AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

RESEARCH ARTICLE
Host-Microbe Biology

Pseudomonas aeruginosa Increases the Sensitivity of Biofilm-Grown *Staphylococcus aureus* to Membrane-Targeting Antiseptics and Antibiotics

Giulia Orazi,[a] Kathryn L. Ruoff,[a] George A. O'Toole[a]

[a]Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA

ABSTRACT *Pseudomonas aeruginosa* and *Staphylococcus aureus* often cause chronic, recalcitrant infections in large part due to their ability to form biofilms. The biofilm mode of growth enables these organisms to withstand antibacterial insults that would effectively eliminate their planktonic counterparts. We found that *P. aeruginosa* supernatant increased the sensitivity of *S. aureus* biofilms to multiple antimicrobial compounds, including fluoroquinolones and membrane-targeting antibacterial agents, including the antiseptic chloroxylenol. Treatment of *S. aureus* with the antiseptic chloroxylenol alone did not decrease biofilm cell viability; however, the combination of chloroxylenol and *P. aeruginosa* supernatant led to a 4-log reduction in *S. aureus* biofilm viability compared to exposure to chloroxylenol alone. We found that the *P. aeruginosa*-produced small molecule 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) is responsible for the observed heightened sensitivity of *S. aureus* to chloroxylenol. Similarly, HQNO increased the susceptibility of *S. aureus* biofilms to other compounds, including both traditional and nontraditional antibiotics, which permeabilize bacterial membranes. Genetic and phenotypic studies support a model whereby HQNO causes an increase in *S. aureus* membrane fluidity, thereby improving the efficacy of membrane-targeting antiseptics and antibiotics. Importantly, our data show that *P. aeruginosa* exoproducts can enhance the ability of various antimicrobial agents to kill biofilm populations of *S. aureus* that are typically difficult to eradicate. Finally, our discovery that altering membrane fluidity shifts antimicrobial sensitivity profiles of bacterial biofilms may guide new approaches to target persistent infections, such as those commonly found in respiratory tract infections and in chronic wounds.

IMPORTANCE The thick mucus in the airways of cystic fibrosis (CF) patients predisposes them to frequent, polymicrobial respiratory infections. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are frequently coisolated from the airways of individuals with CF, as well as from diabetic foot ulcers and other wounds. Both organisms form biofilms, which are notoriously difficult to eradicate and promote chronic infection. In this study, we have shown that *P. aeruginosa*-secreted factors can increase the efficacy of compounds that alone have little or no bactericidal activity against *S. aureus* biofilms. In particular, we discovered that *P. aeruginosa* exoproducts can potentiate the antistaphylococcal activity of phenol-based antiseptics and other membrane-active drugs. Our findings illustrate that polymicrobial interactions can dramatically increase antibacterial efficacy *in vitro* and suggest that altering membrane physiology promotes the ability of certain drugs to kill bacterial biofilms—knowledge that may provide a path for the discovery of new biofilm-targeting antimicrobial strategies.

KEYWORDS *Pseudomonas aeruginosa*, *Staphylococcus aureus*, antibiotics, biofilm, membrane

Citation Orazi G, Ruoff KL, O'Toole GA. 2019. *Pseudomonas aeruginosa* increases the sensitivity of biofilm-grown *Staphylococcus aureus* to membrane-targeting antiseptics and antibiotics. mBio 10:e01501-19. https://doi.org/10.1128/mBio.01501-19.

Editor Paul Dunman, University of Rochester

Address correspondence to George A. O'Toole, georgeo@dartmouth.edu.

This article is a direct contribution from a Fellow of the American Academy of Microbiology. Solicited external reviewers: Gerard Wright, McMaster University; Michael Gilmore, Harvard Medical School.

Received 9 June 2019
Accepted 2 July 2019
Published 30 July 2019

Bacterial biofilms are the underlying cause of many chronic, difficult-to-treat infections. The biofilm lifestyle confers high-level tolerance to antibiotics and antiseptics, which is reflected by the requirement of 100- to 1,000-times-higher concentrations of these compounds to treat biofilms compared to their planktonic counterparts (1). As a result, it has proven difficult to find treatments that effectively eradicate biofilms (2–4).

Studies assessing biofilm antibiotic and antiseptic tolerance have typically been performed with single-species biofilms. While such single-species communities are commonly associated with implant infections (5), many infections are caused by polymicrobial biofilms, including respiratory infections, otitis media, urinary tract infections, and infections of both surgical and chronic wounds (6–19). Emerging evidence suggests that growth in these mixed microbial communities can alter antimicrobial tolerance profiles, often in unexpected ways (20–40), but the mechanism(s) underlying such altered tolerance is often poorly understood, with some exceptions. For example, a previous study from our group showed that secreted products of *Pseudomonas aeruginosa* could enhance biofilm tolerance of *Staphylococcus aureus* to vancomycin by 100-fold, likely via interfering with the function of the electron transport chain and slowing growth of *S. aureus* (37).

P. aeruginosa and *S. aureus* coexist in multiple infection settings, and both form biofilms that can be difficult to eradicate. *P. aeruginosa* and *S. aureus* are two of the most prevalent respiratory pathogens in patients with cystic fibrosis (CF) and are both associated with poor lung function and clinical outcomes in these patients (41–45). CF patients who are coinfected with *P. aeruginosa* and *S. aureus* have worse outcomes than those who are infected with either organism alone (46–50). In addition, *P. aeruginosa* and *S. aureus* are often coisolated from chronic wounds, including difficult-to-treat diabetic foot ulcers (51, 52). Furthermore, *in vitro* evidence suggests that *P. aeruginosa* and *S. aureus* coinfection delays wound healing (53).

In this study, we have identified several compounds that alone have little activity against *S. aureus* biofilms, but when combined with secreted products from *P. aeruginosa*, these agents can effectively decrease *S. aureus* biofilm viability. We propose a model whereby the *P. aeruginosa* exoproduct 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) interacts with the *S. aureus* cell membrane, which leads to increased membrane fluidity and potentiates the ability of membrane-active compounds to more effectively target *S. aureus* biofilms.

RESULTS

***P. aeruginosa* supernatant increases *S. aureus* sensitivity to multiple antibiotic compounds.** In a previous study, we found that *P. aeruginosa* exoproducts decrease the efficacy of vancomycin against *S. aureus* biofilms (37). To test whether *P. aeruginosa* might impact *S. aureus* sensitivity to other antibiotics, we screened Biolog Phenotype MicroArray panels for changes in *S. aureus* antibiotic sensitivity in the presence versus absence of *P. aeruginosa* cell-free culture supernatant. Specifically, we tested MicroArray panels 11 to 20, which contain 240 antibacterial compounds. We identified many compounds that became either less effective, as reported previously (37), or, as we show here, more effective at killing *S. aureus* when in the presence of *P. aeruginosa* exoproducts (see Table S1 in the supplemental material). Increased efficacy of a drug was defined as at least a 10-fold decrease in CFU between *S. aureus* exposed to the antibiotic alone and *S. aureus* exposed to *P. aeruginosa* supernatant plus the antibiotic. Of the 240 compounds tested, 107 became more effective against *S. aureus* biofilm populations in the initial screen (Table S1).

Among the several classes of antimicrobial agents that became more effective at killing *S. aureus* in the presence of *P. aeruginosa* supernatant are nucleic acid synthesis inhibitors, membrane-active antibiotics, and antiseptics. Additionally, we identified other compounds not typically used to treat bacterial infections that became more effective at decreasing *S. aureus* viability, including anticholinergic agents, antipsychotic drugs, and ion channel blockers (Table S1).

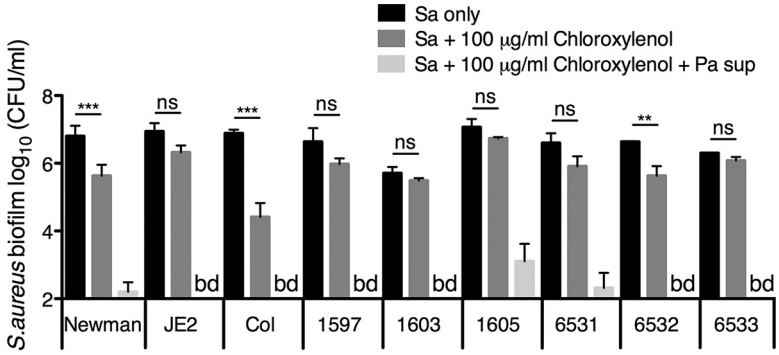

FIG 1 *P. aeruginosa* supernatant increases *S. aureus* biofilm sensitivity to chloroxylenol. Biofilm disruption assays on plastic were performed with the specified *S. aureus* clinical isolate, *P. aeruginosa* PA14 supernatant (Pa sup), and chloroxylenol at 100 μg/ml. Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm CFU were determined. Each column displays the average from two biological replicates, each with three technical replicates. Error bars indicate standard deviation (SD). Sa, *S. aureus*; bd, below detection; ns, not significant; **, $P < 0.01$; ***, $P < 0.001$, by ordinary one-way ANOVA and Bonferroni's multiple-comparison posttest.

***P. aeruginosa* supernatant increases *S. aureus* biofilm sensitivity to chloroxylenol.** In the experiments described above using the Biolog Phenotype MicroArray panels, the compounds tested were added at the same time as the microbes were inoculated into the medium; thus, there was limited time for the bacteria to form a biofilm before exposure to the candidate agents. Therefore, we next tested whether *P. aeruginosa* supernatant could increase the efficacy of the compounds we identified in the Biolog screen against preformed early (6-h) *S. aureus* biofilms. In these experiments, the biofilm of *S. aureus* Newman was allowed to form for 6 h, and fresh medium supplemented with the indicated compound and/or *P. aeruginosa* supernatant was added to this preformed biofilm. This method is what we refer to as the biofilm disruption assay, described in more detail in the supplemental materials and methods (Text S1). Previously, we showed that by 6 h postinoculation (p.i.), the adherent population of *S. aureus* Newman cells is tolerant to vancomycin; at this time point, there is a difference of 3 logs between the cell viability of the biofilm population and that of the planktonic population for a given dose of antibiotic (37). Thus, these communities have one of the key phenotypic traits of a biofilm.

