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RESEARCH ARTICLE

Intracellular Accumulation of Staphylopine Can Sensitize *Staphylococcus aureus* to Host-Imposed Zinc Starvation by Chelation-Independent Toxicity

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ABSTRACT The host restricts the availability of zinc to prevent infection. To overcome this defense, *Staphylococcus aureus* and *Pseudomonas aeruginosa* rely on zincophore-dependent zinc importers. Synthesis of the zincophore staphylopine by *S. aureus* and its import are both necessary for the bacterium to cause infection. In this study, we sought to elucidate how loss of zincophore efflux impacts bacterial resistance to host-imposed zinc starvation. In culture and during infection, mutants lacking CntE, the staphylopine efflux pump, were more sensitive to zinc starvation imposed by the metal-binding immune effector calprotectin than those lacking the ability to import staphylopine. However, disruption of staphylopine synthesis reversed the enhanced sensitivity phenotype of the Δ*cntE* mutant to calprotectin, indicating that intracellular toxicity of staphylopine is more detrimental than the impaired ability to acquire zinc. Unexpectedly, intracellular accumulation of staphylopine does not increase the expression of metal importers or alter cellular metal concentrations, suggesting that, contrary to prevailing models, the toxicity associated with staphylopine is not strictly due to intracellular chelation of metals. As *P. aeruginosa* and other pathogens produce zincophores with similar chemistry, our observations on the crucial importance of zincophore efflux are likely to be broadly relevant.

IMPORTANCE *Staphylococcus aureus* and many other bacterial pathogens rely on metal-binding small molecules to obtain the essential metal zinc during infection. In this study, we reveal that export of these small molecules is critical for overcoming host-imposed metal starvation during infection and prevents toxicity due to accumulation of the metal-binding molecule within the cell. Surprisingly, we found that intracellular toxicity of the molecule is not due to chelation of cellular metals.

KEYWORDS *Staphylococcus aureus*, calprotectin, nutritional immunity, pseudopaline, staphylopine, zincophore

Transition metals such as zinc (Zn) are essential for all living organisms, with approximately 50% of enzymes predicted to utilize a metal cofactor for biological function (1–4). This essentiality poses a significant challenge for invading microbes, as Zn bioavailability is restricted during infection (5–8). Recently, a new class of Zn importers that relies on a bacterially produced small molecule, or zincophore, was reported (9–11). This broadly distributed family of transporters contributes to the ability of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to cause infection (12–15). The Centers for Disease Control and Prevention and the World Health Organization have both called antibiotic-resistant pathogens, such as *S. aureus* and *P. aeruginosa*, signif-

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icant threats to human health (16, 17). One strategy to combat these pathogens is to target their nutrient acquisition mechanisms, such as Zn import pathways (18, 19). While this new family of zincophore-driven importers is critical for infection, the relative importance of the individual components to resisting host-imposed metal starvation remains unclear as is the nature of the impact arising from their disruption (9, 10, 12–15). Addressing this knowledge gap will provide opportunities for targeting this new class of zinc importer in therapeutic development.

During infection, the host limits the ability of pathogens to obtain iron (Fe), manganese (Mn), and Zn (7, 20, 21). Host-mediated restriction of Zn and Mn is exemplified by the staphylococcal abscess, which is rendered virtually deplete of both metals during infection (5, 6, 22). A key mediator of this response is the metal-binding protein calprotectin (CP), which can be found in the abscess at concentrations exceeding 1 mg/ml and can bind Mn, Zn, and other metals (5, 23–30). In culture, metal binding enables CP to inhibit the growth of numerous pathogens, including *S. aureus*, *Enterococcus faecalis*, *P. aeruginosa*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, and *Aspergillus nidulans* (25, 31–34). Loss of CP reduces the ability of mice to sequester Mn during infection, with CP-deficient mice showing increased susceptibility to infection with *S. aureus* and other pathogens (5, 6, 33, 35–39).

High-affinity metal import pathways increase the ability of *S. aureus* and other pathogens to compete with the host for metals and are frequently important for infection (31, 33, 40–45). Acquisition of the metal by bacterial importers can be achieved by a variety of strategies, including direct recruitment of the metal ion, import of a metal-chelate complex, or through capture of an extracellular metalloprotein (20, 46, 47). Until recently, the latter two strategies were thought to be used only by Fe uptake systems, comprising metal-chelate uptake in the form of siderophores and capture of host Fe-containing proteins, namely, hemoglobin and transferrin (46, 48–50). However, it has become apparent that pathogens also use all three strategies to obtain Zn during infection (12–14, 31, 33, 44, 51). Direct recruitment of Zn ions occurs via the ATP-binding cassette permeases within the Adc/Znu subgroup, which are found in almost all known bacteria (31, 41, 43, 45, 52–54). In addition to this ubiquitous family, *Neisseria* species possess transporters that can obtain Zn from CP, while *S. aureus* and *P. aeruginosa* utilize a zincophore-based system (9, 10, 12, 51, 55). The zincophore produced by *S. aureus* is staphylopine (StP); the *P. aeruginosa* analog is pseudopaline (9–11, 13). In both species, their respective zincophore-based systems contribute to their ability to cause infection despite each species also possessing an Adc/Znu system (12–14). Notably, in *S. aureus*, the StP system, not the Adc system, is the primary importer that enables successful competition with CP for Zn (12). Loci encoding for analogous zincophore-based importers are present in a wide array of Gram-positive and Gram-negative pathogens, suggesting that zincophores are a common strategy used to obtain Zn in a metal-limited environment, such as the host (10–12, 56).

The zincophores produced by *S. aureus* and *P. aeruginosa* are chemically similar, consisting of histidine, aminobutyric acid, and an alpha-keto acid. (9–11). In *S. aureus*, D-histidine and pyruvate are used, while in *P. aeruginosa*, L-histidine and alpha-ketoglutarate are used (9–11). *Yersinia pestis* and *Paenibacillus mucilaginosus* also produce putative zincophores that are chemically similar to but distinct from StP and pseudopaline (56, 57). In *S. aureus*, all of the machinery necessary for synthesis (*cntKLM*), import (*cntABCDF*), and export (*cntE*) of StP is present at a single locus (9). StP synthesis is initiated by the conversion of L-histidine into D-histidine by CntK, followed by attachment of aminobutyric acid by CntL, which generates the intermediate xNA (aminobutyrate attached to D-histidine). Subsequently, CntM adds a pyruvate to xNA to produce StP (9).

Loss of StP synthesis (CntKLM) or import (CntA) renders *S. aureus* more sensitive to CP-imposed Zn starvation and reduces the bacterium's ability to cause infection (12). Loss of CntO/ZrmA in *P. aeruginosa*, the outer membrane porin responsible for pseudopaline import in *P. aeruginosa*, also disrupts the ability of the bacterium to grow in Zn-restricted environments and cause infection (13). In both *S. aureus* and *P. aeruginosa*,

loss of the efflux pump, CntE and CntI, respectively, impairs growth, promotes toxic accumulation of the metallophore, and reduces virulence (10, 15). This has been attributed to toxic intracellular accumulation of the zincophore, wherein it acts as a metal chelator, further enhancing metal restriction, as in both instances, elimination of zincophore synthesis rescued the growth defect (10, 15). However, these studies were conducted in conditions where the second Zn transporter, AdcABC in *S. aureus* and ZnuABC in *P. aeruginosa*, was sufficient for wild-type (WT) growth (10, 15). Therefore, the importance of zincophore efflux to promoting acquisition of Zn when bacteria are Zn starved and to resisting nutritional immunity is unknown.

