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MINIREVIEW

"It Takes a Village": Mechanisms Underlying Antimicrobial Recalcitrance of Polymicrobial Biofilms

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ABSTRACT Chronic infections are frequently caused by polymicrobial biofilms. Importantly, these infections are often difficult to treat effectively in part due to the recalcitrance of biofilms to antimicrobial therapy. Emerging evidence suggests that polymicrobial interactions can lead to dramatic and unexpected changes in the ability of antibiotics to eradicate biofilms and often result in decreased antimicrobial efficacy *in vitro*. In this review, we discuss the influence of polymicrobial interactions on the antibiotic susceptibility of biofilms, and we highlight the studies that first documented the shifted antimicrobial susceptibilities of mixed-species cultures. Recent studies have identified several mechanisms underlying the recalcitrance of polymicrobial biofilm communities, including interspecies exchange of antibiotic resistance genes, β-lactamase-mediated inactivation of antibiotics, changes in gene expression induced by metabolites and quorum sensing signals, inhibition of the electron transport chain, and changes in properties of the cell membrane. In addition to elucidating multiple mechanisms that contribute to the altered drug susceptibility of polymicrobial biofilms, these studies have uncovered novel ways in which polymicrobial interactions can impact microbial physiology. The diversity of findings discussed highlights the importance of continuing to investigate the efficacy of antibiotics against biofilm communities composed of different combinations of microbial species. Together, the data presented here illustrate the importance of studying microbes as part of mixed-species communities rather than in isolation. In light of our greater understanding of how interspecies interactions alter the efficacy of antimicrobial agents, we propose that the methods for measuring the drug susceptibility of polymicrobial infections should be revisited.

KEYWORDS biofilm, polymicrobial, antibiotics, resistance, tolerance, recalcitrance

BIOFILMS ARE RECALCITRANT TO ANTIMICROBIAL TREATMENT

Biofilms are communities of microbial cells that are attached to a surface and to one another and are surrounded with a self-produced matrix. The biofilm lifestyle is incredibly common in all environments, from natural settings to the human body, both in health and in disease (1, 2). Furthermore, it is estimated that the majority of chronic infections are caused by biofilms (3). Biofilms are 100- to 1,000-fold more tolerant to antibiotics than their planktonic counterparts (2, 4) and promote persistence in the host, which makes successful treatment challenging.

The reason that biofilms are able to withstand such high concentrations of antimicrobial agents is multifactorial and incompletely understood. Antibiotic resistance refers to genetically encoded mechanisms that allow microbes to grow in the presence of a drug, whereas tolerance is the ability to survive transient exposure to an otherwise lethal dose of an antibiotic, by either genetically encoded or phenotypic, nonheritable mechanisms (5–8). Biofilms exhibit both resistance and tolerance to antibiotics, a combination referred to as "recalcitrance" (5–7).

The many genetically encoded resistance mechanisms used by planktonic cells to withstand antimicrobial treatment contribute to the recalcitrance of biofilms. Resis-

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tance can arise in various ways, such as by modifying an antibiotic target, enzymatic deactivation, or active efflux of a drug once it has entered the cell. Furthermore, resistance can be acquired by spontaneous mutations or by exchanging antibiotic resistance genes between other cells. These mechanisms are at play within biofilms and are often compounded by other aspects of biofilm physiology to confer even greater levels of recalcitrance. For example, β-lactamases concentrated within the extracellular matrix can degrade the antibiotic before it can reach the cells within the biofilm (4). Additionally, the biofilm lifestyle has been found to facilitate rates of horizontal gene transfer (HGT); bacteria within biofilms exchanged plasmids containing antibiotic resistance genes at a higher rate than their planktonic counterparts (8–10).

In addition to the resistance mechanisms outlined above, multiple aspects of a microbe's physiology contribute to the elevated antibiotic tolerance of cells within a biofilm relative to planktonic cells. These features include reduced growth rate (11–13) due to oxygen-depleted microenvironments (14), nutrient limitation and the subsequent activation of stress responses (15–17), limited diffusion of certain antibiotics, such as via chelation of cationic compounds by extracellular matrix components (18–20), and the frequent emergence of so-called "persister" cells—dormant or slow-growing cells that are genetically identical to the rest of the population but display several phenotypic differences, such as elevated multidrug tolerance and high induction of stress responses (6). In addition, bacterial biofilms upregulate efflux pumps even in the absence of antibiotic exposure, conferring high levels of drug tolerance (21, 22). As another example, the production of periplasmic cyclic glucans in biofilm-grown bacteria can enhance biofilm tolerance to aminoglycoside antibiotics (23). Thus, multifaceted resistance and tolerance mechanisms contribute to the overall recalcitrance of biofilms to antimicrobial therapy.

THE ETIOLOGY OF MOST CHRONIC INFECTIONS IS POLYMICROBIAL

Multispecies biofilm-like communities are responsible for causing persistent infections in a wide range of body sites, including the lung (24–31), oral cavity (32, 33), middle ear (34–36), urinary tract (37–39), and both surgical and chronic wounds (40–45). In several disease settings, polymicrobial infections have been reported to cause worse outcomes than single-species infections (46–54). In particular, polymicrobial bloodstream infections are associated with higher rates of mortality than monospecies infections (46–50). Similarly, polymicrobial lung infections have been shown to lead to worse prognoses. Cystic fibrosis (CF) patients who are coinfected with *Pseudomonas aeruginosa* and *Staphylococcus aureus* demonstrate more rapid lung function decline than patients with monospecies infections (55–59). Furthermore, *P. aeruginosa* and *S. aureus* have been detected in the same lobe of the lung (60, 61), and they engage in a plethora of interactions *in vitro* (62–68). Thus, it is possible that these microbes interact during infection.