Of the 106 compounds that became more effective at killing *S. aureus* biofilms in the original screen, 42 compounds were tested against preformed early (6-h) *S. aureus* biofilms, which were representatives of a variety of drug classes (Table S1). Out of the 42 compounds tested, 6 became more effective at killing preformed *S. aureus* biofilms when in the presence of *P. aeruginosa* supernatant (Table S1).

We found that *P. aeruginosa* supernatant increased the sensitivity of early (6-h) *S. aureus* biofilms to the topical antibiotic chloroxylenol (Fig. 1). Similarly to other phenol-based antiseptics, this compound impacts bacterial cell membranes, leading to increased fluidity and membrane permeability (54–56). Alone, chloroxylenol displayed modest activity against *S. aureus* biofilms. Strikingly, the ability of the antiseptic chloroxylenol to kill early *S. aureus* Newman biofilms was enhanced by 4 logs compared to the activity of chloroxylenol alone when combined with *P. aeruginosa*-secreted products (Fig. 1). We evaluated whether this phenotype is specific to the Newman strain or a more general phenomenon by testing multiple *S. aureus* laboratory strains and clinical isolates—both methicillin sensitive and methicillin resistant (Table S2). In all cases, we observed that *P. aeruginosa* supernatant dramatically increased the efficacy of chloroxylenol against *S. aureus* biofilms (Fig. 1). Chloroxylenol is dissolved in ethanol; we confirmed that the volume of ethanol used does not decrease *S. aureus* viability in either the presence or absence of *P. aeruginosa* supernatant (Fig. S1A). Moreover, the impact of supernatant on *S. aureus* sensitivity to chloroxylenol could be observed as early as 3 h after addition of the compounds to a 6-h-old biofilm, and the reduction in

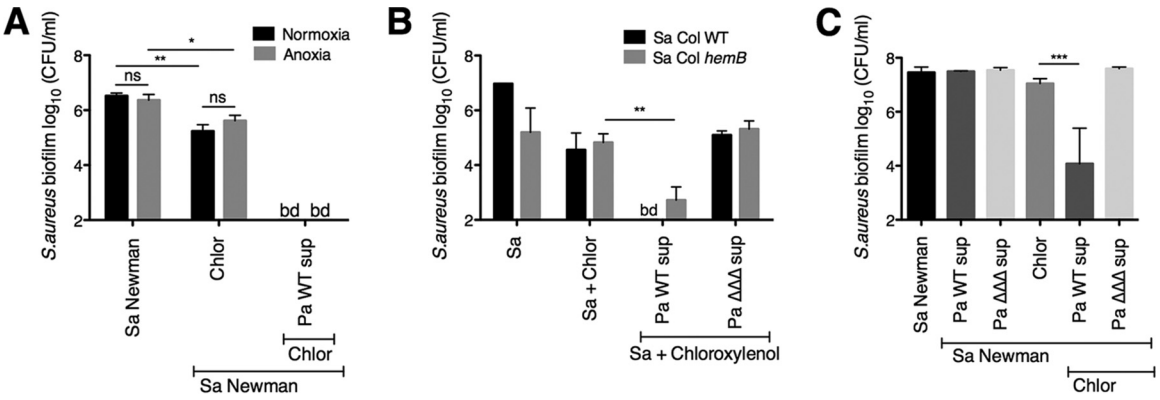

FIG 2 *P. aeruginosa* supernatant enhances the ability of chloroxylenol to kill difficult-to-treat *S. aureus* biofilms. (A) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Newman, *P. aeruginosa* PA14 supernatant (Pa sup), and chloroxylenol (Chlor) at 100 μg/ml under normoxic or anoxic conditions. Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm CFU were determined. (B) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Col parental strain or *hemB* mutant, supernatants from wild-type *P. aeruginosa* PA14 and the Δ*pqsL* Δ*pvdA* Δ*pchE* mutant (Pa ΔΔΔ sup), and chloroxylenol (Chlor) at 100 μg/ml. Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm CFU were determined. (C) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Newman, supernatants from wild-type *P. aeruginosa* PA14 and the Δ*pqsL* Δ*pvdA* Δ*pchE* mutant (Pa ΔΔΔ sup), and chloroxylenol (Chlor) at 100 μg/ml. Biofilms were grown for 24 h and exposed to the above treatments for 24 additional hours, and *S. aureus* biofilm CFU were determined. Each column displays the average from three biological replicates, each with three technical replicates. Error bars indicate standard deviations. bd, below detection; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, by ordinary one-way ANOVA and Tukey's multiple-comparison posttest.

viability continued for 24 h posttreatment, wherein the assay was reaching its limit of detection (Fig. S1B).

***P. aeruginosa* supernatant increases the ability of chloroxylenol to eradicate difficult-to-treat *S. aureus* biofilms.** We then determined whether *P. aeruginosa* could enable chloroxylenol to kill especially difficult-to-treat *S. aureus* biofilms. *S. aureus* grown in anoxia and respiration-deficient *S. aureus* small colony variants (SCVs) both exhibit high tolerance to many classes of antibiotics (57–59), likely because the bacteria need to be actively growing in order for many antibacterial compounds to be effective. Depending on the antibiotic class, either the antibiotic target needs to be produced or electron transport is required for drug uptake (57, 60), but membrane-targeting agents are an exception; the target is present whether or not the organism is actively growing (61). Indeed, *P. aeruginosa* supernatant increased the efficacy of chloroxylenol against *S. aureus* Newman biofilms to similar degrees in anoxia and normoxia (Fig. 2A).

To test whether the combination of *P. aeruginosa* supernatant and chloroxylenol is effective against biofilm-grown *S. aureus* SCVs, we used an *S. aureus* Col strain that has a mutation in *hemB*, a gene involved in hemin biosynthesis. The *S. aureus hemB* mutant is defective in electron transport and has the typical characteristics of clinical SCVs (62). We observed that *P. aeruginosa* supernatant enhanced chloroxylenol's activity against the Col *hemB* mutant as well as the parental strain (Fig. 2B).

Furthermore, we tested whether more mature *S. aureus* biofilms could be effectively targeted by the *P. aeruginosa* supernatant-chloroxylenol combination. When we grew *S. aureus* Newman biofilms for 24 h before exposure to the combination treatment, we observed a striking 4-log-fold enhancement of chloroxylenol's antimicrobial activity (Fig. 2C), similar to what was seen for 6-h-grown biofilms (Fig. 1).

The *P. aeruginosa* exoproducts HQNO and siderophores increase *S. aureus* biofilm and planktonic sensitivity to chloroxylenol. To explore the mechanism underlying *P. aeruginosa* supernatant-mediated enhancement of chloroxylenol's anti-staphylococcal activity, we sought to identify *P. aeruginosa* mutants that were unable to increase the sensitivity of *S. aureus* Newman biofilms to this drug. Previously, we showed that 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and siderophores contribute to the ability of *P. aeruginosa* to protect *S. aureus* from vancomycin (37). Thus, we tested *P. aeruginosa* PA14 strains with mutations in genes encoding components of the *Pseudomonas* quinolone signal (PQS) quorum sensing system (*pqsA*, *pqsH*, and *pqsL*)

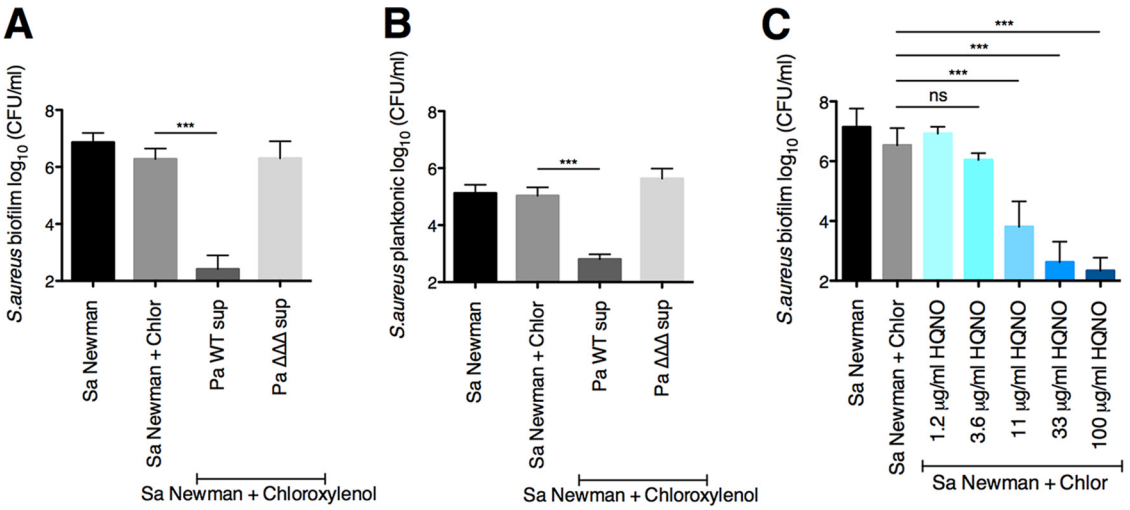

FIG 3 The *P. aeruginosa* exoproducts HQNO and siderophores increase *S. aureus* biofilm and planktonic sensitivity to chloroxylenol. (A and B) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Newman, supernatants from wild-type *P. aeruginosa* PA14 and the Δ*pqsL* Δ*pvdA* Δ*pchE* deletion mutant (Pa ΔΔΔ sup), and chloroxylenol (Chlor) at 100 μg/ml. Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm (A) and planktonic (B) CFU were determined. Data in panels A and B were from the same experiments. (C) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Newman, chloroxylenol (Chlor) at 100 μg/ml, and the specified concentrations of HQNO (dissolved in DMSO). Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm CFU were determined. Each column displays the average from at least three biological replicates, each with three technical replicates. Error bars indicate SD. ns, not significant; ***, $P < 0.001$, by ordinary one-way ANOVA and Tukey's multiple-comparison posttest.

and biosynthesis of the siderophores pyoverdine (*pvdA*) and pyochelin (*pchE*). Supernatants from *P. aeruginosa* PA14 Δ*pqsA*, Δ*pqsH*, Δ*pqsL*, and Δ*pvdA* Δ*pchE* mutants each had a defect in the ability to increase *S. aureus* Newman biofilm sensitivity to chloroxylenol relative to the wild-type *P. aeruginosa* PA14 (Fig. S2A and B).

