Antimicrobial Activity of Clinically Isolated Bacterial Species Against Staphylococcus aureus

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Bacteria often exist in polymicrobial communities where they compete for limited resources. Intrinsic to this competition is the ability of some species to inhibit or kill their competitors. This phenomenon is pervasive throughout the human body where commensal bacteria block the colonization of incoming microorganisms. In this regard, molecular epidemiological and microbiota-based studies suggest that species-specific interactions play a critical role in the prevention of nasal colonization of the opportunistic pathogen Staphylococcus aureus. Despite this, S. aureus exists as part of the microbiota of ∼25% of the population, suggesting that the interplay between S. aureus and commensals can be complex. Microbiota studies indicate that several bacterial genera are negatively correlated with S. aureus colonization. While these studies paint a broad overview of bacterial presence, they often fail to identify individual species-specific interactions; a greater insight in this area could aid the development of novel antimicrobials. As a proof of concept study designed to identify individual bacterial species that possess anti-S. aureus activity, we screened a small collection of clinical isolates from the Walter Reed National Military Medical Center for the ability to inhibit multiple S. aureus strains. We found that the majority of the isolates (82%) inhibited at least one S. aureus strain; 23% inhibited all S. aureus strains tested. In total, seven isolates mediated inhibitory activity that was independent of physical contact with S. aureus, and seven isolates mediated bactericidal activity. 16S rRNA based-sequencing revealed that the inhibitory isolates belonged to the Acinetobacter, Agromyces, Corynebacterium, Microbacterium, Mycobacterium, and Staphylococcus genera. Unexpectedly, these included seven distinct Acinetobacter baumannii isolates, all of which showed heterogeneous degrees of anti-S. aureus activity. Defined mechanistic studies on specific isolates revealed that the inhibitory activity was retained in conditioned cell free medium (CCFM) derived from the isolates. Furthermore, CCFM obtained from S. saprophyticus significantly decreased mortality of S. aureus-infected...
INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that, due to its ability to quickly adapt to harsh conditions and evade the host’s immune system, can colonize virtually any niche throughout the human body. S. aureus causes a variety of diseases, most frequently skin and soft tissue infections, but also systemic and toxin-mediated disease (Otto, 2010). To further exacerbate matters, numerous S. aureus strains are resistant to multiple antibiotics, which subsequently makes treatment more difficult. Even amongst otherwise healthy individuals, the lack of appropriate treatment often leads to more severe morbidity and higher mortality rates (Lowy, 2003). Accordingly, methicillin-resistant S. aureus (MRSA)-mediated disease was responsible for approximately 10,000 deaths from 2005 to 2013 in the United States (Klevens et al., 2007). Furthermore, the worldwide pervasiveness of multidrug-resistant S. aureus strains has led the World Health Organization to designate MRSA as a “high” threat to the global population (WHO, 2017).

Despite the propensity to cause significant morbidity and mortality, S. aureus exists as a part of the microbiota of approximately one-quarter of the population (Sakr et al., 2018); however, colonized individuals are more likely to develop S. aureus-mediated disease (Kluytmans and Wertheim, 2005). In thinking about the dynamics of colonization of the host, S. aureus must interact and compete with the other resident flora as a means to establish itself as a part of the microbiota of a particular niche (Burian et al., 2017). This is undoubtedly a complicated process. However, even the vast amount of currently available microbiota data has not substantially increased our current understanding of the molecular mechanisms underlying the complex interactions between resident flora and incoming pathogens like S. aureus. It is well-established that commensal microbes play a critical role in decreasing and preventing pathogen colonization. A well-known example of this can be found with the ability of fecal transplants from healthy donors to treat patients with recurrent Clostridium difficile infections; restoration of the normal gastrointestinal microbiota eliminates and prevents C. difficile colonization (Buffie et al., 2015).

The ability of commensal bacteria to block pathogen colonization is true at other anatomical locations as well. S. aureus nasal colonization in particular is greatly dependent on molecular interactions with the nasal flora (Brugger et al., 2016; Sakr et al., 2018). Indeed, the nasal cavity is a high salinity and nutrient scarce niche where resident and incoming bacteria compete for limited resources and space in a type of “bacterial warfare” (Krismer et al., 2014). These interactions are often species-specific, and commensal bacterial have been found to use a variety of mechanisms to block pathogen colonization, including the production and secretion of toxic compounds that directly kill or inhibit competitors. For example, various species from within the Streptococcus and Corynebacterium genera are inversely correlated with the presence of S. aureus in the nasal cavity or have been found to directly antagonize S. aureus (Lemon et al., 2010; Bomar et al., 2016). Even other members of the Staphylococcus genus have been found to negatively impact S. aureus viability; several coagulase-negative Staphylococcus (CoNS) species have evolved mechanisms to inhibit S. aureus colonization. Specifically, some S. epidermidis strains secrete a serine protease that is capable of disrupting S. aureus biofilm formation and blocking nasal colonization (Iwase et al., 2010). S. hominis and S. epidermidis both secrete strain-specific antimicrobial peptides that have potent selective bactericidal activity against S. aureus (Nakatsuji et al., 2017). Moreover, lugdunin, a novel cyclic peptide antibiotic produced by S. lugdunensis, has bactericidal properties against several Gram-positive pathogens, including S. aureus, and can prevent S. aureus nasal colonization (Zipperer et al., 2016). It is clear that within the context of the human nose, there is a selective pressure, even amongst closely related commensal species, to block or eliminate S. aureus.

Despite recent advancements detailing the negative molecular interactions that occur between S. aureus and the resident nasal flora, little is known about S. aureus interactions with bacteria isolated from other anatomical locations. Given this deficit and the fact that S. aureus can colonize the human body virtually ubiquitously, as a proof of concept study we set out to characterize S. aureus interactions with clinical bacterial isolates obtained from a variety of body sites from a diverse patient population at the Walter Reed National Military Medical Center. Herein, we show that the majority (82%, 28/34) of clinical isolates possessed some degree of in vitro anti-S. aureus activity when tested against multiple strains of S. aureus, including MRSA. Moreover, eight clinical isolates showed anti-S. aureus activity against all tested strains. Several of the clinical isolates that belonged to the Staphylococcus and Corynebacterium genera mediated contact-independent inhibitory activity against S. aureus. Furthermore, a portion of the clinical isolates (7/28) showed bactericidal activity against S. aureus. Unexpectedly, Acinetobacter baumannii isolates represented the most commonly identified species that produced heterogenous strain-specific anti-S. aureus activity. Finally, analysis of conditioned cell free medium (CCFM) from several isolates revealed that inhibitory activity was often mediated contact-independent mechanisms, our current findings support the study of polymicrobial interactions as a strategy to understand bacterial competition and to identify novel therapeutics against S. aureus and other pathogens.

Keywords: Staphylococcus aureus, MRSA, polymicrobial interactions, bacterial interaction, clinical isolates
present in the CCFM. Furthermore, CCFM derived from
*S. saprophyticus* was able to reduce mortality of *S. aureus-
infected Galleria mellonella* caterpillars. These findings
suggest that *S. aureus* interactions with other bacteria are
far more multifaceted than previously recognized, and strongly
support the study of these interactions at the molecular
level as a means to reveal novel *S. aureus* molecular targets
or therapeutics.

