HIJACKING ANAEROBIC METABOLISM TO RESTORE ANTIBIOTIC EFFICACY IN *Pseudomonas aeruginosa*

by

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A THESIS

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Aggressive antibiotic treatment often fails to resolve chronic infections. Most antibiotics target fast-growing bacteria, however, pathogens like *Pseudomonas aeruginosa* grow slowly in chronic infection environments because of oxygen limitation (hypoxia). Thus, antibiotic treatment failure occurs in oxygen limited (hypoxic) environments when slow bacterial growth facilitates tolerance to antibiotics or because many bacteria are resistant to antibiotics. The goal of this thesis is to identify new therapeutic strategies to kill pathogens under hypoxic conditions like those in chronic infections. Chlorate is a drug that kills *P. aeruginosa* growing in conditions with no oxygen by hijacking a metabolic process known as nitrate respiration. This pathway converts nontoxic chlorate into toxic chlorite, which kills *P. aeruginosa*. Chlorate and antibiotic treatment are most effective against *P. aeruginosa* growing in low oxygen and high oxygen conditions, respectively, but neither is effective at killing *P. aeruginosa* cells under hypoxic conditions. We found that chlorate interacts synergistically with different classes of antibiotics to eliminate hypoxic *P. aeruginosa* populations, overcoming both antibiotic tolerance and resistance. Chlorate has a unique ability to synergize with different classes of drugs because most antibiotic-antibiotic combinations did not display synergy. Future work will focus on understanding the mechanism of chlorate-antibiotic synergy and why antibiotic-antibiotic

combinations are not synergistic. Identifying synergistic drug combinations holds promise for curing recalcitrant infections, where current antibiotic-only treatments often fail patients. This thesis includes co-authored material and collaboratively produced work.

Acknowledgements

I want to thank the Spero Lab, my friends, and my family for all their support during my time at UO. I have come a very long way from four years ago and it is largely because of my incredible support system. The Spero Lab has provided the perfect environment for me to mature as a scientist, have lots of fun, and laugh at my ridiculous animals. My mentor, Dr. Melanie Spero, has been a patient, kind, supportive, and enthusiastic pillar both in the lab and out, and for that I thank you. I am so grateful for many of my other professors at UO who have fielded so many of my questions, whether on-topic or otherwise. Many of you have inspired me to pursue science and attend graduate school and have helped me get there.

I also want to acknowledge the research fellowship from The Office of the Vice President for Research and Innovation that funded my research during the Summer of 2023 and the Clark Honors College Mentored Research Program that funded my research during the Winter of 2024.

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Preface

This thesis contains co-authored material and collaborative work which I believe is key to doing good science. The purpose of this preface is to orient the reader to the structure of the thesis and acknowledge the invaluable contribution of both my collaborator and mentor. The majority of this work is a co-written manuscript for a scientific audience, so I have also included preface, background, and conclusion sections that cater to a lay audience and were written solely by myself.

Collaboration

I completed this project in collaboration with Celine Lopez Padilla, who was involved from my very first day in the lab. Celine is a fellow undergraduate student who has been integral to completing this research project. Celine and I began working on this project in the summer of 2022 when we were accepted into the lab of Dr. Melanie Spero, our mentor and principal investigator (PI). Dr. Spero laid out the motivation for our project based on work she had conducted during her postdoc, and Celine and I were very excited to get started. During our first summer, we spent a lot of time gathering preliminary data and deciding which methods would be best for our project. At times, Celine and I performed the same experiment in hopes of getting results that were consistent from two different hands. We had weekly meetings with Dr. Spero to discuss our progress and make a plan for the next week. Through the next school year, Celine and I continued to collect and analyze data and we both grew more confident in our research skills. We kept our weekly meeting with Dr. Spero to discuss our project and we presented during lab meetings to get feedback from others.

During Spring 2023, Celine and I created a poster for the Undergraduate Research Symposium. We both learned a lot making that poster, particularly because it was one of the first

times we shared our project with people outside of our lab. We both worked tirelessly to make our poster accessible and beautiful with the oversight of Dr. Spero. We ended up winning an award for our co-presented poster at the symposium, which was a great way to justify our hard work.

Over the Summer of 2023, I received a research fellowship that enabled me to work full-time on our project for 10 weeks. I continued to perform the same experiments Celine and I had conducted during the school year, and I was able to collect a large amount of data. Celine also started a new branch of our project that required a lot of preliminary work over the summer. We both encountered setbacks in our respective experiments during the summer. When I repeated the experiments I had run before, I got slightly different results which would impact our scientific conclusions. With the help of Dr. Spero and our weekly meetings, we both continued experimenting for the rest of the summer and into the Fall of 2023. I was able to modify conditions to get consistent results from my experiments. Whether fortunately or unfortunately, we both got to experience how difficult it can be to work in science.

I was able to collect all of the data for our project by the end of Fall term which meant it was time to analyze and write our manuscript. During our weekly meetings in the Winter of 2024, Dr. Spero, Celine, and I would outline a section of the paper, then Celine and I would split up our weekly writing goal. At the next meeting, we would review what we had written and start another section. Between the three of us, we went through many versions of each section to keep making improvements. I also prepared the figures for our paper and every week I made edits to make them even better. Throughout the Winter, I was funded by the Clark Honors College Mentored Research Program. Toward the end of the Winter term, we finally put all of our sections into one document and read through it. Seeing the whole manuscript put together was

pretty incredible! It represents many hours of work and thought from all of us. We are continuing to perfect our manuscript for publication, but what is included in this thesis represents a thoughtful scientific paper co-written by Celine, Dr. Spero, and me.

Background

The Problem: Chronic Infections Are Not Resolved by Current Antibiotic Treatments

Bacteria can cause infections all over the human body that are either short-lived (acute) or long-lasting (chronic) (1). Chronic infections can last for months to decades despite the extensive use of antibiotics (2, 3). On the longer end of this spectrum are Cystic Fibrosis (CF) lung infections. Patients with CF have less water in the mucus that coats their airways, which causes it to be thicker than normal and build up in the lungs (4). Mucus buildup presents a nice environment for bacteria to grow, which results in chronic lung infections in patients with CF. Chronic wound infections are another common type of chronic infection (5). Chronic wounds cause problems for patients because the normal wound healing process has stalled, keeping wounds open for months to years (6). When chronic wounds get infected, they can become so unmanageable that they must be resolved by limb amputation (5). Diabetic foot ulcers are a key example of chronic wounds. Diabetic patients experience compromised blood circulation, foot deformities, and decreased sensation in their feet all of which contribute to the formation of diabetic foot ulcers (7). Each year, 19 million people around the world suffer from diabetic foot ulcers (7). Half of all diabetic foot ulcers are infected and around 20% of infected ulcers require limb amputation (7). Diabetic foot ulcers and amputation resulting from infections adversely affect racial minorities and older patients (7). These chronic infections and many more are not being successfully resolved with antibiotics, and, therefore necessitate finding new treatments.

Pseudomonas aeruginosa is a bacterium that is commonly isolated from both chronic wound infections and CF lung infections (1). *P. aeruginosa* is prone to antibiotic treatment failure leaving patients to suffer with long-lasting infections (8–11). Understanding the nature of

antibiotic treatment failure will inform treatment solutions, so we must identify the cause of the failure.

Antibiotic Treatment Failure Results from Tolerance or Resistance

Most antibiotics target bacterial growth processes and can be sorted into different classes based on their cellular targets (12). For instance, fluoroquinolones are antibiotics that inhibit DNA replication, polymyxins are antibiotics that disrupt cell membranes, aminoglycosides are antibiotics that inhibit bacterial protein synthesis, and β-lactams are antibiotics that target the bacterial cell wall (12). The antibiotics used in this project belong to four different antibiotic classes and are commonly used to treat *P. aeruginosa* infections (13, 14). The antibiotics include Ciprofloxacin, Colistin, Tobramycin, and Ceftazidime (Table 1).

