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RESEARCH ARTICLE
Host-Microbe Biology

# *Corynebacterium pseudodiphtheriticum* Exploits *Staphylococcus aureus* Virulence Components in a Novel Polymicrobial Defense Strategy

**Britney L. Hardy,[a] Seth W. Dickey,[b] Roger D. Plaut,[c] Daniel P. Riggins,[c] Scott Stibitz,[c] Michael Otto,[b] D. Scott Merrell[a]**

[a]F. Edward Hébert School of Medicine, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA
[b]Pathogen Molecular Genetics Section, Laboratory of Bacteriology, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland, USA
[c]Division of Bacterial, Parasitic, and Allergenic Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, USA

**ABSTRACT** Commensal bacteria in the human nasal cavity are known to suppress opportunistic pathogen colonization by competing for limited space and nutrients. It has become increasingly apparent that some commensal bacteria also produce toxic compounds that directly inhibit or kill incoming competitors. Numerous studies suggest that microbial species-specific interactions can affect human nasal colonization by the opportunistic pathogen *Staphylococcus aureus*. However, the complex and dynamic molecular interactions that mediate these effects on *S. aureus* nasal colonization are often difficult to study and remain poorly understood. Here, we show that *Corynebacterium pseudodiphtheriticum*, a common member of the normal nasal microbiota, mediates contact-independent bactericidal activity against *S. aureus*, including methicillin-resistant *S. aureus* (MRSA). Bacterial interaction assays revealed that *S. aureus* isolates that were spontaneously resistant to *C. pseudodiphtheriticum* killing could be recovered at a low frequency. To better understand the pathways associated with killing and resistance, a *S. aureus* transposon mutant library was utilized to select for resistant mutant strains. We found that insertional inactivation of *agrC*, which codes for the sensor kinase of the Agr quorum sensing (Agr QS) system that regulates expression of many virulence factors in *S. aureus*, conferred resistance to killing. Analysis of the spontaneously resistant *S. aureus* isolates revealed that each showed decreased expression of the Agr QS components. Targeted analysis of pathways regulated by Agr QS revealed that loss of the phenol-soluble modulins (PSMs), which are effectors of Agr QS, also conferred resistance to bactericidal activity. Transmission electron microscopy analysis revealed that *C. pseudodiphtheriticum* induced dramatic changes to *S. aureus* cell surface morphology that likely resulted in cell lysis. Taken together, these data suggest that *C. pseudodiphtheriticum*-mediated killing of *S. aureus* requires *S. aureus* virulence components. While *S. aureus* can overcome targeted killing, this occurs at the cost of attenuated virulence; loss of Agr QS activity would phenotypically resemble a *S. aureus* commensal state that would be unlikely to be associated with disease. Commensal competition resulting in dampened virulence of the competitor may represent an exciting and unexplored possibility for development of novel antimicrobial compounds.

**IMPORTANCE** While some individuals are nasally colonized with *S. aureus*, the underlying factors that determine colonization are not understood. There is increasing evidence that indicates that resident bacteria play a role; some commensal species can eradicate *S. aureus* from the nasal cavity. Among these, *Corynebacterium pseudodiphtheriticum* can eliminate *S. aureus* from the human nose. We sought to understand this phenomenon at a molecular level and found that *C. pseudodiphtheriticum* produces a factor(s) that specifically kills *S. aureus*. While resistant *S. aureus* isolates

**Citation** Hardy BL, Dickey SW, Plaut RD, Riggins DP, Stibitz S, Otto M, Merrell DS. 2019. *Corynebacterium pseudodiphtheriticum* exploits *Staphylococcus aureus* virulence components in a novel polymicrobial defense strategy. mBio 10:e02491-18. https://doi.org/10.1128/mBio.02491-18.

**Editor** Nina R. Salama, Fred Hutchinson Cancer Research Center



Address correspondence to D. Scott Merrell, douglas.merrell@usuhs.edu.

**Received** 9 November 2018
**Accepted** 27 November 2018
**Published** 8 January 2019

were recovered at a low frequency, resistance came at the cost of attenuated virulence in these strains. Molecular dissection of the specific strategies used by *C. pseudodiphtheriticum* to kill *S. aureus* could lead to the development of novel treatments or therapies. Furthermore, commensal competition that requires virulence components of the competitor may represent an exciting and unexplored possibility for development of novel antimicrobial compounds.

**KEYWORDS** commensal bacteria, *Corynebacterium*, nasal microbiota, polymicrobial interactions, *Staphylococcus aureus*

*Staphylococcus aureus* is both a commensal bacterium and a versatile opportunistic pathogen that is the causative agent of numerous types of disease, including skin and soft tissue infections (SSTIs), systemic infections, and toxin-mediated diseases (1). While typically not life-threatening, SSTIs are by far the most common *S. aureus*-mediated disease presentation worldwide (2, 3). Moreover, annual health care costs associated with *S. aureus*-mediated disease approach $14 billion in the United States alone (4, 5). To further complicate matters, many circulating *S. aureus* strains are highly resistant to multiple antibiotics, which compromises treatment. In turn, the lack of suitable treatment leads to more severe illness and higher mortality rates (6). Indeed, in the United States methicillin-resistant *S. aureus* (MRSA) infections were responsible for nearly 10,000 deaths from 2005 to 2013, representing more deaths than those caused by HIV and tuberculosis combined (7). Clearly, hospital-acquired MRSA and, increasingly, community-acquired MRSA infections are serious public health concerns (8). In fact, due to the high prevalence of circulating multidrug-resistant *S. aureus* strains, the World Health Organization and the Centers for Disease Control and Prevention have designated MRSA and vancomycin-resistant *S. aureus* as "high" and "serious" threats, respectively (9, 10).

While *S. aureus* can cause significant morbidity and mortality, this bacterium is a part of the nasal microbiota of approximately one-third of the population; thus, *S. aureus* is considered to be a commensal bacterium in these individuals (11, 12). However, asymptomatic *S. aureus* nasal carriage is a known risk factor for subsequent *S. aureus*-mediated disease. In fact, patients with invasive disease are often infected with their commensal strain (13). For example, in patients diagnosed with *S. aureus*-induced sepsis, 82% of blood isolates were shown by multilocus sequence typing (MLST) to match nasal isolates from the same patient (14). Thus, transmission of *S. aureus* from the nasal cavity to other vulnerable anatomical locations likely precedes more serious disease. Additionally, these *S. aureus* carriers likely serve as a reservoir for transmission of the bacterium to other individuals (14). As a result of this, the decolonization of persistent and intermittent *S. aureus* carriers, especially in vulnerable populations (i.e., military personnel and individuals in long-term-care facilities), has been proposed as one approach that could significantly reduce *S. aureus* transmission and incidence of disease (15, 16).

*S. aureus*-mediated disease has been extensively studied, yet the specific factors that promote *S. aureus* commensalism are not well understood. However, it is clear that *S. aureus* expresses an expansive set of virulence and colonization factors that are needed to establish itself within the host's nasal niche. These factors include adhesin molecules that promote attachment to host nasal epithelial cells, as well as pore-forming toxins and other proteins that help the bacterium to evade immune clearance (17, 18). The expression of many of the genes that encode these factors is regulated by the well-characterized accessory gene regulator quorum sensing (Agr QS) system (1). The Agr QS system is encoded ubiquitously among *S. aureus* strains by the *agrBDCA* operon (19). The various genes in this operon encode AgrB, a hydrolase that processes AgrD, releasing the autoinducing peptide (AIP); AgrD, the propeptide precursor of AIP; AgrC, a sensor kinase; and the corresponding response regulator, AgrA. When cell density is high, AIP binds to AgrC, and a phosphorelay cascade is initiated that culminates with phosphorylation and activation of AgrA (19). Activated AgrA positively autoregulates

the expression of *agrBDCA* as well as the important effector regulatory molecule, RNAIII. RNAIII and, in some instances, AgrA modulate expression of several *S. aureus* virulence and colonization genes (20, 21). Included among the list of activated genes are six *psm* genes: four *psm*$\alpha$ and two *psm*$\beta$ genes. These genes encode the phenol-soluble modulins (PSMs), small cyclic protein toxins which are important for *S. aureus* virulence and are widely synthesized across strains (22). In the United States, the predominant *S. aureus* disease-causing genetic lineage, USA300, typically shows high levels of *agrBDCA* transcription (23). Therefore, it is hypothesized that elevated Agr QS activity directly influences *S. aureus* virulence (23). This hypothesis is in keeping with the fact that USA300 causes severe disease in both immunocompromised and healthy individuals (24). Therefore, not only are many *S. aureus* isolates resistant to multiple antibiotics, but many also show high levels of expression of the Agr QS-regulated virulence factors that mediate severe disease. The importance of the Agr QS system in modulation of the *S. aureus* pathogenic/commensal state is further evidenced by the fact that *S. aureus* isolates that are recovered after invasive infection are typically Agr QS defective (25, 26); this fact suggests that there is a selective pressure against the expression of virulence factors following invasive infection, as the bacterium continues to exist within these sites (27). Furthermore, constitutive RNAIII/*agrBDCA* expression results in reduced nasal colonization in a cotton rat model of *S. aureus* nasal colonization (28). Thus, it has been hypothesized that the reduction or complete loss of *agrBDCA*/RNAIII expression or genes activated by these systems represents a fundamental shift from the pathogenic state to a commensal one (29, 30).

The nasal cavity is a nutrient-poor, high-salinity niche where bacteria must compete for limited resources. This microbial competition may be considered a type of bacterial "warfare" whereby various species utilize a variety of tactics to overcome their competitors. Mounting evidence suggests that *S. aureus* nasal colonization is heavily dependent on intricate molecular interactions with the resident nasal microbiota (31). For example, several high-throughput sequencing analyses of the composition of the nasal microbiota have revealed an inverse relationship between the presence of the *Firmicutes* and *Actinobacteria* phyla; i.e., individuals who are colonized with a high abundance of *Actinobacteria* generally have a low abundance of *Firmicutes*, including *S. aureus* (32). More strikingly, the presence of the *Corynebacterium* genus alone appears to impact *Staphylococcus* nasal colonization (33). In our prior studies that examined the nasal microbiota of U.S. military recruits, we found that individuals who were colonized with *Corynebacterium* species were less likely to be colonized with *Staphylococcus,* particularly *S. aureus* (2, 3). Similar results have been seen in other cohorts; Yan et al. found that individuals who were colonized with a single *Corynebacterium* species, *C. pseudodiphtheriticum*, had a very low probability of being colonized with *S. aureus* (31). Moreover, they found that coculture of *C. pseudodiphtheriticum* and *S. aureus* on agar plates resulted in visible inhibition of *S. aureus* (31).

Though few publications have looked mechanistically at the polymicrobial interactions between *S. aureus* and *Corynebacterium* species, some studies do suggest that the Agr QS system may impact the interaction of *S. aureus* and *Corynebacterium* species. There are four possible *agr* alleles (I to IV) that are differentiated by nucleotide changes in the hypervariable region within *agrD* and *agrC*. Lina et al. utilized multiple logistic regression analysis to show that the probability of isolating *S. aureus* strains bearing *agr* allele I or II from the nasal cavity is extremely low in the presence of *Corynebacterium* (34). Interestingly, no such correlation was seen with *S. aureus* strains bearing *agr* types III and IV. Thus, *agr* allele type may influence the ability of *S. aureus* to coexist with *Corynebacterium* in the nasal cavity (34).

