpressed in the $_p$*pst* mutant was evaluated. In the $_p$*pst* mutant, expression of *pstSCAB* was approximately 2- and 4-fold higher than that of wild-type bacteria in the absence and presence of CP, respectively (Fig. 1B). To evaluate if overexpression of *pstSCAB* explains the $_p$*pst* phenotype, *pstSCAB* was expressed from a plasmid under the control of an anhydrotetracycline-inducible promoter (P_{Tet}-*pstSCAB*). Overexpression of *pstSCAB* in wild-type bacteria sensitized *S. aureus* to CP, similarly to the $_p$*pst* mutant, without reducing growth of the bacteria in the absence of CP (Fig. 1C and Fig. S1). Cumulatively, these observations suggest that aberrant overexpression of *pstSCAB*, not its loss, sensitizes *S. aureus* to CP.

Overexpression of *pstSCAB* sensitizes *S. aureus* to manganese starvation. CP can bind multiple first-row transition metals, but in the standard tryptic soy broth (TSB)-based medium used to assess staphylococcal sensitivity, Mn, and to some extent Zn, sequestration drives the antimicrobial activity (26, 36, 38). Therefore, the growth of strains overexpressing *pstSCAB* was assessed in the presence of CP variants lacking either site 1 (which binds both Mn and Zn) or site 2 (binds Zn). In the presence of the site 1 mutant that can no longer bind Mn, the increased sensitivity of the $_p$*pst* mutant was largely abolished (Fig. 2A). The P_{Tet}-*pstSCAB*-possessing strain was more sensitive than wild-type bacteria, raising the possibility that sufficient overexpression of PstSCAB could reduce the ability of *S. aureus* to cope generally with metal limitation. In the presence of the site 2 mutant that retains its ability to bind Mn, both strains remained more sensitive than the wild type to CP (Fig. 2A). To further test if inability to resist Mn limitation was responsible for the phenotype, *pstSCAB* was overexpressed in the Δ*mntCH* strain, which lacks functional copies of the two known staphylococcal Mn

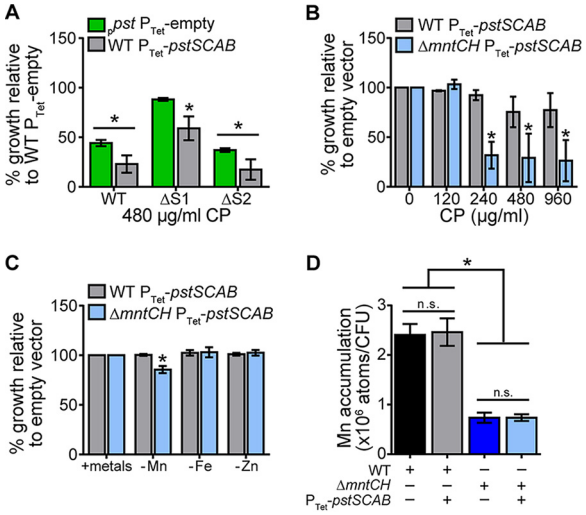

FIG 2 Overexpressing *pstSCAB* sensitizes *S. aureus* to Mn limitation without reducing Mn acquisition via MntABC and MntH. (A) Growth (OD_{600}) of the indicated strains was normalized to that of the wild type (WT) carrying empty vector after 10 h in rich medium in the presence of 480 μg/ml of wild-type CP and its ΔS1 or ΔS2 site mutants. *, $P < 0.05$ compared to WT carrying empty vector by two-way ANOVA with Tukey's multiple-comparison test. $n = 7$ biological replicates; error bars indicate SEM. Anhydrotetracycline (25 ng/ml) was added to induce *pstSCAB* expression. (B) A CP growth assay was performed in rich medium in the presence of increasing CP concentrations. Growth (OD_{600}) of the indicated strains was normalized first to untreated bacteria and then to the parent strain carrying empty vector after 10 h. *, $P < 0.05$ compared to parent carrying empty vector by two-way ANOVA with Tukey's multiple-comparison test. (C) Growth (OD_{600}) of the indicated strains in divalent metal-defined medium with or without 25 μM $MnCl_2$, $FeSO_4$, or $ZnSO_4$ was measured after 7 h. *, $P < 0.05$ compared to WT P_{Tet}-*pstSCAB* by two-way ANOVA with Sidak's multiple-comparison test. (D) The indicated strains were grown in divalent metal-defined medium without added Mn, and cellular metal content was measured using inductively coupled plasma mass spectrometry (ICP-MS). *, $P > 0.05$ comparing Δ*mntCH* strains to WT strains by unpaired *t* test. (B to D) Anhydrotetracycline (12.5 ng/ml) was added to induce *pstSCAB* expression. $n = 3$ biological replicates; error bars indicate SEM. (A to C) Similar results were observed at other time points (data not shown).