Interspecies interactions profoundly influence microbial physiology in ways that may alter disease progression and outcome, including the upregulation of virulence factors (69–73), altered biofilm formation (67, 74–76), impaired wound healing (73, 77), and shifted antibiotic susceptibility profiles (63, 67, 74, 75, 77–88). Additionally, interactions between a host and one microbial species may modify immune responses to a coinfecting species (76, 89, 90). Therefore, as others have postulated (91, 92), perhaps the consequences of these interactions on microbial and host physiology contribute to the worse patient outcomes that are frequently observed for coinfections than for monoinfections.

THE DISCONNECT BETWEEN *IN VITRO* ANTIMICROBIAL SUSCEPTIBILITY AND TREATMENT SUCCESS

An important clinical outcome to consider is whether a given antimicrobial therapy can successfully treat an infection. Various studies have evaluated whether *in vitro* MIC—still considered the gold standard for drug susceptibility testing—correlated with the success or failure of antimicrobial treatment. Surprisingly, these studies found little

or no association between clinical antimicrobial susceptibility testing results (specifically, a pathogen's *in vitro* MIC value for a specific drug) and clinical outcomes measured following antibiotic treatment, even in the context of single-species infections (93–98). In other words, patients did no better when they were infected by susceptible organisms (low MIC) than by resistant organisms (high MIC). An even more worrisome observation was made by authors studying the association between *Candida* fluconazole MICs and treatment outcomes of bloodstream infections. Contrary to expectation, low *in vitro* MICs actually correlated with treatment failure (99). Approximately one-third of isolates with low MICs ($<$16 μg/ml) failed to respond to fluconazole therapy, indicating that a drug judged to be effective *in vitro* was unable to eradicate infections in multiple patients. The reverse was also true, whereby the treatment of four *Candida* isolates was successful despite having MICs of $\geq$32 μg/ml (99). A similar correlation was observed for *Candida* caspofungin MICs and candidiasis outcomes (100). Therefore, these studies illustrate that while the susceptibility methods currently used in the clinical microbiology laboratory are often quite useful, such *in vitro* tests are not always able to predict a patient's response to antimicrobial treatment.

While alarming, these findings are not entirely surprising, given the enormous difference between controlled laboratory conditions and an infection site within a patient, and others have questioned the clinical predictive value of MIC tests (2, 101). Standard antibiotic susceptibility testing guidelines recommend that sensitivity should be measured when a microbe is grown planktonically, in rich medium, in monoculture. Therefore, test results only actually indicate whether an organism is sensitive to an antimicrobial compound under those precise conditions. These laboratory tests do not consider the conditions that microbes experience within an infection site, including constant assault by the host immune system. In addition, antimicrobial efficacy is also influenced by immunosuppression (102) and drug-drug interactions (103), which may contribute to differences between laboratory results and clinical outcomes. Furthermore, in most chronic infections, microorganisms likely form biofilms and probably interact with a multitude of neighbors (including other microbes and the host) within that infection niche.

Importantly, as we discuss below, in addition to adopting a biofilm lifestyle, interacting with other microbes in these sessile communities can contribute to drug sensitivity profiles that are vastly different from when an organism is grown planktonically in pure culture. Therefore, it is possible that interactions between microbes could influence the success of antimicrobial treatment. Here, we discuss various mechanisms underlying how interspecies interactions alter antimicrobial sensitivity profiles within polymicrobial biofilm communities, which may in part explain why antimicrobial therapies often fail to eradicate chronic infections.

MECHANISMS OF ANTIMICROBIAL RESISTANCE IN POLYMICROBIAL BIOFILMS

Some of the same genetic mechanisms that can cause planktonic cells to become antibiotic resistant also contribute to the ability of biofilm-forming microbes to withstand antibiotic treatment. Here, we review the contributions of HGT and antibiotic-inactivating enzymes to drug resistance within multispecies biofilms.

INTERSPECIES GENETIC EXCHANGE CONFERS ANTIMICROBIAL RESISTANCE

In addition to the occurrence of spontaneous mutations that are genetically inherited by daughter cells, horizontal gene transfer (HGT) is another means whereby microbes can acquire new sources of antibiotic resistance genes. The biofilm lifestyle has been found to promote HGT by increasing the rates of conjugation (8–10, 104, 105) and transformation (106), and to increase the stability of plasmids (107) relative to the planktonic setting. It has been proposed that the ordered structure and high density of cells within a biofilm promote efficient conjugation, although the underlying mechanisms remain unclear (107). However, genetic exchange may not occur uniformly within biofilms. For example, high conjugation frequencies may be confined to subpopulations of cells during initial stages of biofilm development (108, 109). Additionally,

conjugative pili were found to promote attachment and biofilm formation by *Escherichia coli* (110, 111), raising the possibility that maintenance and transfer of conjugative plasmids within biofilms benefit cells growing within these communities.

In addition to conjugation, rates of transformation are also elevated within biofilms. In one study, the transformation frequencies of biofilm-grown *Streptococcus mutans* were found to be 10- to 600-fold higher than for planktonic populations (112). The induction of competence in many species is influenced by a variety of environmental cues, including cell density, environmental pH, antibiotic exposure, starvation, and the presence or absence of particular carbon sources (113–116). In addition to its role in the induction of competence, quorum sensing plays a role in regulating biofilm formation in many bacterial species (117); therefore, competence and biofilm formation are linked by virtue of being regulated (at least in part) by a common pathway. Furthermore, the presence of extracellular DNA within the biofilm matrix has been proposed to induce competence (118) and may in part contribute to the high rates of transformation observed within biofilms.