**MATERIALS AND METHODS**

**Strains, Culture and Bacterial Interaction Assays**

All deidentified clinical isolates were obtained as a part of a
memorandum of understanding (MOU) between the Uniformed
Services University of the Health Sciences (USU) and the
Walter Reed National Military Medical Center (WRNMMC),
Department of Clinical Microbiology. The described studies
represent research Not Involving Human Subjects since all
isolates were obtained from discarded clinical microbiology
plates that contained samples that were obtained during routine
diagnostic testing and treatment of WRNMMC patients. Both
USU and WRNMMC agree and acknowledge that the activities
and projects pursued under the MOU complied with the
applicable rules and regulations governing human subjects
research within the Department of Defense; the Institutional
Review Board at WRNMMC was the IRB of record for the
collection of all patient samples. Strains were maintained as
−80°C freezer stocks and were cultured under the following
conditions unless otherwise noted: Clinical isolates were streaked
from frozen glycerol stocks on Brain Heart Infusion (BHI)
agar (Becton Dickinson) supplemented with 1% Tween 80 (BHI,
Sigma-Aldrich). *S. aureus* strains were streaked from glycerol
stocks on BHI agar. Each isolate was incubated overnight at
37°C. Bacterial interaction assays were performed as previously
described (Yan et al., 2013; Hardy et al., 2019). Briefly, 40 mg
of *S. aureus* or a clinical isolate was directly harvested from an
agar plate with a sterile inoculating loop and then re-suspended
in 200 µL of sterile saline solution (Fisher Chemicals). Eight
microliters of the *S. aureus* cell suspension was inoculated into
15 mL of sterile BHI agar that had been cooled to
55°C; inoculated agar was poured into a sterile petri dish, and
was allowed to dry for 40 min under sterile conditions. The
resulting plates were incubated at 37°C with shaking for
24–48 h. Overnight broth cultures were pelleted by
centrifugation and re-suspended in 0.2 mL of Phosphate
Buffer Solution (PBS, Fisher Chemicals). Cell suspensions were
lysed in a Bullet Blender Homogenizer for 5 min by mechanical
disruption in bead-beater tubes that contained 0.1 mm sterile
glass beads. Genomic DNA was extracted from lysed cells
suspensions with the Wizard Genomic DNA Purification Kit
(Promega) according to the manufacturer’s instructions.

Purified genomic DNA from each sample was subjected to
PCR amplification of the 16S rRNA gene using the
8F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′
GGTTACCTTGTTACGACTT 3′) primers. PCR mixtures (25 µL)
contained 5X Phusion HF buffer, 200 mM of each
dNTP, 0.5 µM of each primer, and 0.02 U/µL of Phusion DNA
polymerase. PCR amplification was performed with the following
reaction conditions: 98°C for 30 s, 30 cycles of 98°C for 5 s,
51°C for 30 s, 72°C for 1 min 30 s, with a final elongation step
of 72°C for 5 min. The PCR amplified products were visualized
on a 1% agarose gel to confirm the presence of an approximately
1,500 base pair band.

PCR products were purified using the QIAquick PCR
purification kit according to the manufacturer’s instructions.

**DNA Extraction, Amplification, Cloning,**
and **16S rRNA Gene Sequencing**

All clinical isolates that possessed anti-*S. aureus* activity (28/34)
were streaked from frozen glycerol stocks on BHIT agar and
incubated overnight at 37°C. Single colonies of each isolate were
subcultured in 2 mL of BHIT broth and incubated at 37°C with
shaking for 24–48 h. Overnight broth cultures were pelleted
by centrifugation and re-suspended in 0.2 mL of Phosphate
Buffer Solution (PBS, Fisher Chemicals). Cell suspensions were
lysed in a Bullet Blender Homogenizer for 5 min by mechanical
disruption in bead-beater tubes that contained 0.1 mm sterile
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of 72°C for 5 min. The PCR amplified products were visualized
on a 1% agarose gel to confirm the presence of an approximately
1,500 base pair band.

PCR products were purified using the QIAquick PCR
purification kit according to the manufacturer’s instructions. Purified
PCR products were polyadenylated utilizing the
A-tailing procedure; reaction components (10 µL), including
PCR-amplified DNA, 10X ThermoPol Buffer, 1mM dATP, and
Taq DNA Polymerase, were incubated at 70°C for 30 min.
A-tailed PCR products were subsequently cloned into the pGEM-
T Easy vector according to the manufacturer’s instructions
(Promega). Ligation products were transformed into *E. coli*
TOP10 CaCl₂ chemically competent cells. Transformants with
the desired insert were isolated via “blue/white” selection
on LB (Luria-Bertani) agar supplemented with ampicillin
(100 µg/mL), X-gal (40 µg/mL) and IPTG (1 µM). To
confirm the presence of the correct insert, colony PCR was
performed on at least five white colonies per transformation
using the GoTaq Green Master Mix (Promega) and pGEM-T
Easy specific T7 (5′ GGGTTTTCCCCAGTACGAGA 3′) and SP6 (5′
GGACCCCCAGGCTTACAC 3′) primers with the following PCR
reaction conditions: 95°C for 3 min, 30 cycles of 95°C for 30 s, 45°C for
30 s, 72°C for 1 min 30 s, with a final elongation step of 72°C
for 5 min. White colonies that contained the correct insert were
cultered overnight in LB Broth plus ampicillin (100 µg/mL) with
shaking. Plasmids were purified using QIAprep Spin Miniprep
Kit (Qiagen) according to the manufacturer’s instructions and
then used for sequencing.

As previously described (Johnson et al., 2016), to
ensure near full-length coverage of the 16S rRNA gene, six
individual sequencing reactions were performed on purified
plasmids using the following primers: T7, SP6, 8F, 1492R,
515F (5′ GTGYCACCMGGCGGTAA 3′), and 806R (5′
806R (5′

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### TABLE 1 | Clinical isolates and S. aureus strains assayed.