Additionally, we tested *P. aeruginosa* PA14 strains with mutations in genes encoding the following secreted products: hydrogen cyanide (*hcnA* and *hcnB*), LasA protease (*lasA*), elastase (*lasB*), and rhamnolipids (*rhlA*). Supernatants from these mutants retained the ability to increase the sensitivity of *S. aureus* biofilms to chloroxylenol (Fig. S2A and B).

To investigate whether HQNO, pyoverdine, and pyochelin all contributed to the phenotype, we tested whether the supernatant from *P. aeruginosa* strains with mutations in the genes encoding all three factors was deficient in enhancing chloroxylenol's activity against *S. aureus*. Indeed, supernatant from the *P. aeruginosa* PA14 Δ*pqsL* Δ*pvdA* Δ*pchE* mutant (designated the ΔΔΔ mutant) was unable to increase the sensitivity of *S. aureus* Newman biofilms to chloroxylenol (Fig. 3A and Fig. S2B). Supernatant from the *P. aeruginosa* PA14 Δ*pqsL* Δ*pvdA* Δ*pchE* mutant was unable to potentiate the ability of chloroxylenol to kill difficult-to-treat SCVs and 24-h-grown biofilms (Fig. 2B and C; Pa ΔΔΔ sup). Similarly to the biofilm population, we observed that *P. aeruginosa* PA14 wild-type supernatant, but not the Δ*pqsL* Δ*pvdA* Δ*pchE* mutant, enhances the ability of chloroxylenol to kill planktonic *S. aureus* Newman by approximately 3 logs (Fig. 3B). Thus, our data indicate that HQNO and both siderophores are required for *P. aeruginosa*-mediated enhancement of chloroxylenol's activity against both planktonic and biofilm populations of *S. aureus*.

HQNO alone enhances the activity of chloroxylenol against *S. aureus* biofilms. To test whether HQNO alone could enhance to the ability of chloroxylenol to kill *S. aureus* in biofilm, we performed a biofilm disruption assay using commercially available HQNO. We used concentrations of HQNO that are in the range of those produced by *P. aeruginosa* PA14 under our experimental conditions (37), as well as those produced by stationary-phase *P. aeruginosa* cultures grown in rich medium (63, 64). Previously, we quantified the level of HQNO produced by *P. aeruginosa* PA14 after 24 h of growth in minimal medium on plastic plates, which is the source of *P. aeruginosa* supernatants

used throughout this study (37). We found that the level of HQNO in these *P. aeruginosa* supernatants is ~10 μg/ml. Additionally, *P. aeruginosa* PA14 produced ~15 μg/ml HQNO when grown on CF-derived epithelial cells for 6 h (37). We observed a dose-response whereby increasing concentrations of exogenous HQNO corresponded with enhanced ability of chloroxylenol to kill *S. aureus* Newman biofilms (Fig. 3C). These results indicate that the presence of a single secreted factor, HQNO, is sufficient to alter *S. aureus* biofilm sensitivity to chloroxylenol.

HQNO likely does not increase *S. aureus* sensitivity to chloroxylenol via inhibition of the ETC. HQNO is well known to inhibit electron transport chain (ETC) complexes II and III in both mammalian and bacterial cells (65–68). To investigate whether HQNO shifts *S. aureus* sensitivity to chloroxylenol by inhibiting respiration, we tested the following ETC inhibitors: 3-nitropropionic acid (3-NP; complex II inhibitor), antimycin A (complex III inhibitor), sodium azide (azide; complex IV inhibitor), and oligomycin (ATP synthase inhibitor) or mutations in components of ATP synthase. All but one of the compounds tested, antimycin A, had little to no impact on *S. aureus* sensitivity to chloroxylenol, nor did mutations in the ATPase (Fig. S3A to E).

It is possible that HQNO and antimycin A are changing antibiotic sensitivity not by inhibiting the ETC but via a different mechanism entirely. Thus, we took a different approach to investigate whether ETC inhibition changes *S. aureus* susceptibility to chloroxylenol. Exposure to anoxic conditions is a way to inhibit respiration that does not require the use of chemical compounds. Anoxia did not enhance chloroxylenol's efficacy against *S. aureus* Newman biofilms in the absence of *P. aeruginosa* supernatant (Fig. 2A). Also, despite lacking a functional ETC, *S. aureus* SCVs are not hypersensitive to chloroxylenol (Fig. 2B). Furthermore, as we observed above, *P. aeruginosa* supernatant is able to potentiate the activity of chloroxylenol to kill SCVs even though these cells are respiration deficient (Fig. 2B). Together, these data indicate that HQNO likely alters *S. aureus* antibiotic sensitivity via a mechanism independent of its effects on the ETC.

We next considered several possible mechanisms underlying HQNO-mediated enhancement of chloroxylenol's antistaphylococcal activity. Specifically, we tested the following models: (i) HQNO-mediated changes in membrane potential increase antibiotic sensitivity, (ii) HQNO-induced generation of reactive oxygen species leads to enhanced bacterial killing, (iii) HQNO alters the ability of *S. aureus* to efflux chloroxylenol, and/or (iv) HQNO changes properties of the *S. aureus* cell membrane. Experiments testing the first three of these models, which did not support these models, are presented in the supplemental results (Text S1) and in Fig. S3 and S4.

Exogenous HQNO increases *S. aureus* membrane fluidity. Previous studies have found that changes in the cell membrane fatty acid composition, which influences membrane fluidity, alter the susceptibility of bacterial cells to phenolic compounds (69). Thus, we tested whether HQNO might cause heightened susceptibility to chloroxylenol by altering the fluidity of the *S. aureus* cell membrane. To measure membrane fluidity, we performed Laurdan generalized polarization (GP) assays. Laurdan is a fluorescent dye that is sensitive to changes in membrane fluidity; the emission spectrum changes depending on the physical state of lipids within a bilayer. A decrease in Laurdan GP values corresponds to an increase in membrane fluidity. This dye has been previously used to measure the cell membrane fluidity of *S. aureus* (70–72).

We used benzyl alcohol, a well-established membrane fluidizing agent (73–75), as a positive control. Exposure to 500 mM or 1 M benzyl alcohol for 1 h led to a significant decrease in Laurdan GP relative to *S. aureus* exposed to minimum essential medium (MEM), indicating an increase in membrane fluidity (Fig. 4A). We observed that treatment of *S. aureus* Newman with HQNO at all concentrations tested led to a significant reduction in Laurdan GP relative to exposure to MEM alone, indicating that HQNO has a fluidizing effect on the *S. aureus* membrane (Fig. 4B). Additionally, we found that exposure to antimycin A also led to a significant increase in fluidity (Fig. 4C), albeit to a lesser extent than HQNO (Fig. 4B). Furthermore, we showed that the solvents for

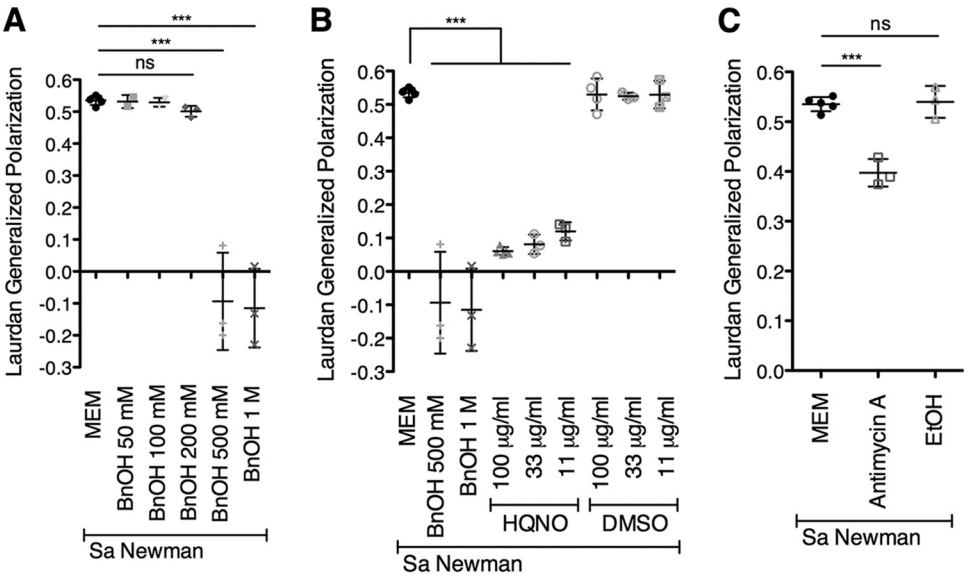

FIG 4 Exogenous HQNO increases *S. aureus* membrane fluidity. (A to C) Laurdan generalized polarization (GP) was performed with *S. aureus* (Sa) Newman, benzyl alcohol (BnOH) (A and B), HQNO (B), and the DMSO control (solvent for HQNO) (B) at the indicated concentrations and antimycin A at 100 μg/ml along with the ethanol (EtOH) control (solvent for antimycin A) (C). *S. aureus* was exposed to the above treatments for 1 h, and GP values were determined. Each column displays the average from at least three biological replicates, each with three technical replicates. Error bars indicate SD. ns, not significant; ***, $P < 0.001$, by ordinary one-way ANOVA and Tukey's multiple-comparison posttest.

HQNO and antimycin A, dimethyl sulfoxide (DMSO) and ethanol, respectively, did not cause the observed increase in *S. aureus* membrane fluidity (Fig. 4B and C).