There have been multiple reports of HGT of antibiotic resistance genes between species within polymicrobial biofilms. One study found that a plasmid harboring a carbapenemase resistance gene (bla_{NDM-1}) can be transferred from *E. coli* to either *P. aeruginosa* or *Acinetobacter baumannii* via conjugation within dual-species biofilms (119), which was not observed between these organisms in planktonic settings (120). These results highlight the enhanced propensity of genetic exchange within biofilms. Additionally, conjugative transposons have been implicated in transferring antimicrobial resistance genes within a multispecies oral biofilm *in vitro* (121, 122). For example, *Streptococcus* spp. acquired a transposon from *Veillonella dispar* by two distinct mechanisms: either conjugation with *V. dispar* (121) or transformation of purified *V. dispar* DNA (122). These data indicate that in addition to conjugation, which requires intimate cell-to-cell contact, uptake of extracellular DNA is another way in which members of a mixed-species community can exchange genetic information while in close proximity.

Moreover, there is evidence for HGT occurring between pathogens *in vivo* in the context of a mixed microbial community. It was discovered that the multidrug resistance of an *S. aureus* isolate within a polymicrobial infection of an indwelling tube was mediated by interspecies interactions (123). The already methicillin-resistant *S. aureus* isolate likely became resistant to vancomycin *in vivo* via the acquisition of a *vanA*-containing plasmid from *Enterococcus faecium*, one of the constituents of the polymicrobial biofilm. Additionally, the *S. aureus* isolate harbored a tetracycline resistance gene (*tetU*) that was not previously detected in *S. aureus* and was also likely transferred by *E. faecium* within this mixed-species biofilm during infection.

The studies described above determined that conjugation and transformation can mediate the spread of antimicrobial resistance between species. These findings underscore the potential for interspecies transfer of antibiotic resistance genes within an infection site, which can alter the antimicrobial susceptibility of an entire polymicrobial community. Importantly, a single conjugative event can have wide-ranging consequences for the spread of antimicrobial resistance, both within a single infection and between patients.

β-LACTAMASE-PRODUCING STRAINS PROTECT THEIR NEIGHBORS

Bacteria produce multiple genetically encoded factors that inactivate antibiotics. An important example is the group of proteins called β-lactamases, which are microbially produced enzymes that hydrolyze β-lactam antibiotics, a clinically important class of cell-wall-targeting drugs. These enzymes can be either chromosomally or plasmid encoded and have played a major role in the emergence of multidrug resistance among Gram-negative bacteria (124). β-Lactamases can be either localized to the periplasmic space of the producing cell or released into the extracellular space. Additionally, β-lactamases have been found within the outer membrane vesicles (OMVs) of multiple Gram-negative bacteria (125, 126) as well as the extracellular vesicles of *S. aureus* (127).

Importantly, inactivation of antibiotics by β-lactamases can protect not only the

producing cell but other cells in the vicinity. In addition to protecting sensitive cells of the same species from antibiotics (128, 129), β-lactamase-producing bacteria can alter the antimicrobial sensitivity profiles of entire polymicrobial communities, allowing coinfecting species to survive otherwise lethal doses of such antibiotics (75, 126, 127, 130–132). In particular, the production of β-lactamases was shown to influence the sensitivity of multispecies biofilms involving three predominant etiologic agents of otitis media: *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (34). β-Lactamases produced by *M. catarrhalis* conferred protection on both *H. influenzae* (75) and *S. pneumoniae* (130, 132) in dual-species biofilms. Furthermore, these interactions were shown to be mediated by *M. catarrhalis* OMVs (126). Additionally, *H. influenzae*-produced β-lactamases were shown to influence the sensitivity of *S. pneumoniae* biofilms to amoxicillin *in vitro* and in a chinchilla model of otitis media (131). Together, these studies illustrate that three pathogens that are commonly coisolated from inner ear infections and form mixed-species biofilms can greatly influence one another's susceptibility to β-lactams in a passive, contact-independent manner via antibiotic-degrading enzymes.

Importantly, β-lactamases have been detected in clinical samples from polymicrobial infections, including CF pulmonary infections (133) and otitis media (134, 135). Additionally, one study detected β-lactamase activity in 89% of ear aspirates from patients with infections that failed to respond to amoxicillin (134). These findings indicate the potential for this interspecies mechanism of antimicrobial resistance to occur *in vivo*, thus potentially contributing to treatment failure.

The above studies demonstrate that enzymatic inactivation of β-lactams can confer antibiotic resistance within mixed-species biofilms. This community-wide mechanism does not require direct interaction between two coinfecting species and has been shown to protect entire biofilms from antibiotic challenge (4). Thus, combined with the threat of plasmid-mediated exchange of resistance genes between species, this type of community-level resistance may have potentially far-reaching impacts on the outcomes of polymicrobial infections.

MECHANISMS UNDERLYING DRUG TOLERANCE OF POLYMICROBIAL BIOFILMS

In addition to the mechanisms leading to antimicrobial resistance typically associated with planktonic organisms (which are discussed above), recent studies have elucidated novel mechanisms explaining how interspecies interactions contribute to the antimicrobial tolerance of polymicrobial biofilm communities. We discuss these mechanisms below in the context of biofilms or biofilm-like infections.

MICROBIALLY SECRETED PRODUCTS ALTER ANTIBIOTIC TOLERANCE

Primary metabolites. Metabolic interactions between members of a polymicrobial community have been found to influence antibiotic sensitivity of the community's constituent microbes. For example, *P. aeruginosa* and anaerobic bacteria, organisms frequently coisolated from CF respiratory infections, were shown to engage in cross-feeding whereby *P. aeruginosa* consumes metabolites that are produced upon mucin fermentation by anaerobes (136). Interestingly, *P. aeruginosa* became more susceptible to ampicillin when in the presence of the drug-sensitive anaerobic community than when grown in monoculture (87). These data suggest that the drug sensitivity of an organism can increase when it is participating in particular metabolic interactions. In contrast, the interactions between members of a cross-feeding community composed of *E. coli*, *Salmonella enterica*, and *Methylobacterium extorquens* produced an opposite result, whereby *E. coli* sensitivity to tetracycline was lower in coculture than in monoculture (87). Together, these data illustrate that polymicrobial interactions can produce unexpected antibiotic sensitivity profiles. These findings also have potentially important implications for patient treatment—when a pathogen is part of a microbial community, the antibiotic dose needed for eradication may be different than what was predicted from monoculture experiments. Examples of such metabolite-driven changes in biofilm tolerance are outlined in the subsequent paragraphs.

