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Interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* during co-cultivations and polymicrobial infections

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Abstract

Pseudomonas aeruginosa and *Staphylococcus aureus* are versatile bacterial pathogens and common etiological agents in polymicrobial infections. Microbial communities containing both of these pathogens are shaped by interactions ranging from parasitic to mutualistic, with the net impact of these interactions in many cases resulting in enhanced virulence. Polymicrobial communities of these organisms are further defined by multiple aspects of the host environment, with important implications for disease progression and therapeutic outcomes. This mini-review highlights the impact of these interactions on the host and individual pathogens, the molecular mechanisms that underly these interactions, and host-specific factors that drive interactions between these two important pathogens.

Keywords

Pseudomonas aeruginosa, *Staphylococcus aureus*; cystic fibrosis; polymicrobial infections; alkylquinolones

INTRODUCTION

Polymicrobial communities are prevalent in many infectious disease states, including surgical and diabetic foot wounds, otitis media, oral infections, and cystic fibrosis (CF) lung disease (Peters et al. 2012). While the importance of these communities in infectious disease has long been understood, the advent of cutting-edge technologies is yielding new insights into the complexities and impacts of multi-species infections on human health (Marsland and Gollwitzer 2014; Surette 2013; Lynch and Bruce 2013; Price et al. 2013; Lipuma 2010; Rogers et al. 2010; Lyczak, Cannon, and Pier 2002; Filkins and O'Toole 2015). Despite these advances, the impact of polymicrobial interactions on infectious disease remains

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COMPLIANCE WITH ETHICAL STANDARDS

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difficult to dissect in a laboratory environment. Contributing to the complexity of these studies is a dearth of appropriate models that accurately reflect the host environment. In particular, models for studying polymicrobial interactions must take into account the availability of required nutrients, impacts of host immunity factors, and changes in host physiology that occur as a result of infection and/or the underlying disease state.

Biological interactions in polymicrobial communities are most commonly defined based on the outcome of the interaction on each of the two participating species: parasitism refers to a relationship in which one organism benefits at the cost of the other, commensalism indicates that one organism benefits from the relationship with no effect on the other, and mutualism is defined as a relationship where both organisms benefit. Regardless of their outcome on the individual species, each of these interactions has the potential to accelerate disease progression when it occurs at the site of an infection. In these cases, the term “synergism” has been applied to denote cooperativity – *i.e.* enhanced virulence capacity during co-infection as compared to that of either microbe alone (Murray et al. 2014). In contrast, antagonism occurs when the presence of two or more species protects the host from disease that occurs when only one species is present. As will be discussed in this review, defining specific interactions as mutualistic or antagonistic can be subjective, as negative effects on growth rates of one species may promote survival of that species in specific host environments.

In this mini-review, we will provide an overview of interactions that have been described between *Staphylococcus aureus* and *Pseudomonas aeruginosa*, two versatile bacterial pathogens that commonly inhabit the CF lung and chronic wound infections (Stacy et al. 2016; Peters et al. 2012; Foundation 2014). Synergism between *S. aureus* and *P. aeruginosa* has been observed in multiple models of infection, including wounds and chronic lung infection (Sibley et al. 2008; Korgaonkar et al. 2013; Korgaonkar and Whiteley 2011; DeLeon et al. 2014; Dalton et al. 2011). Numerous studies have revealed both mutualistic and parasitic interactions that drive the synergistic impact of these two pathogens on infectious disease progression. We will specifically discuss the molecular mechanisms guiding these interactions, as well as their implications for the progression and outcome of polymicrobial infections.