transporters, the NRAMP family transporter MntH, and the ABC permease MntABC (29). The Δ*mntCH* P_{Tet}-*pstSCAB* strain was more sensitive than the Δ*mntCH* mutant with an empty vector to CP (Fig. 2B). Cumulatively, these results indicate that strains overexpressing *pstSCAB* are significantly more sensitive to CP-mediated Mn sequestration.

Manganese accumulation is not disrupted by *pstSCAB* overexpression. The enhanced sensitivity of the $_{p}$*pst* mutant to Mn limitation could potentially be explained by a defect in Mn accumulation. To evaluate this possibility, Mn levels were assessed in wild-type *S. aureus* and in the Δ*mntCH* mutant overexpressing *pstSCAB*. Consistent with its enhanced sensitivity to CP-mediated Mn depletion, the Δ*mntCH* mutant overexpressing *pstSCAB* had a growth defect in this medium when Mn, but not Zn or Fe, was omitted (Fig. 2C). Under the Mn-depleted growth conditions, inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the total Mn content of the strains. Consistent with previous reports (29, 36), the Δ*mntCH* mutant accumulated less Mn than did the wild type, regardless of *pstSCAB* expression (Fig. 2D). However, overexpression of *pstSCAB* did not impact Mn levels in either the wild-type or Δ*mntCH* mutant backgrounds, indicating that *pstSCAB* overexpression does not affect accumulation of Mn (Fig. 2D).

The sensitivity of *pstSCAB*-overexpressing strains is independent of PhoPR activation. In the absence of an apparent defect in Mn uptake, the possibility that overexpression of *pstSCAB* was altering the activity of PhoPR and phosphate homeostasis was evaluated. Expression of *pstSCAB* is dependent on PhoPR in wild-type bacteria. It therefore follows that transcriptional activity of a wild-type copy of the *pstSCAB* promoter can be used to assess PhoPR activation (48). Using a reporter plasmid (pP_{pstS}-*yfp*) in which *yfp* expression is controlled by a wild-type copy of the *pstSCAB*