Given our lack of understanding of the molecular details that govern the interaction between *C. pseudodiphtheriticum* and *S. aureus*, we set out to characterize this process. Here, we show that *C. pseudodiphtheriticum* mediates contact-independent bactericidal activity against *S. aureus*, including MRSA. *S. aureus* strains bearing mutations in components of the Agr QS or showing decreased expression of the Agr QS system were able to survive exposure to *C. pseudodiphtheriticum* killing activity. Absence of the PSMs

also conferred resistance to killing activity, independently of Agr QS activity. Thus, *C. pseudodiphtheriticum* directly kills *S. aureus*; in turn, *S. aureus* can indirectly resist this assault by surrendering expression of its important virulence factors, most notably the *psm* genes. Commensal competition that requires virulence components of the competitor may represent an exciting and unexplored possibility for development of novel antimicrobial compounds.

## RESULTS

***C. pseudodiphtheriticum* mediates bactericidal activity against *S. aureus* via a contact-independent mechanism.** Data suggest that *C. pseudodiphtheriticum* is an important community determinant of *S. aureus* nasal colonization; there is an inverse relationship between nasal carriage of *C. pseudodiphtheriticum* and *S. aureus* (31). Given the lack of information concerning the reason for this negative correlation, we sought to characterize the molecular interactions of these bacterial species. We began with the prior observation that coincubation of *C. pseudodiphtheriticum* and *S. aureus* on an agar plate results in visible inhibition of *S. aureus* growth (31) (Fig. 1). For these initial characterization experiments, we performed modified bacterial interaction assays (31) with two strains of *C. pseudodiphtheriticum*: 10700, a lab-adapted strain, and USU1, a new nasal isolate recently acquired from a healthy volunteer. From these experiments, we found that *C. pseudodiphtheriticum* USU1 mediated a larger zone of clearance (ZOC) against *S. aureus* COL than *C. pseudodiphtheriticum* 10700 (data not shown). This result suggests that the potency of *C. pseudodiphtheriticum*-mediated activity against *S. aureus* differs among *C. pseudodiphtheriticum* strains. We chose to utilize the more active *C. pseudodiphtheriticum* strain, USU1, throughout the remainder of this study. To determine whether this inhibition occurred against multiple *S. aureus* strains, we tested a collection of isolates, including strains that fall within the clinically relevant USA300 pulse field type (Table 1), in the bacterial interaction assay. Furthermore, to determine whether growth inhibition was species specific, we also tested the sensitivity of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. *C. pseudodiphtheriticum*-mediated inhibitory activity was temporally quantified by measuring the size of the ZOC that appeared around the *C. pseudodiphtheriticum* spot every 24 h for 120 h total. We observed that the ZOC steadily increased over time (Fig. 1a and b) and that this was true for both MRSA and methicillin-sensitive *S. aureus* (MSSA) strains. Strain sensitivity fell into three categories: highly sensitive, moderately sensitive, and resistant. *S. aureus* 2014.N, an uncharacterized recently acquired nasal isolate, was highly sensitive to *C. pseudodiphtheriticum*-mediated inhibition; the entire agar plate was completely cleared after 72 h of incubation. In comparison, *S. aureus* strains A950085, COL, LAC, MW2, and NRS384 were moderately sensitive (ZOC range of 2 to 10 mm) and *S. aureus* strains A970377, Mu50, and N315 were completely resistant (Fig. 1a and b). In addition, *S. epidermidis* and *S. saprophyticus* were resistant to *C. pseudodiphtheriticum*-mediated inhibition (Fig. 1a and data not shown). To determine whether other species within the *Corynebacterium* genus could also inhibit *S. aureus* in this bacterial interaction assay, we also assessed the ability of *Corynebacterium accolens* and *Corynebacterium diphtheriae* to inhibit *S. aureus* 2014.N, which was the most sensitive of the tested strains. No ZOC was observed with either of these other *Corynebacterium* species (data not shown). Taken together, these results suggested that *C. pseudodiphtheriticum* was able to specifically inhibit many, but not all, *S. aureus* strains via inhibition of *S. aureus* growth or by killing and lysing the *S. aureus* cells; the latter seemed the more plausible, since a lawn of *S. aureus* was visible at early time points, and the size of the ZOC increased with time.

To our knowledge, there is no published evidence that any *Corynebacterium* species possesses bactericidal activity against *S. aureus*. Thus, to determine if *C. pseudodiphtheriticum*-mediated inhibition was due to *S. aureus* growth inhibition (bacteriostatic) or due to killing of the *S. aureus* cells (bactericidal), we attempted to recover surviving *S. aureus* directly from the ZOC. Agar punches were taken from inside the ZOC for highly sensitive (2014.N), intermediate sensitive (LAC) and resistant (Mu50)

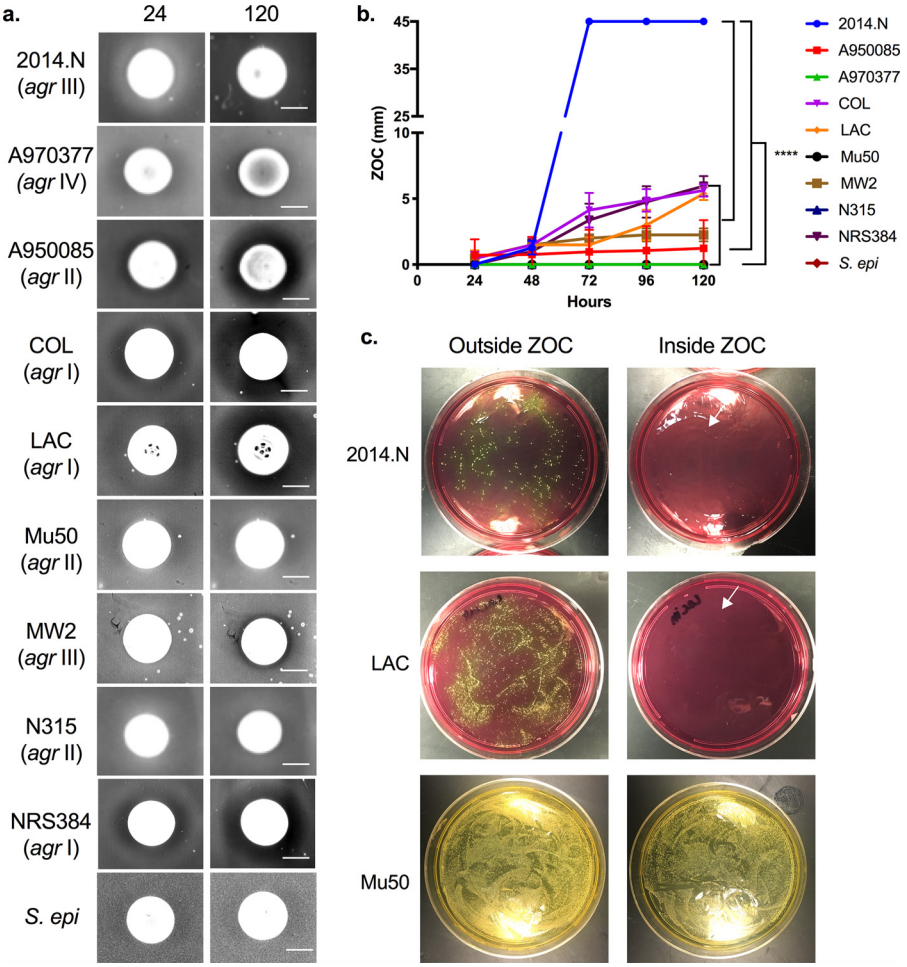


**FIG 1** *C. pseudodiphtheriticum* mediates bactericidal activity against *S. aureus*. (a) *C. pseudodiphtheriticum* was spotted on agar plates that had been seeded with various *S. aureus* strains (2014.N, A970377, A950085, COL, LAC, Mu50, MW2, N315, and NRS384) or *S. epidermidis* 1457 (*S. epi*). Plates were incubated at 28°C, and images were taken every 24 h (24- and 120-hour images are shown). The *agr* allele type of each strain is given in parentheses. Bar, 10 mm. (b) The ZOC was defined as the distance between the edge of the *C. pseudodiphtheriticum* spot and the visible edge of the *S. aureus* ring of clearance. The ZOC was measured using ImageJ software (NCBI). Each symbol represents the arithmetic mean from 3 independent biological replicates measured temporally; error bars represent ±1 standard deviation. A two-way ANOVA with Dunnett's correction for multiple comparisons was performed on the ZOC lengths at the 120-hour time point. The symbol **** on the graph indicates a statistically significant difference ($P < 0.0001$) in ZOC lengths for 2014.N versus all other strains and for Mu50 versus all other sensitive strains. (c) Punches of agar from the ZOC directly adjacent to the *C. pseudodiphtheriticum* or outside the ZOC were plated and grown for 48 h to recover surviving *S. aureus* CFU. White arrows indicate rare surviving colonies.

strains; we reasoned that even though a ZOC did not form around a resistant strain, the *S. aureus* cells might still be dead within that region. Similar punches were taken from outside the ZOC for qualitative comparison and to estimate the frequency of any spontaneous resistance. While large numbers of bacteria were recovered from outside the ZOC, only rarely were colonies recovered from inside the ZOC for strain 2014.N or LAC. Conversely, similar numbers of colonies were recovered from outside and within the ZOC for Mu50, a resistant isolate (Fig. 1c). Since some colonies were recovered from the ZOC of sensitive strains, we hypothesized that these bacteria could represent spontaneously resistant isolates. To test this, we randomly selected five recovered 2014.N isolates and assayed them individually for sensitivity to *C. pseudodiphtheriticum*-mediated inhibition. All five isolates (Survivors A to E) were completely resistant to inhibition (see Fig. S1 in the supplemental material), as no ZOC formed. Thus, sensitive *S. aureus* strains can develop spontaneous resistance to *C. pseudodiphtheriticum*-

**TABLE 1** Strains used in this study[a]

| Strain | Lab strain designation | Origin | Yr isolated | Notes | Reference(s) |
|---|---|---|---|---|---|
| *C. pseudodiphtheriticum* USU1 | DSM1434 | Nose | 2014 | Commensal | This study |
| *C. pseudodiphtheriticum* ATCC 10700 | DSM1447 | Throat | 1980s | Commensal | 72 |
| *C. accolens* ATCC 49725 | DSM1448 | Cervix | 1991 | Commensal | 73 |
| *C. diphtheriae* | DSM1380 | — | — | Commensal | This study |
| *S. aureus* COL | DSM1450 | Nose | 1961 | Early MRSA isolate | 74 |
| *S. aureus* NRS384 | DSM1471 | Abscess | Early 1990s | CA-MRSA, USA300 | — |
| *S. aureus* NRS384 transposon library | DSM1470 | — | 2015 | Mariner transposon library | This study |
| *S. aureus* LAC | DSM1485 | Blood | 2005 | CA-MRSA | 75 |
| *S. aureus* JE2 | DSM1513 | — | — | *S. aureus* LAC derivative | 37, 76 |
| *S. aureus* MW2 | DSM1483 | Blood | 1998 | CA-MRSA | 77 |
| *S. aureus* 2014.N | DSM1416 | Nose | 2012 | Commensal | This study |
| *S. aureus* 2014.N Survivor A | DSM1652 | — | 2015 | Spontaneously resistant isolate | This study |
| *S. aureus* 2014.N Survivor B | DSM1653 | — | 2015 | Spontaneously resistant isolate | This study |
| *S. aureus* N315 | DSM1634 | Throat | 1982 | HA-MRSA | 78 |
| *S. aureus* Mu50 | DSM1633 | Abscess | 1997 | HA-MRSA | 78 |
| *S. aureus* A950085 | DSM1685 | — | — | Clinical isolate | 79 |
| *S. aureus* A970377 | DSM1686 | — | — | Clinical isolate | 79 |
| *S. epidermidis* 1457 | DSM1637 | — | — | Commensal | 80 |
| *S. epidermidis* 1457 $pTX_{\Delta}psm\alpha1$-4 | DSM1687 | — | — | — | This study |
| *S. epidermidis* 1457 $pTX_{\Delta}16$ | DSM1688 | — | — | Empty vector control | This study |
| *S. saprophyticus* | DSM1655 | Urine | — | — | This study |
| *S. aureus* JE2 *Tn::agrA* | DSM1640 | — | — | — | 37 |
| *S. aureus* JE2 *Tn::agrB* | DSM1641 | — | — | — | 37 |
| *S. aureus* JE2 *Tn::agrC* | DSM1639 | | — | — | 37 |
| *S. aureus* JE2 *Tn::agrC* $pCL15\text{-}P_{spac}$-*agrC* | DSM1654 | — | This study | Complemented strain | This study |
| *S. aureus* JE2 *Tn::agrC* $pTX_{\Delta}psm\alpha1$-4 | DSM1689 | — | This study | $pTX_{\Delta}psm\alpha1$-4, carries $\alpha$-*psm* genes | This study |
| *S. aureus* LAC Δ*agrBDCA* | DSM1486 | — | 2013 | Transduction of *agr* deletion from RN6911, $Tet^r$ | 23 |
| *S. aureus* MW2 Δ*agrBDCA* | DSM1484 | — | 2013 | Transduction of *agr* deletion from RN6911, $Tet^r$ | 23 |
| *S. aureus* LAC Δ*psm* | DSM1520 | — | 2012 | Deletions of *psmβ* and *psmα* operons, mutation of start codon of *hld* to nonsense, $Spec^r$ | 39 |
| *S. aureus* LAC Δ*psm* $pTX_{\Delta}psm\alpha1$-4 | DSM1523 | — | 2014 | Complemented strain | 39 |