To evaluate how loss of StP efflux impacts *S. aureus* when metal starved, we leveraged the ability of CP to impose Zn starvation and a strain lacking the AdcABC system, both of which necessitate the use of the Cnt system to obtain Zn. We show that loss of CntE rendered bacteria more sensitive to Zn sequestration by CP than either wild-type *S. aureus* or an import-deficient mutant. Further investigations revealed that the more pronounced growth defect of the Δ*cntE* mutant was due to the accumulation of StP and that CntE was less important for Zn acquisition than the StP importer. Unexpectedly and contrary to the current model of intracellular StP toxicity (10), heterologous production of StP did not induce expression of metal importers or lead to an increase in cell-associated metals. These observations suggest that the toxicity of StP is not strictly due to intracellular metal sequestration but also includes the inherent toxicity of this molecule and its intermediates. The reduced ability of a strain lacking CntE to compete with the host for Zn combined with intracellular accumulation of a toxic molecule resulted in a virulence defect comparable to a strain lacking both Zn importers. Given the wide range of bacteria predicted to produce StP-like molecules, these findings are likely to be broadly applicable.

RESULTS

Loss of staphylopine export sensitizes *S. aureus* to calprotectin-imposed metal starvation. We first investigated how loss of StP efflux impacted *S. aureus* growth in environments where the Cnt system is necessary for Zn acquisition. This was addressed by growing wild-type *S. aureus*, as well as Δ*cntE* (which cannot efflux StP), Δ*cntA* (which lacks the StP solute binding protein), and Δ*cntKLM* (which cannot produce StP) mutants in the presence of CP. While all three mutants grew worse than the wild type in the presence of CP, the growth defect of the Δ*cntE* mutant was more pronounced than that of the Δ*cntA* (Fig. 1A; see also Fig. S1 in the supplemental material) or Δ*cntKLM* (Fig. 1B) mutant at 120 and 240 μg/ml of CP. The enhanced sensitivity of the Δ*cntE* mutant relative to that of the Δ*cntA* or Δ*cntKLM* mutant was abrogated upon deletion of *cntKLM* (Fig. 1B). This is consistent with recent work (15) and suggests that the toxicity of StP significantly contributes to the enhanced sensitivity of the Δ*cntE* mutant relative to that of the Δ*cntA* or Δ*cntKLM* mutant even when *S. aureus* is metal starved. Building on this framework and to eliminate the confounding contribution of the AdcABC Zn import system, the ability of the Δ*cntE* Δ*adcA* and Δ*cntA* Δ*adcA* mutants to grow in the presence of CP was assessed. At CP concentrations of $>$120 μg/ml, both the Δ*cntE* Δ*adcA* and Δ*cntA* Δ*adcA* mutants failed to grow (Fig. 1A). However, at 60 μg/ml, the Δ*cntA* Δ*adcA* mutant, but not the Δ*cntE* Δ*adcA* mutant, grew worse than the wild type (Fig. 1A). This latter result is similar to what has been observed for a Δ*cntKLM* mutant (12) and suggests that the Cnt importer contributes more to Zn uptake when *S. aureus* is modestly metal limited than either the synthesis machinery or CntE. Ectopic expression of *cntE* reversed the enhanced sensitivity of the Δ*cntE* and Δ*cntE* Δ*adcA* strains to CP (Fig. 1C). Similarly, expression of *adcA* in the Δ*cntE* Δ*adcA* double mutant results in sensitivity comparable to that of the Δ*cntE* single mutant (Fig. 1C). Collectively, these observations show that CntE is necessary for staphylococcal growth when metal starved. However, distinct from the other components of the Cnt system, the importance of CntE arises from its role in facilitating Zn uptake and in preventing StP toxicity.

Zinc and iron suppress the growth defect of a strain lacking CntE. CP binds a variety of first-row transition metals (6, 25, 26, 29, 30). Accordingly, use of wild-type CP

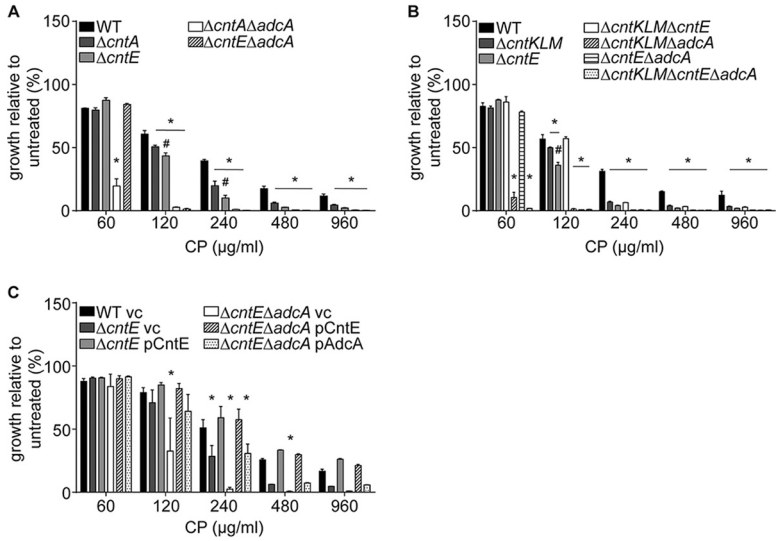


FIG 1 CntE promotes resistance to calprotectin-imposed metal starvation. (A) Wild-type *S. aureus* and Δ*cntA*, Δ*cntE*, Δ*cntA* Δ*adcA*, and Δ*cntE* Δ*adcA* mutants were incubated in the presence of increasing concentrations of CP, and growth was assessed by measuring optical density at 600 nm (OD_{600}) at 12 h. (B) Wild-type *S. aureus* and Δ*cntA*, Δ*cntE*, Δ*cntKLM* Δ*cntE*, Δ*cntA* Δ*adcA*, Δ*cntE* Δ*adcA*, and Δ*cntKLM* Δ*cntE* Δ*adcA* mutants were incubated in the presence of increasing concentrations of CP, and the growth was assessed by measuring the OD_{600} at 12 h. (C) Wild-type *S. aureus* and Δ*cntE* and Δ*cntE* Δ*adcA* mutants with the indicated plasmid were incubated in the presence of increasing concentrations of CP, and the growth was assessed by measuring the OD_{600} at 12 h. (A and B) *, $P < 0.05$ compared to wild type at the same CP concentration via two-way analysis of variance (ANOVA) with Dunnett's posttest; #, $P < 0.05$ compared to the Δ*cntA* (A) or Δ*cntKLM* (B) mutant at the same CP concentration via two-way ANOVA with Dunnett's posttest. $n \geq 3$. (C) *, $P < 0.05$ compared to wild-type vector control (VC) at the same CP concentration via two-way ANOVA with Dunnett's posttest. Error bars in all panels are standard errors of the mean, and each panel represents an independent series of experiments performed the indicated number (*n*) of times on independent days. See also Fig. S1 in the supplemental material.