Indole, an aromatic compound derived from tryptophan, has been described to shift the antibiotic sensitivity profiles of neighboring organisms both within and between species via transcriptional changes. Indole can impact the antibiotic sensitivity of single-species *E. coli* populations by inducing the expression of drug transporters (137). Additionally, indole was shown to mediate an interaction between the human commensal *E. coli* and *Salmonella enterica* serovar Typhimurium, a pathogen of the gastrointestinal tract. Indole produced by *E. coli* altered the antibiotic sensitivity of *S.* Typhimurium during *in vitro* coculture and in a coinfection model of intestinal infection in part via the induction of the *S.* Typhimurium OxyR oxidative stress pathway (83). Indole was hypothesized to protect *S.* Typhimurium from ciprofloxacin by increasing the expression of OxyR-regulated genes that confer protection from oxidative stress, including the catalase gene *katG*. This interaction illustrates how the production of a metabolite by a commensal can protect a pathogen from antimicrobial treatment by altering its physiology. Since indole is produced in high quantities by the mammalian intestine (138), the host may also influence bacterial drug susceptibility profiles in the same manner. Thus, indole can promote antibiotic tolerance in multiple ways.

The volatile fermentation product 2,3-butanedione has also been implicated in shifting antimicrobial sensitivity profiles by inducing changes in gene expression. 2,3-Butanedione is produced by streptococci as a consequence of acetoin metabolism, and it can be consumed by *P. aeruginosa* (139). Exposure to 2,3-butanedione was found to protect *E. coli* from various antibiotics by strongly inducing the expression of *hipA* (140), which is proposed to promote the generation of persister cells (141). Importantly, this volatile metabolite was detected in high levels in the airways of CF patients by breath gas analysis (139), indicating the possibility that microbially produced volatiles can exert long-range effects on other microbes within the lung.

Finally, the volatile metabolic by-product ammonia was also shown to modulate the antibiotic sensitivity of other microbes at a distance. Exposure to ammonia altered the susceptibility of *E. coli* to tetracycline (82). Bernier et al. propose that ammonia altered the antibiotic sensitivity of spatially distant bacteria by stimulating the production of polyamines (82)—compounds known to influence bacterial membrane permeability and drug susceptibility profiles (142–144). Additionally, ammonia has the ability to alkalize the surrounding growth medium, which was shown to inactivate ampicillin (145). Thus, it is possible that ammonia and other metabolites can interfere with the activity of antibiotics more generally by chemically modifying the environment. Moreover, the microbially produced gases hydrogen sulfide and nitric oxide were also observed to decrease the antibiotic sensitivity of multiple bacterial species (146, 147), suggesting the common ability of microbially produced volatiles to influence antimicrobial efficacy *in vitro* (148).

Together, these findings suggest that by-products of microbial metabolism can have diverse effects on the drug susceptibility of other organisms, both within and between species. However, it remains unclear in many instances how exposure to metabolites leads to altered antibiotic sensitivity profiles. Is it by directly influencing microbial physiology or the growth environment, or both? Further research is needed to elucidate the mechanisms underlying these observations. Additionally, multiple compounds, including ammonia, nitric oxide, and indole, were also reported to influence biofilm formation (149–152), which can further exacerbate the recalcitrance of microbes. Finally, the detection of volatile metabolites *in vivo* suggests that they have the potential to influence bacteria at a distance from the producing cell, which may lead to widespread changes in antibiotic sensitivity within an infection site.

Quorum sensing signals and other secondary metabolites. Quorum sensing molecules have been shown to mediate interactions between microbes residing within multispecies biofilms. It is thought that microbes can distinguish between signals from different species, since different concentrations and combinations of autoinducers lead to distinct behaviors in responding species (153–155). Interspecies communication using quorum sensing signals was shown to impact drug sensitivity within mixed-

species biofilms. Ryan et al. (74) discovered that *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, two organisms that coinfect CF patients, can communicate using quorum sensing molecules. The *S. maltophilia*-derived diffusible signal factor (DSF) was shown to be recognized by a *P. aeruginosa* sensor kinase and to cause the induction of *P. aeruginosa* PmrAB, a two-component system that regulates resistance to cationic peptides (74). As a result, *P. aeruginosa* biofilms became less sensitive to polymyxins. In addition, *P. aeruginosa* exposed to DSF adopts a striking filamentous phenotype (74).

A different interspecies interaction involving quorum sensing molecules was found to influence biofilm formation as well as to confer protection from antibiotic challenge (75). The authors observed that the thickness of *M. catarrhalis* biofilms, as well as the number of viable cells within the biofilm, increased in response to the quorum sensing signal AI-2 produced by *H. influenzae* (75). Whether and how *M. catarrhalis* senses and processes the AI-2 signal remain to be elucidated. Furthermore, the *H. influenzae*-mediated enhancement in *M. catarrhalis* biofilm biomass benefited both organisms, resulting in increased tolerance of both species to multiple antibiotics (75), perhaps by inhibiting drug diffusion through the biofilm. In addition, *H. influenzae* promoted enhanced persistence of *M. catarrhalis* in a chinchilla model of infection in an AI-2-dependent manner (75), suggesting that quorum sensing signals are mediating interactions between these bacterial pathogens *in vivo*. Together, the above studies suggest that interspecies communication via quorum sensing can impact antibiotic efficacy within multispecies biofilms.