| Strain                                      | Lab strain designation | Origin   | Accession #                  | Year isolated | Anti-S. aureus activity<sub>4</sub> | Contact dependent vs. Independent | Bactericidal vs. Bacteriostatic | References                      |
|---------------------------------------------|------------------------|----------|------------------------------|---------------|-------------------------------------|----------------------------------|----------------------------------|---------------------------------|
| *Staphylococcus aureus* test strains        |                        |          |                              |               | 2014.N LAC Mu50                     |                                   |                                  |                                 |
| S. aureus LAC                               | DSM1485                | Blood    | NC_002758.2                  | 2005          | N/A                                 | N/A                              | N/A                              | Voyich et al. (2005)             |
| S. aureus 2014.N                            | DSM1416                | Nose     | N/A                          | 2012          | N/A                                 | N/A                              | N/A                              | Hardy et al. (2019)             |
| S. aureus Mu50                              | DSM1633                | Abscess  | NC_002758.2                  | 1997          | N/A                                 | N/A                              | N/A                              | Kuroda et al. (2001)            |
| *Acinetobacter* clinical isolates           |                        |          |                              |               |                                     |                                  |                                  |                                 |
| A. baumannii-1                              | DSM1675                | Wound    | MN175920                     | 2016          | Strong                              | Weak                             | Weak                             | Independent                    |
| A. baumannii-2                              | DSM1676                | Wound    | MN175921                     | 2016          | Strong                              | Weak                             | Not Tested                      | Dependent                     |
| *Corynebacterium* clinical isolates         |                        |          |                              |               |                                     |                                  |                                  |                                 |
| C. amycolatum-1                             | DSM1914                | Nasal    | MN175942                     | 2016          | Weak                                | None                             | None                             | Not Tested                     |
| C. amycolatum-2                             | DSM1567                | Nasal    | MN175937                     | 2016          | Weak                                | None                             | Strong                           | Not Tested                     |
| C. aurimucosum-1                            | DSM1560                | Urine    | MN175936                     | 2016          | Weak                                | None                             | Weak                             | Independent                    |
| C. aurimucosum-2                            | DSM1678                | Wound    | MN175938                     | 2016          | Weak                                | None                             | Strong                           | Independent                    |
| C. aurimucosum-3                            | DSM1912                | Wound    | MN175945                     | 2016          | None                                | None                             | Weak                             | Independent                    |
| C. aurimucosum-4                            | DSM1913                | Wound    | MN175932                     | 2016          | None                                | None                             | Weak                             | Not Tested                     |
| C. jeikeium                                  | DSM1915                | Wound    | MN175945                     | 2016          | None                                | None                             | None                             | Not Tested                     |
| C. striatum-1                               | DSM1564                | Wound    | MN175927                     | 2016          | Weak                                | None                             | Weak                             | Not Tested                     |
| C. striatum-2                               | DSM1566                | Blood    | MN175947                     | 2016          | Strong                              | None                             | None                             | Independent                    |
| C. tuberculosislareum                       | DSM1925                | Nasal    | MN175944                     | 2016          | None                                | None                             | None                             | Not Tested                     |
| *Microbacterium* clinical isolates          |                        |          |                              |               |                                     |                                  |                                  |                                 |
| M. paraoydans-1                             | DSM1919                | Nasal    | MN175940                     | 2016          | None                                | Weak                             | Not Tested                      | Not Tested                     |
| M. paraoydans-2                             | DSM1920                | Wound    | MN175935                     | 2016          | None                                | Weak                             | Not Tested                      | Not Tested                     |
| *Staphylococcus* clinical isolates          |                        |          |                              |               |                                     |                                  |                                  |                                 |
| S. epidermidis-1                            | DSM1679                | Wound    | MN175939                     | 2016          | Strong                              | Weak                             | Independent                     | Bacteriostatic<sub>1</sub>      |
| S. epidermidis-2                            | DSM1759                | Wound    | MN175929                     | 2016          | Strong                              | Weak                             | Dependent                       | Bactericidal<sub>1</sub>       |
| S. epidermidis-3                            | DSM1760                | Wound    | MN175930                     | 2016          | Strong                              | Weak                             | Dependent                       | Bactericidal<sub>1</sub>       |
| S. epidermidis-4                            | DSM1922                | Wound    | MN175931                     | 2016          | Strong                              | Weak                             | Dependent                       | Bactericidal<sub>1</sub>       |
| S. epidermidis-5                            | DSM1761                | Nasal    | MN175933                     | 2016          | Weak                                | None                             | Not Tested                      | Not Tested                     |
| S. hominis                                  | DSM1916                | Wound    | MN175934                     | 2016          | Strong                              | Weak                             | Dependent                       | Bactericidal<sub>1</sub>       |
| S. saprophyticus                            | DSM1655                | Urine    | MN175941                     | 2016          | Weak                                | None                             | Dependent                       | Bactericidal<sub>2</sub>       |
| Other clinical isolates                     |                        |          |                              |               |                                     |                                  |                                  |                                 |
| Agromyces sp. 3098BRFJ                      | DSM1921                | Wound    | MN175928                     | 2016          | None                                | None                             | Not Tested                      | Not Tested                     |
| Mycobacterium yunnanensis                  | DSM1677                | Wound    | MN175946                     | 2016          | None                                | Weak                             | Not Tested                      | Not Tested                     |

Subscript "1" indicates assay was tested against S. aureus 2014.N. Subscript "2" indicates assay was tested against S. aureus Mu50. Subscript "4" indicates strong anti-S. aureus activity defined as follows: ZOC was completely transparent, ≥2 mm, and a defined edge at 72 h. Weak anti-S. aureus was defined as follows: ZOC was not completely transparent, with a hazy and undefined edge at 72 h. None indicates a ZOC was not present at 72 h. A "-" indicates the ZOC was too small to perform Bactericidal vs. Bacteriostatic assays. "N/A" denotes information that was unavailable or not applicable.

Sequence reads were manually assembled into a double stranded near full length 16S rRNA gene sequence, and taxonomic information was assigned after comparison with other 16S rRNA gene sequences in the Ribosomal Database Project (RDP)<sup>1</sup> and GenBank<sup>2</sup> using the Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene sequences of all the strains speciated in this study were deposited in GenBank and assigned accession numbers. Strain descriptions, species identification, and accession numbers can be found in Table 1.

### Contact-Dependent Assays

Strongly inhibitory clinical isolates were defined as follows: a ZOC that was visibly transparent, at least 2 mm in length,

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<sup>1</sup>http://rdp.cme.msu.edu  
<sup>2</sup>https://blast.ncbi.nlm.nih.gov
and with a defined edge. These isolates (17/28) were assayed to determine if anti-\textit{S. aureus} activity was dependent on direct physical contact between the bacteria; in each case, the activity of each clinical isolate was tested against the \textit{S. aureus} strain for which the strongest ZOC was obtained in the absence of a filter disk. A sterile 0.2 \(\mu\)m filter disk was placed on top of the BHIT agar that had been seeded with \textit{S. aureus}; each clinical isolate was then individually spotted on top of the filter disk so that none of the cell suspension physically touched the \textit{S. aureus} seeded agar plate. Plates were incubated at 28°C and were visually assessed at 24, 72, and 120 h for the absence or presence of a ZOC. The absence of a ZOC in the presence of a filter disk indicates that physical contact is necessary for anti-\textit{S. aureus} activity against the corresponding most sensitive \textit{S. aureus} strain. Clinical isolates were assessed in three independent biological replicates.