limits the ability to determine the metal responsible for the phenotype. To address this issue, two CP variants with altered metal-binding capacity can be used, including the following: (i) ΔS1, which lacks the hexahistidine metal-binding site, and (ii) ΔS2, which lacks the three histidine and aspartate metal-binding sites. Both mutant variants retain Zn binding. The ΔS1 mutant lacks the ability to bind Mn or Fe, while the ΔS2 mutant is still capable of binding Mn or Fe (25–28). The CP variants were assessed for their impact on the growth of the *S. aureus* Δ*cntE*, Δ*cntA*, Δ*cntA* Δ*adcA*, and Δ*cntE* Δ*adcA* mutants. The Δ*cntE* and Δ*cntE* Δ*adcA* mutants showed a reduction in growth in the presence of both the ΔS1 and ΔS2 CP variants compared to that of wild-type bacteria. Growth of the Δ*cntE* mutant was inhibited by the ΔS1 and ΔS2 CP variants to a greater extent than that of the Δ*cntA* mutant, while the Δ*cntE* Δ*adcA* mutant phenocopied the Δ*cntA* Δ*adcA* mutant at the CP concentration tested (Fig. 2A; see also Fig. S2A and B in the supplemental material). Together, these observations suggest that the reduced ability of strains lacking CntE to grow in the presence of CP is driven by an inability to cope with Zn starvation.

To further evaluate how the strains lacking CntE respond to metal limitation, the growth of Δ*cntE* and Δ*cntA* mutants in a metal-defined medium (NRPMI) from which Mn, Fe, and Zn were omitted was evaluated. Similar to previous studies (12, 58), the Δ*cntA* mutant did not have a growth defect in NRPMI lacking all three metals (Fig. 2B). In contrast, the Δ*cntE* mutant had a modest growth defect (Fig. 2B; see also Fig. S2C to G), consistent with a recent report (15). However, the growth defect was substantially less pronounced compared to that of the Δ*cntE* Δ*adcA* mutant. Addition of either Zn, Fe, or Mn reversed the growth defect of the Δ*cntE* mutant (Fig. 2B). However, the modest phenotypic impact and the general growth improvement upon metal supplementation limits the ability to draw robust conclusions from the observations with the Δ*cntE* single mutant. As the Adc permease alone is sufficient for Zn uptake in NRPMI

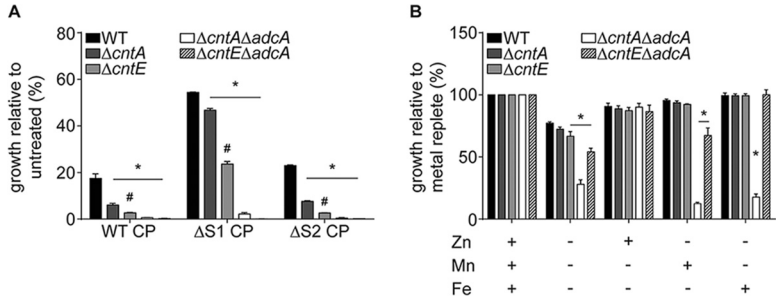


FIG 2 Zinc and iron reverse the growth defect of strains lacking CntE. (A) Wild-type *S. aureus* and Δ*cntA*, Δ*cntE*, Δ*cntA* Δ*adcA*, and Δ*cntE* Δ*adcA* mutants were grown in medium containing 480 μg/ml of wild type, ΔS1, or ΔS2 CP. Growth was assessed by measuring the OD_{600} at 12 h. *, $P < 0.05$ compared to wild-type bacteria grown in the same variant of CP via two-way ANOVA with Dunnett's posttest. #, $P < 0.05$ compared to the Δ*cntA* mutant grown at the same CP concentration via two-way ANOVA with Dunnett's posttest. $n \geq 3$. (B) Wild-type *S. aureus* and Δ*cntA*, Δ*cntE*, Δ*cntA* Δ*adcA*, and Δ*cntE* Δ*adcA* mutants were grown in NRPMI containing 25 μM $ZnSO_4$, $MnCl_2$, or $FeSO_4$ as indicated. Growth was assessed by measuring the OD_{600} at 7 h. *, $P < 0.05$ compared to wild type in the same medium via two-way ANOVA with Dunnett's posttest. $n \geq 3$. Error bars in all panels are standard errors of the mean. See also Fig. S2 in the supplemental material.

medium (12), it would not be expected that the single mutants would experience Zn limitation. Therefore, double mutants lacking *adcA* and either *cntE* or *cntA* were assessed for growth in NRPMI medium and the associated impact, if any, of metal supplementation. In the absence of Mn, Fe, and Zn, the Δ*cntE* Δ*adcA* and Δ*cntA* Δ*adcA* mutants had growth defects compared to wild-type bacteria. However, similar to exposure to low concentrations of CP, the growth defect of the Δ*cntE* Δ*adcA* mutant was significantly less than that of the Δ*cntA* Δ*adcA* mutant in this medium. The addition of Mn did not affect the growth of either strain (Fig. 2B). Consistent with a defect in Zn uptake, the addition of Zn reversed the growth defect of both the Δ*cntE* Δ*adcA* and Δ*cntA* Δ*adcA* mutants (Fig. 2B). Similar to previous studies (12), the addition of Fe did not reverse the growth defect of the Δ*cntA* Δ*adcA* mutant. Conversely, the addition of Fe reversed the growth defect of the Δ*cntE* Δ*adcA* mutant (Fig. 2B). While expression of the *cnt* locus is predominantly regulated by Zn availability, Fe also suppresses its expression (12, 59). Additionally, simultaneous loss of the Cnt-StP and Adc systems does not reduce the ability of *S. aureus* to overcome Fe limitation; in fact, the addition of Fe to medium lacking Zn further suppresses the growth of a Δ*cntA* Δ*adcA* mutant (12). Cumulatively, these results suggest that the ability of Fe to rescue the Δ*cntE* Δ*adcA* mutant is due to a reduction in the expression of the StP synthesis machinery, reducing accumulation of StP, a toxic molecule (15). Collectively, these observations indicate that loss of CntE diminishes the ability of *S. aureus* to grow when Zn restricted and that the toxicity of StP significantly contributes to this phenotype.