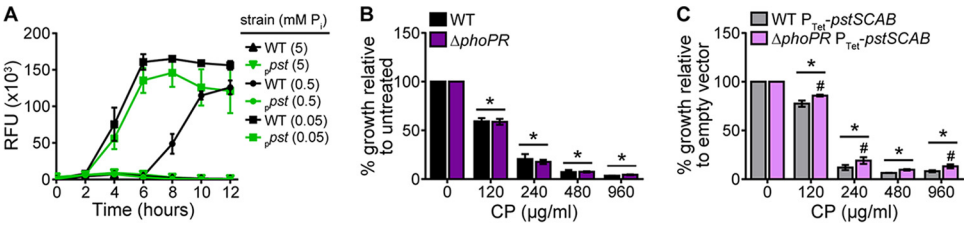

FIG 3 The Mn starvation sensitivity associated with *pstSCAB* overexpression is independent of PhoPR activation. (A) Expression of *pstS* as a marker for PhoPR activation was monitored in the wild type (WT) and the $_p$*pst* mutant during growth in phosphate-defined medium supplemented with 5 mM (excess), 0.5 mM (intermediate), or 0.05 mM (limiting) P_i. Expression (relative fluorescence units [RFU]) was assessed by measuring fluorescence from the pP_{pstS}-*yfp* reporter plasmid, normalizing to optical density (OD_{600}), and subtracting empty vector (pP_{empty}-*yfp*) values. (B) *S. aureus* WT and Δ*phoPR* were grown in the presence of CP in rich medium, and optical density (OD_{600}) was assessed after 7 h. *, $P < 0.05$ compared to untreated parent by two-way ANOVA with Sidak's multiple-comparison test; no significant differences were measured between WT and Δ*phoPR*. (C) A CP growth assay was performed in rich medium in the presence of increasing CP concentrations. Growth (OD_{600}) of the indicated strains was normalized first to untreated bacteria and then to the parent strain carrying empty vector after 10 h. *, $P < 0.05$ compared to parent carrying empty vector; #, $P < 0.05$ Δ*phoPR* P_{Tet}-*pstSCAB* compared to WT P_{Tet}-*pstSCAB* (two-way ANOVA with Tukey's multiple-comparison test). (A to C) $n = 3$ biological replicates; error bars indicate SEM. (B and C) Similar results we observed for other time points (data not shown).

promoter (48), activity of PhoPR was assessed in wild-type bacteria and in the $_p$*pst* mutant when grown in medium containing high, intermediate, and low concentrations of P_i. Consistent with prior results (48), *pstS* expression is undetectable in wild-type bacteria under P_i-replete conditions (5 mM P_i) and is highly induced under P_i-limiting conditions (0.05 mM P_i) (Fig. 3A). A similar expression pattern was seen in the $_p$*pst* mutant, suggesting that overexpression of the *pst* operon does not result in inappropriate activation of PhoPR (Fig. 3A). However, at an intermediate P_i concentration (0.5 mM P_i), while *pstS* expression in the wild type was activated after several hours of growth (presumably because P_i was being depleted from the medium), *pstS* was not induced in the $_p$*pst* mutant over the course of the growth curve (Fig. 3A). This raised the possibility that altered activation of PhoPR could explain the enhanced sensitivity of the $_p$*pst* mutant. However, a Δ*phoPR* mutant was not more sensitive than wild-type *S. aureus* to CP (Fig. 3B), suggesting that reduced activity of the system was not responsible for the increased sensitivity of the $_p$*pst* mutant to metal starvation. Additionally, loss of PhoPR did not prevent overexpression of *pstSCAB* from sensitizing *S. aureus* to CP (Fig. 3C). Together, these data indicate that while PhoPR activity is diminished in strains overexpressing *pstSCAB*, enhanced sensitivity to metal starvation is not dependent on PhoPR.

Overexpression of *pstSCAB* sensitizes *S. aureus* to elevated P_i levels but does not result in P_i overaccumulation. The diminished activity of PhoPR in the $_p$*pst* mutant raised the possibility that *pstSCAB* overexpression results in overaccumulation of P_i. To test this possibility, the wild type and the $_p$*pst* mutant were grown in P_i-replete (5 mM) and P_i-depleted (0.05 mM) defined medium. In P_i-limited medium, the $_p$*pst* mutant grew similar to the wild type, reaching nearly the same terminal optical density at 600 nm (OD_{600}) (Fig. 4A). However, in P_i-replete medium, the $_p$*pst* mutant had a severe growth defect compared to the wild type (Fig. 4A). Wild-type bacteria expressing *pstSCAB* from a plasmid also had a growth defect in 5 mM P_i but not in 0.05 mM P_i (Fig. 4B). These results suggest that P_i overaccumulation could potentially be responsible for the sensitivity to CP. To assess if *pstSCAB*-overexpression resulted in P_i overaccumulation in CP assay medium, total cell P_i was assessed. However, neither the $_p$*pst* mutant carrying empty vector nor the wild type carrying P_{Tet}-*pstSCAB* accumulated more P_i than that accumulated by the wild type with empty vector, even at inducer levels that resulted in Mn starvation phenotypes (Fig. 4C). Total cell-associated phosphorous was also assessed using ICP-MS following growth in the cation-defined P_i-replete medium previously used to assess Mn accumulation. Similarly to the P_i accumulation assays, no difference in cell-associated phosphorous was observed (Fig. 4D). Cumulatively, these