[a]Symbols and abbreviations: —, specific information was unavailable or not applicable. CA, community acquired; HA, hospital acquired.

mediated inhibition. Using the number of bacterial colonies obtained from outside and within the ZOC for both 2014.N and LAC, we estimated that the frequency of spontaneous resistance was approximately $10^{-8}$. *En masse*, these data indicate that *C. pseudodiphtheriticum* selectively mediates bactericidal activity against many *S. aureus* strains and that spontaneous resistance to this activity occurs at a low frequency.

In order to further characterize this interaction at a molecular level, we next performed two variations of the bacterial interaction assay. To determine whether *C. pseudodiphtheriticum*-mediated bactericidal activity required live bacteria, heat-killed *C. pseudodiphtheriticum* cells were utilized; no ZOC was formed (Fig. 2a). To determine whether bactericidal activity was dependent on direct contact, *C. pseudodiphtheriticum* and *S. aureus* cells were separated with a permeable 0.2-$\mu$m filter disk; the ZOC formed as before (Fig. 2b). Given this indication that bactericidal activity was mediated by a secreted factor(s), we next asked whether this factor(s) would be present in conditioned cell-free medium (CCFM) in which *C. pseudodiphtheriticum* had been grown overnight. Activity of concentrated CCFM was tested via disk diffusion assays against *S. aureus* strains that fell into the various *C. pseudodiphtheriticum* sensitivity categories: 2014.N (highly sensitive), LAC (moderately sensitive), and Mu50 (resistant). Mimicking the degree of bactericidal activity mediated by live *C. pseudodiphtheriticum* (Fig. 1a), a

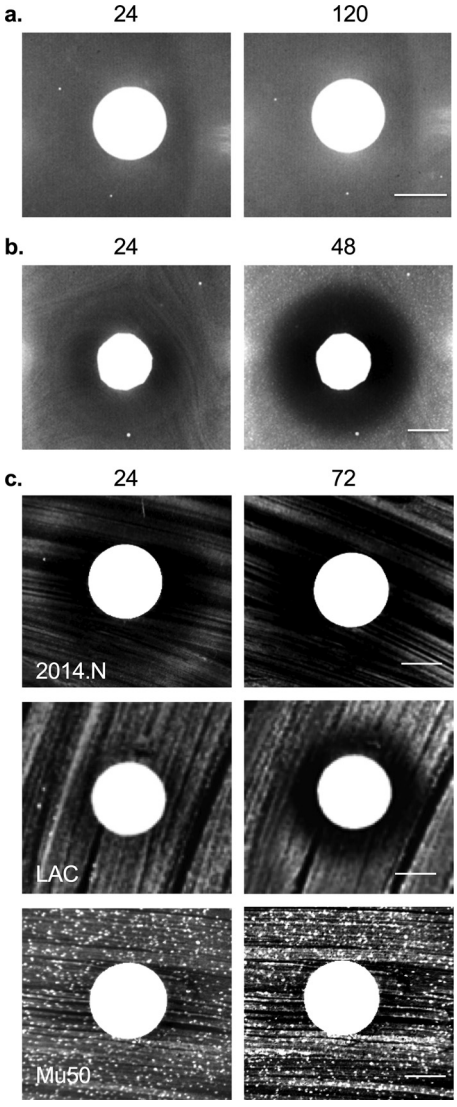


**FIG 2** Bactericidal activity against *S. aureus* requires live *C. pseudodiphtheriticum* and is contact independent. (a) *C. pseudodiphtheriticum* cells were heated to 90°C for 10 min and then spotted on BHIT agar seeded with *S. aureus* 2014.N. (b) A 0.2-$\mu$m filter was placed on top of BHIT agar seeded with *S. aureus* 2014.N, and *C. pseudodiphtheriticum* was spotted on top of the filter. Images of the ZOC were taken after 24, 48, and 120 h of incubation at 28°C; 24- and 120-hour images are shown in panel a, and 24- and 48-hour images are shown in panel b. Images are representative of three independent biological replicates. Bar, 10 mm. (c) *S. aureus* strains 2014.N, LAC, and Mu50 were spread on the agar surface, and a sterile disk was placed in the center of the plate. Fifty microliters of concentrated CCFM prepared from *C. pseudodiphtheriticum* was inoculated onto the disk and allowed to dry. Images of the ZOC were taken after 24 and 72 h of incubation. Images are representative of three independent biological replicates. Bar, 10 mm.

visible ZOC formed with 2014.N and LAC but not with Mu50 (Fig. 2c). Overall, these data indicate that *C. pseudodiphtheriticum*-mediated bactericidal activity is an active process that is mediated by a secreted factor(s).

**Identification of potential target(s) of bactericidal activity using a *S. aureus* Tn mutant library.** Given that we observed spontaneous resistance of *S. aureus* sensitive strains to *C. pseudodiphtheriticum*-mediated bactericidal activity at a relatively low frequency ($10^{-8}$), we reasoned that we should be able to use a *S. aureus* transposon (Tn) library to select for surviving transposants. Therefore, we repeated the bacterial interaction assay using a pooled *S. aureus* NRS384 *H1 mariner* transposon library; we recovered resistant transposant isolates from the ZOC. The experiment was independently repeated three times and yielded a total of 31 recovered resistant transposon

**TABLE 2** Recovered resistant transposon mutant strains

| Gene | No. of colonies recovered | Location | Pathway | No. of independent insertion sites |
|---|---|---|---|---|
| *agrC* | 14 | Chromosome | Quorum sensing | 3 |
| *bacA* | 5 | Plasmid | Peptidoglycan synthesis | 2 |
| *carA* | 3 | Chromosome | *De novo* pyrimidine synthesis | 1 |
| *purL* | 4 | Chromosome | *De novo* purine synthesis | 1 |
| *purK* | 3 | Chromosome | *De novo* purine synthesis | 1 |
| *pyrF* | 2 | Chromosome | *De novo* pyrimidine synthesis | 1 |

isolates. The site of transposon insertion was identified using a rescue cloning strategy and revealed insertions within 6 separate genes (Table 2). The most frequently recovered transposants (14/31) had insertions in *agrC*, which encodes AgrC, the sensor kinase that is a part of the Agr QS system (Table 2). These 14 recovered isolates represented three independent transposon insertion events within the *agrC* open reading frame (Fig. 3a), strongly suggesting that disruption of *agrC* reduced sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. The remaining recovered resistant transposon mutants had insertions in genes predicted to be involved in *de novo* nucleotide synthesis (*carA*, *purL*, *purK*, and *pyrF*) (35) and peptidoglycan synthesis (*bacA*) (36) (Table 2). A representative isolate bearing a transposon insertion in each of the six genes was independently retested in the bacterial interaction assay and was confirmed to be resistant to *C. pseudodiphtheriticum*-mediated bactericidal activity (Fig. S2). Given the importance of the Agr QS system for *S. aureus* virulence and because previous studies have demonstrated that some *Corynebacterium* species, including *C. pseudodiphtheriticum*, can induce changes in *S. aureus* Agr QS activity (36), we focused the remainder of our studies on gaining a more thorough understanding of the role of Agr QS in the context of *C. pseudodiphtheriticum*-mediated bactericidal activity.

**Absence of or decreased expression of *agrBDCA* confers resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity.** The NRS384 transposon mutant screen described above revealed that insertional inactivation of *agrC* conferred resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity. However, we noted that we did not recover resistant mutant isolates that had insertions in the other genes of the *agrBDCA* operon. The NRS384 *H1 mariner* pooled transposon library that was initially used was not fully characterized at the time of this study; thus, transposants with insertions in *agrA*, *agrB*, and *agrD* may not exist within the transposon pool. As expression of *agrBDCA* is autoregulated by AgrA, we hypothesized that insertional

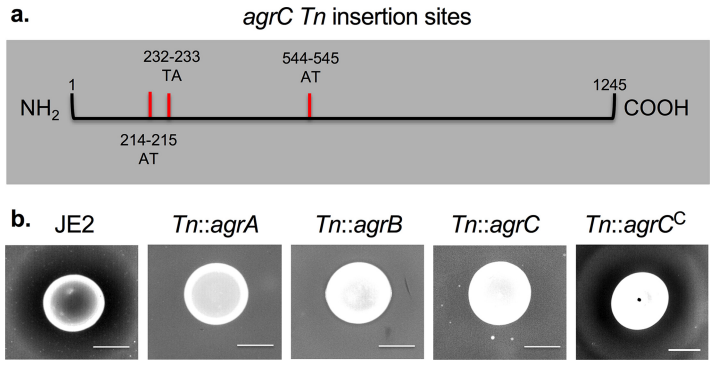


**FIG 3** Absence of *agrBDCA* confers resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity. (a) *agrC* insertion sites were mapped against the NCTC 8325 *S. aureus* reference genome (NCBI). The sites of insertion are indicated as hatch marks, and the nucleotide position (number) and bases flanking the transposon insertion site (letters) are shown. (b) *S. aureus* JE2 and JE2 strains containing Tn insertions in *agrA*, *agrB*, *agrC*, and *agrC*$^{C}$ were tested for sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. Images of the ZOC were taken after 120 h of incubation at 28°C and are representative of three independent biological replicates. Bar, 10 mm.

inactivation of any gene in the *agrBDCA* operon should confer resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity. To test this, we analyzed the sensitivity of *S. aureus* JE2 (USA300, LAC derivative) and JE2 transposon mutant strains that contained Tn insertions in *agrB*, *agrA*, and *agrC* to *C. pseudodiphtheriticum*-mediated bactericidal activity (Fig. 3b). We observed that insertional inactivation of any of these genes conferred resistance to bactericidal activity. Furthermore, this phenotype was complementable; complementation of *agrC* in the *Tn*::*agrC* mutant strain (*Tn*::*agrC*$^{C}$) restored sensitivity to bactericidal activity (Fig. 3b). To further ensure that complementation of *agrC* alone restored Agr QS activity in the *Tn*::*agrC* mutant strain, hemolysis assays were also performed; red blood cell hemolysis is a crude measure of Agr QS activity (37). Indeed, hemolysis activity was restored in *Tn*::*agrC*$^{C}$ (data not shown). To extend these results to other *S. aureus* strain backgrounds, we also tested the sensitivity of isogenic Δ*agrBDCA* mutant strains constructed in the MW2 and LAC strains. While each of the parental strains was sensitive to *C. pseudodiphtheriticum*-mediated bactericidal activity, no ZOC formed with either Δ*agrBDCA* mutant strain (Fig. S3). Thus, loss of Agr QS activity conferred resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity across multiple *S. aureus* strain backgrounds.