Intracellular toxicity of staphylopine can occur independent of metal chelation. StP is a broad-spectrum metallophore with high affinity for a variety of first-row transition metals. Intracellular accumulation of StP has been proposed to starve bacteria for essential metal ions (10). Nevertheless, recent transcriptional analyses have challenged the assumption that the toxicity of StP is driven by chelation, as StP accumulation does not induce the expression of metal importers in a Δ*cntE* mutant relative to the wild type (15). Notably, the study used a broad-spectrum chelator in the growth medium, which may have influenced the relative baseline of expression of metal import pathways being interrogated and prevented further induction. Therefore, the possibility that intracellular chelation of metals by StP mediates toxicity could not be excluded. All of the prior studies examining the toxicity of StP-like zincophores have relied on native expression of the synthesis machinery requiring that the bacteria be Zn starved (10, 15), which in turn makes it difficult to determine if metallophore accumulation induces metal starvation. To overcome the regulatory issues and resolve whether StP induces metal starvation, a plasmid in which *cntKLM* expression is controlled by an

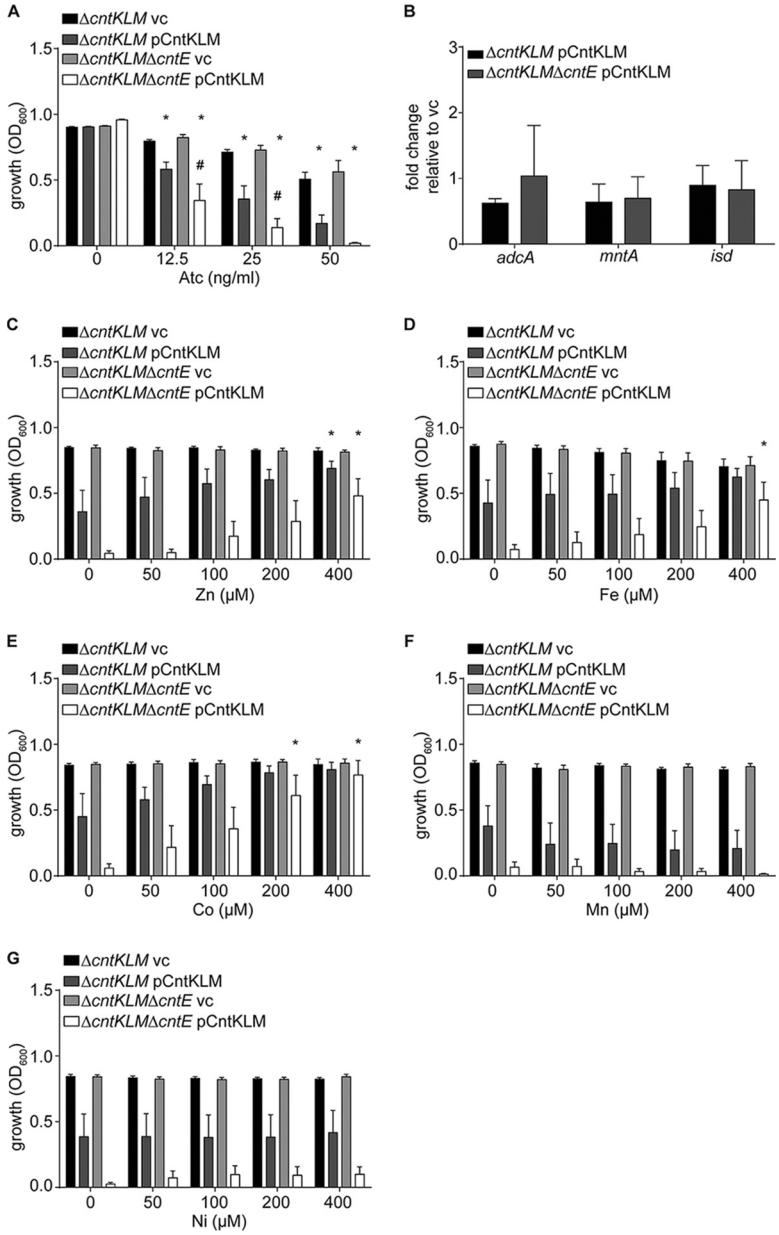


FIG 3 Over accumulation of staphylopine is toxic in metal-replete medium. *S. aureus* Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutants containing the indicated plasmids were grown in medium supplemented with anhydrotetracycline (Atc). (A) Growth was assessed by measuring the OD_{600}. *, $P < 0.05$ compared to the same strain carrying the vector control (VC) plasmid at the same concentration of Atc via two-way ANOVA with Tukey's posttest. #, $P < 0.05$ compared to the Δ*cntKLM* pCntKLM mutant via two-way ANOVA with Tukey's posttest. $n \geq 3$. (B) Bacteria were grown in the presence of 6.25 ng/ml Atc, and gene expression was assessed via qRT-PCR. $n \geq 3$. (C to G) The growth medium was supplemented with Zn (C), Fe (D), Co (E), Mn (F), and Ni (G). Growth was assessed by measuring the OD_{600} at 8 h. *, $P < 0.05$ compared to the same strain grown in the absence of additional metals via two-way ANOVA with Dunnett's posttest. Error bars in all panels are standard errors of the mean. See also Fig. S3 in the supplemental material.

inducible promoter was created and introduced into the Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutant strains. This construct allows the production of StP to be induced in Zn-replete medium. Overexpression of *cntKLM* reduced the growth of the Δ*cntKLM* mutant and the Δ*cntKLM* Δ*cntE* mutant in a metal-replete medium (Fig. 3A; see also Fig. S3 in the supplemental material). This toxicity was enhanced in the Δ*cntKLM* Δ*cntE* mutant compared to that of the Δ*cntKLM* mutant. Subsequently, transcription of *adcA* (a

component of the AdcABC Zn importer), *mntA* (a component of the MntABC Mn importer), and *isdC* (a component of the *S. aureus* heme uptake system), which are regulated by the metal-responsive regulators Zur, MntR, and Fur, respectively, was monitored as reporters of intracellular Zn, Mn, and Fe limitation (6, 12, 60–62). Consistent with the previous study (15), none of the metal importers were induced in the Δ*cntKLM* Δ*cntE* strain relative to the Δ*cntKLM* strain when CntKLM was ectopically expressed, nor were they induced compared to the vector control (Fig. 3B). This suggests that StP toxicity arises from accumulation, independent of metal sequestration. To test this inference, the ability of Zn, Fe, Mn, nickel (Ni), and cobalt (Co) supplementation to suppress the growth defect of the Δ*cntKLM* Δ*cntE* mutant was evaluated. Supraphysiological levels of Zn, Fe, and Co partially relieved the growth defect of the Δ*cntKLM* Δ*cntE* mutant overexpressing StP from an inducible plasmid (Fig. 3C to E). In contrast to the prior study, which relied on expression from the native promoter (15), addition of Mn, which is relatively weakly bound by StP (9), did not reverse the growth defect (Fig. 3F). Ni also failed to reverse the growth defect of the StP-overexpressing strains (Fig. 3G). Although not all metals reversed the StP-mediated growth defect in the Δ*cntE* mutant, these data are not inconsistent with chelation-driven toxicity. However, 200 μM or more of each metal was necessary to reverse the growth defect; therefore, it is probable that ablation of the StP-mediated growth defect occurs through another mechanism, such as reducing the activity of StP dehydrogenase, which can be suppressed by the presence of excess metals (63).