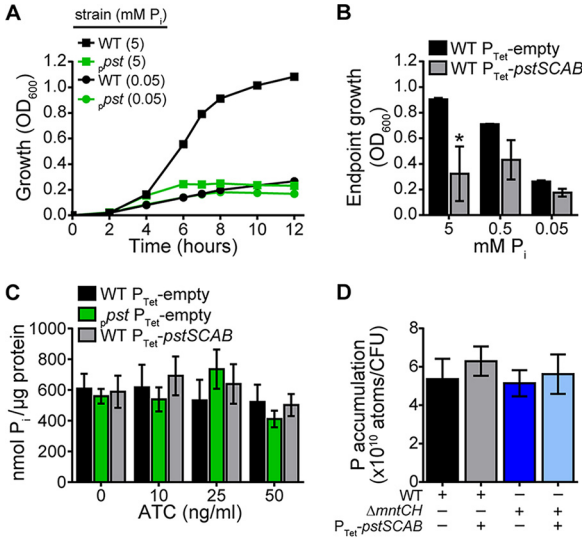

FIG 4 *pstSCAB*-overexpressing cells overimport but do not overaccumulate P_i. (A) Growth (OD_{600}) of wild type (WT) and the $_p$*pst* mutant in phosphate-defined medium supplemented with 5 mM (excess) or 0.05 mM (limiting) P_i. $n = 3$ biological replicates; error bars indicate SEM and are often smaller than the symbols. (B) Growth (OD_{600}) after 12 h of the indicated strains in phosphate-defined medium supplemented with 5 mM (excess), 0.5 mM (intermediate), or 0.05 mM (limiting) P_i. Anhydrotetracycline (200 ng/ml) was added to induce *pstSCAB* expression. *, $P < 0.05$ compared to empty vector by two-way ANOVA with Sidak's multiple-comparison test. $n = 3$; error bars indicate SEM. (C) Intracellular P_i accumulation was measured and normalized to protein content in the indicated strains grown in CP assay medium supplemented with increasing concentrations of anhydrotetracycline (ATC) to induce *pstSCAB* expression. $n = 6$ biological replicates; error bars indicate SEM. No significant differences were found between strains by two-way ANOVA. (D) Total cell-associated P was measured via ICP-MS in the indicated strains grown in divalent metal-defined medium with or without 25 μM $MnCl_2$. Anhydrotetracycline (12.5 ng/ml) was added to induce *pstSCAB* expression. No statistical differences were observed between the indicated strains via unpaired *t* test. $n = 3$ biological replicates; error bars indicate SEM.

results suggest that overaccumulation of P_i is unlikely to explain the diminished ability of *pstSCAB*-overexpressing strains to resist Mn starvation.

Overexpression of *pstSCAB* increases the sensitivity of *S. aureus* to manganese starvation during infection. To determine if aberrant *pstSCAB* overexpression disrupts the ability of *S. aureus* to cause disease, wild-type C57BL/6 mice were retro-orbitally infected with wild-type *S. aureus* or the $_p$*pst* mutant. Over the course of 4 days, mice infected with the $_p$*pst* mutant lost less weight than those infected with wild-type *S. aureus* (Fig. 5A). After 4 days of infection, fewer bacteria were recovered from the kidneys and livers of mice infected with the $_p$*pst* mutant than from those infected with the wild type (Fig. 5B and C). Taken together, these data show that overexpression of