Antibiotic	**Class**	**Cellular Target**
Ciprofloxacin	Fluoroquinolone	Inhibits DNA replication of bacteria
Colistin	Polymyxin	Disrupts cell membrane
Tobramycin	Aminoglycoside	Inhibits bacterial protein synthesis
Ceftazidime	Cephalosporin (β-lactam)	Targets the bacterial cell wall

Table 1. Antibiotics used in this project, their class, and their mechanism of action (12, 15).

Because bacterial growth processes are the main targets of antibiotic action, most antibiotics are highly effective at killing fast-growing bacteria (16). However, if bacteria are not growing quickly, many antibiotics become less toxic. Antibiotic tolerance refers to a reversible metabolic state that allows bacteria to survive short-term antibiotic exposure (16). Generally, bacteria can achieve antibiotic tolerance through slow growth (16). Slower growth means slower growth processes which limit the ability of antibiotics to target and kill bacterial cells. Slow bacterial growth readily occurs in infection environments implicating antibiotic tolerance may be responsible for a portion of antibiotic treatment failure (17–19).

In addition to antibiotic tolerance, antibiotics also fail to kill bacteria when they are resistant to these drugs. Bacteria display antibiotic resistance and survive antibiotic exposure when they acquire a genetic change(s) that let cells survive or even grow in the presence of the antibiotic (16). Rates of antibiotic resistance are rising, and in 2019, an estimated 5 million antibiotic resistance-associated deaths occurred (8). The antibiotic resistance crisis is further compounded by the limited development of new antibacterial compounds, particularly those with novel biological targets (20–22). The increasing impact of antibiotic resistance has garnered much attention and research, but treatment failure due to antibiotic tolerance has received comparatively less attention.

Oxygen-Limited *P. aeruginosa* Grows Slowly and Uses Anaerobic Respiration

Oxygen availability is an important environmental factor for bacteria like *P. aeruginosa* that can change their physiology. There is a range of oxygen availability within chronic infection environments, but they are frequently limited for oxygen because of the activity of host cells, host immune cells trying to fight off the infection, and bacterial cells (23–26). Environments where oxygen concentrations are either high (e.g. atmospheric oxygen) or completely lacking are termed oxic and anoxic, respectively, whereas hypoxic environments have intermediate oxygen concentrations (Figure 1A). In oxic environments, *P. aeruginosa* grows using aerobic respiration, similar to how humans conserve energy. In other words, the bacterial cells use oxygen to capture energy from sugars (Figure 1B). Electrons harvested from metabolizing sugars are put onto the electron acceptor oxygen, thereby forming water. In hypoxic or anoxic environments like chronic infections, *P. aeruginosa* captures energy by using nitrate as an electron acceptor in the place of oxygen (Figure 1C). Nitrate respiration is a form of anaerobic metabolism. In *P. aeruginosa,* the Nar enzyme transfers electrons to nitrate, thereby generating

nitrite. Generally, aerobic respiration supports faster growth than anaerobic respiration because it harnesses more energy.

We hypothesize that antibiotics fail to resolve chronic infections because oxygen limitation causes slow bacterial growth which facilitates antibiotic tolerance. *P. aeruginosa* uses Nar to grow in hypoxic or anoxic environments where the bacterial cells display antibiotic tolerance. Thus, the *nar* gene is expressed by *P. aeruginosa* in those conditions and represents a potential drug target for killing bacterial cells that are tolerant to antibiotic treatment.

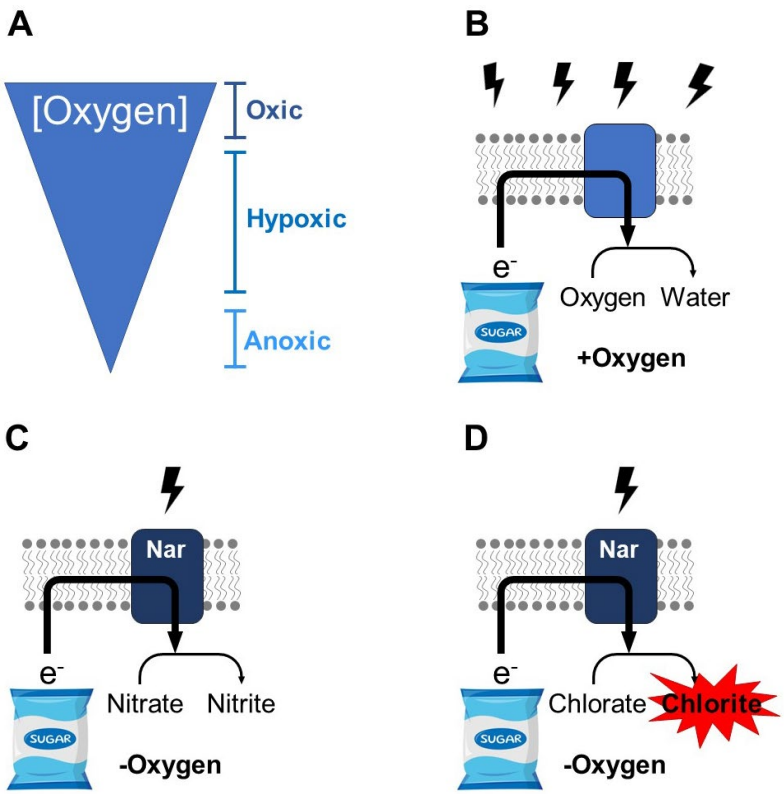

Figure 1. *P. aeruginosa* grows in all oxygen concentrations and anaerobic respiration can be hijacked by chlorate.

A. Oxic conditions have high oxygen availability, hypoxic conditions have an intermediate amount of oxygen, and anoxic conditions lack oxygen. **B.** *P. aeruginosa* uses aerobic respiration to grow when there is oxygen in the environment. The reduction of oxygen to water captures energy from sugar metabolism that is used for cell growth. **C.** *P. aeruginosa* can use anaerobic nitrate respiration to grow when there is limited or no oxygen in the environment. The Nar enzyme facilitates using nitrate instead of oxygen to capture energy for cell growth, although it conserves less energy than in aerobic respiration. **D.** The Nar enzyme can also reduce non-toxic chlorate to chlorite which is a toxic agent that can kill *P. aeruginosa* by bleaching the cells.

Chlorate and Drug Synergy: A Potential Solution

The Nar enzyme that enables nitrate respiration in *P. aeruginosa* can also reduce chlorate because nitrate and chlorate are structurally similar. Chlorate is a small, non-toxic chemical but when it accepts electrons it is converted into chlorite, which is a toxic oxidizing agent that is similar to bleach (Figure 1D) (27). The ability of Nar to reduce nitrate and chlorate has been known for decades (28, 29), but chlorate has only recently been investigated as a possible treatment for infections (27, 30, 31). Because of the location of the Nar enzyme, chlorite is generated inside *P. aeruginosa* cells, which kills *P. aeruginosa* without harming nearby host cells (Figure 1D) (27, 32). Chlorate is most effective at killing *P. aeruginosa* under anoxic conditions when Nar is most active (27). Thus, chlorate is toxic to *P. aeruginosa* under anoxic conditions when the pathogen is tolerant to many antibiotics.

Chlorate is a particularly exciting therapeutic candidate because it bears many qualities that would make it applicable to a range of bacterial infections. First, many bacterial pathogens make the Nar enzyme and likely use nitrate respiration to grow in host environments including *Salmonella enterica, Corynebacterium diphtheriae, Mycobacterium tuberculosis, Staphylococcus aureus, Escherichia coli*, *Burkholderia* spp., and *Brucella* spp. (32–45). These bacteria cause a wide range of infections and take a large toll on the medical system and patients. Second, chlorate treatment does not cause toxicity to the host, but does to the Nar-containing bacteria causing the infection. The toxic chlorite generated by Nar remains within the bacterium until it is converted to harmless chloride (32, 46). Humans and animals do not have the Nar enzyme, so chlorate will only be turned into chlorite inside Nar-containing bacterial cells. Livestock animals have been treated with chlorate without showing signs of toxicity up to high concentrations (30, 31, 47–49). Third, chlorate has been shown to effectively kill *P. aeruginosa*

in the lab (27) and in chronic wounds infected with *P. aeruginosa* in diabetic mice (49). Finally, *P. aeruginosa* requires Nar to establish a chronic wound infection in mice (49). Because Nar is required for *P. aeruginosa* to grow in these environments, it is particularly exciting that the primary mechanism for *P. aeruginosa* to develop chlorate resistance is by decreasing Nar activity (50). Thus, whether *P. aeruginosa* is sensitive or resistant to chlorate treatment, its growth in infection environments will be negatively impacted. These traits make chlorate a promising drug candidate that is worth further exploration, which was the main motivation for this project.