Shifting membrane fluidity alters *S. aureus* biofilm sensitivity to chloroxylenol. Next, we investigated whether the observed HQNO-mediated increase in membrane fluidity can lead to increased sensitivity to chloroxylenol. To test this hypothesis, we exposed *S. aureus* biofilms to various compounds that are known to influence membrane fluidity. Benzyl alcohol and 1-heptanol both impart higher fluidity, whereas dimethyl sulfoxide (DMSO) causes membranes to become less fluid (73–77). We observed that benzyl alcohol and 1-heptanol both increased *S. aureus* Newman biofilm sensitivity to chloroxylenol (Fig. 5A and B). In contrast, the membrane-rigidifying agent DMSO did not increase *S. aureus* Newman biofilm sensitivity to chloroxylenol (Fig. 5C). These results suggest that alterations in *S. aureus* membrane fluidity impact sensitivity to chloroxylenol, whereby increased fluidity leads to higher sensitivity.

Next, we showed that manipulating *S. aureus* fatty acid composition either by adding exogenous unsaturated fatty acids (Fig. S5A and Text S1) or by increasing the proportion of branched-chain fatty acids (BCFAs) relative to short-chain fatty acids (SCFAs) by mutation (Fig. S5B and Text S1) leads to increased *S. aureus* sensitivity to chloroxylenol. Additionally, we showed that decreasing levels of BCFAs relative to SCFAs by introducing the *lpd* mutation does not increase sensitivity to chloroxylenol (Fig. S5C) and that cardiolipin is not required for altered *S. aureus* sensitivity to this drug (Fig. S5D and Text S1).

Together, our data suggest that changes in membrane fatty acid composition influence the efficacy of chloroxylenol and are consistent with our model that an increase in membrane fluidity promotes chloroxylenol's ability to kill *S. aureus* biofilms.

Prolonged exposure to *P. aeruginosa* exoproducts alters *S. aureus* membrane fatty acid profiles. Our data above suggest that HQNO increases *S. aureus* membrane fluidity, which leads to heightened sensitivity of *S. aureus* to chloroxylenol. Thus, we explored whether HQNO induces changes in *S. aureus* membrane fatty acid composition. We performed a time course to track *S. aureus* fatty acid composition over time in the presence of *P. aeruginosa* exoproducts. Briefly, *S. aureus* Newman cells were

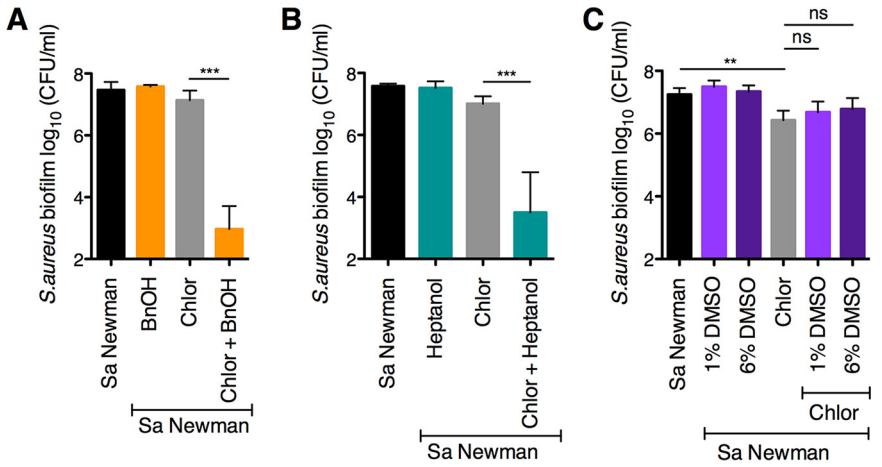

FIG 5 Shifting membrane fluidity alters *S. aureus* biofilm sensitivity to chloroxylenol. (A to C) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Newman, chloroxylenol (Chlor) at 100 μg/ml, benzyl alcohol (BnOH) at 50 mM (A), 1-heptanol at 50 mM (B), and dimethyl sulfoxide (DMSO) at 1% and 6% (C). Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm CFU were determined. Each column displays the average from at least three biological replicates, each with three technical replicates. Error bars indicate SD. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$, by ordinary one-way ANOVA and Tukey's multiple-comparison posttest.

exposed to medium alone (MEM + L-Gln) or *P. aeruginosa* PA14 wild-type supernatant for differing lengths of time (30 min, 1 h, 3 h, 6 h, or 10 h). Subsequently, fatty acid methyl ester (FAME) analysis was performed to measure the membrane fatty acid composition.

By 30 min or 1 h, the membrane fatty acid profile of *S. aureus* cells grown in medium alone appeared similar to the profile of *P. aeruginosa* supernatant-exposed *S. aureus* cells (Fig. S6A to C and Table S3). However, prolonged treatment with *P. aeruginosa* supernatant led to a shift in *S. aureus* membrane fatty acid profiles. In particular, *S. aureus* cells incubated with *P. aeruginosa* exoproducts for 24 h had significantly reduced relative BCFA levels compared to *S. aureus* grown in medium alone (Fig. S6D and E and Text S1). Above, we found that HQNO significantly increases *S. aureus* membrane fluidity after 1 h (Fig. 4B). Because the fluidizing effect of HQNO occurs more rapidly than the effect of *P. aeruginosa* supernatant on *S. aureus* membrane fatty acid composition, it is likely that the HQNO-mediated increase in *S. aureus* membrane fluidity that we observe does not occur via changes in membrane fatty acid profiles.

***P. aeruginosa* supernatant increases *S. aureus* biofilm sensitivity to multiple membrane-targeting compounds.** Given the effects of *P. aeruginosa* exoproducts on *S. aureus* sensitivity to chloroxylenol, we explored whether *P. aeruginosa* alters the antistaphylococcal efficacy of other membrane-active antibiotics. Here, we tested the efficacy of the phenol-based antiseptic biphenyl, as well as the topical peptide antibiotic gramicidin in combination with *P. aeruginosa* supernatant. Both of these compounds are thought to kill bacteria by ultimately causing an increase in cell membrane permeability. We discovered that *P. aeruginosa*-secreted products enhance the ability of the membrane-active drugs biphenyl and gramicidin to kill *S. aureus* Newman biofilms (Fig. 6A and B). We also made the interesting observation that *P. aeruginosa* supernatant increases *S. aureus* biofilm sensitivity to two nontraditional antibiotics, trifluoperazine, an antipsychotic, and amitriptyline, an antidepressant (Fig. 6C and D). Strikingly, the combination of either of these drugs and *P. aeruginosa* supernatant led to a 2.5- to 3-log reduction in *S. aureus* biofilm viability compared to exposure to the drug alone (Fig. 6C and D). Supernatants from *P. aeruginosa* PA14 Δ*pqsL* and Δ*pvdA* Δ*pchE* mutants each had defects in the ability to increase *S. aureus* Newman biofilm sensitivity to trifluoperazine and amitriptyline relative to the wild-type *P. aeruginosa* PA14 (Fig. 6C and D), suggesting that HQNO and siderophores both contribute to this phenotype. In

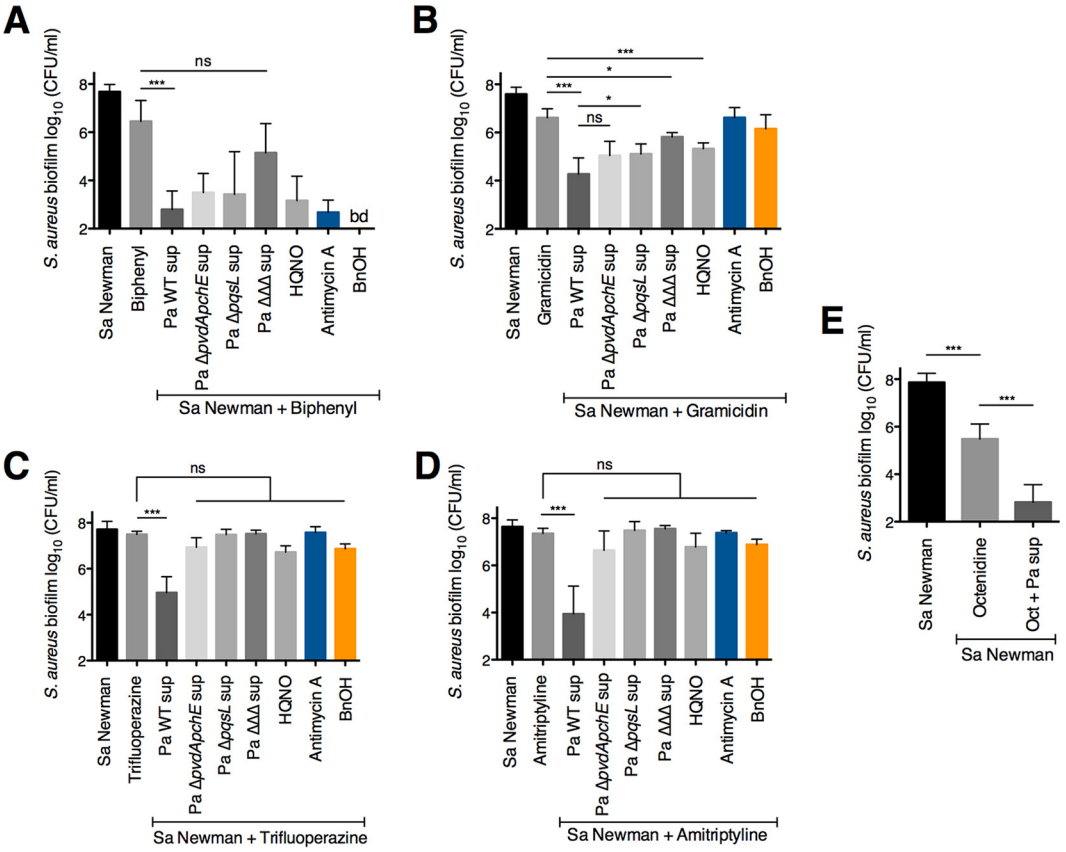

FIG 6 *P. aeruginosa* supernatant increases *S. aureus* biofilm sensitivity to other membrane-targeting compounds. (A to E) Biofilm disruption assays on plastic were performed with *S. aureus* (Sa) Newman; supernatants from wild-type *P. aeruginosa* PA14 and the specified mutants (Pa sup); and either biphenyl at 200 μg/ml (A), gramicidin at 100 μg/ml (B), trifluoperazine at 100 μg/ml (C), amitriptyline at 100 μg/ml (D), or octenidine dihydrochloride (Oct) at 5 μg/ml (E). Biofilms were grown for 6 h and exposed to the above treatments for 18 h, and *S. aureus* biofilm CFU were determined. Each column displays the average from at least three biological replicates, each with three technical replicates. Error bars indicate standard deviation (SD). ns, not significant; *, $P < 0.05$; ***, $P < 0.001$, by ordinary one-way ANOVA and Tukey's multiple-comparison posttest.

contrast, it appears than another *P. aeruginosa*-produced factor is involved in enhancing the activity of gramicidin against *S. aureus* biofilms (Fig. 6B).