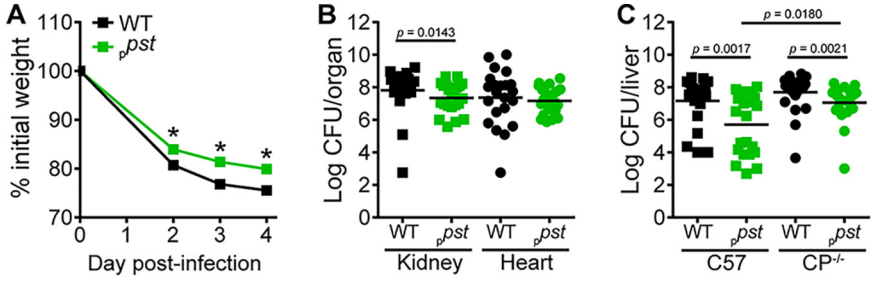

FIG 5 Overexpression of *pstSCAB* decreases the virulence of *S. aureus* and renders it more sensitive to nutritional immunity. Wild-type C57BL/6 (C57) and CP-deficient C57BL/6 S100A9$^{-/-}$ (CP$^{-/-}$) mice were retro-orbitally infected with *S. aureus* wild type (WT) or the $_p$*pst* mutant. (A) Average weight loss of wild-type mice was monitored over the course of infection. *, $P < 0.05$ via two-way ANOVA with Sidak's multiple-comparison test. (B and C) Bacterial burdens in (B) heart and kidneys and (C) liver were enumerated 4 days postinfection. *P* values were determined by the Mann-Whitney test. The lines indicate means. The data are results from two independent infections.

pstSCAB reduces the ability of *S. aureus* to cause infection. To assess whether maintaining appropriate expression levels of *pstSCAB* is important for resisting host-imposed Mn limitation, CP-deficient mice (C57BL/6 S100A9$^{-/-}$), which fail to sequester Mn away from staphylococcal liver abscesses, were infected. In mice lacking CP (CP$^{-/-}$), the $_{P}$*pst* mutant had increased bacterial burdens relative to those of wild-type mice (Fig. 5C). However, the bacterial burdens remained lower than those recovered from CP-deficient mice infected with wild-type *S. aureus*. These data suggest that the virulence defect associated with *pstSCAB* overexpression is due, at least in part, to an inability to cope with Mn starvation.

DISCUSSION

During infection, pathogens must obtain all inorganic nutrients from the host (11, 12). As a result, pathogens express an array of importers dedicated to the uptake of nutrients, including metals and P_i. While essential, these nutrients can also be toxic when consumed in excess (51–54). Therefore, the expression of nutrient importers is carefully controlled. In the current work, investigation of how *S. aureus* overcomes host-imposed Mn and Zn limitation revealed an unexpected link with phosphate homeostasis. Specifically, overexpression of the P_i transporter PstSCAB sensitizes *S. aureus* to Mn starvation both in culture and during infection. A few connections between phosphate and metal homeostasis have been made in a variety of organisms. In the pathogen *Edwardsiella tarda*, expression of two virulence-associated secretion systems is coregulated by P_i and Fe availability, with maximum expression achieved under conditions when both nutrients are limiting, such as would be encountered in the intestine (55). In *Candida albicans* and *Saccharomyces cerevisiae*, perturbation of intracellular P_i levels results in significant changes to cellular metal content (56, 57). In a more direct sense, P_i and inorganic polyphosphate interact with and can effectively sequester intracellular metals (58, 59), a function that has been demonstrated to mitigate heavy metal toxicity in diverse organisms (60). However, to our knowledge, this is the first report describing an intersection between phosphate homeostasis and resistance to nutritional immunity.

Previously, mutations that disrupt phosphate homeostasis in *S. aureus* have been shown to result in increased P_i accumulation (61). Surprisingly, the current investigations found that overexpressing *pstSCAB* does not increase total cell-associated phosphate or the fraction of which that is P_i. This suggests that either increased expression of *pstSCAB* does not increase P_i uptake or that P_i exits *pstSCAB*-overexpressing cells at a similar rate to that of its entry. Biochemical characterization of the P_i transporters PitA and NptA in other organisms, including *E. coli*, has shown that both transporters are capable of mediating P_i efflux from the cell in addition to P_i import (62–65). Considering the observation that overexpression of *pstSCAB* sensitizes *S. aureus* to elevated levels of P_i, the data cumulatively suggest that the second possibility is more likely. An alternative, but related, possibility is that the import activity of PitA or NptA is reduced in response to increased expression of PstSCAB.