Furthermore, quorum sensing signals and related small molecules can shift the antimicrobial susceptibility of neighboring microbes via mechanisms other than classical quorum sensing-mediated transcriptional changes. It has been recognized that quorum sensing signals can impact multiple aspects of microbial physiology (156), and emerging evidence suggests that these physiological changes can, in turn, lead to unexpected changes in drug tolerance. One study showed that farnesol, a quorum sensing molecule produced by *Candida albicans*, protected *S. aureus* from vancomycin within a polymicrobial biofilm (157). The authors propose a mechanism whereby exposure to farnesol induces the production of reactive oxygen species in *S. aureus*, which activates a general stress response and causes the upregulation of efflux pumps, thereby enhancing *S. aureus* antibiotic resistance (157). These data suggest that a quorum sensing molecule can shift drug sensitivity within polymicrobial biofilms by stimulating a cascade of physiological changes.

A different secondary metabolite has been described to mediate polymicrobial interactions and alter antibiotic susceptibility. The *P. aeruginosa*-secreted small molecule 2-heptyl-4-hydroxyquinolone *N*-oxide (HQNO) has been shown to strongly influence *S. aureus* physiology *in vitro*. HQNO is part of the *Pseudomonas* quinolone signal (PQS) quorum sensing system pathway and acts as a potent inhibitor of the *S. aureus* electron transport chain (ETC) (158). Inhibition of the *S. aureus* ETC causes a reduction in the electrochemical gradient, which is required for the cellular uptake of multiple classes of protein synthesis inhibitors (159). Consequently, exposure to HQNO decreases the susceptibility of *S. aureus* to the aminoglycosides dihydrostreptomycin and tobramycin (78, 80) and to multiple tetracycline and macrolide antibiotics (85).

Furthermore, HQNO has been shown to protect *S. aureus* biofilms from cell wall-targeting antibiotics, including the front-line drug vancomycin and multiple β-lactams (85). HQNO-mediated inhibition of the ETC forces *S. aureus* to grow by fermentation (64), leading to a decrease in ATP levels (86) and reduction in growth (85). Since many drug classes are effective against only actively growing cells, it is likely that slow growth renders *S. aureus* less susceptible to cell wall-targeting antibiotics. Additionally, prolonged exposure to HQNO has been shown to select for small-colony variants (SCVs) of *S. aureus* (80). SCVs have a nonfunctional ETC, which renders them highly tolerant to several classes of antibiotics and promote persistence within the host (160). Infection sites are often oxygen limited (161, 162), which can also induce a metabolic shift to fermentation and slow growth (163) and may further contribute to antibiotic tolerance

within chronic infections. Together, these data illustrate that inhibition of electron transport can have wide-ranging effects on drug efficacy and can explain how the same microbe grown under different conditions might display differential susceptibility to the same antimicrobial agent.

Moreover, quorum sensing signals and related small molecules can impact antimicrobial efficacy by altering properties of bacterial membranes. For example, HQNO was shown to increase *S. aureus* cell membrane fluidity, rendering *S. aureus* biofilms more susceptible to several membrane-targeting antibiotics and antiseptics, including chloroxylenol (88). Additionally, the *C. albicans* quorum sensing signal farnesol can disrupt the integrity of the *S. aureus* cell membrane (164, 165), which, in turn, was hypothesized to contribute to farnesol's ability to shift *S. aureus* drug sensitivity profiles (164). It has been proposed that the hydrophobic character of both of these molecules may allow for their accumulation within the *S. aureus* membrane (88, 164). These findings raise the possibility that other hydrophobic molecules with well-known roles in interbacterial signaling also have the ability to alter membrane properties and influence antibiotic sensitivity.

Two additional *P. aeruginosa* quorum sensing-regulated products, LasA and rhamnolipids, have been found to shift the susceptibility of *S. aureus* planktonic populations to various antibiotics. The endopeptidase LasA was observed to potentiate the efficacy of vancomycin, while rhamnolipids were proposed to increase the efficacy of tobramycin by facilitating uptake of the antibiotic (86, 166). In the future, it will be important to investigate whether LasA and rhamnolipids are able to modulate the drug susceptibility of biofilm cells in addition to that of planktonic cells.

Together, the above studies illustrate that quorum sensing signals and downstream products regulated by these pathways can elicit a suite of physiological changes that greatly alter the antimicrobial susceptibility profiles of polymicrobial biofilm communities. It is important to note that a single molecule can act via more than one mechanism to alter antibiotic sensitivity, as in the case of HQNO or farnesol. Finally, it is possible that other quorum sensing molecules modulate drug efficacy via mechanisms other than their canonical roles in signaling.

BIOFILM MATRIX COMPONENTS WITHIN POLYMICROBIAL BIOFILMS MODULATE DRUG SENSITIVITY

Multiple studies have described cross-domain interactions that modify the structure of microbial biofilms and lead to altered sensitivity to antimicrobial agents. *C. albicans* is well known to form robust, polymicrobial biofilms with several bacterial species, including staphylococci (81, 84, 167–169). In particular, *S. aureus* cells can use *C. albicans* hyphae as a scaffold for attachment and subsequent microcolony formation (81). As a consequence, residence within this mixed-species biofilm protected *S. aureus* from miconazole (84) and from elevated doses of vancomycin (up to 1,600 μg/ml) (81). In contrast, the drug susceptibility of *C. albicans* to multiple antifungal drugs was unaltered (81, 84). Extracellular DNA and β-1,3-glucan components of the fungal matrix were shown to contribute to the observed protection of *S. aureus* within the mixed-species biofilm (81, 84, 170), but the underlying mechanisms are still unclear. Similarly, protection of *E. coli* from ofloxacin within a dual-species biofilm with *C. albicans* is also dependent on the presence of the polysaccharide β-1,3-glucan (171).