**Recovery of \textit{S. aureus} From ZOC**

To determine if anti-\textit{S. aureus} activity was bacteriostatic (growth inhibition) or bactericidal (killing), \textit{S. aureus} survival and growth was monitored as compared to the original inoculum. Immediately after the plates solidified and before a clinical isolate was spotted, five-milligram punches of \textit{S. aureus}-seeded agar were taken with a sterile pipette tip as a means to enumerate \textit{S. aureus} colony forming units (CFU) present at T0. Bacterial interaction assays were performed with 15/28 strongly inhibitory clinical isolates as described above. Two isolates that produced a defined and transparent ZOC, but exactly 2 mm in length, were excluded from these experiments as the ZOC produced against \textit{S. aureus} was too small to accurately extract agar punches. Each strongly inhibitory clinical isolate was tested against the \textit{S. aureus} strain for which the strongest ZOC was produced. After 48 h (T48) of incubation at 28°C, five-milligram punches of agar directly adjacent to the clinical isolate spot (Inside ZOC) or at the edge of the petri dish (Outside ZOC) were again taken with a sterile pipette tip. To determine the number of \textit{S. aureus} CFU present in an agar punch, punches were resuspended in 1 mL of BH agar broth and heated to 55°C for 10 min. 10-fold serial dilutions of each suspension were prepared in PBS and then plated on Mannitol Salt Agar (MSA, Criterion). Plates were incubated at 37°C overnight, and recovered colonies were quantified. The number of CFU present in the 1 mL original suspension was calculated, and the fold change from T0 was calculated as follows: (Number of CFU present Inside or Outside ZOC at T48/Number of CFU present at T0). Fold change values less than 1 indicate bactericidal activity; \textit{S. aureus} CFU recovered in an agar punch at T48 was less than the \textit{S. aureus} CFU recovered in an agar punch at T0. Contact-dependent experiments were completed in three independent biological replicates.

**Conditioned Cell Free Medium (CCFM) Preparation and Disk Diffusion Assays**

Clinical isolates that produced contact-independent bactericidal anti-\textit{S. aureus} activity (\textit{C. amy}-2, \textit{S. sap}, and \textit{S. epi}-3) were independently cultured in 10 mL BHIT broth overnight at 37°C with shaking at 190 rpm. Cultures were pelleted by centrifugation, and the supernatant was filter sterilized with a2 \(\mu\)m filter (Corning). One-milliliter of sterile supernatant was retained, and the remaining supernatant was concentrated (50X) with ammonium sulfate precipitation as previously described (Hardy et al., 2019). For heat-treatments, 50 \(\mu\)L aliquots of unconcentrated or 50X CCFM were incubated at 90°C for 10 min, then allowed to cool. For the disk diffusion assays, the \textit{S. aureus} strain that was most sensitive to the corresponding inhibitory activity (\textit{C. amy}-2/Mu50, \textit{S. sap}/LAC, and \textit{S. epi}-3/LAC) was cultured on BH agar overnight at 37°C. The following day, the plate-grown cells were recovered and diluted to \(1 \times 10^8\) cells/mL (OD\text{600} 0.1) in BH broth. A sterile swab was then used to spread the \textit{S. aureus} cell suspension on BH agar as a lawn. The plate was allowed to dry in a laminar flow hood for 30 min. Next, a sterile 5 mm diffusion disk was placed on top of the \textit{S. aureus} lawn, and 50 \(\mu\)L of unconcentrated CCFM or 50X CCFM was inoculated onto the disk. Plates were incubated at 28°C, and images were taken after 72 h of incubation. Disk diffusion assays were conducted in three independent biological replicates.

**\textit{S. aureus} Infection and CCFM Treatment of \textit{Galleria mellonella} Caterpillars**

\textit{Staphylococcus aureus} strains 2014.N, Mu50, and LAC were cultured overnight on BH agar at 37°C. The following day, \textit{S. aureus} cells were recovered and diluted to \(1 \times 10^8\) cells/mL (OD\text{600} 0.1) in PBS. Total CFU were then further adjusted to obtain the required doses; i.e., \(10^7\) CFU or \(10^6\) CFU in 5 \(\mu\)L of PBS + 0.01% bromophenol dye. For infections, \textit{Galleria mellonella} caterpillars (Vanderhorst Wholesale Inc) were utilized within 1 day of receipt. Caterpillars between 200 and 300 mg were chosen for infection. The injections were carried out as described previously (Desbois and Coote, 2011) with minor adaptations. Briefly, 5 \(\mu\)L of inoculum that contained \(10^7\) or \(10^8\) total CFU of \textit{S. aureus} was injected into the last left proleg using a 10 \(\mu\)L glass syringe (Hamilton) fitted with a 31G needle. For caterpillars that were treated with CCFM, the caterpillars were maintained at room temperature for 1 h following the \textit{S. aureus} injection, then refrigerated at 4°C for 12 min and then injected with 5 \(\mu\)L of freshly prepared 50X CCFM from \textit{S. sap} or \textit{S. epi}-3 (treated) or 50X concentrated BHIT (sham treated). These injections were into the last right proleg. All caterpillars were incubated at 37°C, and survival was monitored over 120 h. Untouched, and PBS injected caterpillars were included as controls. Data found in Figures 7A,B represent two completely independent biological replicates (\(n = 15\) caterpillars) performed with different batches of caterpillars. Data found in Figure 7C represent a single batch of caterpillars, but two independently derived batches of CCFM (\(n = 15\) caterpillars)/CCFM preparation. Kaplan–Meier survival curves were compared between groups using the Mantel–Cox test with Holm’s correction for multiple comparisons (excluding Untouched and PBS negative controls). An alpha value of 0.05 was considered statistically significant.
RESULTS

Activity of Clinical Bacterial Isolates Against S. aureus

Polymicrobial interactions within the human host are complex and dynamic. Numerous studies have shown that several genera that inhabit the skin and nasal cavity prevent the colonization of opportunistic pathogens (Jarraud et al., 2002; Bomar et al., 2016). However, these studies often focus on specific anatomical locations and do not represent the host as one environmental niche. Given this, we questioned whether bacterial species isolated from a diverse patient population and a variety of body sites would display antagonistic interactions against S. aureus. To this end, we obtained a collection of clinical isolates (Table 1) from the WRNMMC Clinical Microbiology Lab and assayed in vitro anti-S. aureus activity utilizing a bacterial interaction assay (Hardy et al., 2019). As prior studies have shown that antagonistic polymicrobial interactions are often strain-specific and because we previously showed that Corynebacterium pseudodiphtheriticum, a common skin and nasal commensal microbe, mediates heterotypic bactericidal activity against specific S. aureus strains (Hardy et al., 2019), we assayed anti-S. aureus activity against three phenotypically different S. aureus strains: S. aureus LAC (Community-Acquired, MRSA), S. aureus Mu50 (Hospital-Acquired, MRSA) and 2014.N (Methicillin-Sensitive S. aureus), a recently acquired nasal isolate (Table 1). To this end, 34 individual clinical isolates were assessed against each S. aureus strain in the bacterial interaction assays; appearance of a visible zone of clearance (ZOC) around the clinical isolate was considered a positive indicator of anti-S. aureus activity. While we found that six clinical isolates showed no anti-S. aureus activity, the majority (28/34, 82%) of tested clinical isolates possessed inhibitory activity against at least one of the S. aureus strains (Figure 1A). Furthermore, eight of the clinical isolates were able to inhibit the growth of all tested S. aureus strains. As expected, many of the clinical isolates mediated inhibitory activity in a S. aureus strain-specific manner: three clinical isolates only inhibited 2014.N, five only inhibited Mu50, and two only inhibited LAC (Figure 1A).