Additionally, we examined whether altering membrane fluidity influenced *S. aureus* biofilm sensitivity to the above compounds. We observed that benzyl alcohol did not appreciably alter *S. aureus* sensitivity to gramicidin, trifluoperazine, or amitriptyline (Fig. 6B to D). In contrast, the fluidizing agent led to a striking increase in the antibacterial efficacy of biphenyl; the combination of these compounds led to a decrease in *S. aureus* Newman biofilm viability to below the level of detection of this assay (~200 CFU/ml [Fig. 6A]). These results suggest that a more fluid membrane increases the susceptibility of *S. aureus* biofilms to biphenyl, which is a compound similar to chloroxylenol in structure and function.

Finally, we tested whether *P. aeruginosa* secreted products could increase the antistaphylococcal efficacy of octenidine dihydrochloride, a surfactant-based antiseptic that is approved for treatment of wound infections and has low cytotoxicity (78, 79). We observed that *P. aeruginosa* supernatant potentiates the activity of octenidine against *S. aureus* biofilms by 2.5 logs (Fig. 6E).

DISCUSSION

In this study, we found that the interactions between two bacterial pathogens that are frequently coisolated from infections can cause striking and unexpected changes in antimicrobial susceptibility profiles. We showed that *P. aeruginosa* potentiates the ability of various antibacterial agents to kill *S. aureus* biofilms, which are often difficult

to eradicate. In particular, we found that *P. aeruginosa*-secreted products increase the sensitivity of *S. aureus* biofilms to the topical antiseptic chloroxylenol. Alone, chloroxylenol at a concentration of 100 μg/ml is not effective at eradicating *S. aureus* biofilms; however, in combination with *P. aeruginosa* cell-free culture supernatant, which alone does not impact *S. aureus* viability, the efficacy of chloroxylenol increased 4-log-fold. Moreover, we have shown that *P. aeruginosa* supernatant can increase the ability of chloroxylenol to kill multiple strains and clinical isolates of *S. aureus*. Furthermore, we found that the small molecule HQNO and the siderophores pyoverdine and pyochelin contribute to the *P. aeruginosa*-mediated increase in the efficacy of chloroxylenol against *S. aureus* biofilms. In addition, we showed that HQNO alone recapitulated the effect of *P. aeruginosa* supernatant. Thus, the addition of a small molecule alone can greatly influence the efficacy of this antiseptic.

Previous studies have detected HQNO in expectorated sputum from CF patients infected with *P. aeruginosa*, and these levels are highly variable (29, 80). *P. aeruginosa* isolates from chronic CF pulmonary infections frequently have loss-of-function mutations in the quorum sensing regulator *lasR* and often overproduce alginate (81, 82). LasR inactivity and mucoidy each can lead to decreased HQNO production *in vitro* (64, 83). Therefore, quorum sensing activity and mucoidy may modulate the levels of HQNO produced by *P. aeruginosa* during infection and, in turn, influence the ability of HQNO to modify *S. aureus* drug sensitivity profiles *in vivo*.

HQNO has been shown to inhibit the *S. aureus* electron transport chain (ETC) (65). To investigate whether HQNO influences *S. aureus* susceptibility to chloroxylenol via inhibition of respiration, we treated *S. aureus* with chemical inhibitors of the ETC alone or in combination with the antibiotic. We found that only a subset of the ETC inhibitors tested increased the efficacy of chloroxylenol. However, anoxia did not increase *S. aureus* chloroxylenol sensitivity in the absence of HQNO. Additionally, despite having a defective ETC, *S. aureus* SCVs became more susceptible to chloroxylenol in the presence of HQNO, suggesting that inhibition of respiration is not required for this phenotype.

Since it is known that changes in membrane lipid profiles impact sensitivity to membrane-targeting compounds (69), we hypothesized that HQNO might cause heightened susceptibility to chloroxylenol by altering one or more properties of the *S. aureus* cell membrane. Like other phenol-based antiseptics, chloroxylenol is thought to insert into the cell membrane and cause an increase in membrane fluidity and permeability (54–56). Thus, an increase in membrane fluidity mediated by HQNO may allow for greater accumulation of chloroxylenol within the membrane and subsequently cause an increase in efficacy of the antibiotic. Manipulating the fluidity of *E. coli* membranes has been previously demonstrated to alter sensitivity to phenols, whereby decreasing membrane fluidity conferred increased tolerance to these compounds (69). Therefore, we tested whether HQNO changes the fluidity of the *S. aureus* cell membrane, potentially explaining the increased antimicrobial sensitivity we observe. We found that exogenous HQNO causes a striking increase in *S. aureus* membrane fluidity. Due to its hydrophobic character, it is plausible that HQNO directly interacts with the membrane to increase fluidity. In light of this result, we hypothesized that antimycin A and oligomycin, both hydrophobic compounds, also increase *S. aureus* sensitivity to chloroxylenol by altering membrane fluidity; the other ETC inhibitors tested, 3-NP and sodium azide, which did not enhance sensitivity to chloroxylenol, are both hydrophilic compounds. We showed that treatment of *S. aureus* with antimycin A also leads to an increase in membrane fluidity. These findings suggest that the observed HQNO-mediated increase in antibiotic efficacy is independent of the effect of HQNO on the *S. aureus* ETC. Furthermore, we showed that modulating membrane fluidity via either genetic or chemical approaches shifts *S. aureus* chloroxylenol sensitivity profiles. Together, these results are consistent with a model whereby HQNO increases *S. aureus* membrane fluidity, which greatly enhances the ability of chloroxylenol to kill *S. aureus* biofilms.

We also found that treatment with *P. aeruginosa* supernatant or pure HQNO influenced the membrane fatty acid composition of *S. aureus*. Specifically, *S. aureus*

grown in medium alone had a significantly higher proportion of BCFA than did *S. aureus* cells exposed to *P. aeruginosa* supernatant or HQNO for 24 h. Given these results, we hypothesize that HQNO-mediated inhibition of the *S. aureus* ETC leads to decreased rates of fatty acid synthesis. Previous work from our laboratory has shown that when these organisms are in coculture, *P. aeruginosa* forces *S. aureus* to grow by fermentation (84), which leads to a reduction in growth of *S. aureus* (37). Furthermore, during coculture with *P. aeruginosa*, *S. aureus* downregulates multiple genes involved in fatty acid synthesis, including the cardiolipin synthase (*cls1*) and branched-chain amino acid transporters (*brnQ1*, *brnQ2*, *brnQ3*, and *bcaP*) (84). Additionally, it has been shown that anaerobically grown *S. aureus* has lower protein synthesis rates for multiple enzymes involved in metabolism, including FabG1, which is required for fatty acid synthesis (85).

Together, our results are consistent with the following two models, which are not mutually exclusive: (i) HQNO increases *S. aureus* membrane fluidity, potentially via direct interaction with the membrane, and (ii) exposure to HQNO slows or halts *S. aureus* fatty acid synthesis, leading to altered membrane lipid composition, perhaps via ETC inhibition. Our data suggest that the first model may explain how HQNO potentiates the activity of chloroxylenol against *S. aureus* biofilms. In contrast, our data do not support a role for the second model in explaining the altered chloroxylenol susceptibility profiles we observe. Specifically, the HQNO-mediated increase in *S. aureus* membrane fluidity occurs more rapidly than the *P. aeruginosa* supernatant-induced changes in fatty acid profiles. Therefore, we hypothesize that HQNO increases fluidity via direct interaction with the membrane, rather than via inducing a shift in membrane fatty acid composition. The second model could explain other potential consequences of this interspecies interaction, such as an impaired ability to adapt to changing environmental conditions.

We observed that *P. aeruginosa* exoproducts can potentiate the activity of multiple membrane-active compounds, including the phenol biphenyl and gramicidin, which forms channels within the membrane (86–88). Interestingly, we also showed that *P. aeruginosa*-secreted factors enhanced the activity of two nontraditional antibiotics, trifluoperazine and amitriptyline. Both of these drugs have a fused tricyclic structure and have been found to possess antibacterial activity (89–93). Additionally, trifluoperazine was found to synergize with fluconazole against multiple fungal species (94). Due to its high degree of hydrophobicity, trifluoperazine has been shown to interact with cell membranes and cause increased fluidity and permeability (95, 96); it has been hypothesized that amitriptyline acts in a similar manner (93).

Importantly, we found that the combination of *P. aeruginosa* supernatant and chloroxylenol was effective against multiple slow-growing *S. aureus* populations, namely, anaerobically grown biofilms and SCVs. Infection sites can have steep oxygen gradients (97, 98), which may lead to slow microbial growth *in vivo* (99). Slow-growing pathogens are difficult to eradicate because many antibiotic classes are effective against only actively growing cells; in contrast, antibacterial agents that target membranes are effective whether or not bacteria are growing. Thus, our discovery that an interspecies interaction can potentiate the activity of membrane-active drugs could be used to inform the treatment of recalcitrant mixed-species infections involving bacterial biofilms in oxygen-depleted sites.