Antibiotics are the most effective at killing *P. aeruginosa* cells under oxic conditions when the cells are growing quickly. Conversely, chlorate is the most effective at killing *P. aeruginosa* cells under anoxic conditions. However, antibiotics and chlorate are each only marginally effective at killing hypoxic *P. aeruginosa* populations. Occasionally, when drugs are combined, they exhibit drug synergy. Drug synergy occurs when the toxic effect of two drugs in combination is greater than the sum of the individual drug toxicities (51). Thus, we predicted that combined antibiotic and chlorate treatment could interact synergistically to effectively kill hypoxic *P. aeruginosa* populations.

We found that chlorate is indeed able to synergize with antibiotics to kill hypoxic *P. aeruginosa*. Chlorate displays synergy when combined with antibiotics from various classes. Treating hypoxic *P. aeruginosa* with antibiotics alone revealed that antibiotic treatment failure occurs due to both antibiotic resistance and tolerance, suggesting that chlorate-antibiotic synergy can overcome both antibiotic resistance and tolerance. Chlorate has a unique ability to synergize with other drugs because antibiotic-antibiotic combinations displayed fewer instances of synergy compared to antibiotic-chlorate combinations. Our findings support the promise of antibiotics

combined with chlorate to treat chronic infections, where current antibiotic-only treatments routinely fail patients.

Data Presentation

All of the data presented in this thesis was collected using the same technique, a drug treatment assay followed by a plate count to quantify survival of the bacterial culture. These procedures are described in more detail in the methods section. In brief, liquid bacterial cultures are treated with or without drug(s) for 24 hours, after which the cultures are plated to determine the number of bacteria that survived the drug treatment relative to the untreated control (i.e. calculate percent survival). The percent survival data were log transformed, which is shown as the y-axis in graphs presented here: $\log_{10}$(% survival). The y-axis can be interpreted as 100% bacterial survival at y = 2, 10% survival at y = 1, ultimately reaching the detection limit for these experiments of 0.0001% survival at y = -4. A drug that reduces bacterial survival to our detection limit (y = -4) represents the most effective treatment identified in our studies.

Introduction

Bacterial pathogens routinely encounter hypoxic microenvironments within the human body. In healthy adults, oxygen concentrations range from 1-10% across different tissues and concentrations can be even lower within the gastrointestinal or urinary tract (52–54). Oxygen tensions will drop below physiologically normal levels when rates of oxygen delivery and/or consumption are altered, which happens in a variety of circumstances (25, 26, 55). During infection, oxygen concentrations are locally depleted by both host cells and microbes through aerobic respiration, and immune cells rapidly consume oxygen through processes like the respiratory burst (24, 56). Additionally, within the host environment, pathogens typically grow adhered to one another as multicellular aggregate biofilms (57, 58); these high-cell density aggregates generate steep oxygen gradients, where biofilm-interior populations experience hypoxia/anoxia due to rapid oxygen consumption by biofilm-exterior populations (59).

Because pathogens frequently inhabit hypoxic microenvironments in the host, there is a need for antimicrobial therapies that are highly effective under hypoxia. However, pathogens can exhibit tolerance to conventional antibiotics under hypoxic/anoxic conditions (60–62). Many antibiotics are less effective at killing slow- or non-growing bacteria, and oxygen availability is a key determinant of growth rate for many pathogens (16). The relationship between environmental hypoxia and antibiotic treatment failure is thought to underpin different types of recalcitrant infections. In chronic wounds, which affect ~2% of the U.S. population (63), tissue hypoxia stems from insufficient blood supply and oxygen consumption by microbes and overactive immune cells (26). Chronic wounds cannot heal so long as there is an active infection, yet antibiotic treatments often fail to resolve wound infections, which can ultimately lead to further complications like limb amputation (2, 64–67). The airways of people with cystic fibrosis

(CF) are coated with a thick mucus that is largely hypoxic/anoxic due to immune cell activity (19, 24). Pathogens grow slowly in the CF airway environment (18, 68, 69), which likely contributes to the decades-long persistence of CF lung infections despite aggressive antibiotic regimens (4). Finally, biofilms are a hallmark of recalcitrant infections, and pathogens are known to form biofilms in both chronic wound tissue and CF sputum (17, 70, 71). Biofilms are notoriously tolerant to high antibiotic concentrations because they harbor slow-growing, oxygen-limited interior populations (59, 72).

To overcome hypoxia-associated antibiotic treatment failure, we propose identifying drugs that target anaerobic bacterial metabolisms. Nitrate respiration is a widespread form of anaerobic energy metabolism that supports the growth or survival of pathogens in hypoxic host environments (33, 73). During nitrate respiration, nitrate reductase reduces nitrate to nitrite. Although there are several types of nitrate reductases, only the Nar enzyme directly contributes to energy conservation by coupling nitrate reduction to the formation of a proton motive force (74). Low but appreciable nitrate concentrations (~400 μM) are available at infection sites (34, 75, 76) as a byproduct of inflammation: reactive oxygen and reactive nitrogen species generated by immune cells react nonenzymatically to form nitrate (77). There is also strong, often direct evidence that Nar-mediated nitrate respiration supports pathogen survival or growth in the host. Enteric pathogens, including *Salmonella enterica* and *Escherichia coli*, respire host-derived nitrate to boost their growth in the inflamed gut (34–38). In *Mycobacterium* spp., nitrate respiration supports persistence in different host models, and there is evidence that this nitrate is generated as a byproduct of immune cell activity (39–41). *Brucella suis* also appears to use nitrate respiration to replicate in macrophages and hypoxic environments (42, 43), and *Burkholderia* spp. were shown to upregulate *nar* within the host environment (44) and when

grown as a biofilm (45). Finally, we recently showed that the opportunistic pathogen, *Pseudomonas aeruginosa*, requires nitrate respiration to perpetuate chronic wound infections (49). Taken together, nitrate respiration is a promising therapeutic target for killing pathogens in hypoxic/anoxic host environments.

It has long been known that the small molecule, chlorate, is toxic to bacteria when they use Nar-dependent nitrate respiration (28, 29). Chlorate (ClO_3^-) is a nitrate (NO_3^-) analog that acts as a prodrug: chlorate itself is relatively nontoxic, but Nar can bind and reduce chlorate to generate chlorite (ClO_2^-), which is a toxic oxidizing agent (28, 30, 78) (Figure 2A). Because mammals lack Nar, it is unsurprising that chlorate shows low toxicity against mammals (30), with an estimated lethal oral dose of 20-35 grams for humans (79, 80). We previously showed that chlorate kills the oxygen-limited, antibiotic-tolerant populations of *P. aeruginosa* biofilms via Nar activity (27). In addition to those *in vitro* studies, we recently found that topical chlorate treatment supports the healing of *P. aeruginosa*-infected wounds using a diabetic chronic wound mouse model (49). Our exciting *in vitro* and *in vivo* findings encourage our continued exploration of chlorate's therapeutic potential.

Here, we investigate chlorate's remarkable ability to synergize with various classes of antibiotics to eradicate hypoxic, antibiotic recalcitrant populations of *P. aeruginosa*. In some cases, the addition of chlorate substantially reduced the effective dose of an antibiotic by >100-fold. We also tested the toxicity of different antibiotic-antibiotic combinations against hypoxic *P. aeruginosa*, finding that most antibiotics did not exhibit synergistic interactions across multiple drug classes. Our results demonstrate that combined chlorate-antibiotic treatment holds promise for combatting antibiotic treatment failure in hypoxic host environments. The identification of synergistic drug combinations is considered an essential strategy in the fight against antibiotic

treatment failure. In this context, our findings highlight how little we know about the mechanisms that underpin drug synergy, which stymies our ability to identify new, powerful drug combinations.