Given that Agr QS conferred sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity, we next wondered if the level of sensitivity of the various *S. aureus* strains (Fig. 1) would correlate with the level of expression of *agrBDCA* transcript found in these strains. Therefore, we measured *agrBDCA* expression in *S. aureus* strains 2014.N (highly sensitive), LAC (moderately sensitive), and Mu50 (resistant) relative to the 16S rRNA gene. The levels of *agrBDCA* expression perfectly correlated with the overall sensitivity to *C. pseudodiphtheriticum*-mediated killing (Fig. 4a); resistant Mu50 showed a very low level of *agrBDCA* expression, moderately sensitive LAC showed an intermediate level of expression, and highly sensitive 2014.N showed a significantly higher level of *agrBDCA* expression. Furthermore, transcript levels of RNAIII, an important effector molecule of Agr QS, mirrored the relative transcription level of *agrBDCA* (Fig. 4a). Given this, transcript levels of *agrBDCA* and RNAIII (data not shown) were also measured in strain A970377, which was also resistant to *C. pseudodiphtheriticum*-mediated killing activity (Fig. 1a and b). Expression of these genes was higher than in Mu50 but lower than in LAC. Thus, there may be a threshold of *agrBDCA* expression that is required for *C. pseudodiphtheriticum*-mediated bactericidal activity. Given these results, we next wondered if the spontaneously resistant *S. aureus* isolates that were recovered from the ZOC in the bacterial interaction assay (Fig. S1) showed some form of defect in *agrBDCA* or RNAIII expression. Therefore, we assessed *agrBDCA* and RNAIII transcripts in two of the spontaneously resistant 2014.N isolates that were previously recovered: Survivors A and B. Expression of *agrBDCA* and RNAIII transcript level was significantly reduced in both Survivor A and Survivor B (Fig. 4b) compared to the parental strain. These results strongly suggest that absence of or lower expression of *agrBDCA* results in protection from *C. pseudodiphtheriticum*-mediated bactericidal activity.

It was previously observed that *S. aureus* strains that carry *agr* allele I or II rarely coexist with *Corynebacterium* species in the nasal cavity (38). This observation, combined with the variability in the degree of sensitivity seen with the various strains (Fig. 1a and b), led us to ask whether the *agr* allele type of those strains corresponded to the overall degree of sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. All three strains carrying *agr* I alleles showed intermediate sensitivity. The three strains carrying *agr* II alleles showed either resistance (two strains) or intermediate sensitivity (one strain). The two *agr* III-containing strains showed high and intermediate sensitivity. The one available *agr* IV allele-containing strain was resistant. Overall, there appeared to be no direct correlation between *C. pseudodiphtheriticum*-mediated bactericidal activity and *agr* allele type. Thus, again we concluded that a threshold level of *agrBDCA*/RNAIII expression must exist to confer sensitivity to bactericidal activity, regardless of what *agr* allele type is encoded.

**Loss of *psm* expression confers resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity.** The finding that *C. pseudodiphtheriticum* appears to affect *S.*

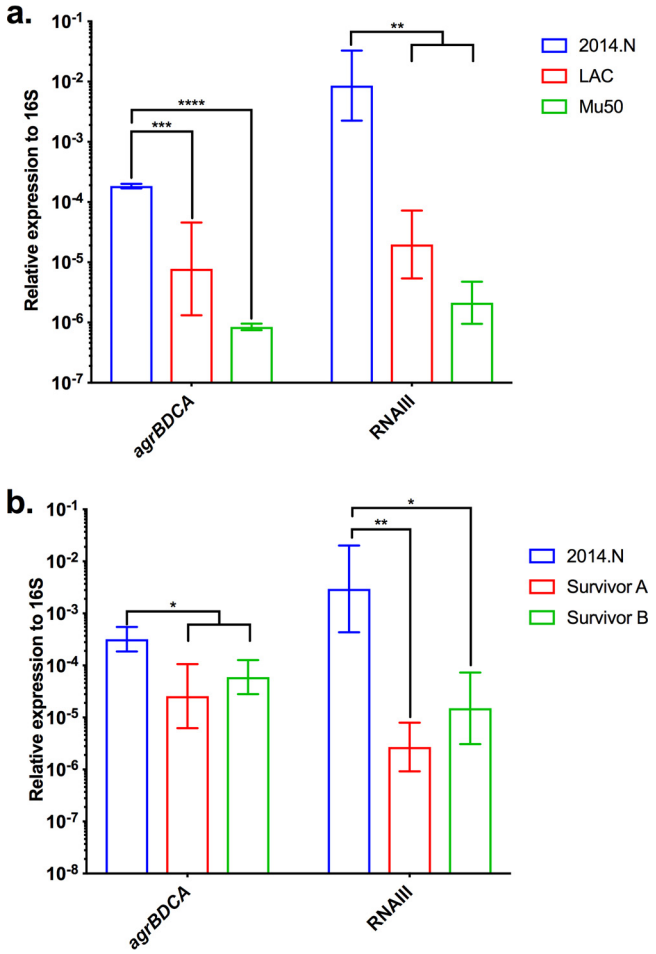


**FIG 4** Expression of *agrBDCA* is decreased in *S. aureus* isolates that are resistant to *C. pseudodiphtheriticum*-mediated bactericidal activity. Expression of *agrBDCA* was measured by qRT-PCR using primers specific to *agrB*, the first gene in the operon. RNA was isolated from (a) *S. aureus* LAC, Mu50, and 2014.N and (b) two spontaneously resistant 2014.N mutant strains (Survivors A and B) after 24 h of incubation at 28°C on BHIT agar. *agrB* and RNAIII expression are shown relative to 16S rRNA gene expression for three independent biological replicates. The data were plotted as follows: the geometric mean is represented as a bar, and error bars represent ±1 geometric standard deviation. A two-way ANOVA with Dunnett's corrections for multiple comparisons was performed. The symbols above the graph indicate the following: *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$; and ****, $P < 0.0001$, for differences in *agrBDCA* or RNAIII gene expression for 2014.N versus the other strains.

*aureus* Agr QS as a mechanism to mediate bactericidal activity was unexpected given that, to our knowledge, there is no evidence that directly connects Agr QS activity to cell death. Hence, we focused on genes that are known to be regulated by Agr QS as a means to understand the link between the Agr QS system and *C. pseudodiphtheriticum*-mediated bactericidal activity. Of the known Agr-regulated factors, the PSMs were of particular interest. The PSMs are downstream effectors of Agr QS, and data indicate that aberrant buildup of the PSMs inside the *S. aureus* cell via disruption of the gene encoding their dedicated transporter, PMT, leads to membrane instability and eventual cell lysis (39). Thus, we questioned whether the PSMs were required for *C. pseudodiphtheriticum*-mediated bactericidal activity. To test this, we assayed the sensitivity of wild-type *S. aureus* LAC compared to mutant strains that contained isogenic deletions of all six *psm* genes (LACΔ*psm*), or a complemented derivative containing the α-*psm* genes (LACΔ*psm* pTX$_{\Delta}$*psm*α1-4) under the control of a constitutively expressed promoter. The deletion of the *psm* genes resulted in resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity, and sensitivity was restored upon complementation of the α-*psm* genes (Fig. 5a and b).

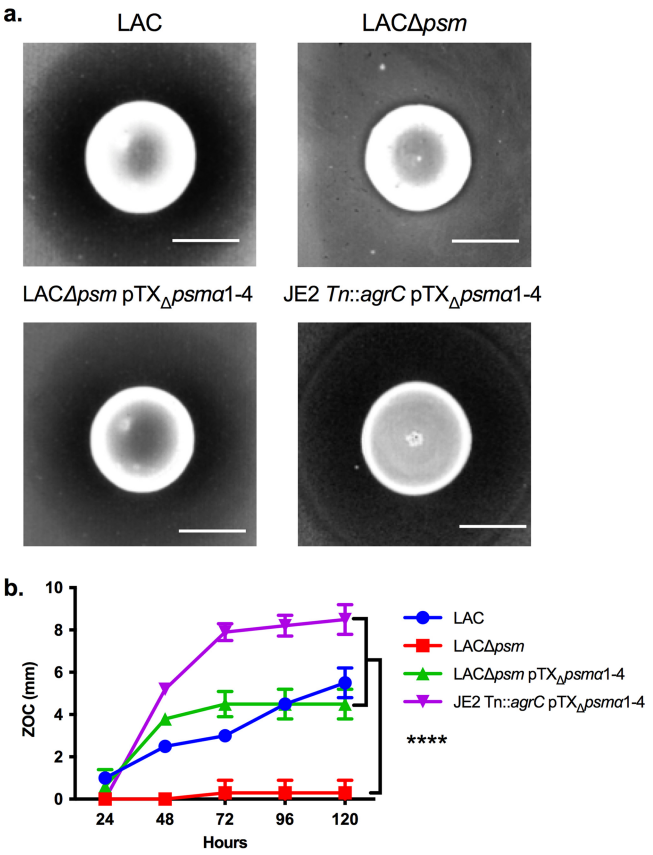


**FIG 5** Absence of *psm* confers resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity. (a) *S. aureus* LAC, LACΔ*psm*, LACΔ*psm* pTX$_\Delta$*psm*α1-4, and JE2 *Tn*::*agrC* pTX$_\Delta$*psm*α1-4 were assayed for sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. Images of the ZOC were taken after 120 h of incubation at 28°C and are representative of three independent biological replicates. Bar, 10 mm. (b) The ZOC distance was measured using ImageJ software. Each symbol represents the arithmetic mean from three independent biological replicates, and errors bars represent ±1 standard deviation. A two-way ANOVA with Dunnett's correction for multiple comparisons was performed on the ZOC lengths at the 120-hour time point. The symbol **** on the graph indicates statistically significant differences ($P < 0.0001$) of ZOC lengths for the LACΔ*psm* strain versus all other strains.

Given that *agr* mutations are pleiotropic and result in changes in expression of many genes, we next asked whether resistance of the *agr* mutant strains (Fig. 3) was specifically due to loss of expression of the α-*psm* genes. To this end, we transformed the JE2 *Tn*::*agrC* strain with the α-*psm* complementation vector (pTX$_\Delta$*psm*α1-4) and tested sensitivity of the strain to *C. pseudodiphtheriticum*-mediated bactericidal activity; the α-*psm* genes are constitutively expressed in the pTX$_\Delta$*psm*α1-4 vector and are not subject to Agr-mediated regulation. JE2 *Tn*::*agrC* pTX$_\Delta$*psm*α1-4 was fully sensitive to *C. pseudodiphtheriticum*-mediated bactericidal activity (Fig. 5). Furthermore, when *S. epidermidis* 1457, which was previously resistant to killing (Fig. 1), was transformed with the pTX$_\Delta$*psm*α1-4 vector, *S. epidermidis* 1457 pTX$_\Delta$*psm*α1-4 became sensitive to *C. pseudodiphtheriticum*-mediated killing; no ZOC was formed with the *S. epidermidis* pTX$_\Delta$16 empty vector control (Fig. 6). Thus, resistance of the *agr* mutant strains (Fig. 3) appeared not to be due to any larger pleiotropic effect, and expression of the *psm* genes in *S. epidermidis* was sufficient to induce susceptibility.