As another example, formation of a mixed-species biofilm can also shield both microbes from antimicrobial treatment. A matrix-nonproducing strain of *Staphylococcus epidermidis* is normally highly sensitive to vancomycin; however, when grown in coculture with *C. albicans*, *S. epidermidis* was protected from the drug, while *C. albicans* was protected from fluconazole (172). Thus, while it is well appreciated that biofilm matrix components enable intimate associations between coinhabiting species and that the biofilm lifestyle confers protection from antimicrobial insults, the above studies suggest that structural components produced by one microbe can greatly influence the drug sensitivity of a coexisting microbe within a mixed-species biofilm.

Interactions between bacterial species have also been shown to influence biofilm

structure. Staphylococcal protein A (SpA), an adhesin known to mediate *S. aureus* biofilm formation (173), can also bind to the *P. aeruginosa* exopolysaccharide Psl and inhibit initial *P. aeruginosa* biofilm formation (174). In a different study, the interaction between these two adhesins led to striking changes in the architecture of mature *P. aeruginosa* biofilms (67). Specifically, exposure to SpA transformed *P. aeruginosa* biofilms into densely packed aggregates that occupied less surface area and were much less susceptible to tobramycin than untreated biofilms. While the observed shifts in biofilm structure and drug tolerance were shown to require the presence of both adhesins, it is currently unclear how the interaction between SpA and Psl causes the formation of aggregates. Together, these data indicate that interspecies interactions can modify biofilm architecture in diverse ways and, as a consequence, can greatly influence the drug susceptibility of a polymicrobial community.

INTERSPECIES INTERACTIONS WITHIN POLYMICROBIAL BIOFILMS INDUCE CELL WALL CHANGES

Several recent studies indicate that polymicrobial interactions can influence susceptibility to antibiotics by altering characteristics of their neighbor's cell wall. One study examined the drug susceptibility profiles within a three-species biofilm community composed of *Streptococcus anginosus*, *P. aeruginosa*, and *S. aureus*. When part of this polymicrobial biofilm, *S. anginosus* became more tolerant to vancomycin, while *S. aureus* became less tolerant (175). Additionally, the authors found that exposure to *S. aureus* culture supernatant protected *S. anginosus* biofilm cells, but not planktonic cells, from vancomycin (175). In a subsequent study, the same group found that residence within the multispecies biofilm led to the upregulation of *S. anginosus* cell wall biosynthesis genes and caused an increase in cell wall thickness, which was hypothesized to protect *S. anginosus* biofilms from the drug (176). These data are consistent with the well-documented association between increased cell wall thickness and decreased susceptibility to vancomycin in *S. aureus* (177–180). Together, these findings suggest that interspecies interactions can alter properties of bacterial cell walls, which may contribute to changes in antimicrobial susceptibility within biofilm communities.

CONCLUSIONS

As we have reviewed in this article, biofilms are recalcitrant to antimicrobial therapy due to a combination of genetic and phenotypic mechanisms. These same mechanisms operate within polymicrobial biofilms, but with added layers of complexity produced by interspecies interactions. There are many examples of polymicrobial interactions influencing the antibiofilm efficacy of antibiotics. Here, we highlight the few, better-understood mechanisms that have been uncovered (Fig. 1), including those involved in a well-studied interspecies interaction that shift the drug susceptibility of *S. aureus* biofilms (Fig. 2). Antibiotic recalcitrance mechanisms within polymicrobial biofilms include horizontal gene transfer of antibiotic resistance genes, enzymatic degradation of antibiotics (by β-lactamases), the induction of transcriptional changes by primary metabolites (e.g., indole and 2,3-butanedione) or quorum sensing signals (e.g., DSF and farnesol), inhibition of electron transport (by HQNO), and changes in membrane fluidity (by HQNO). Chronic polymicrobial infections, such as those associated with airway infections in patients with CF, are likely impacted by many of these mechanisms, as has been highlighted in a recent literature review (181).

Based on the studies reviewed above, it is clear that interspecies interactions can modulate the sensitivity of entire polymicrobial communities in dramatic and highly unpredictable ways, often enabling mixed-species biofilms to withstand even greater antibiotic challenge compared to single-species biofilms. Therefore, we believe that the results of monoculture experiments cannot always be extrapolated to predict the behavior of organisms when they are part of a community composed of other pathogens or members of the microbiota. Unfortunately, that is exactly how we currently measure the antimicrobial susceptibility of disease-causing organisms in the clinical setting. Indeed, a recent publication called into question the utility of using MIC testing