Overall, our work demonstrates that polymicrobial interactions can profoundly shift the antibiotic sensitivity profiles of bacteria growing as biofilms. Furthermore, we discovered that interspecies interactions can lead to changes in the fluidity and composition of the bacterial cell membrane, which may influence other aspects of bacterial physiology as well as responses to environmental stressors. Additionally, our results suggest that manipulating membrane fluidity can influence the efficacy of various membrane-targeting drugs against bacterial biofilms. We propose that these findings could inspire new strategies for eradicating recalcitrant infections.

Because of its ability to inhibit mitochondrial respiration, HQNO is not a good candidate for a therapeutic; however, it is possible that other membrane-altering compounds could be used as adjuvants to antibacterial therapy. Together, our findings

may have important consequences for the treatment of polymicrobial infections in multiple disease contexts, including nonhealing wounds and pulmonary infections in patients with cystic fibrosis.

MATERIALS AND METHODS

See the supplemental materials and methods in Text S1 in the supplemental material for additional details regarding the methods.

Bacterial strains and culture conditions. A list of all strains used in this study is included in Table S2. *S. aureus* was grown in tryptic soy broth (TSB), and *P. aeruginosa* was grown in lysogeny broth (LB). All overnight cultures were grown with shaking at 37°C for 12 to 14 h, except for the *S. aureus* Col *hemB* mutant, which was grown statically at 37°C for 20 h.

Biolog MicroArray antibiotic susceptibility assay. Biolog Phenotype MicroArray bacterial chemical sensitivity assay panels were used to test *S. aureus* antimicrobial sensitivities as previously described (37). See the supplemental materials and methods in Text S1 for additional details.

Biofilm disruption assay on plastic. *S. aureus* biofilms were treated with antimicrobial agents, followed by enumeration of viable cell counts, as previously described (37). See the supplemental materials and methods in Text S1 for additional details.

Membrane potential measurements. *S. aureus* membrane potential was determined using the fluorescent dye $DiOC_2$ as previously described (100, 101) with some modifications. See the supplemental materials and methods in Text S1 for additional details.

Laurdan membrane fluidity analysis. *S. aureus* membrane fluidity was determined by Laurdan generalized polarization (GP) as previously described (101, 102) with some modifications. See the supplemental materials and methods in Text S1 for additional details.

Fatty acid methyl ester analysis. Whole-cell direct fatty acid methyl ester (FAME) analysis of *S. aureus* pellets was performed by Microbial ID, Inc. (Newark, DE), as previously described (103). See the supplemental materials and methods in Text S1 for additional details.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01501-19.

TEXT S1, PDF file, 0.2 MB.
FIG S1, PDF file, 0.2 MB.
FIG S2, PDF file, 0.1 MB.
FIG S3, PDF file, 0.4 MB.
FIG S4, PDF file, 0.3 MB.
FIG S5, PDF file, 0.3 MB.
FIG S6, PDF file, 0.3 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, XLSX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank Ambrose Cheung, Deborah Hogan, Vineet Singh, and David Heinrichs for providing bacterial strains.

This work was supported by National Institutes of Health grant R37 AI83256-06 and a Cystic Fibrosis Foundation (OTOOLE16G0) grant to G.A.O. and a Microbiology and Molecular Pathogenesis training grant (T32-AI007519) to G.O. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35:322–332. https://doi.org/10.1016/j.ijantimicag.2009.12.011.
2. Bhattacharya M, Wozniak DJ, Stoodley P, Hall-Stoodley L. 2015. Prevention and treatment of *Staphylococcus aureus* biofilms. Expert Rev Anti Infect Ther 13:1499–1516. https://doi.org/10.1586/14787210.2015.1100533.
3. Wu H, Moser C, Wang H-Z, Høiby N, Song Z-J. 2015. Strategies for combating bacterial biofilm infections. Int J Oral Sci 7:1–7. https://doi.org/10.1038/ijos.2014.65.
4. Penesyan A, Gillings M, Paulsen IT. 2015. Antibiotic discovery: combatting bacterial resistance in cells and in biofilm communities. Molecules 20:5286–5298. https://doi.org/10.3390/molecules20045286.
5. Campoccia D, Montanaro L, Arciola CR. 2006. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials 27:2331–2339. https://doi.org/10.1016/j.biomaterials.2005.11.044.
6. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, Kaess H, Deterding RR, Accurso FJ, Pace NR. 2007. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proc Natl Acad Sci U S A 104:20529–20533. https://doi.org/10.1073/pnas.0709804104.
7. Filkins LM, Hampton TH, Gifford AH, Gross MJ, Hogan DA, Sogin ML,

Morrison HG, Paster BJ, O'Toole GA. 2012. Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol 194:4709–4717. https://doi.org/10.1128/JB.00566-12.
8. Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, Wolfgang MC. 2012. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLoS One 7:e45001. https://doi.org/10.1371/journal.pone.0045001.
9. Stressmann FA, Rogers GB, van der Gast CJ, Marsh P, Vermeer LS, Carroll MP, Hoffman L, Daniels TWV, Patel N, Forbes B, Bruce KD. 2012. Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. Thorax 67:867–873. https://doi.org/10.1136/thoraxjnl-2011-200932.
10. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, Young VB, LiPuma JJ. 2012. Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A 109:5809–5814. https://doi.org/10.1073/pnas.1120577109.
11. Lim YW, Schmieder R, Haynes M, Willner D, Furlan M, Youle M, Abbott K, Edwards R, Evangelista J, Conrad D, Rohwer F. 2013. Metagenomics and metatranscriptomics: windows on CF-associated viral and microbial communities. J Cyst Fibros 12:154–164. https://doi.org/10.1016/j.jcf.2012.07.009.
12. Filkins LM, O'Toole GA. 2015. Cystic fibrosis lung infections: polymicrobial, complex, and hard to treat. PLoS Pathog 11:e1005258. https://doi.org/10.1371/journal.ppat.1005258.
13. Giacometti A, Cirioni O, Schimizzi AM, Del Prete MS, Barchiesi F, D'Errico MM, Petrelli E, Scalise G. 2000. Epidemiology and microbiology of surgical wound infections. J Clin Microbiol 38:918–922.
14. Citron DM, Goldstein EJC, Merriam CV, Lipsky BA, Abramson MA. 2007. Bacteriology of moderate-to-severe diabetic foot infections and in vitro activity of antimicrobial agents. J Clin Microbiol 45:2819–2828. https://doi.org/10.1128/JCM.00551-07.
15. Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA, Wolcott RD. 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol 8:43. https://doi.org/10.1186/1471-2180-8-43.
16. Post JC, Preston RA, Aul JJ, Larkins-Pettigrew M, Rydquist-White J, Anderson KW, Wadowsky RM, Reagan DR, Walker ES, Kingsley LA, Magit AE, Ehrlich GD. 1995. Molecular analysis of bacterial pathogens in otitis media with effusion. JAMA 273:1598–1604. https://doi.org/10.1001/jama.1995.03520440052036.
17. Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ. 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J Clin Microbiol 35:2854–2858.
18. Ronald A. 2003. The etiology of urinary tract infection: traditional and emerging pathogens. Dis Mon 49:71–82. https://doi.org/10.1067/mda.2003.8.
19. Kline KA, Lewis AL. 2016. Gram-positive uropathogens, polymicrobial urinary tract infection, and the emerging microbiota of the urinary tract. Microbiol Spectr 4(2):UTI-0012-2012. https://doi.org/10.1128/microbiolspec.UTI-0012-2012.
20. Lightbown JW. 1954. An antagonist of streptomycin and dihydrostreptomycin produced by *Pseudomonas aeruginosa*. J Gen Microbiol 11:477–492. https://doi.org/10.1099/00221287-11-3-477.
21. Shahidi A, Ellner PD. 1969. Effect of mixed cultures on antibiotic susceptibility testing. Appl Microbiol 18:766–770.
22. Barry AL, Joyce LJ, Adams AP, Benner EJ. 1973. Rapid determination of antimicrobial susceptibility for urgent clinical situations. Am J Clin Pathol 59:693–699. https://doi.org/10.1093/ajcp/59.5.693.
23. Ellner PD, Johnson E. 1976. Unreliability of direct antibiotic susceptibility testing on wound exudates. Antimicrob Agents Chemother 9:355–356. https://doi.org/10.1128/aac.9.2.355.
24. Hollick GE, Washington JA. 1976. Comparison of direct and standardized disk diffusion susceptibility testing of urine cultures. Antimicrob Agents Chemother 9:804–809. https://doi.org/10.1128/aac.9.5.804.
25. Johnson JE, Washington JA. 1976. Comparison of direct and standardized antimicrobial susceptibility testing of positive blood cultures. Antimicrob Agents Chemother 10:211–214. https://doi.org/10.1128/aac.10.2.211.
26. Linn BS, Szabo S. 1975. The varying sensitivity to antibacterial agents of micro-organisms in pure vs. mixed cultures. Surgery 77:780–785.
27. Lebrun M, de Repentigny J, Mathieu LG. 1978. [Diminution of the antibacterial activity of antibiotics in cultures and in experimental mixed infections.] Can J Microbiol 24:154–161. https://doi.org/10.1139/m78-028.
28. Mirrett S, Reller LB. 1979. Comparison of direct and standard antimicrobial disk susceptibility testing for bacteria isolated from blood. J Clin Microbiol 10:482–487.
29. Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI. 2006. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 103:19890–19895. https://doi.org/10.1073/pnas.0606756104.
30. Ryan RP, Fouhy Y, Garcia BF, Watt SA, Niehaus K, Yang L, Tolker-Nielsen T, Dow JM. 2008. Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. Mol Microbiol 68:75–86. https://doi.org/10.1111/j.1365-2958.2008.06132.x.
31. Harriott MM, Noverr MC. 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. Antimicrob Agents Chemother 53:3914–3922. https://doi.org/10.1128/AAC.00657-09.
32. Armbruster CE, Hong W, Pang B, Weimer KED, Juneau RA, Turner J, Swords WE. 2010. Indirect pathogenicity of *Haemophilus influenzae* and *Moraxella catarrhalis* in polymicrobial otitis media occurs via interspecies quorum signaling. mBio 1:e00102-10. https://doi.org/10.1128/mBio.00102-10.
33. Bernier SP, Létoffé S, Delepierre M, Ghigo J-M. 2011. Biogenic ammonia modifies antibiotic resistance at a distance in physically separated bacteria. Mol Microbiol 81:705–716. https://doi.org/10.1111/j.1365-2958.2011.07724.x.
34. Vega NM, Allison KR, Samuels AN, Klempner MS, Collins JJ. 2013. *Salmonella typhimurium* intercepts *Escherichia coli* signaling to enhance antibiotic tolerance. Proc Natl Acad Sci U S A 110:14420–14425. https://doi.org/10.1073/pnas.1308085110.
35. DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP. 2014. Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *in vitro* wound model. Infect Immun 82:4718–4728. https://doi.org/10.1128/IAI.02198-14.
36. Beaudoin T, Yau YCW, Stapleton PJ, Gong Y, Wang PW, Guttman DS, Waters V. 2017. *Staphylococcus aureus* interaction with *Pseudomonas aeruginosa* biofilm enhances tobramycin resistance. NPJ Biofilms Microbiomes 3:25. https://doi.org/10.1038/s41522-017-0035-0.
37. Orazi G, O'Toole GA. 2017. *Pseudomonas aeruginosa* alters *Staphylococcus aureus* sensitivity to vancomycin in a biofilm model of cystic fibrosis infection. mBio 8:e00873-17. https://doi.org/10.1128/mBio.00873-17.
38. Kean R, Rajendran R, Haggarty J, Townsend EM, Short B, Burgess KE, Lang S, Millington O, Mackay WG, Williams C, Ramage G. 2017. *Candida albicans* mycofilms support *Staphylococcus aureus* colonization and enhances miconazole resistance in dual-species interactions. Front Microbiol 8:258. https://doi.org/10.3389/fmicb.2017.00258.
39. Radlinski L, Rowe SE, Kartchner LB, Maile R, Cairns BA, Vitko NP, Gode CJ, Lachiewicz AM, Wolfgang MC, Conlon BP. 2017. *Pseudomonas aeruginosa* exoproducts determine antibiotic efficacy against *Staphylococcus aureus*. PLoS Biol 15:e2003981. https://doi.org/10.1371/journal.pbio.2003981.
40. Adamowicz EM, Flynn J, Hunter RC, Harcombe WR. 2018. Cross-feeding modulates antibiotic tolerance in bacterial communities. ISME J 15:555. https://doi.org/10.1038/s41396-018-0212-z.
41. Cystic Fibrosis Foundation. 2015. Cystic Fibrosis Foundation patient registry 2015 annual data report. Cystic Fibrosis Foundation, Bethesda, MD.
42. Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, Karaoz U, Andersen GL, Brown R, Fujimura KE, Wu B, Tran D, Koff J, Kleinhenz ME, Nielson D, Brodie EL, Lynch SV. 2010. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. PLoS One 5:e11044. https://doi.org/10.1371/journal.pone.0011044.
43. Wolter DJ, Emerson JC, McNamara S, Buccat AM, Qin X, Cochrane E, Houston LS, Rogers GB, Marsh P, Prehar K, Pope CE, Blackledge M, Deziel E, Bruce KD, Ramsey BW, Gibson RL, Burns JL, Hoffman LR. 2013. *Staphylococcus aureus* small-colony variants are independently associated with worse lung disease in children with cystic fibrosis. Clin Infect Dis 57:384–391. https://doi.org/10.1093/cid/cit270.
44. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. 2002. *Pseudomonas aeruginosa* and other predictors of mortality and mor-