PstSCAB is not required for systemic *S. aureus* infection, and indeed, a strain forced to rely on PstSCAB is attenuated compared to the wild type and strains dependent on NptA or PitA (46). While this observation suggests that P_i is not limiting during systemic infection, subsequent experiments revealed that the loss of PhoPR results in a substantial virulence defect (48), indicating that *S. aureus* contends with P_i limitation in the host. As *pstSCAB* is regulated by PhoPR, overactivation of this two-component system would lead to overexpression of *pstSCAB* (48). In conjunction with the current observations, this suggests that overactivation of PhoPR, in addition to loss, should reduce the ability of *S. aureus* to cause infection. Supporting this idea, aberrantly high or low activity of PhoPR also attenuates the virulence of other organisms, including *E. coli*, *Vibrio cholerae*, and *Mycobacterium tuberculosis* (50, 66–68).

PitA requires a divalent cation to translocate P_i across the membrane (62), and *E. coli* has been shown to leverage this requirement to export toxic amounts of metal (69). One can therefore envision a model in which excess P_i imported through PstSCAB is

TABLE 1 Strains used in this study

Strain	Description	Source or reference
Newman	Wild-type *S. aureus*	
$_{P}$*pst*	$_{P}$*pst*::*erm* allele phage-transduced into Newman	This study
Δ*pstSCAB*	Clean deletion of *pstSCAB* in Newman	46
Δ*mntCH*	Clean deletions of *mntC* and *mntH* in Newman	29
Δ*phoPR*	Clean deletion of *phoPR* in Newman	48

exported via PitA, resulting in a "P_i cycling" phenomenon. Since P_i chelates Mn intracellularly and PitA will transport Mn-P_i complexes (62), it is possible that such P_i cycling could deplete cells of Mn when the availability of this metal is restricted. However, our ICP-MS analyses indicate that both the wild type and the Δ*mntCH* mutant overexpressing *pstSCAB* contain similar Mn levels as empty vector controls. This suggests that the Mn starvation phenotype is not due to Mn export or to a failure to obtain this metal. The observation that PhoPR activation is delayed in the $_{P}$*pst* mutant suggests that PstSCAB may be contributing more to P_i uptake in the mutant than in wild-type bacteria. The delayed activation of PhoPR is likely an indirect effect of elevated expression, as expression would enable the $_{P}$*pst* mutant to obtain phosphate more efficiently than wild-type bacteria as the availability of this nutrient became limiting. The $_{P}$*pst* mutant thus would have a reduced need to activate PhoPR. When phosphate is replete, *S. aureus* normally relies on PitA and NptA to obtain this nutrient (46). This leads to the hypothesis that the increased use of a more energy-intensive mechanism to obtain P_i, PstSCAB, in the $_{P}$*pst* mutant, while not normally detrimental, becomes so under metal-restricted conditions. This idea is supported by prior observations that forcing *S. aureus* to rely solely on PstSCAB to obtain P_i reduces the growth rate (46) and that Mn starvation reduces the ability of *S. aureus* to use glucose as its preferred carbon source (38). Although the specific mechanism remains unclear, the current results demonstrate that overexpression of *pstSCAB* sensitizes *S. aureus* to nutritional immunity.

All pathogenic organisms must balance expression of nutrient transporters to optimize acquisition and mitigate toxicity. Mutations that diminish phosphate acquisition, eliminate the ability to respond to phosphate limitation, or result in overexpression of phosphate importers are deleterious to the ability of *S. aureus* and other pathogens to cause infection (46, 48, 50, 54, 66–68). Many biological systems have a high degree of plasticity that enables adaptive evolution to preserve function, which reduces their ability to be disrupted by therapeutic interventions. Unlike other systems, phosphate homeostasis appears more rigid, as either over- or underactivation of PhoPR ablates staphylococcal virulence. This reduced plasticity suggests that adaptive evolution to drugs targeting this two-component system or phosphate homeostasis in general would be more difficult to achieve. Thus, targeting phosphate homeostasis in pathogenic bacteria may represent a new opportunity for therapeutic development.