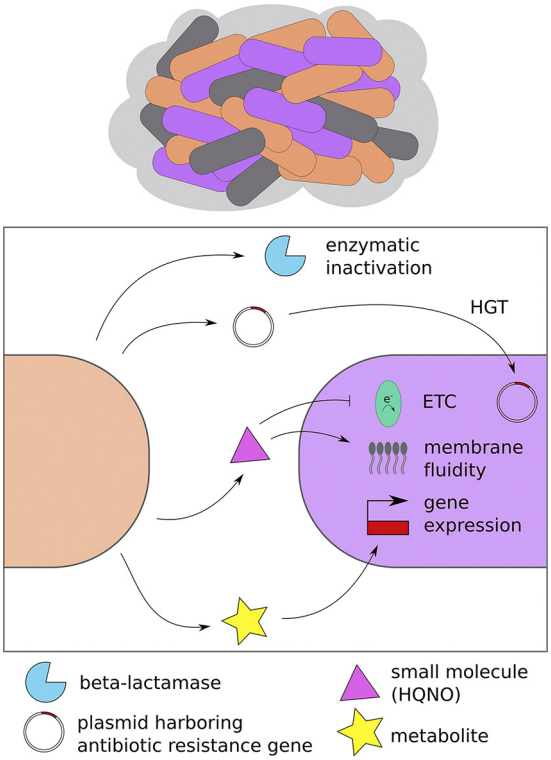

FIG 1 Multiple mechanisms contribute to the recalcitrance of polymicrobial biofilms to antimicrobial treatment. Interspecies interactions can shift the drug sensitivity profiles of microbes within multispecies biofilms, as represented by the differently colored microbes in a biofilm community at the top of the panel, and can do so via several mechanisms, including enzymatic inactivation of antibiotics by β-lactamases, interspecies exchange of antibiotic resistance genes, inhibition of electron transport, altered membrane fluidity, and metabolite-induced transcriptional changes. Abbreviations: HGT, horizontal gene transfer; ETC, electron transport chain; HQNO, 2-heptyl-4-hydroxyquinolone *N*-oxide.

to guide the treatment of the chronic, polymicrobial communities that are characteristic of CF airway infections (182).

In the past, multiple studies have made the striking observation that a microorganism's antimicrobial sensitivity profile can be dramatically different when it is grown in mixed culture compared to in pure culture (183–189). As highlighted previously by DeLeon et al., in 1969 Shahidi and Ellner compared the antibiotic sensitivity of an artificial mixed-species community to that of a pure culture (63, 183). Briefly, broth cultures were inoculated either with an individual organism or with different combinations of 10 bacterial species and then spread uniformly onto agar plates. Subsequently, different antibiotic disks were placed on the agar surface, and zones of inhibition were recorded to establish whether an organism was sensitive or resistant to a given drug. To the authors' surprise, they discovered that the ability of a drug to inhibit an organism was often entirely dependent on whether the organism was part of a pure or mixed culture. Specifically, they found multiple instances in which two organisms that were "sensitive" to an antibiotic in pure culture became "resistant" when mixed together. These results did not occur for all combinations tested, and it was not established in these studies what was driving these different outcomes. From these observations, it was concluded that "since reactions of bacterial mixtures are completely unpredictable, the authors emphasize that antibiotic susceptibility testing be limited to pure cultures" (183). This is not an unreasonable conclusion, but the studies cited above also highlight the limitations of such an approach.

Similar results were observed in later studies that assessed whether performing direct susceptibility testing using patient samples could be a way to expedite the communication of test results to clinicians (184–187, 189). Disk diffusion tests were

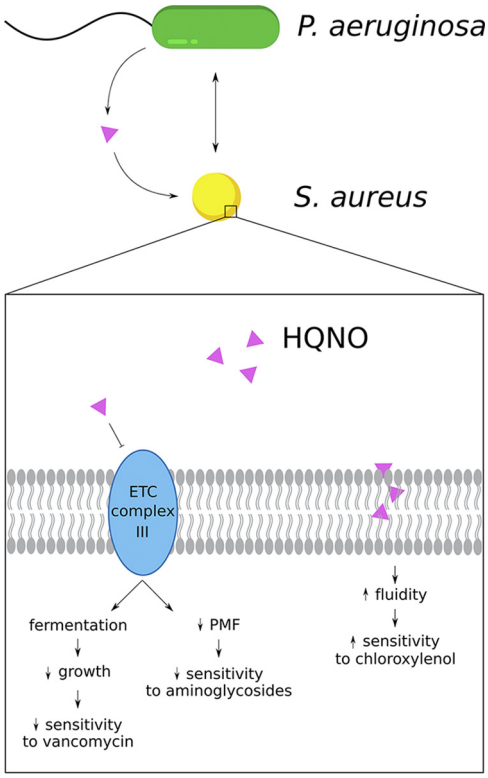

FIG 2 Interspecies interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* influence the efficacy of antibiotics against *S. aureus* biofilms. The *P. aeruginosa*-secreted molecule 2-heptyl-4-hydroxyquinolone *N*-oxide (HQNO) alters the sensitivity of biofilm-grown *S. aureus* to multiple antibacterial agents, including cell wall-targeting drugs (e.g., vancomycin), protein synthesis inhibitors (e.g., aminoglycosides), and membrane-active compounds (e.g., chloroxylenol). HQNO-mediated inhibition of the *S. aureus* electron transport chain (ETC) leads to slow growth and decreases the proton motive force (PMF), which promote tolerance to vancomycin and aminoglycosides, respectively. Additionally, exposure to HQNO increases the fluidity of the *S. aureus* cell membrane, enhancing the ability of the membrane-targeting antiseptic chloroxylenol (and other hydrophobic compounds) to eradicate *S. aureus* biofilms.

performed either by directly swabbing clinical specimens (blood, urine, or wound exudates) onto agar plates or by the traditional method of first isolating individual organisms and growing them in broth to a standard inoculum density before plating. Then, the susceptibility results produced by each method were compared. For specimens that contained a single organism, the susceptibility results from the direct method typically closely mirrored those obtained by the traditional method (7.3% discrepancy in one study [186]). In contrast, for specimens that contained multiple microbes, the results often differed greatly between the two methods (42.6% discrepancy [186]). In other words, the sensitivity profiles for an organism were notably different under the mixed-culture compared to pure-culture conditions. The authors of this study echoed those above by saying the results obtained from mixed-culture experiments are "completely unreliable" and "provide clinically misleading information" (183, 189) and reached the consensus that susceptibility testing should be performed only on organisms grown in pure culture. However, these studies did not consider the intriguing hypothesis suggested by their observations that one species can profoundly influence the antimicrobial susceptibility of another, which may have important implications for the treatment of polymicrobial infections in patients.