The discordant transcriptional and metal rescue observations necessitated an additional, independent approach to evaluate the impact of StP on intracellular metal availability. If StP binds and sequesters metal ions intracellularly, then the total cellular capacity for metals would be predicted to increase proportionally with StP abundance. Accordingly, when StP is produced but not secreted in metal-replete medium, total cellular metal content should increase. To assess whole-cell metal concentrations, we used inductively coupled plasma mass spectrometry (ICP-MS). The analysis revealed that the Zn, Mn, Fe, Co, Ni, and copper (Cu) content of the Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutants overexpressing CntKLM was the same (Fig. 4). To exclude the possibility of insufficient metals in the medium, the experiment was also performed with 400 μM $ZnSO_4$ supplementation, which reverses the growth defect associated with overexpression of CntKLM. ICP-MS revealed that, while supplementation with Zn increased the total cell-associated Zn in all strains, there was no significant difference in total Zn abundance between the StP-overproducing strains and the vector control (Fig. 4A). Therefore, intracellular accumulation of StP does not increase cellular metal capacity and accumulation. This finding, when considered with the transcriptional profiling, strongly suggests that loss of CntE does not increase the extent of metal starvation experienced by *S. aureus* relative to that of other mutants lacking a functional Cnt-StP system.

Accumulation of staphylopine or a synthesis intermediate is toxic. Building on the inference that loss of CntE results in toxicity independent of metal starvation, we investigated the possibility that a synthesis intermediate, and not StP, was responsible for the growth defect of the Δ*cntE* and Δ*cntE* Δ*adcA* mutants. To test this possibility, *cntK*, *cntL*, *cntM*, and *cntKL* were expressed under the control of an inducible promoter in *S. aureus*, and growth was assessed in a metal-replete medium in strains that possess a functional AdcABC system. To ensure that the constructs were the only source of the synthesis machinery, these experiments were performed using the Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutants. Overexpression of either *cntK* or *cntM* did not impact the growth of the Δ*cntKLM* or Δ*cntKLM* Δ*cntE* mutant. This indicates that neither of the proteins are inherently toxic and that the conversion of L-histidine to D-histidine by CntK does not negatively impact the bacterium (Fig. 5). The overexpression of *cntL* resulted in a minor growth defect in the Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutants but only at the highest concentration of inducing agent. Overexpression of CntKL reduced the growth of both the Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutants (Fig. 5). Taken together, these

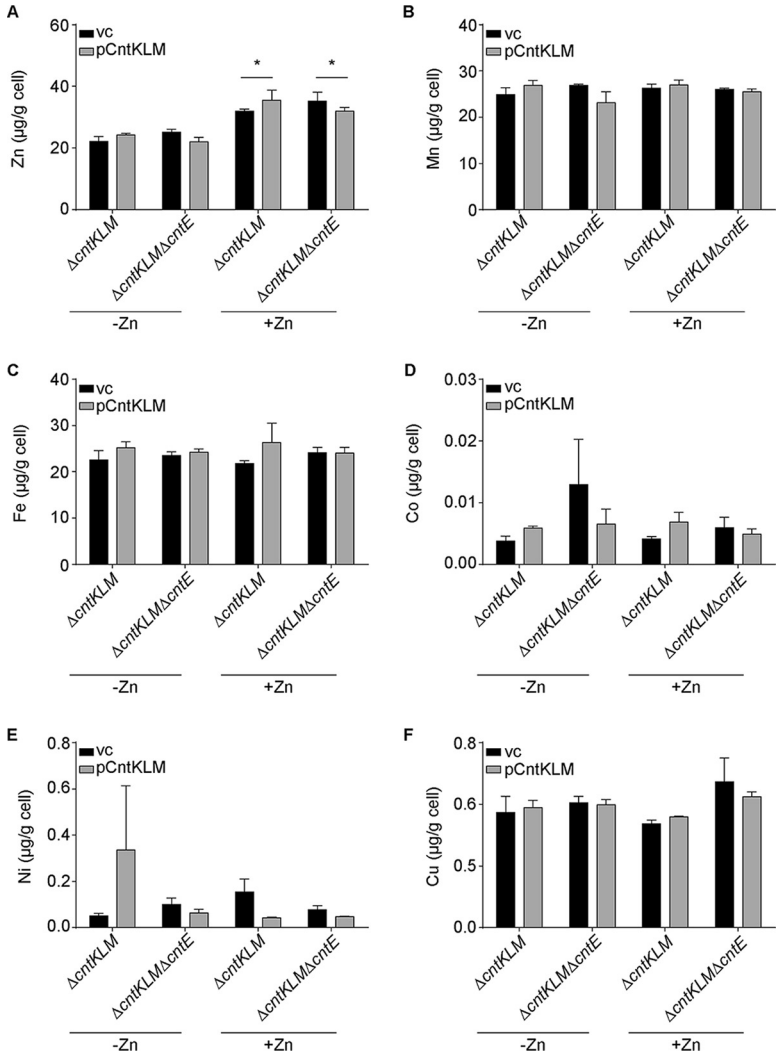


FIG 4 Intracellular staphylopine does not lead to accumulation of metals. *S. aureus* Δ*cntKLM* and Δ*cntKLM* Δ*cntE* mutants containing the indicated plasmids were grown in the presence of 6.25 ng/ml of anhydrotetracycline (Atc) with or without 400 μM $ZnSO_4$. The intracellular metal concentration was assessed using ICP-MS. (A) Zn; (B) Mn; (C) Fe; (D) Co; (E) Ni; (F) Cu. *, $P < 0.05$ compared to the same strain grown with no Zn added via one-way ANOVA using Tukey's posttest. $n \geq 3$. Error bars in all panels are standard errors of the mean.

findings suggest that production and accumulation of the synthesis intermediate xNA is also toxic to *S. aureus*. However, differing from the overexpression of CntKLM, the toxicity associated with CntKL expression was not exacerbated in the absence of CntE (Fig. 5). Collectively, these results show that accumulation of StP or the synthesis intermediate xNA (aminobutyrate attached to D-histidine) results in toxicity to *S. aureus*, although maximal toxicity is achieved with StP accumulation.

The toxicity of staphylopine increases the virulence defect associated with loss of the Cnt-staphylopine system in Zn-limited tissues. While the importance of CntE to staphylococcal infection has been evaluated in a skin abscess model (15, 64), the fact that StP import is not required for infection in this tissue suggests that the bacteria may only be experiencing mild Zn limitation (64). To evaluate the contribution of CntE to infection in which *S. aureus* experiences significant Zn limitation and requires the Cnt-StP system to obtain Zn, a systemic retro-orbital infection model was used. In these experiments, C57BL/6J mice were infected with wild-type *S. aureus* or the Δ*cntA* and Δ*cntE* single mutants. Mice that were infected with the wild type and the Δ*cntA* mutant lost significantly more weight than those infected with Δ*cntA* (Fig. 6A). Consistent with