Methods

Bacterial Strains and Growth Conditions

Strains used in this study include WT *Pseudomonas aeruginosa* UCBPP-PA14 and an isogenic strain with a markerless deletion of the *narGHJI* genes (referred to as *Δnar*) (27). All strains were grown in Miller's LB Broth (Luria Broth Miller's LB Broth; Research Products International) and supplemented with 40 mM potassium nitrate (KNO_3; Sigma Aldrich) where specified to stimulate Nar activity. Oxic cultures were incubated at 37˚C with shaking at 250 rpm. Hypoxic cultures were incubated at 37˚C under static conditions. Anoxic cultures were incubated statically at 37˚C in an anaerobic glove box with a 95% N_2 and 5% H_2 atmosphere.

Antibiotic Treatment Assays

Overnight cultures of *P. aeruginosa* were grown hypoxically for 24 hours in LB with 40 mM potassium nitrate. Overnight cultures were pelleted and resuspended in LB without nitrate to an OD_{500} of 2. Next, 180 µL of resuspended culture was added to the well of a 96-well plate (Genesee Scientific) along with 20 µL of treatment (total volume per well = 200 µL). The 20 µL treatment volume consisted of 20 µL of sterile water for control conditions, 10 µL of sterile water and 10 µL of drug solution for single drug conditions, and 10 µL each of two different drug solutions for drug combination conditions. Cultures were incubated with or without drug(s) for 24 hours at 37˚C under oxic, hypoxic, or anoxic conditions before plating for viable plate counts to determine percent survival. To limit evaporation, cultures exposed to drugs under hypoxic or anoxic conditions were incubated in humidified chambers. Under oxic conditions, evaporation was prevented by covering the edge of the 96-well plate with micropore tape and filling empty wells with 200 µL of sterile water.

The following drug stock solutions (made in water) were added to wells of a 96-well plate in antibiotic treatment assays: 10 µL of 200 mM sodium chlorate (final = 10 mM), 10 µL of 625 µg/mL tobramycin (final = 31.25 µg/mL), 10 µL of 20 µg/mL ciprofloxacin (final = 1 µg/mL), 10 µL of 300 µg/mL colistin (final = 15 µg/mL), 10 µL of 200 µg/mL ceftazidime (final = 10 µg/mL). Additional experiments were conducted using a range of ceftazidime concentrations that were achieved by adding 10 µL of a 20, 2000, or 20000 µg/mL stock solution to wells of a 96-well plate.

Viable Plate Counts for Quantifying Percent Survival

Viable plate counts were determined for untreated or drug-treated samples by serially diluting samples in phosphate-buffered saline (PBS). Six 1:10 serial dilutions were made in PBS, and 10 µL of each dilution and of the undiluted culture were plated onto LB agar, allowing for viability quantification across 7 orders of magnitude. LB plates were incubated for 24 hours at 37˚C and then moved to the bench top to incubate for another 24 hours at room temperature to allow for the growth of slow-growing colonies. Colonies were counted to calculate colony forming units (CFU) per mL for each sample. Percent survival was determined by dividing the CFU/mL value of each treated sample by the CFU/mL value of a control sample and multiplying by 100; the control sample value was the average of triplicate CFU/mL values of the culture at t = 0 (i.e. after washing and resuspending cultures to OD_{500} = 2 and just prior to the 24-hour drug incubation).

Results

P. aeruginosa Exhibits Antibiotic Recalcitrance Under Hypoxic Conditions

Given the prevalence of hypoxia in host environments, we were interested in exploring the relationship between oxygen availability and drug treatment outcomes in *P. aeruginosa*. Prior work from ourselves and others has shown that some antibiotics are less effective at killing pathogens under oxygen-limited, slow-growth conditions (16, 27, 72). We first investigated drug efficacy against *P. aeruginosa* cultures under a range of oxygen tensions. Overnight *P. aeruginosa* cultures were resuspended in fresh LB medium at a high cell density (OD_{500} = 2). Cultures were then treated with or without drug(s) and incubated with vigorous shaking (oxic conditions), statically (hypoxic conditions), or in an anaerobic glove box (anoxic conditions) for 24 hours before plating cells for viability.

The Nar enzyme is most active under hypoxic/anoxic conditions, where it reduces chlorate to generate toxic chlorite (Figure 2A). As such, it was unsurprising that chlorate treatment was nontoxic to oxic *P. aeruginosa* cultures, but resulted in ~2.5-log killing under anoxic conditions (Figure 2B). Interestingly, chlorate showed almost no toxicity against hypoxic *P. aeruginosa* cultures (0.2-log killing, Figure 2B), suggesting that either Nar was inactive under these conditions or that the cell was able to defend itself from Nar-generated chlorite stress. We also found that the antibiotic, tobramycin, was highly lethal to *P. aeruginosa* under oxic conditions (> 6-log killing) but much less toxic to *P. aeruginosa* under hypoxic or anoxic conditions (~1.5-log killing; Figure 2B). These results are consistent with prior work, demonstrating that oxygen limitation causes *P. aeruginosa* to grow slowly and adopt an antibiotic-tolerant state (27, 72).

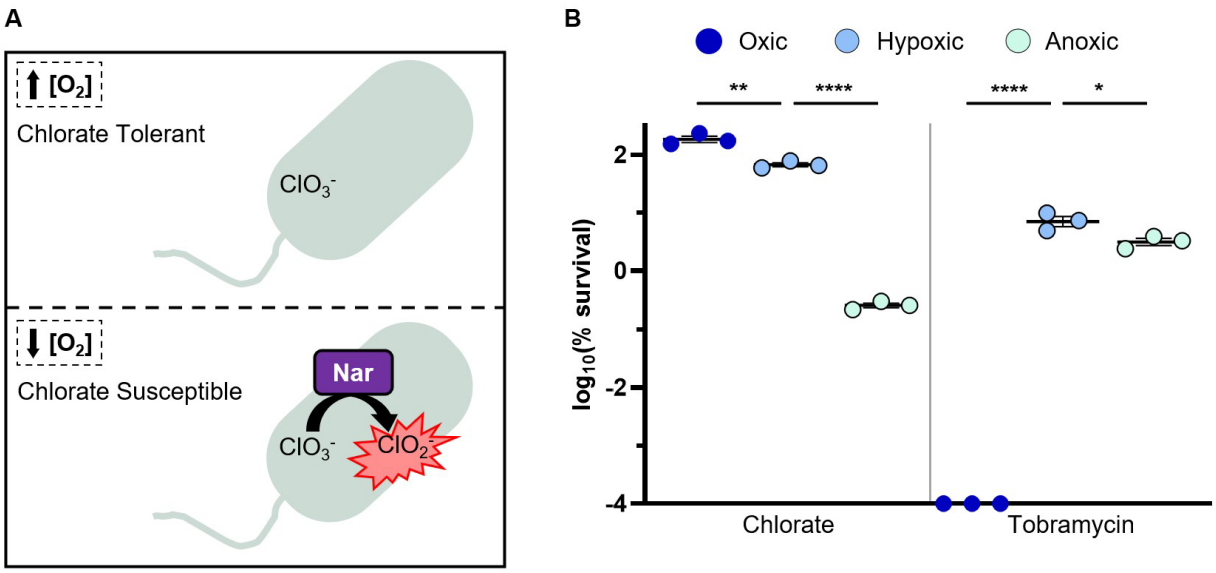

Figure 2. Chlorate treatment hijacks the hypoxically-induced Nar enzyme to kill anoxic, antibiotic-tolerant *P. aeruginosa*.