COMMENSAL AND MUTUALISTIC INTERACTIONS

Both *S. aureus* and *P. aeruginosa* exhibit intrinsic and acquired antibiotic resistance (Carmeli et al. 1999; Lister, Wolter, and Hanson 2009; Chambers and Deleo 2009; Lowy 2003), making infections by these pathogens increasingly difficult to treat. Adding to the complexity of managing these infections is the newly acknowledged phenomenon that co-cultivation of these species enhances survival in the presence of antimicrobials. Synergistic effects of co-culture are observed when assessing tolerance of *S. aureus* to both gentamicin and tetracycline when grown either in planktonic culture or in an *in vitro* wound model (DeLeon et al. 2014). Hoffman, *et al*, further demonstrated that increased tolerance of *S. aureus* to tobramycin during co-culture occurs through the secretion of *P. aeruginosa* exoproducts called alkyl-quinolones (AQs) (Hoffman et al. 2006), secondary metabolites that mediate interactions of this pathogen with several other microbial species (Nguyen et al.

2015; Filkins et al. 2015; Mashburn et al. 2005; Korgaonkar and Whiteley 2011; Heeb et al. 2011) (**Table 1**).

One specific AQ, 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), acts as a quinone analog, binding to and inhibiting activity of cytochrome b in Gram-positive bacteria such as *S. aureus* (Machan et al. 1992; Lightbown and Jackson 1956) (**Fig. 1**). Inhibition of cytochrome b results in decreased electron transport and thus energy production (Lightbown and Jackson 1956; Gotz and Mayer 2013). This reduction in ATP production shifts *S. aureus* metabolism from aerobic respiration to anaerobic or fermentative respiration (Hoffman et al. 2006; Filkins et al. 2015; Biswas et al. 2009). By inhibiting respiration, long term exposure to HQNO selects for *S. aureus* small colony variants (SCVs), producing a similar outcome as either mutations in the terminal oxidases QoxABCD and CydAB (Hammer et al. 2013) or exposure to secondary metabolites such as pyocyanin (Voggu et al. 2006; Biswas et al. 2009). SCV formation in *S. aureus* also increases tolerance to aminoglycoside antibiotics, which require an active electron transport chain to enter the cell (Bryan and Van Den Elzen 1977) (**Fig. 1**). Therefore, SCVs have been shown to display increased tolerance to multiple antimicrobials (Pan et al. 2002; Proctor et al. 2014; Proctor et al. 2006; Lechner, Lewis, and Bertram 2012; Wood, Knabel, and Kwan 2013).

Co-culture with *S. aureus* similarly promotes selection of *P. aeruginosa* SCVs, resulting in increased survival and antimicrobial tolerance of *P. aeruginosa* isolates in the CF lung (Michelsen et al. 2014). This interaction is dependent upon the Agr quorum sensing system, which regulates the expression of multiple virulence factors in *S. aureus* (Novick 2003). The precise mechanism by which *S. aureus* quorum sensing affects *P. aeruginosa* antimicrobial tolerance however remains unclear.

Another major contributor to antibiotic tolerance and virulence during polymicrobial infections is biofilm formation. Mixed microbial biofilms are especially relevant in the context of CF due to the altered physiology of the lung (Costerton 2001; Costerton et al. 1995). Thick, dehydrated mucus and hypoxia in the CF lung provide prime conditions for biofilm formation by both *S. aureus* and *P. aeruginosa* (Costerton 2001). A recent report by Fugere *et al.*, showed that production of HQNO and the pseudomonas quinolone signal (PQS), another AQ produced by *P. aeruginosa*, enhances biofilm formation by *S. aureus* (Fugere et al. 2014). The promotion of mixed biofilms by these species results in decreased susceptibility to multiple classes of antibiotics, complicating eradication of co-colonizing microorganisms.

PARASITIC INTERACTIONS

In 1949, Jacques Monod proposed that limited availability of required nutrients shapes the populations of competing microbes (Monod 1949). Such a change in population dynamics is often observed during chronic CF lung infections. The microbial makeup of the lungs is highly diverse and dynamic in young CF patients, yet this environment becomes dominated by *P. aeruginosa* as disease progresses (Foundation 2014) (**Fig. 2**). Many factors impact the makeup of the bacterial population that inhabit this environment, including changes in nutrient availability and the host immune system (Quinn et al. 2014). The studies

highlighted below outline how specific host factors drive microbial interactions during polymicrobial infections.