Given the data showing the correlation between the level of *agrBDCA* expression and sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity, we also assessed *psm*α$_1$ (first gene in the *psm*α operon) transcript levels in *S. aureus* 2014.N (highly sensitive), LAC (moderately sensitive), and Mu50 (resistant) and in two of the 2014.N spontaneously resistant isolates (Survivors A and B) relative to 16S rRNA gene expression (Fig. 7). The *psm*α$_1$ transcript level perfectly correlated with the overall

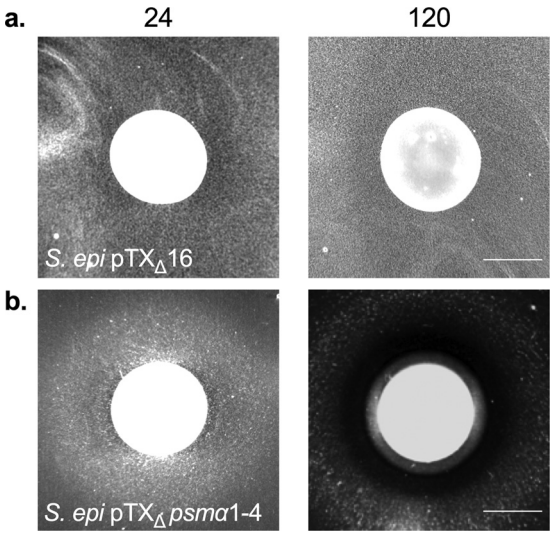


**FIG 6** Expression of the α-*psm* genes in *S. epidermidis* confers sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. *S. epidermidis* 1457 pTX$_\Delta$16 (empty vector) and *S. epidermidis* 1457 pTX$_\Delta$*psm*α1-4 were assayed for sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. Images of the ZOC were taken after 24 and 120 h of incubation at 28°C and are representative of three independent biological replicates. Bar, 10 mm.

sensitivity to *C. pseudodiphtheriticum*-mediated killing; resistant Mu50 showed a very low level of *psm*$\alpha_1$ expression, moderately sensitive LAC showed an intermediate level of expression, and highly sensitive 2014.N showed significantly higher *psm*$\alpha_1$ expression (Fig. 7a). Furthermore, both Survivor A and Survivor B showed significantly decreased levels of *psm*$\alpha_1$ expression (Fig. 7b) compared to 2014.N. To determine if this correlated with decreased levels of α-PSM, we measured total α-PSM concentration from parental strain 2014.N and the surviving derivative, 2014.N Survivor A. The quantity of α-PSMs produced by the spontaneously resistant isolate 2014.N Survivor A was significantly reduced compared to the parental strain (Fig. 7c). While the level of α-*psm* transcript mimicked the *agrBDCA* pattern of expression, the transcript level of *pmt* was not significantly different across strains, with the exception of 2014.N and Mu50 (Fig. 7a) ($P = 0.0001$).

Next, we asked whether defects in expression of *agrBDCA* or *psm*$\alpha_1$ could also account for the resistance seen with mutant strains containing Tn insertions in *purK*, *pyrF*, *carA*, *purK*, or *bacA*. While there was no difference in *agrBDCA* between the parental strain and any of the resistant transposon mutant strains (data not shown), differences in *psm* expression were observed. Specifically, expression of *psm*$\alpha_1$ was significantly decreased in mutant strains bearing Tn insertions in *purL*, *purK*, *pyrF*, and *carA* but not in *bacA* (Fig. S2). Therefore, defects in *psm*α expression correlate with resistance in most of the transposon mutant strains. Taken together, the expression data indicate that absence or reduction of *psm* expression confers resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity, likely independent of Agr QS activity.

**Altered biofilm production is not responsible for resistance to *C. pseudodiphtheriticum*.** Both Δ*agr* and Δ*psm* mutant strains are known to have "hyper-biofilm-producing" phenotypes (40). Indeed, biofilm formation in the *agrC* mutant strain was significantly higher than in the wild-type strain, and this phenotype was lost upon transformation with pTX$_\Delta$*psm*α1-4 (Fig. S4a). It is well established that biofilms provide protection against antimicrobial peptides, antibiotics, and other environmental stresses (41–43). Therefore, it was plausible that the increased biofilm production of the Δ*agr* and Δ*psm* mutant strains was responsible for resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity. To investigate this possibility, we analyzed the available literature on *S. aureus* biofilm production to identify other genes whose mutations

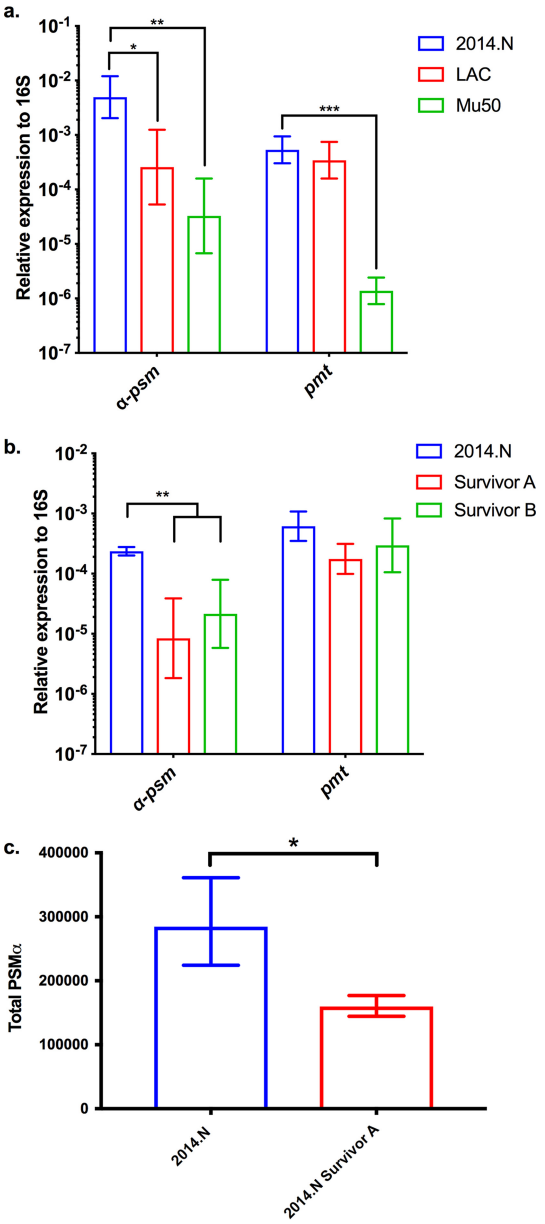


**FIG 7** Expression of α-*psm* is decreased in *S. aureus* isolates resistant to *C. pseudodiphtheriticum*-mediated bactericidal activity. Expression of α-*psm* and *pmt* was measured by qRT-PCR using primers specific to *psmα*$_1$ or *pmtA*, the first gene in each operon, respectively. (a and b) RNA was isolated from *S. aureus* LAC, Mu50, and 2014.N (a) and two spontaneously resistant 2014.N mutant strains (Survivors A and B) (b) after 24 h of incubation at 28°C on BHIT agar. α-*psm* and *pmt* gene expression is shown relative to 16S rRNA gene expression for three independent biological replicates. The data were plotted as follows: the geometric mean is represented as a bar, and error bars represent ±1 geometric standard deviation. A two-way ANOVA with Dunnett's corrections for multiple comparisons was performed. The symbols above the graph indicate the following: *, $P < 0.05$; **, $P < 0.01$; and ***, $P \leq 0.001$, for differences in α-*psm* and *pmt* gene expression for 2014.N versus the indicated strains. (c) 2014.N and 2014.N Survivor A strains were cultured overnight in BHIT broth. The total quantities of PSMs present were analyzed and measured by reverse-phase liquid chromatography–mass spectrometry. Bars represent arithmetic means from three independent biological replicates ±1 standard deviation. A two-tailed Student *t* test with Welch's correction was performed on total PSM quantity. The symbol * above the graph indicated a statistically significant difference between 2014.N and Survivor A ($P = 0.048$).

result in a hyper-biofilm-producing phenotype. We took advantage of the arrayed JE2 transposon library (37) and selected transposon mutant strains that contained mutations in these genes, tested their biofilm-forming ability compared to JE2, and then tested sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity. Transposon

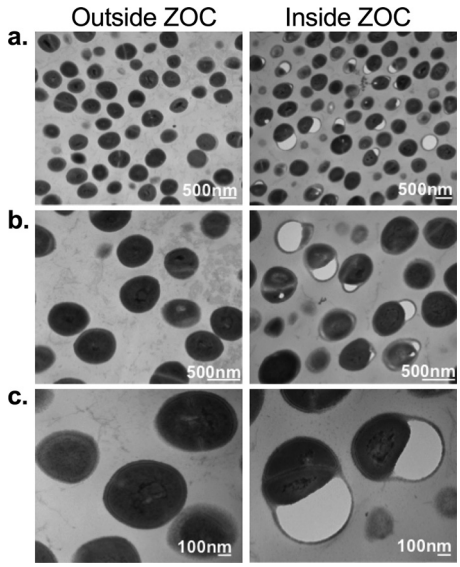


**FIG 8** Coculture with *C. pseudodiphtheriticum* is detrimental to *S. aureus* cell morphology and integrity. *S. aureus* 2014.N was spread in a lawn on top of agar plates, and *C. pseudodiphtheriticum* was spotted directly on top. Plates were incubated at 28°C for 24 h, and cells were recovered directly adjacent to *C. pseudodiphtheriticum* (Inside ZOC) and elsewhere on the plate (Outside ZOC). Changes in *S. aureus* cell morphology were examined by transmission electron microscopy (TEM) at ×25,000 (a), ×50,000 (b), and ×100,000 (c) magnification.

mutant strains bearing Tn insertions in *icaR* (negative regulator of biofilm production), *aur* (metalloprotease with a role in biofilm dispersal), *rsp* (represses biofilm formation), *scpA* (cysteine protease with a role in biofilm dispersal), and *splB* (cysteine protease with a role in biofilm dispersal) were all analyzed. As seen in other strain backgrounds (44–48), biofilm production was significantly increased in 2/5 selected transposon mutant strains and trended higher in the three remaining strains compared to parental JE2 (Fig. S4a and b). Moreover, though the degree of sensitivity did vary across each of the mutant strains, each of the selected hyper-biofilm-producing transposon mutant strains was still sensitive to *C. pseudodiphtheriticum*-mediated bactericidal killing (Fig. S4c and d). These data suggest that increased biofilm production is likely not responsible for the resistant phenotypes of the Δ*agr* and Δ*psm* mutant strains to bactericidal activity.

**Coculture with *C. pseudodiphtheriticum* is detrimental to *S. aureus* cell surface morphology.** Our initial bacterial interactions experiments (Fig. 1) revealed that after 48 to 72 h, a ZOC developed around the *C. pseudodiphtheriticum* spot, and few surviving *S. aureus* cells were recoverable from the ZOC (Fig. 1c). In order to directly visualize *C. pseudodiphtheriticum*-mediated effects on *S. aureus*, we slightly modified the bacterial interaction assay by swabbing *S. aureus* 2014.N (highly sensitive) directly on top of the agar plate and then spotting *C. pseudodiphtheriticum* directly on top of the *S. aureus* lawn. After 24 h of incubation, *S. aureus* cells were recovered from inside and outside the ZOC and were visualized with transmission electron microscopy (TEM). TEM analysis revealed that even after only 24 h of incubation, there were dramatic morphological changes in the *S. aureus* cells that were recovered from inside the ZOC compared to the cells recovered from outside the ZOC (Fig. 8a). A closer view of the cells recovered from inside the ZOC revealed that the *S. aureus* cell surface integrity was severely compromised and that there was some possible leakage of intracellular constituents into the extracellular space (Fig. 8b and c), possibly by plasmolysis. Thus, as suggested by the other interaction assays, *C. pseudodiphtheriticum*-mediated killing of *S. aureus* appeared to be due to significant damage to the *S. aureus* cell surface that likely leads to eventual cell lysis.

