

UNIVERSITY OF CALGARY

Screening and Characterization of Antimicrobial Compounds and Material against *Vibrio*
cholerae

by

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A THESIS

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Abstract

Antibiotic resistance (ABR) is a multisectoral global threat. With an increasing number of critical pathogens acquiring resistance against multiple classes of antibiotics, we face the terror of the post-antibiotic era. *Vibrio cholerae*, the cholera-causing pathogen, is no exception to the menace of multi-drug resistance. Strains from worldwide outbreaks have shown resistance to all antibiotics used to treat cholera, including ciprofloxacin. With cholera being prevalent in developing countries and regions affected by natural and anthropogenic disasters, an increase in fatalities is a rising concern in these areas with the number of effective antibiotics rapidly decreasing.

This thesis explores the alternative approaches to treating cholera and controlling the spread of ABR pathogens in the environment. First, a library of 400 chemical compounds with antimicrobial activities, the Pathogen Box, is screened for compounds that exhibit killing or inhibiting activities against *V. cholerae*. Two compounds, MMV687807 and MMV675968, demonstrated bactericidal and bacteriostatic activity against the pathogen with high efficacy, respectively. RNA-seq analyses of *V. cholerae* treated with each compound revealed that their impact on multiple cellular functions, including carbon metabolism and iron homeostasis. Whole genome sequencing of spontaneous resistance mutants uncovered that activation of an efflux system via mutation to their regulator confers resistance to MMV687807. MMV675968 was identified to function similar to trimethoprim, targeting the dihydrofolate reductase of *V. cholerae*. This compound has the potential to be developed as a trimethoprim replacement with a minimum inhibitory concentration lower by 14-fold.

Next, the antibacterial property of a newly synthesized cellulose aerogel cross-linked with different concentrations of silver/lignin nanoparticles (lignin only, 1Ag/L, 2Ag/L, and

3Ag/L) was studied in collaboration with Xiao He. Each aerogel was incubated with Gram-positive or Gram-negative pathogens, and surviving colonies were counted. Increase in killing efficiency was observed with increasing concentration of Ag/L nanoparticles. The cellulose aerogel with 3 Ag/L exhibited exceptional killing across all tested species: *E. coli*: > 99.99%, *P. aeruginosa*: > 99.9%, *V. cholera*: > 99.99%, *S. aureus*: > 99.99%, *B. subtilis*: > 97.4%.

Through screening small molecules and exploring synthetic material cross-linked with nanoparticles, this thesis demonstrates the significance of developing new tools to inhibit bacterial pathogens and fight the spread of ABR.

Preface

Chapter 2 is a published open-access article as **Kim, H.**, Burkinshaw, B. J., Lam, L. G., Manera, K., and Dong, T. G., “Identification of small molecule inhibitors of the pathogen box against *V. cholerae*”. 2021. Microbiology Spectrum, volume 9, number 3, e00739-21, doi.org/10.1128/Spectrum.00739-21. The journal allows unrestricted reuse of the article under the Creative Commons CC BY license.

Kim, H provided writing contributions, designed, and performed all the experiments except for Figure 2-1 D and E, RNA-sequencing library preparation, and whole-genome sequencing preparation/analysis which were carried out by Burkinshaw B. J. and Lam, L. G. All method and data contributions are mentioned throughout the chapter.

Chapter 3 is original writing with modified figures from a published work He, X., **Kim, H.**, Dong, T. G., Gates, I., and Lu, Q., “Green synthesis of Ag/lignin nanoparticle-loaded cellulose aerogel for catalytic degradation and antimicrobial applications”. 2022. Cellulose, volume 29, pages 9341 – 9360. Kim, H designed and performed experiments related to antimicrobial activity of the Ag/lignin-cellulose aerogel against different species of bacteria. Data and figures that are adapted from the original article is indicated in the figure legends. Letters of permission from the journal and the first author are included in Appendix A.

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List Of Symbols, Abbreviations, and Nomenclature

Name	Definition
°C	Degree Celsius
µL	Microlitre
ABR	Antibiotic resistance
Ag/L	Silver/lignin
ARB	Antibiotic resistant bacteria
CAMHB	Cation-adjusted Mueller-Hinton broth
CCCP	Carbonyl cyanide <i>m</i> -chlorophenylhydrazone
CEC	Contaminants of emerging concern
CFTR	Cystic fibrosis transmembrane receptor
CFU	Colony forming units
CT	Cholera toxin
CTA	Cholera toxin A subunit
CTB	Cholera toxin B subunit
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
ECH	Epichlorohydrin
HepG2	Human hepatoma cells
HGT	Horizontal gene transfer
IC20	Inhibitory concentration of 20%
ICE	Integration conjugative element
LB	Lysogeny broth
MDR	Multidrug resistant
mg	Milligram
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
mL	Millilitre
MMV	Medicine for Malaria Venture