A. Chlorate is a prodrug: while chlorate itself is relatively nontoxic, it is reduced to toxic chlorite by the hypoxically-induced nitrate reductase, Nar. **B.** The $\log_{10}$(% survival) of high-density WT *P. aeruginosa* cultures treated with tobramycin or chlorate for 24 hours incubated under oxic, hypoxic, or anoxic conditions. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by one-way ANOVA; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

We next tested whether other antibiotics exhibit oxygen-dependent toxicity, similar to tobramycin. We focused our studies on different classes of anti-pseudomonal antibiotics, using concentrations that approximate those measured in patient samples (81, 82): tobramycin (aminoglycoside, 31.25 µg/mL), ciprofloxacin (fluoroquinolone, 1 µg/mL), colistin (polymyxin, 15 µg/mL), ceftazidime (cephalosporin, 10 µg/mL), in addition to chlorate (10 mM (1,064 µg/mL)). As before, *P. aeruginosa* cultures were incubated with or without antibiotic treatment under oxic or hypoxic conditions for 24 hours before plating cells to determine viability. Both tobramycin and ciprofloxacin were substantially less toxic to *P. aeruginosa* under hypoxic (~1.5-log killing) compared to oxic (> 6-log killing) conditions. However, colistin was only marginally

less effective at killing hypoxic cultures, and *P. aeruginosa* was completely resistant to ceftazidime under both hypoxic and oxic conditions (Figure 3). Thus, *P. aeruginosa* exhibits antibiotic recalcitrance under hypoxic conditions through two mechanisms: hypoxia-induced tolerance (tobramycin, ciprofloxacin) or via inherent, oxygen-independent resistance (colistin, ceftazidime).

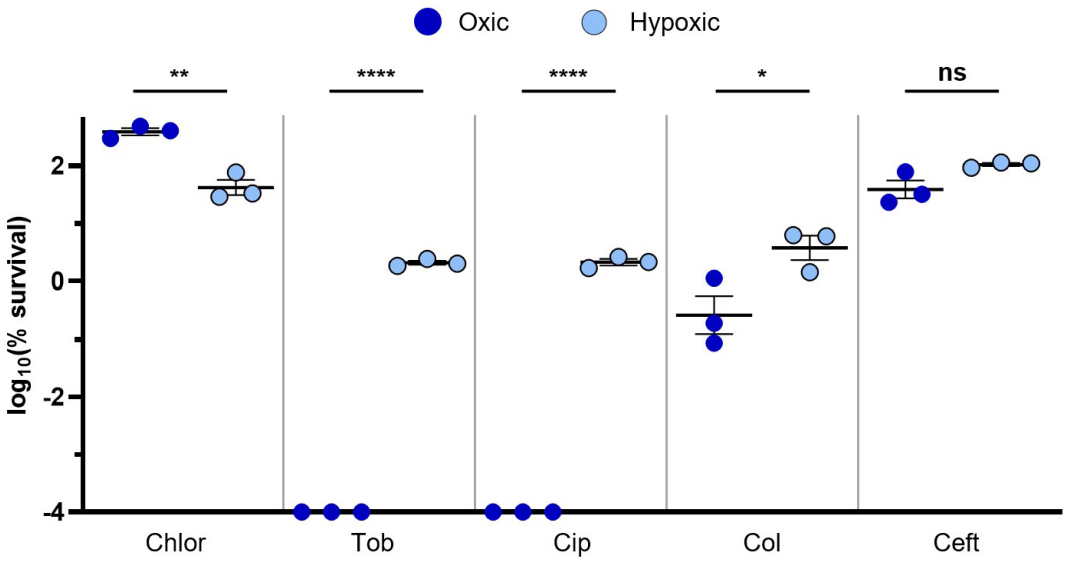

Figure 3: *P. aeruginosa* exhibits antibiotic recalcitrance due to resistance or hypoxia-induced antibiotic tolerance.

The $\log_{10}$(% survival) of high-density WT *P. aeruginosa* cultures treated with chlorate (Chlor), tobramycin (Tob), ciprofloxacin (Cip), colistin (Col), or ceftazidime (Ceft) for 24 hours incubated under hypoxic or oxic conditions. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by two-tailed t-tests; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Chlorate Synergizes with Different Classes of Antibiotics to Eliminate Hypoxic *P. aeruginosa* Populations

Although all tested drug treatments showed modest-to-no toxicity against hypoxic cultures of *P. aeruginosa* (Figure 3), we reasoned that each drug might impose sufficient stress

on the cell such that combined chlorate-antibiotic treatment would interact synergistically to efficiently kill hypoxic *P. aeruginosa* cultures. Synergy occurs when the efficacy of two drugs in combination is greater than the additive effect of each drug on its own. Indeed, we found that all chlorate-antibiotic combinations were synergistic (Figure 4). Despite chlorate-only treatment resulting in little-to-no killing of hypoxic *P. aeruginosa* cultures, chlorate's addition to each antibiotic treatment increased *P. aeruginosa* killing by more than 4 orders of magnitude for all tested classes of antibiotic (Figure 4). To confirm that chlorate reduction (i.e. chlorite generation) is required for chlorate-antibiotic synergy, we performed similar experiments using hypoxic cultures of a *P. aeruginosa* Δ*nar* strain (Figure 5). As expected, synergy is abolished in the Δ*nar* strain, with antibiotic-only and chlorate-antibiotic treatments resulting in the same amount of killing (Figure 5).

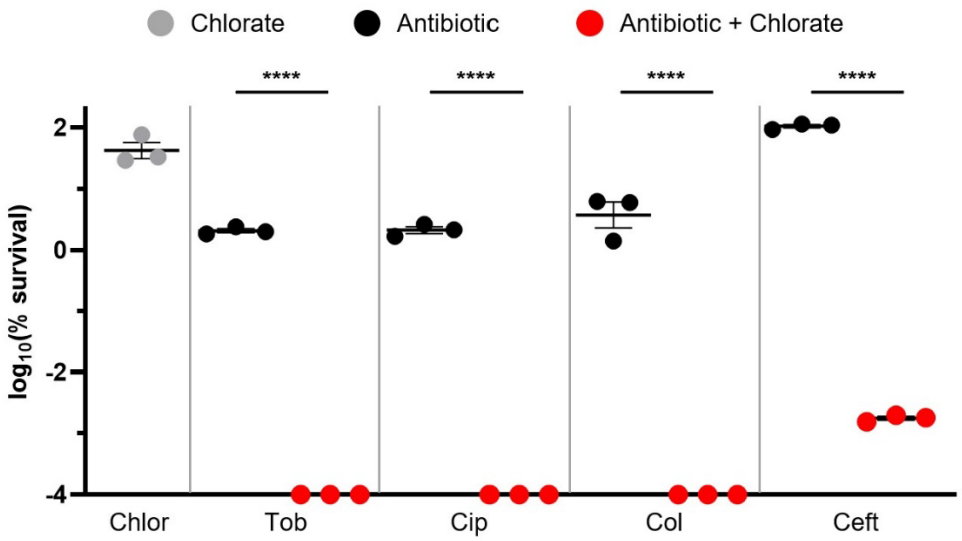

Figure 4. Chlorate synergizes with different classes of antibiotics to kill hypoxic *P. aeruginosa* populations.