bidity in young children with cystic fibrosis. Pediatr Pulmonol 34:91–100. https://doi.org/10.1002/ppul.10127.
45. Com G, Carroll JL, Castro MM, Tang X, Jambhekar S, Berlinski A. 2014. Predictors and outcome of low initial forced expiratory volume in 1 second measurement in children with cystic fibrosis. J Pediatr 164:832–838. https://doi.org/10.1016/j.jpeds.2013.11.064.
46. Hudson VL, Wielinski CL, Regelmann WE. 1993. Prognostic implications of initial oropharyngeal bacterial flora in patients with cystic fibrosis diagnosed before the age of two years. J Pediatr 122:854–860. https://doi.org/10.1016/s0022-3476(09)90007-5.
47. Rosenbluth DB, Wilson K, Ferkol T, Schuster DP. 2004. Lung function decline in cystic fibrosis patients and timing for lung transplantation referral. Chest 126:412–419. https://doi.org/10.1378/chest.126.2.412.
48. Limoli DH, Yang J, Khansaheb MK, Helfman B, Peng L, Stecenko AA, Goldberg JB. 2016. *Staphylococcus aureus* and *Pseudomonas aeruginosa* co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. Eur J Clin Microbiol Infect Dis 35:947–953. https://doi.org/10.1007/s10096-016-2621-0.
49. Maliniak ML, Stecenko AA, McCarty NA. 2016. A longitudinal analysis of chronic MRSA and *Pseudomonas aeruginosa* co-infection in cystic fibrosis: a single-center study. J Cyst Fibros 15:350–356. https://doi.org/10.1016/j.jcf.2015.10.014.
50. Limoli DH, Hoffman LR. 2019. Help, hinder, hide and harm: what can we learn from the interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* during respiratory infections? Thorax 74:684–692. https://doi.org/10.1136/thoraxjnl-2018-212616.
51. Gjødsbøl K, Christensen JJ, Karlsmark T, Jørgensen B, Klein BM, Krogfelt KA. 2006. Multiple bacterial species reside in chronic wounds: a longitudinal study. Int Wound J 3:225–231. https://doi.org/10.1111/j.1742-481X.2006.00159.x.
52. Körber A, Schmid EN, Buer J, Klode J, Schadendorf D, Dissemond J. 2010. Bacterial colonization of chronic leg ulcers: current results compared with data 5 years ago in a specialized dermatology department. J Eur Acad Dermatol Venereol 24:1017–1025. https://doi.org/10.1111/j.1468-3083.2010.03570.x.
53. Pastar I, Nusbaum AG, Gil J, Patel SB, Chen J, Valdes J, Stojadinovic O, Plano LR, Tomic-Canic M, Davis SC. 2013. Interactions of methicillin resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in polymicrobial wound infection. PLoS One 8:e56846. https://doi.org/10.1371/journal.pone.0056846.
54. Silva MT, Sousa JC, Macedo MA, Polónia J, Parente AM. 1976. Effects of phenethyl alcohol on *Bacillus* and *Streptococcus*. J Bacteriol 127:1359–1369.
55. Heipieper HJ, Keweloh H, Rehm HJ. 1991. Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. Appl Environ Microbiol 57:1213–1217.
56. McDonnell G, Russell AD. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 12:147–179. https://doi.org/10.1128/CMR.12.1.147.
57. Miller MH, Edberg SC, Mandel LJ, Behar CF, Steigbigel NH. 1980. Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of *Staphylococcus aureus*. Antimicrob Agents Chemother 18:722–729. https://doi.org/10.1128/aac.18.5.722.
58. Tsuji BT, Eiff von C, Kelchlin PA, Forrest A, Smith PF. 2008. Attenuated vancomycin bactericidal activity against *Staphylococcus aureus hemB* mutants expressing the small-colony-variant phenotype. Antimicrob Agents Chemother 52:1533–1537. https://doi.org/10.1128/AAC.01254-07.
59. Hess DJ, Henry-Stanley MJ, Lusczek ER, Beilman GJ, Wells CL. 2013. Anoxia inhibits biofilm development and modulates antibiotic activity. J Surg Res 184:488–494. https://doi.org/10.1016/j.jss.2013.04.049.
60. Proctor RA, von Humboldt A. 1998. Bacterial energetics and antimicrobial resistance. Drug Resist Updat 1:227–235. https://doi.org/10.1016/S1368-7646(98)80003-4.
61. Hurdle JG, O'Neill AJ, Chopra I, Lee RE. 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9:62–75. https://doi.org/10.1038/nrmicro2474.
62. von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, Götz F. 1997. A site-directed *Staphylococcus aureus hemB* mutant is a small-colony variant which persists intracellularly. J Bacteriol 179:4706–4712. https://doi.org/10.1128/jb.179.15.4706-4712.1997.
63. Lepine F, Deziel E, Milot S, Rahme LG. 2003. A stable isotope dilution assay for the quantification of the *Pseudomonas* quinolone signal in *Pseudomonas aeruginosa* cultures. Biochim Biophys Acta 1622:36–41. https://doi.org/10.1016/S0304-4165(03)00103-X.
64. Deziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG. 2004. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc Natl Acad Sci U S A 101:1339–1344. https://doi.org/10.1073/pnas.0307694100.
65. Lightbown JW, Jackson FL. 1956. Inhibition of cytochrome systems of heart muscle and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-hydroxyquinoline N-oxides. Biochem J 63:130–137. https://doi.org/10.1042/bj0630130.
66. Van Ark G, Berden JA. 1977. Binding of HQNO to beef-heart submitochondrial particles. Biochim Biophys Acta 459:119–137. https://doi.org/10.1016/0005-2728(77)90014-7.
67. Esposti MD. 1989. Prediction and comparison of the haem-binding sites in membrane haemoproteins. Biochim Biophys Acta 977:249–265. https://doi.org/10.1016/S0005-2728(89)80079-9.
68. Miyadera H, Shiomi K, Ui H, Yamaguchi Y, Masuma R, Tomoda H, Miyoshi H, Osanai A, Kita K, Omura S. 2003. Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase). Proc Natl Acad Sci U S A 100:473–477. https://doi.org/10.1073/pnas.0237315100.
69. Keweloh H, Diefenbach R, Rehm H-J. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Arch Microbiol 157:49–53.
70. Domenech O, Dufrêne YF, Van Bambeke F, Tukens PM, Mingeot-Leclercq M-P. 2010. Interactions of oritavancin, a new semi-synthetic lipoglycopeptide, with lipids extracted from *Staphylococcus aureus*. Biochim Biophys Acta 1798:1876–1885. https://doi.org/10.1016/j.bbamem.2010.06.011.
71. Bessa LJ, Ferreira M, Gameiro P. 2018. Evaluation of membrane fluidity of multidrug-resistant isolates of *Escherichia coli* and *Staphylococcus aureus* in presence and absence of antibiotics. J Photochem Photobiol B 181:150–156. https://doi.org/10.1016/j.jphotobiol.2018.03.002.
72. Perez-Lopez MI, Mendez-Reina R, Trier S, Herrfurth C, Feussner I, Bernal A, Forero-Shelton M, Leidy C. 2019. Variations in carotenoid content and acyl chain composition in exponential, stationary and biofilm states of Staphylococcus *aureus*, and their influence on membrane biophysical properties. Biochim Biophys Acta Biomembr 1861:978–987. https://doi.org/10.1016/j.bbamem.2019.02.001.
73. Friedlander G, Le Grimellec C, Giocondi M-C, Amiel C. 1987. Benzyl alcohol increases membrane fluidity and modulates cyclic AMP synthesis in intact renal epithelial cells. Biochim Biophys Acta Biomembr 903:341–348. https://doi.org/10.1016/0005-2736(87)90224-0.
74. Shigapova N, Török Z, Balogh G, Goloubinoff P, Vígh L, Horváth I. 2005. Membrane fluidization triggers membrane remodeling which affects the thermotolerance in *Escherichia coli*. Biochem Biophys Res Commun 328:1216–1223. https://doi.org/10.1016/j.bbrc.2005.01.081.
75. Cebrián G, Condón S, Mañas P. 2016. Influence of growth and treatment temperature on *Staphylococcus aureus* resistance to pulsed electric fields: relationship with membrane fluidity. Innov Food Sci Emerg Technol 37:161–169. https://doi.org/10.1016/j.ifset.2016.08.011.
76. Balogh G, Horváth I, Nagy E, Hoyk Z, Benkõ S, Bensaude O, Vígh L. 2005. The hyperfluidization of mammalian cell membranes acts as a signal to initiate the heat shock protein response. FEBS J 272:6077–6086. https://doi.org/10.1111/j.1742-4658.2005.04999.x.
77. Veerman ECI, Valentijn-Benz M, Nazmi K, Ruissen ALA, Walgreen-Weterings E, van Marle J, Doust AB, van't Hof W, Bolscher JGM, Amerongen A. 2007. Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane. J Biol Chem 282:18831–18841. https://doi.org/10.1074/jbc.M610555200.
78. Daeschlein G. 2013. Antimicrobial and antiseptic strategies in wound management. Int Wound J 10(Suppl 1):9–14. https://doi.org/10.1111/iwj.12175.
79. Assadian O. 2016. Octenidine dihydrochloride: chemical characteristics and antimicrobial properties. J Wound Care 25:S3–S6. https://doi.org/10.12968/jowc.2016.25.Sup3.S3.
80. Barr HL, Halliday N, Cámara M, Barrett DA, Williams P, Forrester DL, Simms R, Smyth AR, Honeybourne D, Whitehouse JL, Nash EF, Dewar J, Clayton A, Knox AJ, Fogarty AW. 2015. *Pseudomonas aeruginosa* quorum sensing molecules correlate with clinical status in cystic fibrosis. Eur Respir J 46:1046–1054. https://doi.org/10.1183/09031936.00225214.
81. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR,