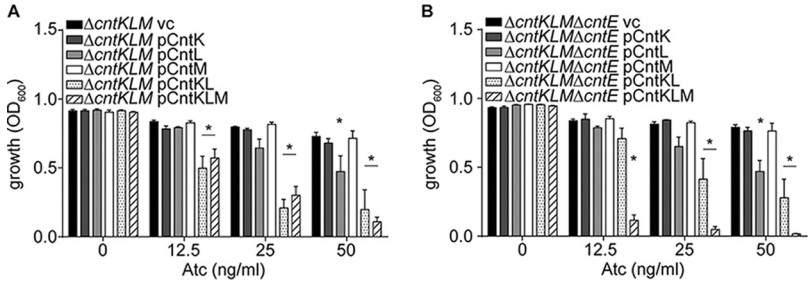


FIG 5 Accumulation of staphylopine intermediates are toxic to *S. aureus*. *S. aureus* Δ*cntKLM* (A) and Δ*cntKLM* Δ*cntE* (B) mutants containing the indicated plasmids were grown in increasing concentrations of anhydrotetracycline (Atc). Growth was assessed by measuring the OD_{600} at 8 h. *, $P < 0.05$ compared to vector control at the same Atc concentration via two-way ANOVA using Dunnett's posttest. $n \geq 3$. Error bars in all panels are standard errors of the mean.

previous results (12), mice infected with the Δ*cntA* mutant had reduced bacterial burdens compared to those of mice infected with the wild-type bacteria in the heart but not in the liver or kidney (Fig. 6B to D). However, mice infected with the Δ*cntE* mutant had significantly reduced bacterial burdens in the liver, heart, and kidneys relative to those infected with the wild-type bacteria (Fig. 6B to D). The bacterial burden of the Δ*cntE* mutant was also significantly reduced compared to that of mice that were infected with the Δ*cntA* mutant. These results suggest that the loss of CntE results in a more pronounced virulence defect than other components of the Cnt-StP system, even in Zn-restricted tissues. As the Δ*cntE* Δ*adcA* mutant is less sensitive to Zn limitation in culture than the Δ*cntA* Δ*adcA* mutant, the ability of the two double mutants to cause infection was also investigated. Mice infected with the Δ*cntA* Δ*adcA* mutant lost less weight and had reduced bacterial burdens in the heart, kidney, and liver compared to those of mice infected with wild-type bacteria (12) (Fig. 6). Infection with the Δ*cntE* Δ*adcA* mutant also resulted in mice losing less weight and having reduced bacterial

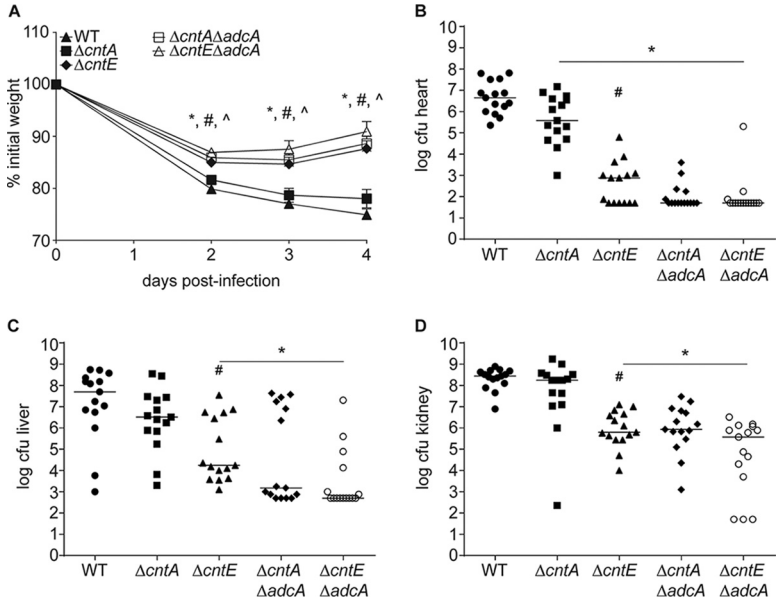


FIG 6 Δ*cntE* is more attenuated than Δ*cntA* during infection. C57BL/6J mice were retro-orbitally infected with 1×10^7 CFU of wild-type *S. aureus* or Δ*cntA*, Δ*cntE*, Δ*cntA adcA*, and Δ*cntE* Δ*adcA* mutants. The weights of the mice (A) as well as the bacterial burdens in the heart (B), liver (C), and kidney (D) were assessed. (A) *, #, and ^, $P < 0.05$ for the Δ*cntE* mutant, the Δ*cntA* Δ*adcA* mutant, and the Δ*cntE* Δ*adcA* mutant, respectively, compared to mice infected with the wild type via two-way ANOVA with Dunnett's posttest. (B to D) *, $P < 0.05$ compared to mice infected with the wild type via Mann-Whitney U test. #, $P < 0.05$ compared to the Δ*cntA* mutant via Mann-Whitney U test. Error bars in panel A are standard errors of the mean.

burdens in the heart, liver, and kidneys compared to those factors in mice infected with wild-type *S. aureus*. Surprisingly, the weight loss and reduction in bacterial burdens of the Δ*cntE* mutant were comparable to those of the two double mutants. These results indicate that even when *S. aureus* is Zn starved during infection, the toxicity associated with intracellular accumulation of StP contributes to the virulence defect of strains lacking CntE.

DISCUSSION

A critical weapon used by *S. aureus*, *P. aeruginosa*, and potentially other pathogens in combating host-imposed Zn starvation is zincophore-dependent Zn uptake systems (12, 13). While the importance of the zincophore importer and synthesis machinery for obtaining Zn has been established (9–13), the contribution of the efflux machinery in obtaining this metal remained unclear. Restriction of Zn abundance in the host results in the *S. aureus* Δ*cntE* mutant being impaired to a greater extent than wild-type bacteria or a mutant lacking the StP importer. However, the more pronounced growth defect relative to that of strains lacking the ability to import StP was not due to an inability to compete with the host for Zn but rather to intracellular toxicity arising from accumulation of StP that does not disrupt metal homeostasis. Thus, our findings contradict the prevailing model of how StP mediates cellular toxicity (10, 15), with our data indicating a mechanism independent of metal starvation. In conjunction with the observation that CntE is necessary to resist host-imposed Zn starvation, this indicates that the pronounced virulence defects of strains that cannot efflux StP are driven by two independent mechanisms.