The $\log_{10}$(% survival) of high-density WT *P. aeruginosa* cultures treated with chlorate (Chlor), tobramycin (Tob), ciprofloxacin (Cip), colistin (Col), ceftazidime (Ceft), or each chlorate-antibiotic combination for 24 hours incubated under hypoxic conditions. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by two-tailed t-tests; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

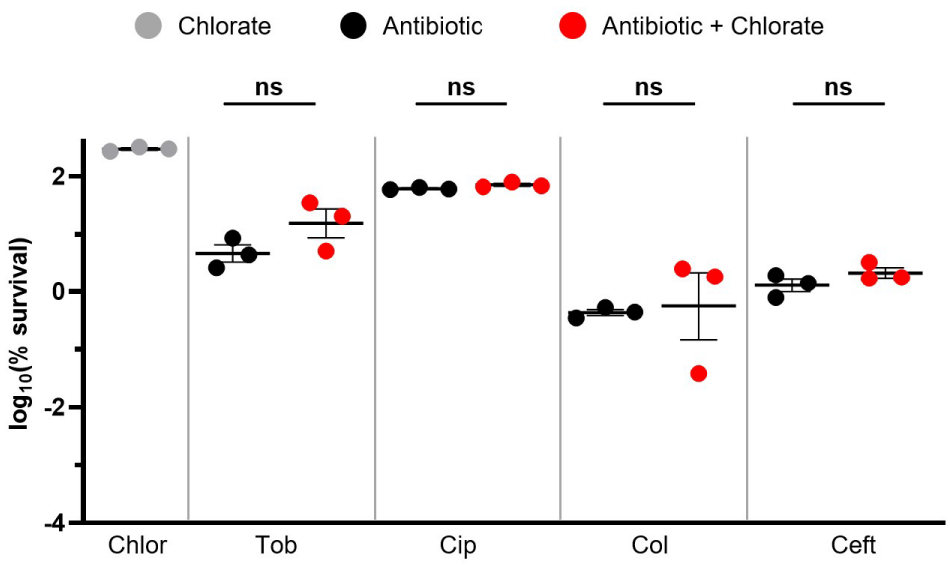

Figure 5. Chlorate-antibiotic synergy is Nar-dependent.

The $\log_{10}$(% survival) of high-density *Δnar P. aeruginosa* cultures treated with chlorate (Chlor), tobramycin (Tob), ciprofloxacin (Cip), colistin (Col), ceftazidime (Ceft), or each chlorate-antibiotic combination for 24 hours incubated under hypoxic conditions. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by two-tailed t-tests; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

We further examined the interaction between chlorate and ceftazidime, given that *P. aeruginosa* was resistant to ceftazidime-only treatment (Figure 3) yet highly susceptible to combined chlorate-ceftazidime treatment (Figure 4). Our initial experiments used ceftazidime at a concentration of 10 µg/mL, but we found that *P. aeruginosa* is resistant to much higher concentrations (Figure 6). By treating hypoxic cultures of *P. aeruginosa* for 24 hours with increasing ceftazidime concentrations, we saw that *P. aeruginosa* is resistant to ceftazidime concentrations as a high as 1,000 µg/mL, which is substantially higher than concentrations that can be achieved in the body when this drug is administered to patients (81, 82). Treating *P. aeruginosa* with the chlorate-ceftazidime combination reduced the effective ceftazidime dose by > 100-fold, since chlorate-ceftazidime is highly toxic to *P. aeruginosa* at a ceftazidime

concentration of just 10 µg/mL (3.5-log killing; Figure 6). Thus, chlorate can reduce the effective dose of an antibiotic to which *P. aeruginosa* is highly resistant.

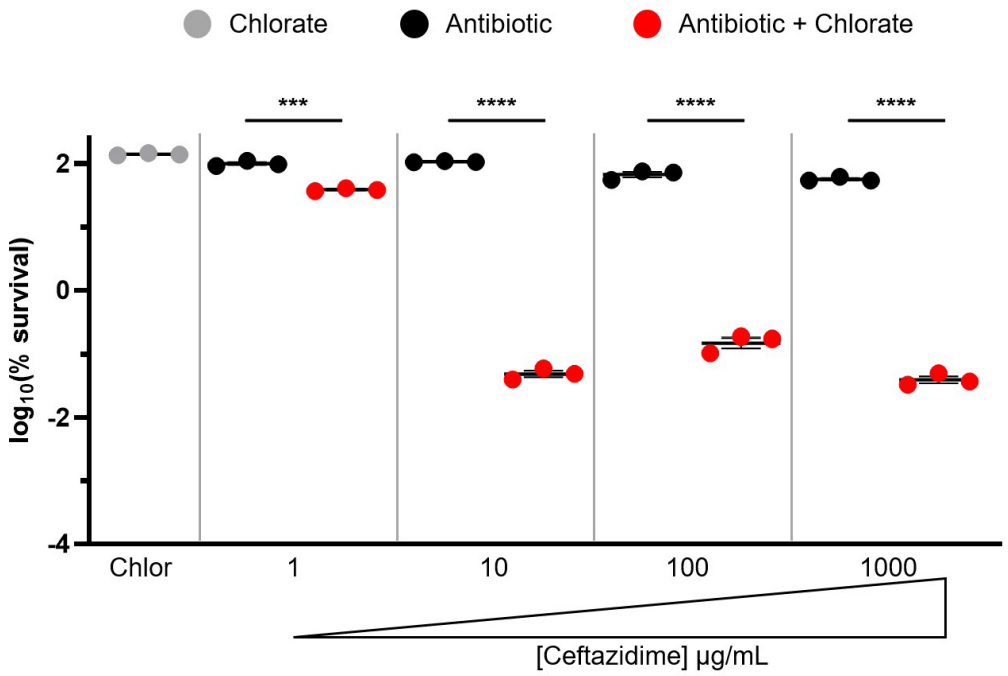

Figure 6: Chlorate substantially lowers the effective ceftazidime dose for killing hypoxic *P. aeruginosa*.

The $\log_{10}$(% survival) of high-density WT *P. aeruginosa* cultures treated with chlorate (Chlor), a range of ceftazidime (Ceft) concentrations, or chlorate-ceftazidime combinations for 24 hours incubated under hypoxic conditions. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by two-tailed t-tests; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Chlorate-Antibiotic Synergy Is Effective Across a Range of Oxygen Availabilities

Given that chlorate-only and some antibiotic-only treatments exhibit oxygen-dependent efficacy, we reasoned that chlorate-antibiotic synergy might also vary across different oxygen tensions. To begin testing this idea, we treated *P. aeruginosa* for 24 hours with different drug combinations under oxic, hypoxic, or anoxic conditions. We first tested chlorate-tobramycin

synergy (Figure 7). These drugs exhibit opposing oxygen-dependent phenotypes (i.e. chlorate and tobramycin are most effective under anoxic and oxic conditions, respectively). Given that tobramycin treatment eliminates *P. aeruginosa* to below our detection limit under oxic conditions, we cannot determine whether chlorate synergizes with tobramycin under these conditions (Figure 7A). However, similar to hypoxic conditions (Figure 7B), we found that chlorate-tobramycin treatment also kills anoxic cultures of *P. aeruginosa* to levels below our detection limit (Figure 7C). Since chlorate-only and tobramycin-only treatments both show some toxicity against anoxic cultures of *P. aeruginosa*, it was unsurprising yet promising to find that these drugs maintain synergy under anoxic conditions.

Next, we examined chlorate-ceftazidime synergy under different oxygen tensions. Unlike tobramycin, ceftazidime does not exhibit oxygen-dependent killing of *P. aeruginosa* (Figure 3). Interestingly, chlorate-ceftazidime treatment showed similar levels of synergistic killing across all oxygen tensions (~3.5- to 4.5-log killing; Figure 7). These results further demonstrate the potential for chlorate-antibiotic synergy under anoxic conditions (Figure 7C). More excitingly, these results suggest that chlorate treatment induces sufficient cellular stress to synergize with antibiotics even under more oxygenated conditions, when Nar is predicted to be less active. Importantly, we recognize that our use of the term “oxic” to describe our experimental conditions is relative, since even a vigorously shaken high-density culture will experience oxygen limitation. Overall, chlorate’s ability to synergize with antibiotics across different oxygen tensions is promising, given that pathogens like *P. aeruginosa* encounter a range of oxygen concentrations in host environments.