D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103:8487–8492. https://doi.org/10.1073/pnas.0602138103.
82. Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI. 2009. *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease progression. J Cyst Fibros 8:66–70. https://doi.org/10.1016/j.jcf.2008.09.006.
83. Limoli DH, Whitfield GB, Kitao T, Ivey ML, Davis MR, Grahl N, Hogan DA, Rahme LG, Howell PL, O'Toole GA, Goldberg JB. 2017. *Pseudomonas aeruginosa* alginate overproduction promotes coexistence with *Staphylococcus aureus* in a model of cystic fibrosis respiratory infection. mBio 8:e00186-17. https://doi.org/10.1128/mBio.00186-17.
84. Filkins LM, Graber JA, Olson DG, Dolben EL, Lynd LR, Bhuju S, O'Toole GA. 2015. Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* drives *S. aureus* towards fermentative metabolism and reduced viability in a cystic fibrosis model. J Bacteriol 197:2252–2264. https://doi.org/10.1128/JB.00059-15.
85. Fuchs S, Pané-Farré J, Kohler C, Hecker M, Engelmann S. 2007. Anaerobic gene expression in *Staphylococcus aureus*. J Bacteriol 189:4275–4289. https://doi.org/10.1128/JB.00081-07.
86. Burkhart BM, Li N, Langs DA, Pangborn WA, Duax WL. 1998. The conducting form of gramicidin A is a right-handed double-stranded double helix. Proc Natl Acad Sci U S A 95:12950–12955. https://doi.org/10.1073/pnas.95.22.12950.
87. Koo SP, Bayer AS, Yeaman MR. 2001. Diversity in antistaphylococcal mechanisms among membrane-targeting antimicrobial peptides. Infect Immun 69:4916–4922. https://doi.org/10.1128/IAI.69.8.4916-4922.2001.
88. Xiong YQ, Mukhopadhyay K, Yeaman MR, Adler-Moore J, Bayer AS. 2005. Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of *Staphylococcus aureus*. Antimicrob Agents Chemother 49:3114–3121. https://doi.org/10.1128/AAC.49.8.3114-3121.2005.
89. Kristiansen JE, Amaral L. 1997. The potential management of resistant infections with non-antibiotics. J Antimicrob Chemother 40:319–327. https://doi.org/10.1093/jac/40.3.319.
90. Mazumder R, Ganguly K, Dastidar SG, Chakrabarty AN. 2001. Trifluoperazine: a broad spectrum bactericide especially active on staphylococci and vibrios. Int J Antimicrob Agents 18:403–406. https://doi.org/10.1016/S0924-8579(01)00324-7.
91. Kristiansen JE, Hendricks O, Delvin T, Butterworth TS, Aagaard L, Christensen JB, Flores VC, Keyzer H. 2007. Reversal of resistance in microorganisms by help of non-antibiotics. J Antimicrob Chemother 59:1271–1279. https://doi.org/10.1093/jac/dkm071.
92. Kristiansen JE, Thomsen VF, Martins A, Viveiros M, Amaral L. 2010. Non-antibiotics reverse resistance of bacteria to antibiotics. In Vivo 24:751–754.
93. Mandal A, Sinha C, Kumar Jena A, Ghosh S, Samanta A. 2010. An investigation on *in vitro* and *in vivo* antimicrobial properties of the antidepressant: amitriptyline hydrochloride. Braz J Microbiol 41:635–645. https://doi.org/10.1590/S1517-83822010000300014.
94. Spitzer M, Griffiths E, Blakely KM, Wildenhain J, Ejim L, Rossi L, De Pascale G, Curak J, Brown E, Tyers M, Wright GD. 2011. Cross-species discovery of syncretic drug combinations that potentiate the antifungal fluconazole. Mol Syst Biol 7:499–499. https://doi.org/10.1038/msb.2011.31.
95. Caetano W, Tabak M. 1999. Interaction of chlorpromazine and trifluoperazine with ionic micelles: electronic absorption spectroscopy studies. Spectrochim Acta A Mol Biomol Spectrosc 55:2513–2528. https://doi.org/10.1016/S1386-1425(99)00043-8.
96. Hendrich AB, Wesołowska O, Michalak K. 2001. Trifluoperazine induces domain formation in zwitterionic phosphatidylcholine but not in charged phosphatidylglycerol bilayers. Biochim Biophys Acta 1510:414–425. https://doi.org/10.1016/S0005-2736(00)00373-4.
97. Kalani M, Brismar K, Fagrell B, Ostergren J, Jorneskog G. 1999. Transcutaneous oxygen tension and toe blood pressure as predictors for outcome of diabetic foot ulcers. Diab Care 22:147–151. https://doi.org/10.2337/diacare.22.1.147.
98. Wattel F, Mathieu D, Coget JM, Billard V. 1990. Hyperbaric oxygen therapy in chronic vascular wound management. Angiology 41:59–65. https://doi.org/10.1177/000331979004100109.
99. Kopf SH, Sessions AL, Cowley ES, Reyes C, Van Sambeek L, Hu Y, Orphan VJ, Kato R, Newman DK. 2016. Trace incorporation of heavy water reveals slow and heterogeneous pathogen growth rates in cystic fibrosis sputum. Proc Natl Acad Sci U S A 113:E110–E116. https://doi.org/10.1073/pnas.1512057112.
100. Nair DR, Monteiro JM, Memmi G, Thanassi J, Pucci M, Schwartzman J, Pinho MG, Cheung AL. 2015. Characterization of a novel small molecule that potentiates β-lactam activity against gram-positive and gram-negative pathogens. Antimicrob Agents Chemother 59:1876–1885. https://doi.org/10.1128/AAC.04164-14.
101. Müller A, Wenzel M, Strahl H, Grein F, Saaki TNV, Kohl B, Siersma T, Bandow JE, Sahl H-G, Schneider T, Hamoen LW. 2016. Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. Proc Natl Acad Sci U S A 113:E7077–E7086. https://doi.org/10.1073/pnas.1611173113.
102. Strahl H, Bürmann F, Hamoen LW. 2014. The actin homologue MreB organizes the bacterial cell membrane. Nat Commun 5:3442. https://doi.org/10.1038/ncomms4442.
103. Zhu K, Bayles DO, Xiong A, Jayaswal RK, Wilkinson BJ. 2005. Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain-keto acid dehydrogenase. Microbiology 151:615–623. https://doi.org/10.1099/mic.0.27634-0.