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 18038) and performed according to NIH guidelines, the Animal Welfare Act, and U.S. federal law.

Bacterial strains and cloning. *S. aureus* Newman and its derivatives were used for all experiments. For routine *S. aureus* cultures, strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA). For routine *E. coli* cultures, strains were grown in Luria-Bertani (LB) broth or on LB agar. Bacteria were grown at 37°C and preserved at −80°C in growth medium containing 30% glycerol. For plasmid propagation, 100 μg/ml ampicillin or 10 μg/ml chloramphenicol was included as needed. Anhydrotetracycline (ATC) was purchased from Acros Organics, resuspended in ethanol at 1 mg/ml, and stored at −20°C. A working stock (100 μg/ml) was made fresh daily by diluting in TSB.

The $_{P}$*pst*::*erm* allele was transduced into Newman via Φ85 phage. The Δ*pstSCAB*, Δ*mntCH*, and Δ*phoPR* mutants were constructed previously (18, 46, 48). The P_{Tet}-*pstSCAB* construct was generated previously (48) in the pRMC2 vector (70). The pP_{empty}-*yfp* and pP_{pstS}-*yfp* plasmids were generated previously (37, 46) in the pAH5 vector (71). All strains were confirmed to be hemolytic. Lists of the strains and plasmids used in these studies are given in Tables 1 and 2.

TABLE 2 Plasmids used in this study

Plasmid	Description	Reference
P_{Tet}-empty	pRMC2 (anhydrotetracycline-inducible expression vector)	70
P_{Tet}-*pstSCAB*	*pstSCAB* in pRMC2	48
pP_{empty}-*yfp*	YFP transcriptional reporter vector (pAH5E) with no promoter driving *yfp* expression	37
pP_{pstS}-*yfp*	pAH5E with *pstS* promoter driving *yfp* expression	46

CP growth assays. CP growth assays were performed as previously described (18, 23) with minor modifications. Bacteria were grown overnight in TSB, subcultured 1:50 into TSB plus the indicated concentration of ATC, and grown on a roller drum at 37°C for 1 or 2 h (strains without and with plasmids, respectively), then subcultured 1:100 into 96-well round-bottom plates containing 100 μl CP assay medium and a gradient of wild-type or site mutant CP. CP assay medium consisted of 38% TSB and 62% CP buffer (20 mM Tris [pH 7.5], 10 mM β-mercaptoethanol, 100 mM NaCl, and 3 mM $CaCl_2$) supplemented with 1 μM $MnCl_2$, 1 μM $ZnSO_4$, and the indicated concentration of ATC. Plates were incubated at 37°C with shaking at 180 rpm. Growth was monitored by measuring OD_{600} or CFU. CP was purified as previously described (18, 23).

Metal- and phosphate-defined growth assays. For metal-defined growth assays, bacteria were grown overnight in TSB, subcultured 1:50 into TSB supplemented with 12.5 ng/ml ATC, and grown on a roller drum at 37°C for 2 h, and then subcultured 1:100 into 96-well round-bottomed plates containing 100 μl of assay medium (Chelex-treated RPMI supplemented with 1% Casamino Acids, 1 mM $MgCl_2$, and 100 μM $CaCl_2$) supplemented with 12.5 ng/ml ATC and, as indicated, 25 μM $FeSO_4$, $ZnSO_4$, and/or $MnCl_2$.