In 1975, Linn and Szabo also observed and considered the problem of the incongruity between monospecies and multispecies antimicrobial sensitivity profiles. Contrary to the previous studies, they argued that "sensitivity testing in mixed cultures may well provide greater clinical relevance since these cultures more closely simulate the

situation in the patient than does the pure culture" (188). Interestingly, they found cases where drugs became more effective when certain organisms were combined. They also observed that the differences in antimicrobial sensitivity between pure and mixed cultures were not randomly distributed but that certain organisms or drugs behaved the same way when in combination, knowledge that may offer therapeutic guidance. The literature above suggests that we have rediscovered and extended the findings of Linn and Szabo. Given the polymicrobial etiology of most chronic infections, and the important influence of interspecies interactions on antimicrobial efficacy *in vitro*, as illustrated by the many findings cited above, we argue that the approaches for measuring the drug susceptibility of mixed-species infections should be reconsidered.

Here, we address several important challenges and propose ideas for testing the antimicrobial susceptibility of microbes within polymicrobial, biofilm infections. A fundamental problem is the lack of effective treatments for polymicrobial infections, such as those of the CF airway or diabetic foot ulcers. Therefore, the first challenge is to direct drug discovery efforts toward identifying antimicrobial agents that can more effectively treat these recalcitrant infections. Furthermore, another key challenge is that we currently do not know how *in vitro* antimicrobial susceptibilities of mixed-species infections correlate with clinical outcomes. Perhaps, one solution could be to design clinical trials that test whether laboratory susceptibility testing results correlate with treatment outcomes (e.g., does the high *in vitro* efficacy of a drug against three bacterial species isolated from a wound infection correlate with the successful eradication of that infection following treatment with the same drug). Additionally, it may be possible to mine data retrospectively to assess if particular antibiotic regimens work better against mixed-species infections caused by certain combinations of organisms. Finally, an important challenge is a lack of knowledge about how antimicrobial efficacy changes with the addition of more than two constituent species. A priority moving forward should be to establish new *in vitro* model systems to interrogate the drug susceptibility profiles of complex polymicrobial communities, as at least one group has begun to do (175).

Once progress has been made in overcoming the above challenges, it may be possible to implement new approaches for susceptibility testing. Here, we propose a few ideas. If and when we reach a stage when we understand which drugs (or drug classes) retain efficacy against particular combinations of microbes, one possible approach could be to develop standardized treatments for particular combinations of microbes within mixed-species infections. As a simple example, if microbes A and B are detected in the same sample, then drug 1 is used, but if microbes A and C are detected together, drug 2 is selected. We envision that these treatments could be used in conjunction with existing MIC testing on pure cultures.

Alternatively, a personalized medicine approach could be a reasonable strategy for evaluating the antimicrobial susceptibility of mixed-species infections. Here, we propose a framework for a potential method. Following the identification of the microbes present in a clinical specimen using rapid, culture-independent methods, it may be possible to select and reconstitute a simplified microbial community (including bacteria and fungi). Perhaps, this community would be composed of the most abundant species, as well as less abundant species that are considered particularly clinically relevant. The selected community would be reconstituted in a microtiter plate and treated with a small number of antimicrobial agents at multiple concentrations, followed by plating onto selective agar for the enumeration of viable counts of each microbial species. This approach would provide a measure of the survival of each species after drug exposure. We recognize that there are many limitations of these proposed approaches for polymicrobial testing in the clinical laboratory. First of all, such strategies might be feasible for sites that are sterile in the absence of infection or sites that contain a relatively small number of resident species. In contrast, this approach could be quite challenging for infections in sites containing a complex microbiota (e.g., the intestine). For example, it is likely that commensals influence the

drug sensitivity profiles of pathogens at these infection sites (83, 87); however, including commensals in the reconstituted community could lead to an intractable number of species for testing. Another important limitation for some of the approaches outlined above would be the high labor costs and time-intensive nature of such testing. However, integrating the routine use of culture-independent techniques could reduce the time and laborious culturing protocols that are commonly used for species identification in clinical microbiology laboratories and allow for more time and resources to be available for performing the proposed drug testing methods. Additionally, recent machine learning approaches have utilized the abundant available genome sequence resources to accurately predict MICs of organisms (190, 191); perhaps such approaches could be applied to polymicrobial communities. Additionally, it may be possible to automate several parts of the process using liquid-handling machines and plate-scanning software. Other concerns are the lack of rapid and reliable methods to quantify fungal viability following coculture with other microbes and the problem of how to tackle the antibiotic susceptibility of biofilms, which adds another layer of complexity.

Finally, perhaps the simplest solution to the challenges outlined here is a return to testing the antibiotic susceptibility of direct clinical samples, as described above (186). For example, following species identification, the original clinical specimen could be transferred to microtiter plates and subjected to different antibiotic treatments. Afterward, the treated sample could be plated on selective medium to determine viability of each species, which can be compared to the starting number of cells before antibiotic treatment to evaluate the success of antimicrobial treatment. Alternatively, for the subset of infections not responding to treatment, the clinical sample could be swabbed directly to permissive medium and a suite of antibiotics could be tested using standard MIC assays to empirically determine the antibiotic(s) that most effectively inhibits the organisms in the infection. As reported previously (87), sometimes only the most susceptible member of the community has to be killed to effectively reduce the growth/viability of the entire community. A major concern of this approach is whether testing on direct clinical samples can be standardized. Thus, because mixed-species infections vary widely from one patient to another, it is possible that a personalized approach is the best strategy to measure susceptibility profiles. To move forward, it is essential to promote discussion and collaboration between the clinical setting, academia, and industry to develop new methods to treat and diagnose polymicrobial infections.

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