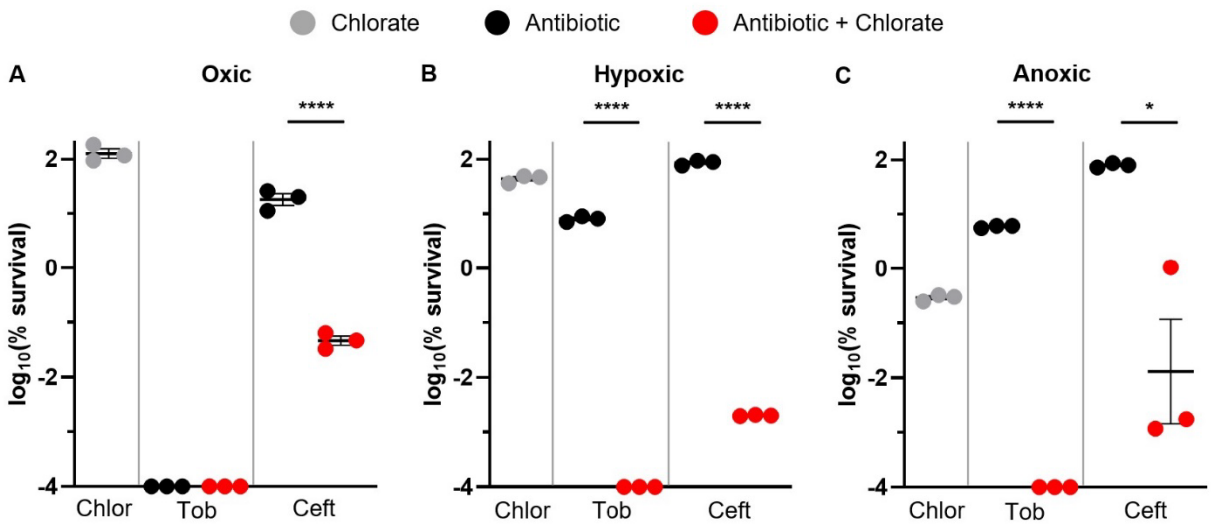

Figure 7: Chlorate-antibiotic synergy is effective across a range of oxygen availabilities.

The $\log_{10}$(% survival) of high-density WT *P. aeruginosa* cultures treated with chlorate (Chlor), tobramycin (Tob), ceftazidime (Ceft), or each chlorate-antibiotic combination for 24 hours incubated under **A.** oxic, **B.** hypoxic, or **C.** anoxic conditions. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by two-tailed t-tests; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Most Antibiotics Do Not Synergize with Other Classes of Antibiotics

Having observed chlorate's ability to synergize with different classes of antibiotics to overcome antibiotic resistance or tolerance, we next sought to determine whether antibiotics display a similar capacity for synergy. After treating hypoxic *P. aeruginosa* cultures for 24 hours with different antibiotic-antibiotic combinations, we found that most classes do not exhibit widespread synergy (Figure 8). In particular, colistin synergized with each of the other tested antibiotics (tobramycin, ciprofloxacin, ceftazidime) to reduce *P. aeruginosa* viability below our detection limit. However, the remaining three antibiotics did not synergize with each other

(Figure 8). Thus, of the five drugs included in our study, only two (chlorate and colistin) displayed a capacity for wide-ranging synergistic interactions.

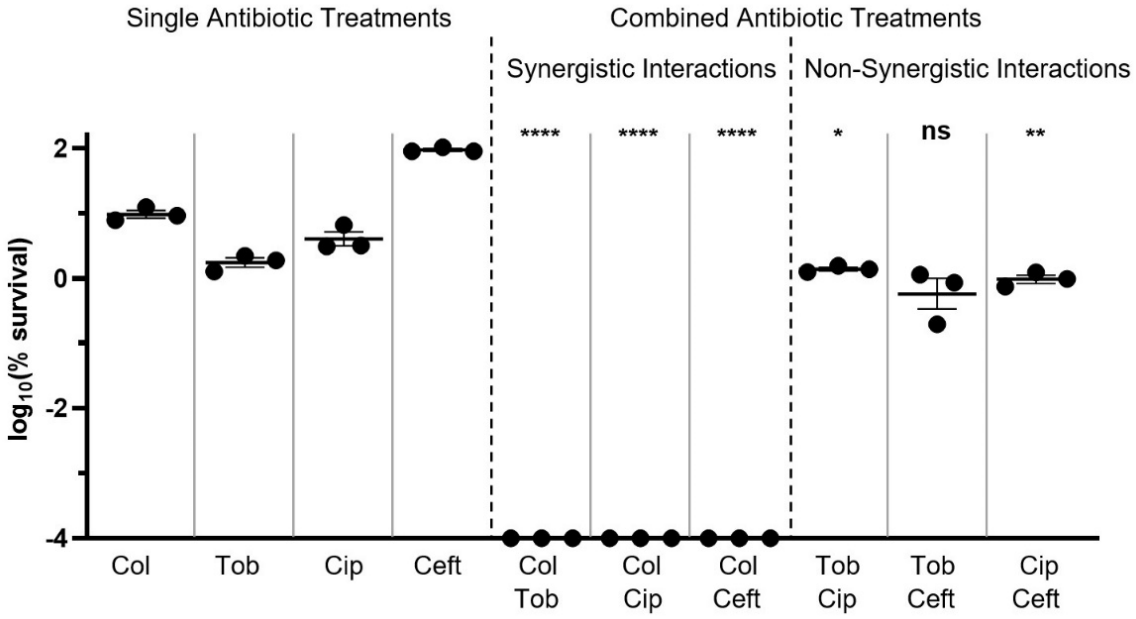

Figure 8: Most antibiotics do not exhibit synergistic interactions across different classes of antibiotics.

Under hypoxic conditions, the $\log_{10}$(% survival) of high-density WT *P. aeruginosa* cultures treated with colistin (Col), tobramycin (Tob), ciprofloxacin (Cip), ceftazidime (Ceft), or each antibiotic-antibiotic combination for 24 hours. Compared to single-antibiotic treatments, only colistin displays synergistic interactions with other classes of antibiotics. All data points on the x-axis are below the limit of detection. Data show the means of 3 replicates and error bars show standard error of the mean. Statistical significance was determined by one-way ANOVA; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ for each antibiotic-antibiotic combination compared to the single-antibiotic treatments that comprise the combination.

Discussion

Here, we have presented chlorate-antibiotic combinations as a method to kill hypoxic *P. aeruginosa* populations (Figure 4) where antibiotics and chlorate alone are only marginally effective (Figure 3). Chlorate synergizes with antibiotics from a range of classes (Figure 4) which is a unique ability considering most antibiotic-antibiotic combinations do not display synergy (Figure 8). Chlorate-antibiotic synergy is Nar-dependent (Figure 5) and reduces the effective dose of ceftazidime by more than 100-fold for efficient killing of hypoxic *P. aeruginosa* (Figure 6). Additionally, chlorate-antibiotic synergy is maintained across a range of oxygen tensions (Figure 7) that are known to occur in chronic infection sites (83, 84). Of note, our experiments used physiologically relevant antibiotic concentrations measured in patient samples (85–87), further supporting chlorate's potential for success in the clinic. Together, our findings underscore the possibility of using chlorate-antibiotic combinations to enhance the efficacy of antibiotic treatments against antibiotic recalcitrant *P. aeruginosa* infections.

In addition to chlorate, colistin also exhibited synergistic interactions with other classes of antibiotics (Figure 8). Colistin was reported to be more lethal to oxygen-limited *P. aeruginosa* compared to oxygen-replete populations (14). Although both chlorate and colistin display broad synergy, we do not expect that these drugs achieve synergy by inducing similar stresses; chlorate-colistin treatment was also synergistic, suggesting that they impose different types of stress on *P. aeruginosa*. Thus, broad-range synergy with different classes of antibiotics can likely be achieved by targeting multiple cellular processes.

Our results also suggest that there may be more than one metabolic state within oxygen-limited *P. aeruginosa* populations, including persister cells. Persister cells are a subset of a clonal population that survives antibiotic exposure by entering into a metabolically inactive,

dormant state (16). The presence of persister cells may account for the observation that increased ceftazidime concentrations did not lead to increased killing during chlorate-ceftazidime treatment (Figure 6). Rather, we observed that the same number of cells survived the treatment regardless of drug concentration, suggesting that a small persister cell population could not be killed by this particular drug combination. Because *P. aeruginosa* populations are known to enter persister cell states during chronic infections (88), enhancing our understanding of the metabolic states that enable bacteria to survive oxygen limitation is valuable for designing therapeutic approaches to resolve recalcitrant infections.