The species of the 28 isolates that exhibited anti-S.aureus activity were next identified via cloning and sequencing of the 16S rRNA gene; sequences were deposited into GenBank and accession numbers are available in Table 1. Analysis of the species information combined with the bacterial interaction assays revealed several types of ZOCs that developed over time (Figure 1B). For example, co-incubation of Corynebacterium aurimucosum (C. aur-1) or Mycobacterium yunnanensis (M. yun) with S. aureus 2014.N or Mu50 resulted in a diffused and moderately sized ZOC; a ZOC did not develop upon co-incubation with S. aureus LAC for either clinical isolate (Figure 1B). In contrast, co-incubation of Staphylococcus saprophyticus (S. sap) with S. aureus LAC resulted in a defined and transparent ZOC, while only a modest and hazy ZOC was produced against S. aureus 2014.N and Mu50.

Temporal quantification of ZOC length additionally revealed distinct patterns of interactions between each clinical isolate and each S. aureus strain. For the majority of the isolates, the ZOC length either remained constant or increased over time (Figure 2A). In support of the literature that suggests that some members of the Corynebacterium genus promote negative interactions with S. aureus (Yan et al., 2013; Hardy et al., 2019), numerous inhibitory isolates were speciated to be members of the Corynebacterium genus. These isolates tended to show anti-S. aureus activity selectively against strains 2014.N and Mu50; only one Corynebacterium isolate, C. jeikeium (C. jei), inhibited S. aureus LAC growth, but neither 2014.N or Mu50. Previous reports have also shown that several CoNS prevent S. aureus colonization by inhibiting growth or by direct killing (Iwase et al., 2010; Zipperer et al., 2016; Nakatsuji et al., 2017). In support of this, numerous Staphylcoccal isolates were identified and possessed activity against S. aureus. These isolates generally mediated robust activity against S. aureus 2014.N and LAC, but only modest anti-S. aureus activity against Mu50 (Figures 2A,B). For example, Staphylococcus epidermidis (S. epi-1) and S. hominis (S. hom) produced defined and transparent ZOCs against 2014.N and LAC, but a comparatively small ZOC was produced against Mu50. Taken together, these results support the current hypothesis that antagonistic interactions with S. aureus are often strain-specific. As it would account for the differences in sensitivity amongst the various S. aureus strains, this may indicate that the S. aureus molecular target(s) of each inhibitory isolate is strain-specific and/or differentially expressed between the various S. aureus strains.

In addition to the expected members of the Corynebacterium and Staphylococcus genera, several clinical isolates that are not typically associated with the human microbiota were found to have anti-S. aureus activity. For example, there are few reports of the clinical isolation of Microbacterium species (Laffineur et al., 2003). However, M. paraoxydans, a pathogen of various fish species (Soto-Rodriguez et al., 2013), was recovered from the clinical isolation of S. hominis (L. hominis-1) and S. hominis (L. hominis), inhibited S. aureus 2014.N and LAC, but a comparatively small ZOC was produced against Mu50. Taken together, these results support the current hypothesis that antagonistic interactions with S. aureus are often strain-specific. As it would account for the differences in sensitivity amongst the various S. aureus strains, this may indicate that the S. aureus molecular target(s) of each inhibitory isolate is strain-specific and/or differentially expressed between the various S. aureus strains.

Heterotypic Inhibitory Activity of A. baumannii Against S. aureus

While Acinetobacter baumannii and S. aureus have been frequently co-isolated from wounds (Furuno et al., 2008; Castellanos et al., 2019), to our knowledge there is no published evidence that A. baumannii possesses any inhibitory activity against S. aureus. Thus, we were surprised that A. baumannii isolates represented ~20% (7/34) of the clinical isolates that showed anti-S. aureus activity (Figures 2A, 3). Though not certain, this large representation of A. baumannii clinical isolates may be a result of the “wounded warrior” patient population that is often treated at WRNMMC. Of the seven A. baumannii
isolates, two (A. baum-1 and A. baum-3) possessed inhibitory activity against all tested S. aureus strains. The remaining five A. baumannii mediated anti-S. aureus activity against at least two strains (Figure 2A).

The type of ZOC produced by A. baumannii varied and was largely dependent on the S. aureus strain being tested. For example, A. baum-2 produced a large and defined ZOC against 2014.N, a large and hazy ZOC against LAC, and no ZOC against Mu50 (Figure 3). In contrast, A. baum-6 produced a moderately sized and very defined ZOC against Mu50, a small and hazy ZOC against LAC, and no ZOC against 2014.N. Taken together, these data indicate that A. baumannii possesses heterogeneous strain-specific anti-S. aureus activity. This may in turn indicate that A. baumannii utilizes multiple independently evolved mechanisms to compete with S. aureus or that the target(s) of anti-S. aureus activity are differentially expressed between S. aureus strains.

**Characterization of Contact-Dependent and Bactericidal Anti-S. aureus Activity**

Commensal bacteria utilize a wide variety of molecular mechanisms to compete with other microbes; these include both contact dependent and independent mechanisms (Brugger et al., 2016). Thus, we sought to determine whether the observed anti-S. aureus activity of the clinical isolates required physical
**FIGURE 2** Clinical isolates mediate strain-specific anti-
*S. aureus* activity. (A) Heat map showing the activity of the indicated clinical isolate against the indicated *S. aureus* strains: The Zone of Clearance (ZOC) was defined as the distance between the edge of the clinical isolate spot to the visible edge of the *S. aureus* ring of clearance. The ZOC was measured using ImageJ software (NCBI) and each value within a box represents the arithmetic mean (in millimeters) of three independent biological replicates measured temporally (24, 72, and 120 h). Clinical isolate species names have been abbreviated as follows: *C. aurimucosum* (*C. aur*), *C. striatum* (*C. str*), *C. amycolatum* (*C. amy*), *C. jeikeium* (*C. jei*), *C. tuberculostearicum* (*C. tub*), *S. epidermidis* (*S. epi*), *S. saprophyticus* (*S. sap*), *S. hominis* (*S. hom*), *A. baumannii* (*A. bau*), *M. paraoxydans* (*M. par*), *M. yunnanensis* (*M. yun*), and Agromyces sp. 3098BRRJ (*Agro* sp.). Additionally, a number preceded by a – is used to indicate an independent isolate of the indicated species. (B) Example of strain-specific activity: *S. epidermidis* (*S. epi*-1) and *S. hominis* (*S. hom*) were co-incubated with agar plates seeded with *S. aureus* strains 2014.N, LAC or Mu50. Images of the ZOC were taken after 24 and 120 h of incubation at 28 °C and are representative of three independent biological replicates. Scale bar = 10 mm and is the same in the corresponding 24 h and 120 images; in some cases the 120 h spots appear larger than the 24 h spots due to growth of the bacteria within the spots.