NP	Nanoparticle
NPET	Nascent peptide exit tunnel
ORS	Oral rehydration solution
PABA	<i>p</i> -aminobenzoic acid
PPCP	Pharmaceutical and personal care product
rpm	Rotations per minute
TCP	Toxin-coregulated pilus
UTI	Urinary tract infection
UV	Ultraviolet
VBNC	Viable but nonculturable cells
VPS	<i>Vibrio</i> polysaccharide
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild type
WWTP	Wastewater treatment plant
XDR	Extensively drug resistant

Chapter One: Introduction

1.1 A tight race between antibiotics and the development of resistance against them

1.1.1 What are antibiotics?

Although antibiotics were introduced less than a century ago for clinical usage, humans have been practicing the concept of fighting infections using natural remedies for many millennia. A great example of this is the use of mouldy bread to treat wounds and burns in ancient Egypt, Greece, and China (1, 2). Since then, significant breakthroughs in the field have been achieved by numerous scientists, starting with Antonie van Leeuwenhoek's first description of micro-organisms in the 1600s, leading to the discovery of a relationship between infections and their causative agents by Robert Koch and Louis Pasteur. In the 1800s, numerous scientists also explored possible treatments to combat infections like syphilis and gonorrhoea (3). These pioneering findings laid the foundation for how we approach infectious diseases, leading to the discovery of the antibiotics.

In the 1900s, Paul Ehrlich's work in finding the "magic bullet" against syphilis marked the beginning of systematic screening of synthetic chemical compounds with antagonistic properties against infectious pathogens. During this time, there was a rapid growth in the German chemical dye industry, and Ehrlich was working on histological stains when he discovered certain dyes had specific killing abilities. The compound 606, active against syphilis and later named "Salvarsan", was the first commercialized antibiotic. Continuing Ehrlich's work, another German pathologist, Gerhard Domagk, followed in his footsteps by searching for antibiotics using a library of dye compounds. In the 1930s, he discovered a novel compound effective against *Streptococcus pyogenes*, which was commercialized as "Prontosil". This was the discovery of a first sulfonamide with minimal human toxicity, and it was widely used during

World War II. This group of antibiotics is still prevalent today (1, 4–6). The following decades, 1940s–1970s, marked the Golden Age of antibiotics: the discovery of penicillin by Alexander Fleming and its mass production by 1945, the work of Selman Waksman and Albert Schatz in finding streptomycin active against *Mycobacterium tuberculosis*, and screening of soil microbes, specifically actinomycetes, as the primary source of earlier groups of antibiotics (7, 8).

The word “antibiotics” comes from “antibiosis”, which refers to any biological relationship where one organism ceases the existence of the other for its survival (3). Originally, the term “antibiotics” was used specifically to refer to antibacterial compounds derived from natural sources. However, in modern days, it has expanded to include compounds active against various micro-organisms and those produced semi-synthetically or fully synthetically (9).

1.1.2 Classification of antibiotics

Antibiotics are divided into two major groups: bactericidal vs. bacteriostatic. Bactericidal drugs kill bacteria, whereas bacteriostatic drugs inhibit growth. The difference is that organisms exposed to bacteriostatic drugs can fully recover once the stress is removed (10). The compounds can be further classified into specific categories depending on their chemical structures and modes of actions. A list of common classes includes β -lactams, aminoglycosides, trimethoprim/sulfonamides, fluoroquinolones, tetracyclines, and macrolides. They can also be grouped into larger categories based on their cellular targets: the cell wall synthesis, the nucleic acid synthesis, and the protein synthesis.

Cell wall:

The synthesis of the cell wall is one of the essential cellular pathways for all species of bacteria for their growth and reproduction. Importantly, this structure is unique to prokaryotic

cells, making it an even better candidate for antibiotics, and β -lactams are undoubtedly the largest group of inhibitors that target its synthesis (11).

β -lactams:

All β -lactams share a common core structure of four-member cyclic amide, the β -lactam ring. Earlier research demonstrated that cells exposed to β -lactams exhibit filamentous physiology, suggesting a disruption in cellular division and shape maintenance. It was later found that β -lactams target the cell envelope, specifically the peptidoglycan (12). The peptidoglycan is a rigid heteropolymer that serves the structural role of the bacterial envelope, protecting the cell from osmotic pressures. The synthesis of peptidoglycan involves polymerizing linear glycan chains and crosslinking these chains with the attached peptides. The enzymes responsible for the latter step of the synthesis, known as transpeptidases or penicillin-binding proteins, are the primary target of the β -lactams. This ultimately results in improper formation of the peptidoglycan leading to cellular lysis and eventually death (13, 14). Penicillin is the first β -lactam discovered, followed by the discovery of cephalosporin from fungus *Cephalosporium acremonium* in 1945. Both were originally introduced as drugs against Gram-positive species, particularly the strepto- and staphylococci. Through semisynthetic chemical modifications, their derivatives and subsequent generations gained activity against a broad-spectrum of species, including Gram-negative species (15). This group still remains one of the most widely used antibiotics in medicine today.