Iron availability

Iron is an essential nutrient for most microbial pathogens and is increasingly appreciated as a critical mediator of CF lung disease (Bouvier 2016; Barnabie and Whiteley 2015; Cassat and Skaar 2013). Iron is limiting during infections due to sequestration by host proteins such as lactoferrin, transferrin, and hemoglobin (Otto, Verweij-van Vught, and MacLaren 1992; Nairz et al. 2010). Microbial pathogens have therefore evolved several means of acquiring this nutrient from the host. *P. aeruginosa* and *S. aureus* obtain iron through the secretion of siderophores (Ochsner, Vasil, and Vasil 1995; Martin et al. 2011; Hammer and Skaar 2011), heme transport and degradation (Ochsner, Johnson, and Vasil 2000; Maresso and Schneewind 2006), and ferrous iron uptake systems (Wang et al. 2011; Ster et al. 2010). While siderophore-mediated iron uptake is clearly important for acute *P. aeruginosa* infections (Takase et al. 2000; Meyer 2000; Meyer et al. 1997), several recent reports demonstrate a decreased reliance on siderophores and an increased utilization of heme and ferrous as an iron source by *P. aeruginosa* during CF infection (Nguyen et al. 2014; Huse et al. 2010; Konings et al. 2013; Hunter et al. 2013) (**Fig. 2**). Skaar *et al.* further showed that *S. aureus* preferentially imports heme over siderophore-mediated scavenging of iron from transferrin (Skaar et al. 2004). Thus, *P. aeruginosa* and *S. aureus* likely compete for limiting and overlapping sources of iron during polymicrobial infections.

While the majority of microbial iron uptake studies have been performed with pure cultures, recent studies are yielding new insights into how co-culture impacts iron homeostasis. *P. aeruginosa* was previously shown to lyse *S. aureus*, thus liberating iron from this competing microbial species during co-culture (Mashburn et al. 2005). This activity requires the *P. aeruginosa* *pqsA* gene, encoding the first enzyme in AQ biosynthesis, demonstrating a role for AQs in this parasitic interaction. More recently, our lab showed that iron deprivation enhances the production of certain AQs, correlating with increased antagonism of *S. aureus* growth in low iron environments (Nguyen et al. 2015). Filkins *et al.*, also showed that HQNO produced by *P. aeruginosa* drives *S. aureus* towards fermentative metabolism when the two species are grown in mixed biofilms, resulting in eventual reduced viability of *S. aureus* (Filkins et al. 2015). The authors additionally showed that this parasitic behavior requires siderophore-mediated iron uptake by *P. aeruginosa*, again demonstrating a role for iron in this parasitic relationship. Release of peptidoglycan upon *S. aureus* lysis during co-culture further enhances AQ production by *P. aeruginosa* (Korgaonkar and Whiteley 2011), presumably allowing for a positive feedback of each of these parasitic behaviors. These studies collectively demonstrate the potential for nutrient availability, as well as microbe-microbe sensing, to enhance parasitic behaviors during polymicrobial infections.

Host immunity

In order to survive during polymicrobial infections, competing microbes must also be able to circumvent the host immune system. Recent studies suggest modulation of the immune system by co-infecting pathogens affects the make up of polymicrobial infections. While the above studies show that changes in nutrient availability, particularly iron, likely contribute to

this shift, modulation of host immunity likely also plays a role. In line with this idea, *P. aeruginosa* induces the production of type-IIA-secreted PLA2 (sPLA2-IIA), a potent bactericidal enzyme, by CF lung cells (Pernet et al. 2014). Notably, the levels detected in CF sputum are potent enough to kill *S. aureus* but have minimal effects on *P. aeruginosa* viability (Pernet et al. 2014). Thus, *P. aeruginosa* appears to induce host bacteriostatic factors to levels that specifically inhibit the growth of competing microbes.