Finally, to determine whether expression of *agrBDCA*, *psm*, and *pmt* changed in response to the presence of *C. pseudodiphtheriticum*, qRT-PCR analysis was performed

on RNA from *S. aureus* cells recovered inside and outside the ZOC after 3 or 24 h of coincubation: highly sensitive (2014.N), intermediately sensitive (LAC), and resistant (Mu50) strains were tested. No significant differences in expression of any of the genes at either time point were observed for 2014.N or Mu50 (Fig. S5). Conversely, *S. aureus* LAC cells recovered inside the ZOC showed higher levels of expression of each gene at 3 h; this was statistically significant for *psm* expression ($P = 0.0052$). This difference in transcript level was lost by 24 h, when killing begins to be readily discernible. These results suggest that though coincubation may alter gene expression in some *S. aureus* strain backgrounds, the overall sensitivity to *C. pseudodiphtheriticum*-mediated killing is not dependent on modulation of expression of any of the tested genes. Instead, the degree of killing is most closely correlated with the overall level of *agr* and *psm* expression seen in the *S. aureus* strain (Fig. 4 and 7).

## DISCUSSION

Bacteria rarely exist as single species in the environments in which they live. This fact has become increasingly appreciated with the emergence of high-throughput sequencing and advances in the study of the human microbiota. Ultimately, efforts to study how bacteria interact within these polymicrobial, and often nutrient-scarce, settings have been challenging due to the fact that many bacterial species are unculturable. Furthermore, when thinking about colonization or infection of human niches, the complexity is further compounded by host factors that influence these interactions. The opportunistic pathogen *S. aureus* is able to successfully persist as commensal flora within the nose of some individuals; this is accomplished by mitigating the host immune response and by outcompeting other resident flora within the nasal niche (18). At any given time, one-third of the population is asymptomatically colonized with *S. aureus* (11, 12). However, a subset of these individuals will ultimately suffer from *S. aureus*-mediated disease that is often caused by the original colonizing strain (14). Therefore, it is imperative to identify the specific molecular factors that foster *S. aureus* commensalism versus pathogenesis. In addition, our current understanding of the complex molecular interactions that occur between the nasal microbiota and *S. aureus* remains limited. Thus, more detailed molecular analyses of the interspecies interactions that have been observed during microbiota studies are warranted.

Molecular profiling studies have revealed that species-specific interactions play critical roles in blocking *S. aureus* nasal colonization and persistence. The *Corynebacterium* genus in particular appears to greatly influence nasal *S. aureus* carrier status (2, 31). Indeed, both *S. aureus* and *Corynebacterium* species colonize the human nose (33), and there is increasing evidence that these microorganisms directly interact. Moreover, inoculation of a collection of *Corynebacterium* species into the nasal cavity was sufficient to completely eradicate *S. aureus* from this niche (49). Furthermore, evidence suggests that a single *Corynebacterium* species, *C. pseudodiphtheriticum*, is sufficient to negatively impact *S. aureus* nasal colonization (31). Indeed, artificial inoculation of *C. pseudodiphtheriticum* was sufficient to eradicate *S. aureus* from the nasal cavity of humans (50). However, the mechanism by which *C. pseudodiphtheriticum* blocks *S. aureus* nasal colonization remains unclear. Here, we set out to characterize the *in vitro* interaction that occurs between *S. aureus* and *C. pseudodiphtheriticum* and have sought to understand the negative interactions that occur between these species. By taking a reductionist approach, we found that *C. pseudodiphtheriticum* mediates specific bactericidal activity against *S. aureus* by ultimately affecting *S. aureus* cell surface integrity. Furthermore, Agr QS system-dependent regulation of the PSMs, which are important virulence factors of *S. aureus*, appears crucial for this activity.

Examination of the sensitivity of various *S. aureus* strains to *C. pseudodiphtheriticum*-mediated bactericidal activity revealed that the majority of tested strains were susceptible and were likely being killed by a secreted factor(s) (Fig. 1 and 2). Interestingly, there were three distinct levels of *S. aureus* susceptibility (Fig. 1b), which could suggest that the target(s) or mediators of bactericidal activity may be differentially expressed or functionally different between the resistant and sensitive strains. Moreover, we found

that *C. pseudodiphtheriticum* bactericidal activity was specific to *S. aureus* (Fig. 1 and data not shown). This finding supports the growing body of literature that indicates that the interactions between normal flora and opportunistic pathogens are highly species specific (32, 48, 51). For example, while *C. pseudodiphtheriticum* mitigates *S. aureus* nasal colonization, the closely related *Corynebacterium* species *C. accolens* is associated with a higher abundance of *S. aureus* in the nasal cavity and appears to promote *S. aureus* growth *in vitro* (31). Intriguingly, *C. accolens* secretes a lipase that converts triacylglycerols that decorate nasal epithelial cells into free fatty acids (FFAs) and glycerol. FFAs, specifically oleic acid, have potent bactericidal activity against *Streptococcus pneumoniae* (51). This finding is significant because an inverse relationship exists between the presence of *S. pneumoniae* and *S. aureus* in the nasal cavity (52). Thus, some *Corynebacterium* species may influence *S. aureus* nasal carrier status by reducing or promoting competition from other incoming opportunistic pathogens.

Our initial characterization experiments and transposon mutant strain screen revealed that *S. aureus* strains that have little to no Agr QS activity, either due to insertional inactivation of *agrBDCA* via a transposition event or due to other spontaneous changes that reduced *agrBDCA* expression, are resistant to bactericidal activity (Fig. 3 and 4; see also Fig. S1 in the supplemental material). *S. aureus* strains N315 and Mu50, which both intrinsically express lower *agrBDCA* and RNAIII transcript levels (53), were also resistant to killing (Fig. 1 and 4). It is worth noting that during infection, Agr QS-defective strains are generally less virulent and show decreased expression of many secreted virulence factors; conversely, these strains show increased expression of many colonization factors (23, 54). The fact that only the phenotypically less virulent *S. aureus* strains can escape *C. pseudodiphtheriticum*-mediated killing perhaps suggests an interesting interplay between bacterial competition, virulence, and commensalism. In this vein, it is worth noting that the ability of *Corynebacterium* species to influence expression of *S. aureus* virulence factors is supported by recent work from Ramsey et al. that showed that a coculture of *S. aureus* and *Corynebacterium striatum*, another common nasal commensal species, led to global changes in *S. aureus* gene transcription (55). These expression changes included significant decreases in transcript levels of *agrBDCA*, *psm*$\beta_1$, and *psm*$\beta_2$. In contrast, transcription of genes that encode proteins that are important for colonization of nasal epithelial cells, including *Staphylococcus* protein A (*spa*) and iron-regulated surface determinant adhesin (*isdA*), was greatly increased; these expression patterns mimic *S. aureus* gene expression seen in the nasal cavity of cotton rats and humans (28, 29). Furthermore, spent medium derived from *C. pseudodiphtheriticum* led to a similar reduction in *agrBDCA* expression, indicating that *S. aureus* modulates gene expression in response to factors remaining in the medium and perhaps suggesting that this is a response to the presence of the *Corynebacterium* genus (55). Given the data presented here, perhaps *S. aureus* turns off virulence factor expression as a means to escape *C. pseudodiphtheriticum*-mediated killing, and thus, an inadvertent consequence of the competition between these two species is selection for *S. aureus* to remain in a phenotypic nonpathogenic state.

We observed that the PSMs are likely the downstream effectors of the Agr QS that are responsible for *C. pseudodiphtheriticum*-mediated bactericidal activity (Fig. 5, 6, and 7); *S. aureus* LAC strains that contained markerless deletions of all 6 *psm* genes were highly resistant to killing activity. In addition, restoration of the $\alpha$-*psm* genes to resistant isolates was sufficient to restore sensitivity to *C. pseudodiphtheriticum*-mediated bactericidal activity, even in a different species, *S. epidermidis* (Fig. 6). Thus, *C. pseudodiphtheriticum* may target the activity of the PSMs or their transporter, PMT, as a mechanism to induce *S. aureus*. While the exact role of the PSMs in *C. pseudodiphtheriticum*-mediated bactericidal activity remains unclear, three plausible mechanisms can be gleaned from the existing literature. First, induction of aberrant accumulation of PSMs within *S. aureus* cells due to decreased expression of *pmt* has been shown to result in rapid killing of *S. aureus*. Indeed, this killing is so robust that PMT appears to be essential for *S. aureus* survival in the presence of the *psm* genes; *pmt* can be deleted only concurrently with deletion of all of the *psm* genes (39). Thus, perhaps *C. pseudodiph-*

*theriticum* causes cell death via induction of an aberrant accumulation of the PSMs within the cell. Second, recent work by Pader et al. (56) found that strains that lack Agr QS activity show enhanced resistance to daptomycin. In Agr QS-defective strains, phospholipid shedding from the cell membrane is increased (56). These phospholipids bind to and inactivate antibiotics like daptomycin, increasing resistance. Mechanistically, this process is directly linked to the lack of PSM synthesis and secretion. Once secreted, the PSMs normally act like detergents that can break up phospholipids. Thus, if the PSMs are not synthesized, more phospholipids are shed and active, which in turn results in increased resistance to daptomycin. Thus, perhaps *S. aureus* increased phospholipid shedding is responsible for the resistance of the Δ*psm* mutant strain; *C. pseudodiphtheriticum* is producing a compound that can be bound and inactivated by shed *S. aureus* phospholipids. Finally, it is possible that the absence of *psm* expression indirectly stabilizes the *S. aureus* cell surface structure through some unknown mechanism, which results in resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity. Future studies will seek to understand and mechanistically define the exact role of the PSMs in this process.

It is tantalizing to explore the idea that products from commensal bacteria, or the use of commensal bacteria as next-generation probiotics, could be used as novel strategies to block opportunistic pathogens. For example, Kanmani et al. found that nasal priming of *C. pseudodiphtheriticum* in mice led to improved resistance to respiratory syncytial virus (RSV) and subsequent *Streptococcus pneumoniae* superinfection (57). The proposed mechanism of resistance was via modulation of the host's immune system, including the influx of lymphocytes. Since *C. pseudodiphtheriticum* can directly kill *S. aureus* and can modulate virulence gene expression (55), our work adds to the growing body of literature that suggests antipathogen properties of this species. Moreover, we observed that *S. aureus* spontaneous resistance to *C. pseudodiphtheriticum*-mediated bactericidal activity occurred at a relatively low frequency (~$10^{-8}$); however, this resistance came with a cost of attenuated virulence gene expression. Deficiencies in *agrBDCA* expression result in virulence attenuation and have been shown to result in less severe *S. aureus*-mediated disease (23). The possible use of compounds that inhibit or decrease virulence of *S. aureus* as a strategy to reduce severity of disease is a relatively unexplored field. However, some work suggests that limiting quorum sensing could be one possible mechanism to decrease *S. aureus* virulence during active infection. Indeed, the host may seek to reduce virulence using this mechanism; human serum has been found to reduce *S. aureus* Agr QS activity via the sequestration of AIP (58). Recently, Paharik et al. found that a commensal *Staphylococcus* species, *S. caprae*, inhibits *S. aureus* Agr QS via the production of an inhibitory AIP (59). This reduction of *S. aureus* Agr QS activity resulted in reduced skin burden and necrosis. Thus, the possibility of targeting a virulence regulon as a means of controlling disease is an attractive approach. Nevertheless, there are several caveats that must be considered with this type of approach. For example, the use of inhibitory *agr* compounds as a method to block virulence does not lead to clearance of *S. aureus* (59). Indeed, it is well established that loss of *agr* expression promotes *S. aureus* biofilm formation and expression of colonization factors (29, 60); this could be particularly problematic for immunocompromised individuals since the local burden of *S. aureus* could be significantly increased. Alternatively, given that *C. pseudodiphtheriticum* directly kills *S. aureus* and that *S. aureus* seeks to escape this killing by turning off virulence gene expression, the use of *C. pseudodiphtheriticum*-derived products as a way to block nasal colonization and/or treat active disease may be superior to antivirulence compounds alone.