|          | 2014.N | LAC | Mu50 |
|----------|--------|-----|------|
|          | 24     | 72  | 120  | 24   | 72  | 120  | 24   | 72  | 120  |
| Agro sp. | 0.00   | 0.00 | 0.00 | 2.47 | 3.41 | 3.63 |
| A. bau-1 | 3.26   | 3.91 | 5.68 | 3.26 | 7.29 | 4.35 | 5.00 | 1.82 | 6.28 |
| A. bau-2 | 3.14   | 5.21 | 4.95 | 4.07 | 5.20 | 2.72 | 0.00 | 0.00 | 0.00 |
| A. bau-3 | 2.79   | 3.15 | 1.95 | 2.60 | 4.88 | 4.23 | 0.00 | 0.00 | 0.00 |
| A. bau-4 | 0.00   | 0.00 | 0.00 | 0.00 | 3.58 | 3.38 | 3.20 | 3.76 | 4.04 |
| A. bau-5 | 0.00   | 0.00 | 0.00 | 2.01 | 2.47 | 2.52 | 0.00 | 1.79 | 2.11 |
| A. bau-6 | 0.00   | 0.00 | 0.00 | 1.30 | 2.70 | 3.14 | 3.47 | 3.93 | 4.31 |
| A. bau-7 | 0.00   | 0.00 | 0.00 | 0.81 | 3.09 | 3.50 | 0.70 | 3.20 | 4.23 |
| C. amy-1 | 2.11   | 3.90 | 4.15 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| C. amy-2 | 0.00   | 4.19 | 3.91 | 0.00 | 0.00 | 0.00 | 0.00 | 5.28 | 7.48 |
| C. aur-1 | 3.50   | 4.50 | 4.40 | 0.00 | 0.00 | 0.00 | 2.28 | 2.49 | 3.31 |
| C. aur-2 | 2.69   | 3.07 | 2.74 | 0.00 | 0.00 | 0.00 | 2.95 | 4.55 | 5.55 |
| C. aur-3 | 0.00   | 0.00 | 0.00 | 0.00 | 1.52 | 2.03 | 2.57 | 1.52 | 2.03 |
| C. aur-4 | 0.00   | 0.00 | 0.00 | 0.00 | 4.07 | 3.25 | 3.33 | 0.00 | 0.00 |
| C. jei   | 0.00   | 0.00 | 0.00 | 0.57 | 2.28 | 2.36 | 0.00 | 0.00 | 0.00 |
| C. str-1 | 2.35   | 3.55 | 3.79 | 0.00 | 0.00 | 0.00 | 3.31 | 3.58 | 3.66 |
| C. str-2 | 3.09   | 2.72 | 4.14 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| C. tub   | 0.00   | 0.00 | 0.00 | 0.00 | 1.44 | 2.76 | 2.60 | 1.44 | 2.76 |
| S. epi-1 | 3.39   | 3.48 | 2.95 | 2.93 | 3.25 | 3.58 | 1.57 | 1.65 | 1.65 |
| S. epi-2 | 3.35   | 4.78 | 3.89 | 3.25 | 4.07 | 4.39 | 2.33 | 3.01 | 3.47 |
| S. epi-3 | 2.94   | 3.35 | 3.50 | 2.68 | 3.74 | 4.72 | 1.27 | 1.76 | 2.57 |
| S. epi-4 | 2.33   | 2.70 | 2.69 | 3.33 | 3.66 | 3.74 | 1.65 | 3.50 | 3.82 |
| S. epi-5 | 3.32   | 2.76 | 5.34 | 1.87 | 2.60 | 4.31 | 0.00 | 0.00 | 0.00 |
| S. hom   | 1.69   | 2.99 | 2.48 | 1.71 | 2.06 | 2.28 | 1.79 | 2.55 | 2.55 |
| S. sap   | 2.83   | 3.24 | 2.66 | 3.90 | 5.37 | 6.02 | 1.73 | 2.98 | 2.98 |
| M. par-1 | 0.00   | 0.00 | 0.00 | 1.63 | 2.28 | 2.36 | 0.49 | 3.04 | 3.82 |
| M. par-2 | 0.00   | 0.00 | 0.00 | 1.14 | 2.28 | 2.28 | 0.49 | 1.46 | 1.68 |
| M. yun  | 2.11   | 2.76 | 3.17 | 0.00 | 0.00 | 0.00 | 2.90 | 2.41 | 2.41 |
bacterial interaction. Of the 28 strains that displayed activity, we focused our efforts on the 17 clinical isolates that showed strong inhibitory activity; these strains produced a defined and transparent ZOC against S. aureus that was greater than or equal to 2 mm. To this end, bacterial interaction assays were repeated, but the clinical isolate was separated from the S. aureus seeded agar with a 0.2 µm filter disk. A ZOC still formed for 41% (7/17) of the tested clinical isolates (Figure 4A), indicating that anti-S. aureus activity was contact-independent. The clinical isolates that mediated contact-independent anti-S. aureus activity were restricted to the Staphylococcus and Corynebacterium genera (Figure 4B and data not shown). For example, two independently recovered S. epidermidis isolates (S. epi-1 and S. epi-3) and S. saprophyticus (S. sap) mediated robust contact-independent inhibitory activity (Figure 4B and data not shown). In addition, four Corynebacterium species (C. amy-2, C. aur-1, C. aur-2, and C. str-1) produced moderate inhibitory activity in the presence of a filter disk (Figure 4B and data not shown). Taken together, these data indicate that the various isolates can use both contact-dependent and contact-independent mechanisms as a means to inhibit S. aureus growth.

Commensal bacteria can compete with other bacteria using mechanisms that either inhibit bacterial growth (bacteriostatic) or directly kill (bactericidal) the competitor. To further characterize the anti-S. aureus activities of the strongly inhibitory clinical isolates, the number of S. aureus CFU were determined from within the ZOC, directly adjacent to the clinical isolate spot (Inside ZOC), and outside of the ZOC, on the edge of the petri dish (Outside ZOC), after 48 h (T48) of incubation. These numbers were then compared to the number of S. aureus CFU seeded within a comparable area of the agar plate at the initiation of the experiment (T0). Of the 17 strongly inhibitory isolates, 15 developed a ZOC that was large enough (greater than 2 mm) to take accurate agar punches that fell fully within the ZOC. Of these 15 isolates, 7 mediated bactericidal activity against S. aureus. Most of these isolates belonged to the Staphylococcus genus (4/7), followed by A. baumannii (2/7), and Corynebacterium (1/7, Figure 5). Combined with the contact dependency assays, a total of 3 clinical isolates (C. amy-2, S. sap, and S. epi-3) produced anti-S. aureus activity that was independent of direct contact and was also bactericidal. This strongly suggests that these isolates directly kill S. aureus via the secretion of toxic compound(s).