P. aeruginosa is also capable of degrading host immune factors, including those involved in the sequestration of iron. Lactoferrin binds to the oxidized, insoluble form of ferric iron (Fe^{3+}) at mucosal surfaces, sequestering this nutrient from infecting microbes (Nairz et al. 2010). The high affinity of secreted siderophores for Fe^{3+} allows this nutrient to be scavenged by microbial pathogens, overcoming this barrier to infection (Otto, Verweij-van Vught, and MacLaren 1992). Lactoferrin is degraded in CF lungs colonized by *P. aeruginosa*, which promotes biofilm formation by this pathogen (Rogan et al. 2004). Biofilm formation in turn contributes to increased local hypoxia of the CF lung, correlating with increased dependency on systems that mediate the uptake of reduced, ferrous iron (Fe^{2+}) by *P. aeruginosa* (Hunter et al. 2013). The impact of decreased lactoferrin on the survival of other microbial species in the CF lung has not yet been investigated. However, one potential hypothesis is that degradation of lactoferrin combined with increased hypoxia of the CF lung confer a specific advantage to *P. aeruginosa* over CF pathogens that are not as adept at ferrous iron uptake.

Chronic polymicrobial infections involving *P. aeruginosa* and *S. aureus* are often the result of a dysfunctional or depressed immune system, and the long-term nature of these infections further alters host physiology and immunity (Peters et al. 2012). Analysis of the DK-2 lineage of *P. aeruginosa*, isolated from multiple CF patients between 1973 and 2008 (Yang et al. 2011), showed that this strain's ability to inhibit *S. aureus* growth was reduced as it adapted to the CF lung environment (Michelsen et al. 2014). A separate analysis of 24 *P. aeruginosa* isolates from 8 distinct CF patients at multiple stages of chronic lung infection showed a similar reduction in the ability of *P. aeruginosa* to inhibit *S. aureus* growth (Baldan et al. 2014). Thus, adaptation to the host environment during chronic CF lung infections shifts the relationship between these two species from parasitic to commensal.

OUTCOMES OF *S. AUREUS*-*P. AERUGINOSA* INTERACTIONS

The above highlighted microbial interactions clearly have the potential to exert synergistic impacts on the progression of polymicrobial infections. The most obvious of these is how increased antimicrobial tolerance during co-cultivation can complicate therapeutic management of co-infections. Enhanced virulence also occurs through the increased production of AQ metabolites that mediate both parasitic and mutualistic interactions. PQS functions as a quorum sensing molecule to activate the production of lytic enzymes that promote tissue invasion and redox-active phenazines that exhibit toxicity against eukaryotic cells (Pesci et al. 1999; Gallagher et al. 2002) (**Table 1**). The precursor to PQS, HHQ, also induces the expression of lytic enzymes and phenazines (Diggle et al. 2007; Deziel et al. 2004) (**Table 1**). As such, enhanced synthesis and secretion of AQS in response to other

microbial pathogens promotes increased production of virulence factors by *P. aeruginosa* (Korgaonkar et al. 2013).

Several infection models have been developed to investigate the impact of co-infections of *P. aeruginosa* and *S. aureus*, including the impact of AOs on virulence. A *Drosophila* model was used to screen 40 oropharyngeal isolates, including some *Staphylococcal* species, for virulence in the presence and absence of *P. aeruginosa*, resulting in the identification of three distinct infection classes (Sibley et al. 2008). Class 3 represented isolates that were avirulent in mono-culture, but when co-cultured with *P. aeruginosa* became pathogenic. This model system was also used to show that *P. aeruginosa* increased AO and virulence factor production in the presence of peptidoglycan released from *S. aureus*, resulting in increased mortality of the fly (Korgaonkar et al. 2013; Korgaonkar and Whiteley 2011). A mammalian polymicrobial infection model, in which mixed *in vitro* biofilms are transplanted into the wounds of mice, has also been used to study co-infections of *S. aureus* and *P. aeruginosa* (Dalton et al. 2011). The authors of this study found that wound closure was delayed, and antimicrobial therapy was less effective, in *S. aureus*-*P. aeruginosa* co-infections as compared to mono-infection with *P. aeruginosa*. Combined, these studies highlight the potential for increased virulence due to co-infections.