While our work sought to elucidate the molecular mechanism that *C. pseudodiphtheriticum* may use to prevent *S. aureus* nasal colonization, it is critical to note that the nasal microenvironment is complex, and bacterial species that negatively affect *S. aureus* nasal colonization are not limited to *Corynebacterium*. There are numerous examples of coagulase-negative *Staphylococcus* (CoNS) species that have evolved strategies to compete with *S. aureus* in the nose. For example, some *S. epidermidis* strains synthesize and secrete a serine protease, Esp, which is capable of disrupting *S.*

*aureus* biofilm formation and is able to block nasal colonization (61). *Staphylococcus hominis*, another common commensal bacterial species, and *S. epidermidis* both secrete strain-specific antimicrobial peptides (AMPs) that have potent selective bactericidal activity against *S. aureus* (62). Furthermore, these novel AMPs were found to work synergistically with the human AMP LL-37 to prevent atopic dermatitis. *Staphylococcus lugdunensis* produces a novel cyclic peptide antibiotic, lugdunin, that has bactericidal properties against several Gram-positive pathogens, including *S. aureus*; lugdunin can prevent *S. aureus* nasal colonization (63). Growing evidence indicates that within the human nose there is a clear selective pressure, even among closely related commensal species, to block or eliminate *S. aureus*. Furthermore, compounds from commensal CoNS have been shown to eliminate *S. aureus* in the nasal cavity in humans (63).

There are limitations to our study. For example, while the data indicate that the PSMs are involved in *C. pseudodiphtheriticum*-mediated killing of *S. aureus*, the exact molecular mechanism by which this occurs remains to be elucidated. Furthermore, it is well recognized that *in vitro* phenotypes do not always correlate with *in vivo* phenotypes. Therefore, even though human studies suggest negative interactions between *Corynebacterium* and *S. aureus* (31, 34, 49, 50, 55), the observed roles of Agr QS and the PSMs in *C. pseudodiphtheriticum*-mediated killing may not be as relevant *in vivo*. Thus, the significance of our findings remains to be tested in the context of nasal colonization in an appropriate animal model. Even then, given that multispecies bacterial communities are complex and dynamic, it will be difficult to confirm that eradication of *S. aureus* in the nasal cavity is due solely to a *C. pseudodiphtheriticum*-derived factor(s); it is possible that a *C. pseudodiphtheriticum*-derived factor(s) may synergize with other host or bacterium-derived compounds to ultimately lead to *S. aureus* killing. Clearly, identification and purification of the *C. pseudodiphtheriticum*-produced bactericidal factor(s) will aid investigation of these possibilities.

In summary, our study has revealed that the molecular interactions between *Staphylococcus* and *Corynebacterium* are far more multifaceted than previously recognized. From the results presented here, we conclude that *C. pseudodiphtheriticum* selectively targets *S. aureus* for killing. This works expands on the well-established negative correlation between the presence of *C. pseudodiphtheriticum* and *S. aureus* in the nose that has been observed in numerous nasal microbiota cohort studies (31, 50). Moreover, given our finding that strains that were deficient in Agr QS were resistant to *C. pseudodiphtheriticum*-mediated killing, there may be a selective advantage for *S. aureus* strains to switch from a pathogenic state to a commensal state. Thus, in order to coexist with *C. pseudodiphtheriticum* and to increase the probability of successful colonization of the nasal niche, *S. aureus* must surrender expression of its primary virulence factors. Future work will pursue the identity of the *C. pseudodiphtheriticum*-derived factor(s) that facilitates bactericidal activity against *S. aureus*. *En masse*, our findings support the continued study of "bacterial warfare" between commensal and pathogenic bacterial species as a way to develop novel therapeutics and/or identify molecular vulnerabilities of pathogens.

## MATERIALS AND METHODS

**Strains, culture, and bacterial interaction assays.** Strains used in this study are listed in Table 1, and primers and plasmids are listed in Table 3. All *S. aureus* strains were typed for the *agr* allele using established protocols (38). *Corynebacterium pseudodiphtheriticum* strain USU1 was isolated as part of a cooperative agreement between the Uniformed Services University of the Health Sciences and the Walter Reed Military Medical Center Clinical Microbiology Lab. Strains were maintained as −80°C freezer stocks and were revived as follows: *Corynebacterium* species were streaked from frozen glycerol stocks on brain heart infusion (BHI) agar (Becton, Dickinson) with 1% Tween 80 (BHIT; Sigma-Aldrich), and *Staphylococcus* species were streaked from glycerol stocks on BHI agar. Each strain was expanded by overnight incubation at 35°C. The bacterial interaction assay was adapted from published protocols (31). Briefly, 0.04 g of *S. aureus* or *S. epidermidis* was directly harvested from agar plates with a sterile inoculating loop and then resuspended in 0.2 ml of sterile 0.9% NaCl (Fisher Chemicals). Eight microliters (~$10^8$ CFU) of the cell suspension was then inoculated into 15 ml of autoclaved BHIT agar that had been precooled to 55°C; inoculated agar was then poured into a sterile petri dish. The agar was allowed to cool in a laminar flow hood for 30 min. Next, 25 $\mu$l of a *C. pseudodiphtheriticum*, *C. accolens*, or *C. diphtheriae* (~$5 \times 10^9$ CFU) cell suspension was spotted onto the center of the agar and was allowed to dry in the laminar flow

**TABLE 3** Primers and plasmids used in this study

| Oligonucleotide or plasmid | Sequence (5′–3′) or notes | Reference |
|---|---|---|
| Oligonucleotides | | |
| Quantitative real-time PCR | | |
| *agrB* FWD | GACCAGTTTGCCACGTATCT | This study |
| *agrB* REV | GCTAAGACCTGCATCCCTAATC | This study |
| RNAIII FWD | GGAGTGATTTCAATGGCACAAG | This study |
| RNAIII REV | GTGAATTTGTTCACTGTGTCGATAA | This study |
| *psm*$\alpha_1$ FWD | GGTATCATCGCTGGCATCATT | This study |
| *psm*$\alpha_1$ REV | CCATGTGAAAGACCTCCTTTGT | This study |
| *pmtA* FWD | CTTGCGCATGTTCTGTTAATCC | This study |
| *pmtA* REV | TCAAGTTGTGAGTGGTGCTATT | This study |
| 16S rRNA FWD | GTGGAGGGTCATTGGAAACT | This study |
| 16S rRNA REV | CACTGGTGTTCCTCCATATCTC | This study |
| *agrC* complementation | | |
| *agrC* FWD BamHI | TGGATCCAGAGGAGAAATTAAATGAT | This study |
| *agrC* REV SstI | CCCGAGCTCCTAGTTGTTAATAATTTCAAC | This study |
| Transposon insertion sequencing | | |
| RP45 | GAGTGTGATGATAAGTGGGAAGGAC | This study |
| SS1905 | GGTAAACTATGATTCACGACGACTAG | This study |
| Plasmids | | |
| pCL15 | *E. coli-S. aureus* shuttle vector | 70 |
| pCL15-P$_{spac}$-*agrC* | *agrC* complementation vector | This study |
| pTX$_\Delta$*psm*$\alpha$1-4 | *psm* complementation vector | 39 |
| pTX$_\Delta$16 | Empty vector | 81 |

hood for 40 min. The resulting plates were incubated at 28°C, and the zone of clearance (ZOC) was examined every 24 h for a total of 120 h. The ZOC was defined as the distance between the edge of the *Corynebacterium* spot and the visible edge of the clearance ring. To measure the ZOC, images were analyzed using ImageJ software (64). Unless otherwise noted, a two-way analysis of variance (ANOVA) statistical test with Dunnett's correction for multiple comparisons was used to assess significance; a confidence interval of 95% and an alpha value of 0.05 were considered significant.

To determine whether live *C. pseudodiphtheriticum* was required to mediate bactericidal activity, ~5 × 10$^9$ CFU of *C. pseudodiphtheriticum* was heated to 90°C for 10 min; loss of viability was confirmed by plating on BHIT agar. The heat-killed *C. pseudodiphtheriticum* was inoculated onto *S. aureus*-seeded plates as described above. Images were taken after 24 and 120 h of incubation at 28°C. To determine if bactericidal activity was dependent on direct physical contact, a sterile 0.2-$\mu$m filter disk was placed on top of the BHIT agar that had been seeded with *S. aureus*; *C. pseudodiphtheriticum* was then spotted on top of the filter disk. Images of the ZOC were taken after 24 and 48 h of incubation at 28°C.

***Corynebacterium* CCFM preparation and disk diffusion assay.** Cell-free conditioned medium (CCFM) was prepared from *C. pseudodiphtheriticum* according to the method of Ramsey et al. (55) with several modifications. One-liter *C. pseudodiphtheriticum* cultures were grown for 24 h in BHIT broth at 37°C with shaking at 190 rpm. Cultures were centrifuged at 13,000 rpm for 10 min, and culture supernatant was then passed through a 0.2-$\mu$m filter (Corning). Sterile CFCM was further concentrated by ammonium sulfate precipitation. Sterile saturated ammonium sulfate was added to sterile CFCM at 1× the original volume. The suspension was centrifuged at 13,000 rpm for 10 min, and the supernatant was decanted. The pellet was air dried and resuspended in 1× PBS (Fisher Chemicals) at a 50× final concentration. For the disk diffusion assay, *S. aureus* was cultured in BHI broth overnight at 37°C. The following day, these cultures were diluted to 1 × 10$^8$ cells/ml (OD$_{600}$ of 0.1) in BHI broth, and a sterile swab was used to spread the *S. aureus* cell suspension on BHIT agar as a lawn. The plate was allowed to dry in a laminar flow hood for 30 min. Next, a sterile 6-mm diffusion disk was place on top of the *S. aureus* lawn, and 50 $\mu$l of CCFM was inoculated onto the disk. Plates were incubated at 28°C, and images were taken after 24 and 72 h of incubation.

**Recovery of resistant *S. aureus* isolates.** *S. aureus* isolates that developed spontaneous resistance to *C. pseudodiphtheriticum*-mediated killing were recovered by extracting 5-mg punches of agar from the ZOC directly adjacent to the *C. pseudodiphtheriticum* spot with a sterile pipette tip. To estimate the frequency of resistance, comparable punches of agar were also taken from an area directly outside the ZOC. Agar punches were resuspended in 1 ml of BHI and heated to 55°C for 10 min. The entire 1-ml cell suspension was plated on mannitol salt agar (MSA; Criterion). Plates were incubated at 37°C overnight, and recovered colonies were visualized. The frequency of spontaneous resistance to bactericidal activity was calculated by dividing the number of *S. aureus* CFU recovered from a 5-mg punch inside the ZOC by the number of *S. aureus* CFU recovered from a 5-mg punch directly outside the ZOC. To confirm that *S. aureus* colonies recovered from the ZOC were stably resistant to *C. pseudodiphtheriticum*-mediated killing, five recovered colonies were chosen at random and reassayed in the described bacterial interaction assay.

**Screen for resistant *S. aureus* transposon mutant strains.** Transposon vector pRP1313 was engineered by adding features of the *mariner* C9 transposase from pMarB (65) to pMAD (66) as described

in Text S1 and Table S1 in the supplemental material. The vector was transformed into RN4220 (yielding strain SAP370) and transduced into NRS384 (yielding strain SAP372) as previously described (67, 68), using chloramphenicol (10 $\mu$g/ml) as the selectable marker and incubating cultures at 30°C.