**Basic Mechanistic Characterization of Contact-Independent Bactericidal Activity**

We hypothesized that clinical isolates that produced contact-independent bactericidal anti-S. aureus activity would do so via a secreted compound(s) that would be present in culture supernatants. To test this hypothesis, C. amy-2, S. sap, and S. epi-3 were independently cultured in BHIT broth, and sterile conditioned cell free medium (CCFM) was prepared. Unconcentrated CCFM and 50X concentrated CCFM were then tested in a disk diffusion assay (Figure 6 and data not shown) against the S. aureus strain for which they showed the most robust bactericidal activity (C. amy-2/Mu50, S. sap/LAC, and S. epi-3/LAC). Each of the 50X concentrated CCFM samples produced a ZOC against the tested S. aureus strain (Figure 6). In addition, unconcentrated CCFM derived from S. sap and S. epi-3 produced a small ZOC against S. aureus LAC (data not shown). To determine the thermostability of the compound(s) found in the concentrated CCFM, aliquots of CCFM were also subjected to heat treatment prior to testing for anti-S. aureus activity. In all cases anti-S. aureus activity was maintained after heat treatment (Figure 6).

To examine the therapeutic potential of the compound(s) found within the CCFM, we next examined the ability of CCFM to rescue S. aureus-infected Galleria mellonella caterpillars. G. mellonella have been established as a simple infection model for several pathogens, including S. aureus.
FIGURE 4 | Select clinical isolates mediate contact-independent anti-S. aureus activity. (A) Strongly inhibitory clinical isolates (17/28) were defined as follows: visibly transparent ZOC of at least 2 mm with a defined edge. (B) A 0.2 µm filter was placed on top of BHIT agar plates seeded with S. aureus (S. epi-1 and S. sap were incubated with S. aureus LAC, C. amy-2 was incubated with S. aureus Mu50, and C. aur-1 was incubated with S. aureus 2014.N). A clinical isolate (as described above) was then spotted on top of the filter paper such that the clinical isolate and the S. aureus seeded agar plate were physically separated. Images of the ZOC were taken after 120 h of incubation at 28°C. Images are representative of three independent biological replicates. Scale bar = 10 mm.

FIGURE 5 | Select clinical isolates mediate bacteriostatic or bactericidal activity against S. aureus. The number of S. aureus CFU seeded within the agar (T0) was compared to the number of S. aureus found after 48 h of incubation. S. aureus CFU numbers were determined from directly adjacent to the clinical isolate spot (Inside ZOC) or from outside the ZOC and the fold change from T0 was calculated. Each box represents the data from three independent biological replicates; the horizontal line is plotted at the arithmetic mean and the length of the box represents the range. A dotted line is indicated at 1, which would represent a purely bacteriostatic interaction where the numbers of S. aureus at T0 and T48 were unchanged. Values below the line indicate a decrease in S. aureus at the 48-h time point, which indicates bactericidal activity.

(Desbois and Coote, 2011; Tsai et al., 2016), and have also been used to test the efficacy of antimicrobials (Desbois and Coote, 2011). Despite the usefulness of this model, little is understood about the relative virulence of different S. aureus strains in G. mellonella. We previously found that in vitro gene expression of important virulence factors broadly varied amongst S. aureus strains 2014.N, LAC, and Mu50; 2014.N expresses the highest levels followed by LAC and then Mu50 (Hardy et al., 2019).
Thus, we first tested the ability of these various strains to induce G. mellonella mortality at various doses. The overall virulence in this model revealed that LAC induced the highest level of death, followed by 2014.N and Mu50. Indeed, infection with LAC or 2014.N killed significantly more G. mellonella than Mu50 at the tested doses (Figures 7A,B). These data support the notion that though in vitro defined virulence factor expression profiles may be helpful, they do not always directly correlate with virulence in every in vivo model.

To examine the therapeutic potential of the compound(s) found within the CCFM, we next tested the ability of CCFM to rescue S. aureus-infected G. mellonella. As we found Mu50 to be essentially avirulent in this model (Figures 7A,B), we focused our efforts on CCFM derived from S. sap and S. epi-3, which was most active against S. aureus LAC (Figure 2A). Treatment with S. sap CCFM, but not S. epi-3 CCFM, 1-h post infection with 10^6 S. aureus LAC significantly reduced mortality of infected G. mellonella compared to sham treated controls (Figure 7C). Taken together, our results indicate that anti-S. aureus activity mediated by the various bacterial species is diverse and suggest that secreted compound(s) derived from S. saprophyticus may have possible future therapeutic value.

DISCUSSION

Humans serve as an incredibly complicated and dynamic environmental niche for microorganisms. Our understanding of this fact has been greatly enhanced by the Human Microbiome Project, which has revealed that most anatomical locations are colonized with dozens, if not hundreds of bacterial species that must compete with each other for limited nutrients (Turnbaugh et al., 2007). While large microbiota-based epidemiological studies have identified the presence of these microbes, they often fail to elucidate the molecular interactions that occur between the resident flora and how these interactions may impact incoming pathogens. In addition, interactions with opportunistic pathogens are difficult to study in particular as the factors that promote commensalism vs. pathogenesis are often ill-defined. This is especially true for S. aureus, which asymptotically colonizes one-quarter of the population at any given time (Kluytmans and Wertheim, 2005; Wertheim et al., 2005), while simultaneously maintaining the ability to cause severe disease. It is well-established that the composition of the host microbiota heavily influences S. aureus carriage (Burian et al., 2017). This is particularly true in the nasal cavity, which serves as a primary reservoir for S. aureus colonization (Sakr et al., 2018). As such, many microbiota studies have focused on S. aureus interactions with the nasal flora. However, little is known about how S. aureus may interact with bacteria commonly found at other anatomical sites. Thus, in a proof of concept study we set out to characterize the basic interactions of S. aureus with bacterial isolates obtained from various sites (wound, blood, urine, and the nasal cavity) from patients at the WRNMMC. By taking a reductionist approach, we found that the majority of clinical isolates we screened displayed some form of in vitro anti-S. aureus activity.

En masse, in vitro bacterial interaction assays against three phenotypically different S. aureus strains revealed that the majority of tested clinical isolates were able to inhibit S. aureus to some degree (Figure 1, 2). Most of the inhibitory isolates were members of the Corynebacterium genera (10/28), which supports well-established findings that show that the Corynebacterium genus heavily impacts S. aureus colonization and viability (Yan et al., 2013; Hardy et al., 2019). For example, we previously showed that C. pseudodiphtheriticum, an important community determinant of S. aureus nasal colonization, mediates potent strain-specific bactericidal activity against S. aureus via production of a secreted factor(s) (Hardy et al., 2019). The results described herein indicate that related Corynebacterium species (C. aurimucosum, C. amycolatum, C. striatum, C. jeikeium, and C. tuberculostearicum) also possess some level of anti-S. aureus activity. Despite this finding, it is not possible to generalize that all Corynebacterium species negatively impact S. aureus. For example, C. accolens has been shown to actually promote S. aureus nasal colonization by reducing competition from other opportunistic pathogens (Yan et al., 2013; Bomar et al., 2016). In our screen, C. accolens possessed no anti-S. aureus activity (Figure 1A). In addition, recent work from Stubbenbeck et al. (2019) showed that some Corynebacterium species can inhibit CoNS growth through the production of siderophores that enable these species to out-compete the CoNS for available iron, and thusly influence S. aureus viability. Therefore, individual Corynebacterium species appear to have evolved independent mechanisms that allow them to either cooperate or compete with S. aureus. Overall, our results combined with the growing body of literature suggest that the relationships observed in microbiota-based studies can be translated into in vitro phenotypes, and that the Corynebacterium genus in particular greatly impacts S. aureus viability and thusly colonization.
Hardy et al. Clinically Isolated Bacteria Inhibit S. epi-