While these studies demonstrate that co-infection often results in enhanced virulence, variations in host age, overall health status, and other microbial inhabitants complicate the ability to predict the outcomes of polymicrobial infections. This is particularly true in the case of CF lung infections, in which both synergistic and antagonistic outcomes of co-infection have been noted. According to a predictive model by Liou *et al.*, the presence of *S. aureus* increases survivorship of patients co-infected with *P. aeruginosa*, suggesting interactions between these two pathogens have beneficial consequences for the infected host (Liou et al. 2001). However, other reports show that the presence of methicillin resistant *S. aureus* (MRSA) with *P. aeruginosa* correlates with increased morbidity and mortality for co-infected CF patients due to increased antibiotic resistance and rate of lung function decline (Hubert et al. 2013). A recent study examined *S. aureus* as a marker for CF lung disease in adult patients. In children with CF disease, the presence of *S. aureus* correlated with increased inflammation and decreased lung function (Ahlgren et al. 2015). In contrast, *S. aureus* in adults could be a marker for milder disease, particularly if it coincides with reduced colonization by *P. aeruginosa*. Thus, host-specific factors play a critical role in the manifestation of polymicrobial infections involving *S. aureus* and *P. aeruginosa*.

Even when analyzing co-cultures *in vitro*, defining whether specific interactions between *S. aureus* and *P. aeruginosa* are parasitic or mutualistic is not as simple or clear as it may first appear. As discussed earlier in this review, *P. aeruginosa* produces and secretes a secondary metabolite, HQNO, that suppresses *S. aureus* growth by inhibiting respiration. While this behavior is seemingly parasitic, as growth suppression would allow *P. aeruginosa* to outcompete *S. aureus* for essential nutrients, it also selects for small colony variants of *S. aureus*, increasing the potential for this competing microbe to persist during infection (Hoffman et al. 2006). Studies cited above from Michelsen, *et al.*, further show that the reduced ability of *P. aeruginosa* CF isolates to inhibit *S. aureus* growth correlates with the selection of *P. aeruginosa* isolates with increased antibiotic tolerance during co-culture

(Michelsen et al. 2014). More studies into the long-term outcomes of chronic polymicrobial infections, relying on appropriate *in vitro* and *in vivo* models, are clearly needed to improve our understanding of how these interactions contribute to infectious disease.

CONCLUSION

Regardless of how interactions between *S. aureus* and *P. aeruginosa* are characterized, the presence of these pathogens together clearly results in distinct disease manifestations and warrants careful consideration for successful management of polymicrobial infections. As highlighted in this review, co-culture of *S. aureus* with *P. aeruginosa* affects the virulence capacity and vulnerability to antimicrobial therapy of both pathogens, exerting both synergistic and antagonistic effects on disease progression. Future studies defining the impacts of interspecies interactions, as well as the host factors that modulate these interactions, are critical for understanding the pathogenesis of polymicrobial infections.