To generate a pooled *S. aureus* transposon library, a 5-ml culture of SAP372 was grown overnight at 30°C in tryptic soy broth (TSB) containing chloramphenicol (10 $\mu$g/ml). The overnight culture was subcultured at 1:1,000 in TSB without antibiotics and was incubated for 1 h at 43°C. Spectinomycin was added (250 $\mu$g/ml), and the culture was incubated for an additional 6 h at 43°C. A spiral plater (Spiral Biotech AP-5000 Autoplate system) was used to spread 20 $\mu$l of this culture onto each of 48 tryptic soy agar plates containing spectinomycin (250 $\mu$g/ml), which were incubated overnight at 43°C. Growth on the plates was pooled (eight pools of six plates each) and resuspended in 30 ml of TSB per pool (~6,000 colonies per pool). Suspensions were centrifuged for 13 min at 4,700 rpm. Supernatants were poured off, and each pellet was resuspended in 32.5 ml of TSB containing 15% glycerol. Aliquots of 1 ml were stored at −80°C.

To identify transposon mutant strains that were resistant to *C. pseudodiphtheriticum*-mediated bactericidal activity, the pooled library was used to seed the agar as described for the bacterial interaction assay and surviving transposon mutant strains were recovered from within the ZOC as described above. Each recovered transposon mutant strain was independently confirmed to be stably resistant to *C. pseudodiphtheriticum*-mediated bactericidal activity. To identify the region of transposon insertion, a rescue cloning strategy was utilized that relied upon the presence of an R6K origin of replication in the *H1 mariner* transposon. Briefly, each resistant transposon mutant strain was grown overnight in 2 ml of BHI broth containing 50 $\mu$g/ml of spectinomycin (Sigma-Aldrich) at 37°C with shaking. Cells were pelleted by centrifugation and resuspended in 0.2 ml of 1× phosphate-buffered saline (PBS; Gibco) with 10 $\mu$g/ml lysostaphin (Sigma-Aldrich). The cell suspension was incubated at 37°C for 1 h. DNA was then extracted using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer's instructions. Recovered DNA was digested with MfeI-HF (New England Biolabs), and a self-ligation was performed via the addition of Quick ligase (New England Biolabs). The resulting material was then transformed into chemically competent DH5$\alpha$ $\lambda$pir, and colonies containing plasmids were recovered by plating on BHI agar that contained 50 $\mu$g/ml spectinomycin. Spectinomycin-resistant colonies were subcultured overnight in BHI broth containing 50 $\mu$g/ml spectinomycin. Plasmid DNA was then extracted from these cultures using the QIAprep Spin miniprep kit according to the manufacturer's instructions (Qiagen). The site of transposon insertion was determined by sequencing each plasmid in the forward (primer RP45) and reverse (primer SS1905) directions. The resulting sequences were then aligned against the *S. aureus* NCTC 8325 reference genome deposited in GenBank (69).

***agrC* complementation.** Purified JE2 genomic DNA was used to amplify *agrC* using primers *agrC* FWD BamHI and *agrC* REV SstI with the following conditions: an initial 30 s denaturation step at 98°C, followed by 35 cycles (10 s of denaturation at 98°C, 30 s annealing at 50°C, and 45 s of extension at 72°C), and a final extension step at 72°C for 10 min. The PCR fragment was digested with BamHI and SstI and was ligated into similarly digested pCL15 (70). In the resulting plasmid, pCL15-$P_{spac}$-*agrC*, *agrC* is under the control of the $P_{spac}$ promoter. The resulting ligation was transformed into chemically competent *Escherichia coli* TOP10 cells, and colonies containing pCL15-$P_{spac}$-*agrC* were selected for on BHI agar containing 100 $\mu$g/ml ampicillin. To confirm plasmid integrity, pCL15-$P_{spac}$-*agrC* was purified and sequenced prior to being electroporated directly into JE2 *Tn*::*agrC*. *S. aureus* transformants were selected on BHI agar containing 25 $\mu$g/ml chloramphenicol. Colony PCR on chloramphenicol-resistant colonies with primers *agrC* FWD BamHI and *agrC* REV SstI was used to confirm the presence of *agrC*. The complemented strain JE2 *Tn*::*agrC*-pCL15-$P_{spac}$-*agrC* is referred to as JE2 *Tn*::*agrC*$^C$ throughout this study.

**Creation of *S. aureus* JE2 *Tn*::*agrC* pTX$_\Delta$*psm*$\alpha$1-4 and *S. epidermidis* pTX$_\Delta$*psm*$\alpha$1-4.** LAC$\Delta$*psm* pTX$_\Delta$*psm*$\alpha$1-4 was cultured overnight at 37°C in BHI broth containing 10 $\mu$g/ml tetracycline (Sigma). Cells were pelleted by centrifugation, and pTX$_\Delta$*psm*$\alpha$1-4 was extracted using the QIAprep Spin miniprep kit according to the manufacturer's instructions (Qiagen), with the following exceptions: 10 $\mu$g/ml lysostaphin was added to the P1 buffer, and the cell suspension was incubated at 37°C for 30 min. Purified plasmid was then transformed into electrocompetent JE2 *Tn*::*agrC* or *S. epidermidis* 1457 cells. Transformants were selected for on LB agar containing 10 $\mu$g/ml tetracycline. To confirm that the plasmid was stably present in transformants, plasmid was then reisolated from JE2 *Tn*::*agrC* pTX$_\Delta$*psm*$\alpha$1-4 and *S. epidermidis* 1457 pTX$_\Delta$*psm*$\alpha$1-4 cells as described above. Purified pTX$_\Delta$16 plasmid was transformed into electrocompetent *S. epidermidis* 1457 to generate *S. epidermidis* 1457 pTX$_\Delta$16, an empty vector control. Transformants were selected for on LB agar containing 10 $\mu$g/ml tetracycline.

**RNA extraction, cDNA synthesis, and qRT-PCR analysis.** Parental *S. aureus* strains and resistant derivative isolates were cultured on fresh BHI agar plates at 28°C overnight (16 to 18 h) or cocultured with *C. pseudodiphtheriticum* on BHIT agar plates for 3 or 24 h at 28°C. A sterile inoculating loop was used to resuspend cells in 1 ml of freshly prepared lysis buffer (1× TE buffer with 10 $\mu$g/ml lysostaphin and 20 $\mu$g/ml proteinase K). The cell suspension was incubated at 37°C until the cell suspension was clear (5 to 15 min). RNA was isolated using TRIzol reagent (Invitrogen) as described previously (71), with the following exceptions: the aqueous phase was used directly in an RNeasy cleanup protocol (Qiagen), and a 60-min DNase (Qiagen) on-column digestion was performed during the cleanup. cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (qRT-PCR) was performed as previously described (71) and involved a Roto-Gene Q instrument (Qiagen); 1× SYBR green RT-PCR master mix (Qiagen); 3 pmol each of the forward and reverse primers (Table 2), which were designed with the IDT PrimerQuest Suite; and 1 $\mu$l of cDNA. qRT-PCR primers were confirmed by sequencing analysis to be 100% homologous to the corresponding gene across all strains tested. A reaction mixture in which no reverse transcriptase enzyme

was added was included as a no-RT control. Cycling conditions were as follows: an initial activation step for 5 min at 95°C and 35 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 10 s. SYBR green fluorescence was measured during each extension step. Transcript level, $2^{-\Delta CT}$, is presented relative to the 16S rRNA gene. Unless otherwise noted, a two-way analysis of variance (ANOVA) statistical test with Dunnett's correction for multiple comparisons was used to assess significance; a confidence interval of 95% and an alpha value of 0.05 were considered significant.

**α-PSM production and quantification.** *S. aureus* strains 2014.N and 2014.N Survivor A were cultured in BHIT broth at 28°C overnight (16 to 18 h) with gentle shaking. Cells were pelleted by centrifugation, and the culture supernatant was filter sterilized with a 0.2-μm filtration unit. Total relative α-PSM concentrations for each culture was determined as described using reverse-phase high-pressure liquid chromatography–electrospray mass spectrometry (RP-HPLC/ESI-MS) (22). Total α-PSM is reported as CEICS peak area. To determine if total α-PSM was significantly different between strains, a two-tailed Student *t* test with Welch's correction was utilized.

**Transmission electron microscopy.** *C. pseudodiphtheriticum* USU1 was streaked on BHIT agar, and *S. aureus* 2014.N was streaked on BHI agar. Each strain was expanded by overnight incubation at 35°C. Following incubation, 0.04 g of *S. aureus* 2014.N was directly harvested from agar plates with a sterile inoculating loop and then resuspended in 0.2 ml of sterile 0.9% NaCl. A sterile swab was used to spread the *S. aureus* cell suspension as a lawn on BHI agar. The plate was allowed to dry in a laminar flow hood for 30 min. Next, 25 μl of a *C. pseudodiphtheriticum* cell suspension, prepared as described above, was spotted onto the center of the agar and was allowed to dry in the laminar flow hood for 40 min. The resulting plates were incubated at 28°C for 24 h to allow for initiation of the development of the ZOC. *S. aureus* 2014.N cells directly adjacent to the *C. pseudodiphtheriticum* spot (inside ZOC) and cells elsewhere on the plate (outside ZOC) were picked up with a sterile cotton swab, directly resuspended in fixing solution (2% formaldehyde freshly prepared from paraformaldehyde crystals and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4), and incubated for 1 h at room temperature. Fixed cells were pelleted by centrifugation, and the supernatant was decanted. The samples were washed three times for 10 min each in cold 0.1 M cacodylate buffer. Samples were incubated in 2% $OsO_4$ in 0.1 M cacodylate buffer (0.1 M, pH 7.4) for 1 h and then washed three times for 10 min each in cacodylate buffer. Samples were then dehydrated in a graduated series of ethanol in water (once for 10 min each in 30%, 50%, 70%, and 95% ethanol and twice for 10 min in 100% ethanol). Following dehydration, samples were infiltrated in a graduated series of Spurr's epoxy resin (Electron Microscopy Sciences) and then polymerized at 70°C for 11 h. Polymerized blocks were sectioned in a Leica UC6 ultramicrotome, and thin sections were collected on 3-mm copper grids. Grids were poststained in a Leica EM AC20 and then examined on a JEOL JEM-1011 transmission electron microscope (JEOL USA). Images were collected on an Advanced Microscopy Techniques (AMT Corp.) digital camera.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02491-18.

**TEXT S1**, DOCX file, 0.03 MB.
**FIG S1**, TIF file, 2.5 MB.
**FIG S2**, TIF file, 2.3 MB.
**FIG S3**, TIF file, 2.6 MB.
**FIG S4**, TIF file, 2.7 MB.
**FIG S5**, TIF file, 1 MB.
**TABLE S1**, DOCX file, 0.02 MB.

## ACKNOWLEDGMENTS

We thank Emad Elassal for providing *S. aureus* strains COL and Mu50 and the staff at Fort Benning, GA, for providing *S. aureus* strain 2014.N. We also thank Ryan C. Johnson and Dennis P. McDaniel for technical assistance and Ian W. Windham and Faith C. Blum for assistance with editing and preparing the manuscript. We thank Abraham L. Sonenshein for providing the pCL15 plasmid and Ken Bayles for the Nebraska *S. aureus* transposon library.

The contents of the manuscript are solely those of the authors and do not reflect the views of the Uniformed Services University, the Department of Defense, the National Institutes of Health, or the Food and Drug Administration.

This work was supported by the Military Infectious Disease Research Program (HU0001-15-0031 to D. S. Merrell), the United States Department of Defense (HT9404-12-10019 to D. S. Merrell), Uniformed Services University of the Health Sciences (T073426618 to B. L. Hardy), and the Intramural Research Programs of the National Institute of Allergy and Infectious Diseases (ZIA AI000904-16 to M.O.) and National Institutes of General Medical Sciences (1FI2GM11999101 to S. W. Dickey).

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