This work and that of others supports the observation that intracellular accumulation of StP mediates toxicity in both *S. aureus* and *P. aeruginosa* (10, 15). Siderophore accumulation in the cytoplasm or periplasm can repress bacterial growth, with the most frequent proposal being that this is due to Fe sequestration (65–67). Therefore, given the broad metal-chelating properties of StP-like molecules, it is unsurprising that intracellular metal sequestration emerged as a readily accepted hypothesis for the molecular basis of their cellular toxicity (10). However, the only studies to examine the impact of StP accumulation on metal homeostasis have provided data inconsistent with this model (15) but without resolving whether toxicity is primarily driven by a chelation-dependent or -independent mechanism. The idea that a metallophore can have chelation-independent toxicity is supported by the Fe-independent toxicity of pyochelin and that trapping mycobacterial siderophores within the cytoplasm results in an Fe-independent growth defect (68, 69). Here, we observe that supraphysiological addition of Zn, Fe, and Co suppress the growth defect of a Δ*cntE* mutant, which supports the idea that StP toxicity is driven by metal sequestration. However, this suppression occurs in medium that is metal replete prior to the addition of metals, and accumulation of StP does not increase cell-associated metals. Loss of CntE, when StP is ectopically expressed in metal-replete medium, also does not induce dedicated metal importers. In *Arabidopsis thaliana*, overaccumulation of nicotianamine, a plant metallophore, disrupts metal homeostasis resulting in both increased expression of metal importers, whose expression responds to metal availability, and causes overaccumulation of metals (70). While chemically distinct from nicotianamine, StP is predicted to bind metals using a similar coordination and with similar affinity (9). Thus, if StP toxicity is driven by metal chelation, one would expect increased metal importer expression and overaccumulation of metals in a CntE mutant. Neither of these were observed in the current or previous (15) studies, strongly supporting a model wherein the intracellular toxicity of StP acts primarily in a chelation-independent manner. However, this does leave unanswered how supplementation with a supraphysiological level of some metals reverses the toxicity associated with loss of CntE. Recently, it was observed that CntM activity is inhibited by excess Zn and Co *in vitro* (63), providing a potential mechanism to resolve the conflicting data regarding why accumulation of StP is toxic. The implication of this finding is that elevated intracellular metal concentrations, which occur upon addition of excess metals to the culture medium, would inhibit the activity

of CntM. This would result in reduced production of StP, thus alleviating the growth defect of strains incapable of exporting the metallophore.

P. aeruginosa mutants that lack the ability to efflux pseudopaline also have more pronounced growth defects than synthesis and import mutants (10). Similar to *S. aureus*, toxicity of pseudopaline is thought to be driven by chelation of intracellular metals. StP and pseudopaline are structurally similar, and efflux mutants have congruent phenotypes (10, 15). In conjunction with the current results, this suggests that the mode of toxicity for pseudopaline may similarly be chelation independent. A wide variety of Gram-positive and Gram-negative microbes possess the ability to make StP-like molecules. Analysis of the synthesis machinery revealed that the metallophores produced from these loci by *Yersinia pestis* and *Paenibacillus mucilaginosus* differ from StP and pseudopaline only by the utilization of D- or L-histidine and pyruvate or alpha-ketoglutarate (56, 57). Combined with recently proposed rules that govern the alpha-keto acid incorporated into the zincophore (56), it seems likely that many of the uncharacterized loci produce molecules similar to StP and pseudopaline. These data, combined with the conserved colocalization of an efflux pump with zincophore synthesis loci (12), suggest that StP-like molecules may be toxic, independent of their metal-chelating ability. As a consequence, there may be a significant potential fitness cost associated with StP production and, hence, the tight regulation of its biosynthesis.

Drug efflux pumps have long been considered potential drug targets to increase the efficacy of current therapies, as their presence frequently increases bacterial resistance to antibiotics (71–74). Prior studies have revealed that CntE is critical for staphylococcal virulence in a skin infection model (15, 64), while the current studies revealed the critical importance of CntE for systemic staphylococcal infection. Notably, in a systemic model of infection, loss of CntE resulted in a strain with a virulence defect comparable to that of a strain lacking both high-affinity staphylococcal Zn importers. The current work suggests that this profound virulence defect is due not simply to disruption of staphylococcal metal acquisition and homeostasis but also to a metal-independent mode of staphylopine toxicity. Two modes of toxicity suggest that it should be more difficult for bacteria to adapt to therapeutic approaches that disrupt efflux pump function. Disruption of pseudopaline efflux also attenuates *P. aeruginosa* virulence (14). All StP-like synthesis loci identified thus far possess an efflux pump (12). Thus, targeting these exporters may be a novel strategy for treating infections by bacteria that rely on these molecules to cause infection. Notably, the efflux pumps encoded by *cnt* loci largely differ between Gram-negative and Gram-positive organisms. *S. aureus* and most other Gram-positive bacteria rely on a pump belonging to the major facilitator superfamily (MFS), while *P. aeruginosa* and other Gram-negative bacteria rely on an EamA family efflux pump. Thus, not only does zincophore efflux represent a potential therapeutic target but one that may minimize the detrimental impact that broad-spectrum antibiotics have on the host's microbiota (75, 76).

The ability of pathogens to acquire essential nutrients during infection is critical for their virulence (7, 20, 77). The use of a zincophore-mediated Zn import system has emerged as an important strategy used by Gram-positive and Gram-negative pathogens to acquire this essential nutrient during infection (10, 12, 13). However, this is not without a trade-off, as they must create a potentially toxic molecule in order to effectively compete with the host. The threat posed by these molecules to the producing bacterium is exemplified by the virulence defects associated with loss of efflux, which exceed that of importer or synthesis mutants (12, 15), highlighting the lengths that invading pathogens must go to overcome host-imposed metal starvation.

MATERIALS AND METHODS

Ethics statement. All animal experiments were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (IACUC license number 18038) and performed according NIH guidelines, the Animal Welfare Act, and U.S. federal law.

Bacterial strains. For routine overnight cultures, *S. aureus* was grown in 5 ml of tryptic soy broth (TSB) in 15-ml conical tubes and grown at 37°C on a roller drum. *S. aureus* Newman and derivatives were used for all experiments. The Δ*cntA*, Δ*cntKLM*, Δ*cntE*, and Δ*cntKLM* Δ*cntE* strains were generated as

TABLE 1 Primers used in this study

Primer	Sequence
CntE F	CTAGCATATGATGAAAGGTGCAATGGCTTGGC
CntE R	TGCAGGATCCGCTTTAATTCACTTCATTAAAGACTACTCGC
CntE KO 5′ F	GGGGACCACTTTGTACAAGAAAGCTGGGTCATGACATTGTTAACAGTTAAACATTTGACG
CntE KO 5′ R	GGTGCAATGGCTTGGCCCCGTCCAGCGAGTAGTCTTTAATGAAGTG
CntE KO 3′ F	AAGACTACTCGCTGGACGGGGCCAAGCCATTGCACCTTTCATTATC
CntE KO 3′ R	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTCTTGATAATCTTTAGTAGTACCG
CntK F	AGCTGGTACCCAGAGGCTCTAAAAATACATCTAAAGGAGTG
CntK R	CGATGAGCTCGTAGGCTCCTTATTCTATATATGCTTTTCC
CntL F	AGCTGGTACCGCTACAGGAAAAGCATATATAGAATAAGGAGCC
CntL R	CGATGAGCTCTAATTTAGACATCCGTAATACCTACCTTTATAGC
CntM F	AGCTGGTACCAAGCAGCTATAAAGGTAGGTATTACGGATGTC
CntM R	CGATGAGCTCCCCTTATTATGAAAGCGTTCTATTGATTTCC
adcA RT F	TCATGCTCTTCACCATGCTC
adcA RT R	CCGGTTGCTAAGAAAGTTGC
mntA RT F	TCTAGATGAGCCGTTTGTCG
mntA RT R	GCTTTTGATAGATCATGGTGGA
isdC RT F	GCGGTACTTTGAATTATGAGG
isdC RT R	GGTTGACAGTTATTTGAACATAC
16S RT F	GCTGCAGCTAACGCATTAAGCACT
16S RT R	TTAAACCACATGCTCCACCGCTTG