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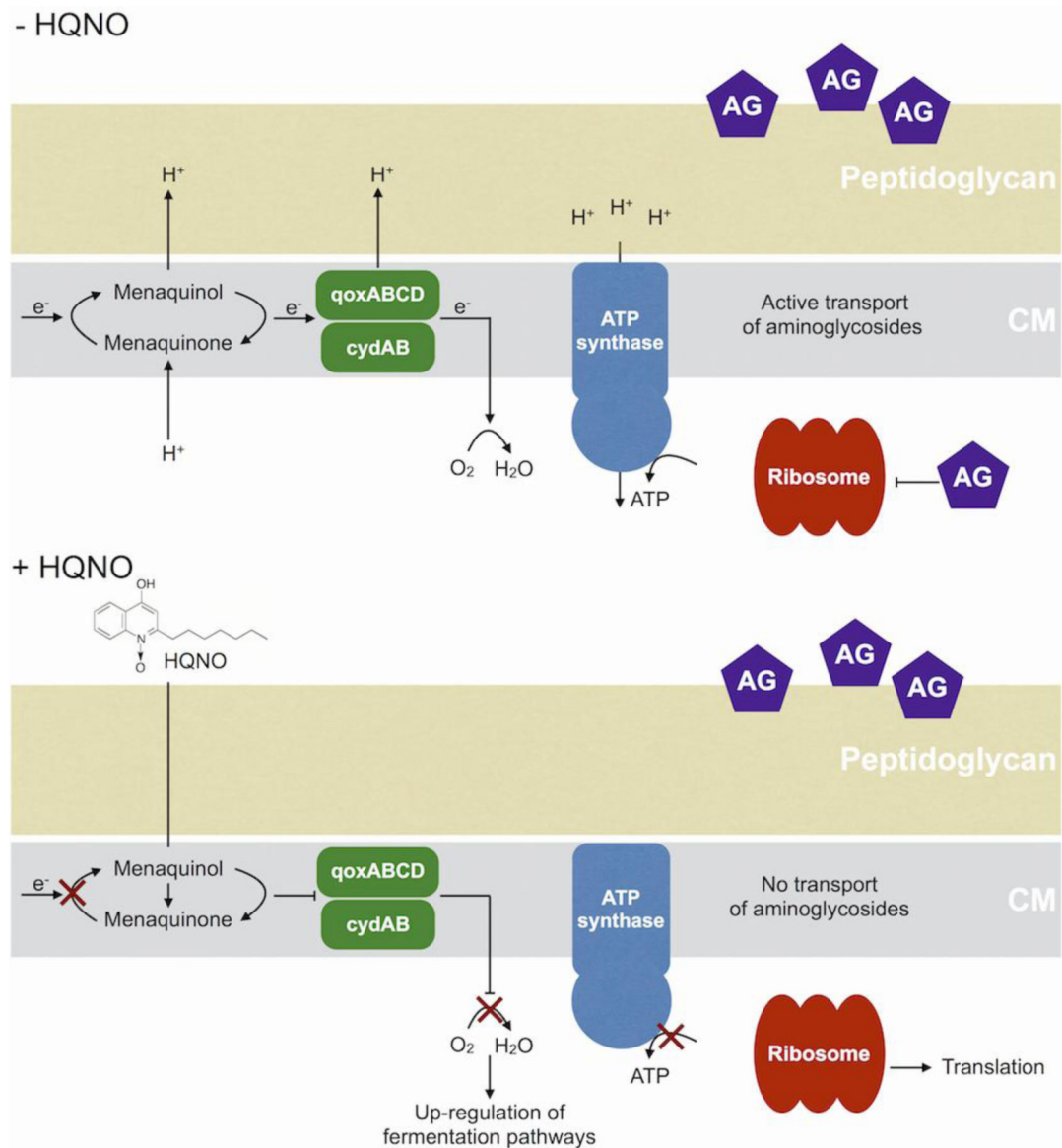


Figure 1. HQNO inhibits cytochrome b and increases tolerance to antimicrobials

Figure showing cytoplasmic membrane (CM) of *S. aureus*. HQNO is a menaquinone analog that inhibits electron transport through cytochrome b. This results in decreased ATP generation and a shift to fermentative metabolism. Reduction of ATP in the cell decreases active transport which is required for uptake of aminoglycoside (AG) antibiotics.