For phosphate-defined growth assays, bacteria were grown in 5 ml TSB for 8 h, subcultured 1:10 into PFM9 medium plus 158 μM P_i for 12 h, and then inoculated 1:100 into a 96-well round-bottomed plate containing 100 μl/well PFM9 and a gradient of P_i. PFM9 medium (described previously in reference 46) consisted of PFM9 salts; 70 mM HEPES, pH 7.4; 0.5% glucose; trace amino acids and vitamins; 6.2 mM β-mercaptoethanol; 2 mM $MgSO_4$; 1 mM $CaCl_2$; and 1 μM $FeSO_4$, $ZnSO_4$, and $MnCl_2$. The P_i source was a mixture of NaH_2PO_4 and Na_2HPO_4 adjusted to pH 7.4. Plates were incubated at 37°C with shaking at 180 rpm. Growth was monitored by measuring OD_{600}. Expression was determined by normalizing fluorescence (excitation/emission wavelengths, 505/535) to OD_{600}, then subtracting empty vector controls.

Quantitative PCR. To assess the expression of *pstS*, *S. aureus* wild-type and $_p$*pst* mutant cultures were grown as for CP growth assays in complex medium in the presence and absence of 120 μg/ml of CP, with the exception that no ATC was added. Bacteria were harvested during log-phase growth (OD_{600} = 0.1 to 0.15), the samples were collected, an equal volume of ice-cold 1:1 acetone-ethanol was then added to the cultures, and samples were frozen at −80°C until RNA extraction. RNA was extracted and cDNA was generated as previously described (29, 72–74). Gene expression was assessed by quantitative reverse transcription-PCR (qRT-PCR) using the indicated primers (pstS RT 5′, AATGGGCTCAAGATCACTCG; pstS RT 3′, GGTCTAGAAGCATCAGCGAAG), and 16S was used as a normalizing control.

Phosphate accumulation assay. Bacteria were grown as for CP assays with the indicated concentrations of ATC, with the exception that no ATC was added to the preculture and no CP was added to the assay. Phosphate accumulation was then measured as previously described (46, 48). Briefly, cells were harvested at similar optical densities (OD_{600} between 0.2 and 0.25), then washed once and lysed in Tris-EDTA buffer by mechanical disruption. Lysates were centrifuged to remove particulates and treated with purified yeast exopolyphosphatase to degrade any polyphosphates. Orthophosphate was measured using the Biomol Green kit (Enzo Life Sciences) according to the manufacturer's instructions. An aliquot was taken from each lysate before treatment with yeast exopolyphosphatase to measure protein concentration using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

Inductively coupled plasma mass spectrometry. For elemental analysis, bacteria were grown overnight in TSB, subcultured 1:50 into TSB supplemented with 12.5 ng/ml ATC, grown for 2 h at 37°C on a roller drum, and then inoculated 1:100 into conical tubes containing 30 ml of assay medium (Chelex-treated RPMI supplemented with 1% Casamino Acids, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 25 μM $FeSO_4$, and 25 μM $ZnSO_4$) supplemented with 12.5 ng/ml ATC. Bacteria were harvested at an OD_{600} of ~0.3, then washed twice with 100 mM EDTA and twice with double-distilled water. Pellets were subsequently resuspended in 1 ml double-distilled water, and a small aliquot was taken to measure CFU. Cells were again pelleted, the supernatant removed, and the pellet was desiccated overnight on a heat block at 96°C. Metal ion content was released by treatment with 1 ml of 7% HNO_3 at 370 K for 60 min. Metal content was analyzed on an Agilent 8900 ICP tandem mass spectrometry (ICP-MS/MS) instrument (75, 76). Bacterial growth for ICP-MS analyses was performed with two technical replicates in three independent biological experiments.

Animal infections. Mouse infections were performed essentially as previously described (46, 48), with key details and differences noted below. Ten-week-old C57BL/6 mice or derivative S100A9$^{-/-}$ mice were retro-orbitally injected with 1×10^7 CFU of *S. aureus* Newman (wild type) or the $_p$*pst* mutant in 100 μl of phosphate-free, carbonate-buffered saline. After 4 days, heart, liver, and kidneys were harvested and homogenized, and bacterial burdens were determined by dilution plating using phosphate-free, carbonate-buffered saline.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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