previously described (12). Briefly, the 5′ and 3′ flanking regions of the genes were amplified using the indicated primers (Table 1). They were then cloned into pKOR1, and the deletion was generated via allelic replacement (78). The *adcA*::*erm* mutant was generated via phage transduction by transducing the allele from USA300 (JE2) via Φ85 phage. All constructs were confirmed to be hemolytic by growth on tryptic soy agar (TSA) blood agar plates. For complementation and overexpression constructs, *cntE*, *cntKLM*, *cntK*, *cntL*, *cntM*, and *cntKL* were amplified using the indicated primers (Table 1). The *cntKLM*, *cntK*, *cntL*, *cntM*, and *cntKL* coding sequences were cloned into pRMC2, a vector containing an anhydrotetracycline-inducible promoter (79). The *cntE* coding sequence was cloned into pOS1 under the control of the *lgt* promoter (80). All PCR-generated constructs were confirmed via sequencing. All strains used in this study are listed in Table 2.

Calprotectin growth assays. CP assays were performed as described previously (6, 12, 37, 81). Briefly, overnight cultures were back-diluted 1:50 in 5 ml of fresh TSB and incubated for 1 h at 37°C on a roller drum. They were then diluted 1:100 in 100 μl of a growth medium consisting of 38% TSB and 62% calprotectin buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 3 mM $CaCl_2$, 10 mM β-mercaptoethanol [BME]) in 96-well round-bottom plates. The medium was also supplemented with 1 μM $ZnSO_4$ and 1 μM $MnCl_2$. Cultures were incubated at 37°C with shaking at 180 rpm. Growth was assessed by measuring the optical density at 600 nm (OD_{600}) at 12 h. CP was purified as previously described (82).

TABLE 2 Strains used in this study

Genotype	Source or reference
Wild type (strain Newman)	This study
Δ*cntA*	12
Δ*cntA adcA*::*erm*	12
Δ*cntE*	This study
Δ*cntE adcA*::*erm*	This study
Δ*cntKLM* Δ*cntE*	This study
Δ*cntKLM* Δ*cntE adcA*::*erm*	This study
Δ*cntE* pOS1plgt	This study
Δ*cntE adcA*::*erm* pOS1plgt	This study
Δ*cntE* pOS1plgt::*cntE*	This study
Δ*cntE adcA*::*erm* pOS1plgt::*cntE*	This study
Δ*cntE adcA*::*erm* pOS1plgt::*adcA*	This study
Δ*cntKLM* pRMC2	12
Δ*cntKLM* pRMC2::*cntKLM*	12
Δ*cntKLM* pRMC2::*cntK*	This study
Δ*cntKLM* pRMC2::*cntL*	This study
Δ*cntKLM* pRMC2::*cntM*	This study
Δ*cntKLM* pRMC2::*cntKL*	This study
Δ*cntKLM* Δ*cntE* pRMC2	This study
Δ*cntKLM* Δ*cntE* pRMC2::*cntKLM*	This study
Δ*cntKLM* Δ*cntE* pRMC2::*cntK*	This study
Δ*cntKLM* Δ*cntE* pRMC2::*cntL*	This study
Δ*cntKLM* Δ*cntE* pRMC2::*cntM*	This study
Δ*cntKLM* Δ*cntE* pRMC2::*cntKL*	This study

NRPMI growth assays. NRPMI growth medium consists of RPMI 1640 tissue culture medium with L-glutamine supplemented with 1% Casamino Acids. Divalent cations are removed from this medium by incubating it with Chelex 100 resin. For growth assays in NRPMI, following overnight growth, bacteria were diluted 1:50 into fresh TSB and grown for 1 h at 37°C. They were then diluted 1:100 into 100 μl of NRPMI containing 1 mM $MgCl_2$ and 100 μM $CaCl_2$ with combinations of 25 μM $ZnSO_4$, $MnCl_2$, and $FeSO_4$. Cultures were incubated at 37°C with shaking at 180 rpm, and the growth was assessed by measuring the OD_{600} at 7 h.

Staphylopine overexpression assays. *S. aureus* and mutants containing pRMC2 with the *cntKLM* coding region were grown overnight in 5 ml of TSB with 10 μg/ml of chloramphenicol at 37°C on a roller drum. The following morning, they were diluted 1:10 into 5 ml of fresh TSB and then further diluted 1:100 in 100 μl of TSB supplemented with 10 μg/ml of chloramphenicol and various concentrations of anhydrotetracycline. For metal add-back experiments, the growth medium was also supplemented with various concentrations of $ZnSO_4$, $MnCl_2$, $FeSO_4$, $CoCl_2$, or $NiSO_4$. Bacteria were grown at 37°C with shaking at 180 rpm, and growth was assessed by measuring the OD_{600} at 8 h.

Expression analysis. Bacteria were grown as for the StP overexpression assays, except in 30 ml of TSB in a 50-ml conical tube. Bacteria were harvested during log phase (OD_{600} of ~0.1). They were suspended in an equal volume of 1:1 ethanol-acetone and frozen at −80°C until RNA extraction. RNA was isolated as described previously (81, 83–85). Reverse transcription-quantitative PCR (qRT-PCR) was performed using the indicated primers (Table 1), with 16S serving as a normalization control.

Elemental analysis. Elemental analysis was performed essentially as described previously (86, 87) using the growth parameters for RNA isolation. Succinctly, bacteria were harvested at an OD_{600} of ~0.1, washed two times with 0.1 M EDTA and then washed two further times with Milli-Q water. The cells were resuspended in 1 ml of Milli-Q water. They were then centrifuged, and the supernatant was removed. The pellet was desiccated overnight at 96°C and then weighed to determine the dry cell mass of the pellet. The pellet was resuspended in 1 ml of 35% HNO_3 and heated at 96°C for 1 h. Insoluble material was removed by centrifugation at 18,000 × *g* for 25 min. Samples were diluted in 1:10 Milli-Q and then analyzed on an Agilent 8900 inductively coupled plasma tandem mass spectrometer (ICP-MS/MS).

Animal infections. All animal experiments were performed as previously described (6, 37, 81). Nine-week-old female C57BL/6J mice were retro-orbitally injected with 1×10^7 CFU of *S. aureus* suspended in 100 μl of phosphate-buffered saline (PBS). Prior to injection, the mice were anesthetized via peritoneal injection of tribromoethanol (Avertin). Four days postinfection, mice were sacrificed, and their livers, kidneys, and hearts were homogenized in and diluted in sterile PBS. Bacterial CFU were determined via dilution plating on TSA plates.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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K.P.G., J.N.R., P.K.P.S., J.R.M., K.A.F., K.G., and S.L.N. conducted the experiments. K.P.G., K.G., S.L.N., C.A.M., and T.E.K.-F. designed the studies and analyzed the data. K.P.G., C.A.M., and T.E.K.-F. wrote the manuscript.

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