The discovery rate of new antibiotics has slowed over the past several decades while rates of antimicrobial resistance are rising (89, 90) which necessitates new approaches for identifying next-generation antimicrobials. Identifying synergistic drug combinations is increasingly recognized as an important method for combatting antibiotic treatment failure (91), but synergistic drug combinations are rare and thus screens are resource-intensive. For instance, testing two-drug combinations of a small molecule library of 1000 drugs will require one million pairwise combinations (92). The most common method for confirming synergistic drug interactions is the checkerboard assay (93), which measures growth inhibition (i.e. minimum inhibitory concentration) of each drug alone or in combination across a range of concentrations (91, 93–95). Using growth inhibition as an output metric is useful for identifying drug combinations that overcome antibiotic resistance, however, it is less likely to identify drug combinations that are effective against slow-growing, antibiotic tolerant bacterial populations.

Our results also demonstrate the necessity of empirical synergy screens, since we show that the synergistic potential of a drug cannot be predicted based on its individual toxicity. For instance, both ceftazidime and chlorate might be discarded as potential therapeutics for killing

slow-growing cells because they are nontoxic to hypoxic cultures of *P. aeruginosa*. However, combined chlorate-ceftazidime treatment achieves substantial killing of *P. aeruginosa* under a range of oxygen tensions. Although each drug on its own is unable to kill *P. aeruginosa*, we assume that each drug still exerts some stress on the cell that, in combination, overwhelms *P. aeruginosa* to become highly lethal.

Ultimately, our ability to identify novel, synergistic drug interactions is limited by our understanding of drug synergy mechanisms. We do not know why some drug combinations are remarkably lethal to bacteria (i.e. synergistic) whereas other combinations exhibit additive or even antagonist interactions (96). We have a mechanistic understanding of just one synergistic antimicrobial drug pairing (trimethoprim-sulfamethoxazole), and this mechanism was only recently elucidated, having been mischaracterized for years (97). A deeper understanding of the biological underpinnings of drug synergy mechanisms will facilitate the rational design of novel synergistic drug pairings. Chlorate's ability to synergize with different drug classes presents an opportunity to understand the mechanisms of drug synergy, which is sorely needed to combat antibiotic failure due to resistance or tolerance.

In addition to its capacity for drug synergy, other attributes recommend continued study of chlorate's therapeutic potential. First, there may be selective pressure against evolving chlorate resistance within chronic infection environments. Our prior work determined that the primary mechanism for chlorate resistance in *P. aeruginosa* is acquiring mutations that reduce nitrate respiration, particularly in *nar* genes (50). However, in other work we showed that *P. aeruginosa* requires *nar* to cause a persistent, chronic infection; this suggests that evolving chlorate resistance will hinder *P. aeruginosa* fitness in oxygen-limited host environments (49). Second, chlorate holds promise for treating infections caused by opportunistic pathogens beyond

P. aeruginosa, since many bacteria respire nitrate using Nar including *E. coli*, *L. pneumophila*, *S. enterica*, *C. diphtheriae*, *M. tuberculosis*, *Burkholderia* spp., *Brucella* spp., and *S. aureus* (32–45). The potential for chlorate treatment to target a range of especially problematic facultative anaerobes motivates the continued study of chlorate and antibiotic-chlorate combinations. Future studies will explore the chlorate-antibiotic synergy across other bacteria and bacterial communities as well as investigate the mechanism(s) of chlorate-antibiotic synergy.

Conclusion

P. aeruginosa is isolated from different types of chronic infections where antibiotic treatments fail, leaving patients to struggle with these infections for months to decades. In this thesis, I have shown data that supports the use of chlorate in combination with different classes of antibiotics for killing hypoxic populations of *P. aeruginosa*. While neither chlorate nor antibiotics on their own can effectively kill hypoxic *P. aeruginosa*, chlorate synergizes with antibiotics that target different bacterial growth processes to effectively kill these bacteria. *P. aeruginosa* displays antibiotic resistance or antibiotic tolerance to each of the antibiotics tested in our studies, highlighting that chlorate addition overcomes both antibiotic resistance and antibiotic tolerance. Chlorate displays synergy with all of the antibiotics we tested, even though most antibiotic-antibiotic combinations did not show synergy, suggesting that chlorate has a unique ability to enhance the toxicity of other drugs. We confirmed that chlorate-antibiotic synergy requires the Nar enzyme, which is responsible for activating chlorate to its toxic form. Surprisingly, we found that synergy between antibiotics and chlorate occurs under a range of oxygen availabilities. These results recommend combined chlorate-antibiotic therapy as a promising new treatment for resolving chronic infections that are difficult to treat with current antibiotic therapies.

Drug synergy has become an important tool in combatting the rise of antibiotic resistance in the face of declining rates of drug discovery (89–91). Currently, we cannot predict events of drug synergy, which are rare, so we must empirically test all possible drug combinations (91, 94, 98). Our current methods for pairwise synergy testing are effective at identifying drug combinations that inhibit bacterial growth (i.e. drugs that combat antibiotic resistance), but do not necessarily identify combinations that kill bacteria, including slow-growing bacteria (i.e.

drugs that combat antibiotic tolerance) (91, 94). Thus, it is critical to consider different methods if the goal of the screen is to find drug combinations that target slow-growing bacteria.

The astonishing piece of this synergy puzzle is that it is, currently, completely unpredictable. Neither chlorate nor antibiotics efficiently kill hypoxic *P. aeruginosa*, yet together they eradicate *P. aeruginosa*. We could not have predicted this outcome based on the limited killing achieved by each drug on its own. For combined chlorate-antibiotic treatment to work so well, each drug must exert some amount of stress that is insufficient to cause cell death on its own, but completely overwhelms the cell when these drugs and stresses are imposed together. If we looked to find synergistic drug pairings based on individual toxicities, we would have completely missed antibiotic-chlorate combinations. In continued efforts to find drugs that synergize with each other, we cannot discount drugs just because they seem ineffective on their own. If we want to predict drug synergy with more accuracy or design better screens for detecting synergistic pairings, we need to improve our understanding of the mechanisms of drug synergy. Our current knowledge of the mechanisms of drug synergy is incredibly limited and is largely speculative (96, 97, 99). Therefore, chlorate represents a useful model for further study to understand how it synergizes well with many antibiotics.

The capacity for nitrate respiration using Nar is shared among many bacteria beyond *P. aeruginosa*. Some of the bacteria that pose the largest global threats to human health because of their antimicrobial resistance (9) have Nar and can use it to respire nitrate in host infection environments, particularly *Salmonella enterica* and *Staphylococcus aureus* (32, 33, 35, 38). These bacteria, and *P. aeruginosa,* are rapidly gaining resistance against additional classes of antibiotics (9). Here, we have shown that chlorate-antibiotic combinations can overcome cases of antibiotic resistance in *P. aeruginosa* and we are hopeful that the same could be true with other

Nar-containing bacteria. While bacterial resistance against chlorate is possible, our previous work supports that chlorate resistance would negatively impact *P. aeruginosa* growth in oxygen-limited host environments (49, 50). We determined that the main mechanism for resistance to chlorate in *P. aeruginosa* is to obtain genetic changes in the *nar* gene that reduce nitrate respiration (50). We also found that *P. aeruginosa* cells need to have *nar* to cause a chronic infection in mice (49). Thus, *P. aeruginosa* will struggle to grow in oxygen-limited environments if the cells evolve chlorate resistance.

Chlorate-antibiotic combinations, as we have shown here, display a unique ability to kill hypoxic *P. aeruginosa* cells that otherwise display antibiotic resistance or tolerance. We next want to determine the mechanism of chlorate-antibiotic synergy. Similarly, we want to test the effectiveness of these synergistic interactions against other bacterial pathogens or bacterial communities. Chlorate-antibiotic combinations have the potential to save the world from many difficult-to-treat chronic infections.

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