Curves were compared (excluding negative controls) using the Mantel–Cox test with Holm’s correction for multiple comparisons. In (A), Mu50 was significantly different than both 2014.N and LAC. In (B), significant differences between the various groups are indicated. In (C), BHIT treated caterpillars were compared to CCFM treated (S. epi-3 or S. sap) Galleria to identify difference; only S. sap was significantly different. Asterisks signifying the P value as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Culture independent-identification methods have revealed that wound infections, rather than being caused by a single species, are often polymicrobial in nature (Bowler et al., 2001; Peters et al., 2012; Tay et al., 2016). Moreover, microbiota-based studies have shown that wounds that are infected with multiple bacterial species tend to have worse outcomes as compared to wounds that are infected with a single species (Dalron et al., 2011; Pastar et al., 2013). It is worth noting that bacteria within wounds have to compete for resources and must contend with the host’s immune system. To aid these processes, bacteria that commonly infect wounds have evolved multiple mechanisms that help in these responses. For example, Pseudomonas aeruginosa and S. aureus are often co-isolated from wounds (Giacometti et al., 2000; Dowd et al., 2008). P. aeruginosa has been found to limit S. aureus growth by sensing the presence of S. aureus peptidoglycan (Korgaonkar et al., 2013; Pastar et al., 2013). P. aeruginosa responds by producing pyocyanin and elastase; both of these molecules have anti-S. aureus properties (Korgaonkar et al., 2013). Similarly, S. aureus and A. baumannii are also commonly co-isolated from wounds. However, to our knowledge there are no published reports of cooperative or competitive interactions between these two species. Thus, we were surprised that our initial screen revealed that A. baumannii was the most frequently isolated species possessing anti-S. aureus activity (7/28, Figures 2, 3). Moreover, the various A. baumannii isolates displayed a wide range of anti-S. aureus activities that were dependent upon both the A. baumannii and S. aureus strains. Future studies that seek to understand these interactions at a molecular level will be of great interest.

In thinking about the types of inhibition that we observed, contact-dependent inhibition can be mediated by variety of different mechanisms. For example, Type VI Secretion Systems (T6SS), which are found in many Gram-negative species, require physical contact and involve injection of toxic compounds directly into competitor cells (Coulthurst, 2019). Similarly, though mechanistically divergent from the T6SS, the Esx secretion pathway, which is broadly distributed amongst Gram-positive bacteria, also requires physical contact between competing bacterial species to mediate growth inhibition via toxic compounds (Whitney et al., 2017). In both these examples, only target cells that are physically touching the inhibitory cells are negatively impacted. In contrast contact-independent growth inhibition is typically mediated by toxic compounds that are synthesized and then secreted by the inhibitory species as a means to kill/prevent the growth of a competitor; no cell-to-cell contact between the two species is required. This approach is a common mechanism that is used by various microbes across...
found to actively compete with *S. saprophyticus*. Our studies identified several isolates that inhibited *S. aureus* and strains utilize a diverse number of mechanisms to inhibit *S. aureus*. Several of the inhibitory isolates identified in this study may produce toxic compounds that have the potential to be used as novel therapeutics or intervention strategies. Our future work will pursue elucidation of the molecular mechanism by which *S. aureus* can also infrequently colonize the urinary tract and cause UTIs, it’s interesting to speculate that *S. saprophyticus* has evolved to kill *S. aureus* as a means to prevent competition for this niche.

We note that secreted bactericidal compound(s) from some of the characterized isolates may have the potential to be developed for use as novel therapeutics to treat or prevent *S. aureus*-mediated infection. This ascertain is supported by the fact that anti-*S. aureus* activity was retained in CCFM from the three tested isolates (Figure 6), suggesting that these species negatively impact *S. aureus* viability most likely through the secretion of a toxic compound(s). The nature of these compound(s) are unclear, they may include compounds like lantibiotics (McAuliffe et al., 2001), which are peptide antibiotics that are produced by a broad range of Gram-positive bacteria, including *Staphylococcus*. Genes that code for lantibiotics are often located on plasmids and other mobile genetic elements, and have a wide range of target-species specificity. Lantibiotics from closely related Staphylococcal species, such as epidermin (Götz et al., 2014), have been found to have potent inhibitory activity against *S. aureus*, including MRSA. It is possible that the anti-*S. aureus* activity we observed from *S. saprophyticus*, and the other Staphylococcal tested species, is the result of a lantibiotic that maintains potent inhibitory properties. Combined, our results indicate that many Staphylococcal species have evolved strategies to compete with *S. aureus*.

While this work was designed as a proof of concept study to explore the extent of anti-*S. aureus* activity exhibited by various microbes, we acknowledge that there are limitations to the study. For example, while the patient population at WRMMC is fairly diverse, given that many of the patients are soldiers that may have incurred traumatic injuries during the course of their service, a substantial proportion of isolates were obtained from wounds; this undoubtedly affected the types of species of bacteria that we ultimately screened. In addition, while this study described the basic molecular mechanisms of these interactions, a more detailed study will be required to clearly identify specific compounds and/or mechanisms of action that are responsible for anti-*S. aureus* activity.

In summary, this proof of concept study indicates that multiple bacterial species possess strain-specific anti-*S. aureus* activity when co-cultured in a bacterial interaction assay. This study further highlights the multifarious nature of polymicrobial interactions, which remain poorly understood. Furthermore, this work expands upon the growing body of literature that supports that the study of 'bacterial warfare' and the toxic compounds created by microbes as a means to compete with one another may be a ‘next best option’ for the identification of novel therapeutics that will help in overcoming the significant increase in antimicrobial resistance that threatens the health and wellbeing of the population (Zipperer et al., 2016; Nakatsuji et al., 2017; Stubbendieck et al., 2019). As such, we hypothesize that several of the inhibitory isolates identified in this study may produce toxic compounds that have the potential to be used as novel therapeutics or intervention strategies. Our future work will pursue elucidation of the molecular mechanism by which...
both A. baumannii and S. saprophyticus inhibit S. aureus. Overall, our findings support the continued study of polymicrobial interactions as a means to identify novel therapeutics and/or molecular targets of S. aureus and other pathogens.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI, GenBank, MN175920–MN175947.

AUTHOR CONTRIBUTIONS

BH and DM designed the research study. EK and JB provided the clinical bacterial isolates utilized in all experiments. BH, GB, KH, AA, and SS performed the experiments. BH, GB, and DM analyzed the data. BH wrote the manuscript. All authors contributed substantially to revisions and approved the final manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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