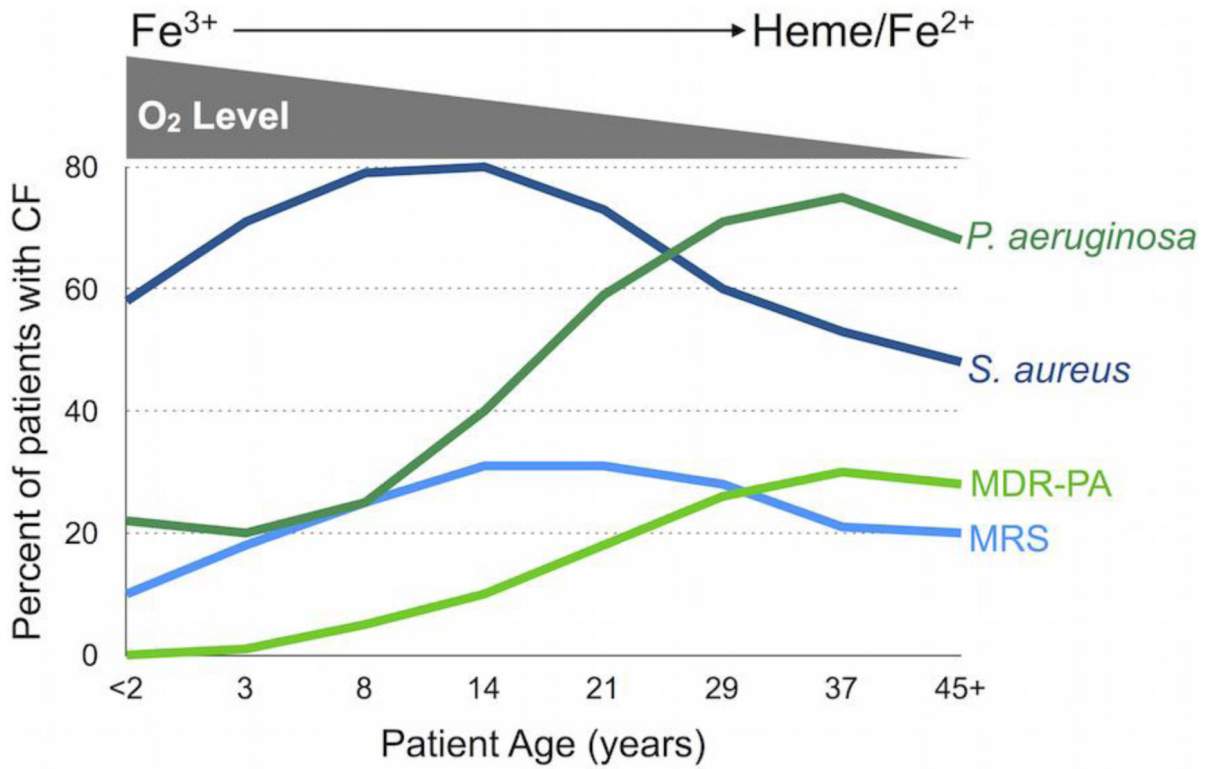
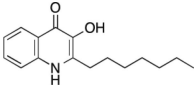
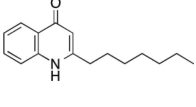
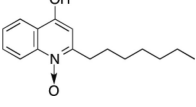


Figure 2. *P. aeruginosa* displaces *S. aureus* in the CF lung

P. aeruginosa and multi-drug resistant *P. aeruginosa* (MDR-PA) outcompete *S. aureus* and methicillin-resistant *S. aureus* (MRSA) over the course of CF lung infection. As CF lung disease progresses, oxygen levels decrease coinciding with the use of heme and ferrous iron as predominant sources of iron.

Table 1

Structure and functions of key alkyl-quinolones

	Nomenclature	Functions	Structure
PQS	2- heptyl-3-hydroxy-4(<i>1H</i>)-quinolone (<i>Pseudomonas</i> quinolone signal)	<ul style="list-style-type: none"> • quorum sensing - virulence gene expression • iron chelation • vesicle formation 	
HHQ	2-heptyl-4-hydroxyquinoline	<ul style="list-style-type: none"> • quorum sensing - virulence gene expression • signaling - phenazine production 	
HQNO	2-heptyl-4-hydroxyquinoline <i>N</i> -oxide	<ul style="list-style-type: none"> • cytochrome <i>bc_L</i> complex inhibitor 	

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