Nucleic acid:

(Fluoro)quinolones:

Quinolones are characterized by their bicyclic core, predominantly consisting of 4-quinolone or 1,8-naphthyridine rings. They are one of the few fully chemically synthetic classes

of antibiotics that exhibit significant rivalry against β -lactams (5, 16). The mechanism of action of quinolones involves targeting two essential enzymes: DNA gyrase and topoisomerase IV. These enzymes belong to the group of type II topoisomerases which are required for regulating topological changes in DNA, especially in introducing negative supercoiling and releasing positive supercoiling. These topological changes are vital for DNA segregation/replication, transcription, and cellular division. Interfering with the activities of these enzymes using quinolones leads to double-strand breaks in DNA, leading to cell death (17, 18).

The first quinolone was identified as a by-product of chloroquine synthesis, an antimalarial compound. Although antimicrobial, the first compound never got commercially developed due to the lack of clinical significance. It wasn't until the discovery of nalidixic acid, the first-generation quinolone, that this class of compounds were seen as an effective treatment against bacterial infections, notably the urinary tract infections (UTIs) (19). Development of second-generation quinolones is marked by the addition of fluorine to C6 position and piperazine ring to C7 position, the beginning of fluoroquinolones. These compounds displayed a broader spectrum of activity against Gram-negative species with enhanced pharmacokinetics and pharmacodynamics. Ciprofloxacin, one of the most frequently prescribed fluoroquinolones, belongs to this generation (20). The third and fourth generations are characterized by their extended spectrum towards Gram-positive species and anaerobes (21). Through chemical modifications, the application of fluoroquinolones has been greatly extended from treating UTIs to many other acute, life-threatening infections. Unfortunately, fluoroquinolones were also found to be associated with serious adverse effects on tendons, muscles, and nervous system causing many countries to re-label and restrict their usage on patients (22, 23).

Sulfonamides and trimethoprim:

As described, the sulfonamides are the oldest and the first class of synthetic antibiotics to be produced at a large commercial scale before the discovery of penicillin. These compounds are characterized by the sulfanilamide core, consisting of sulfonyl group attached to nitrogen, and thousands of derivatives have been synthesized (6). Sulfonamides act as analogues of *p*-aminobenzoic acid (PABA), the enzymatic substrate, and inhibit the activity of dihydropteroate synthase (DHPS). This enzyme is essential in the metabolism of folic acid, specifically in the formation of dihydrofolate before it is reduced into tetrahydrofolate. The end-product of this pathway is crucial in the synthesis of many metabolites, including purines, directly affecting the nucleic acid synthesis (24, 25). Because the activity of sulfonamides is bacteriostatic, they are often used in combination with the trimethoprim (26). Trimethoprim is a synthetic derivative of 2,4-diaminopyrimidine known to inhibit folate reductase, precisely the dihydrofolate reductase (DHFR). It was originally found as a potentiator of sulfamethoxazole since it targets the enzyme responsible for reducing dihydrofolate into tetrahydrofolate, which is a sequential reaction following the enzyme targeted by sulfonamides (27–29). Co-trimoxazole, a combination of trimethoprim and sulfamethoxazole, is also one of the widely used antibiotics in current medicine along with other classes of drugs.

Protein synthesis:

Bacterial protein synthesis requires the formation of ribosomes, a complex machinery composed of small subunit (30S) and large subunit (50S). Ribosomes function as a platform for translation, a process of producing protein via mRNA reading. Because the process is essential for all living organisms, many antibiotics were discovered to target the pathway (30).

Aminoglycosides:

Aminoglycosides are characterized by their amino sugars connected via glycosidic bonds and studied to target 30S subunit of bacterial ribosome (31). Compounds in this class must possess an ability to penetrate the cellular envelope to reach their target. Once reached, the compounds directly bind with the subunit, leading to non-specific binding of tRNAs and the production of defective proteins (32). These proteins incorporated into the cellular membrane increase the uptake of the compounds, resulting in the accumulation of the compounds and non-functional proteins, which cause the bactericidal activity of the drugs (33).

Streptomycin, the first aminoglycoside discovered, was isolated from *Streptomyces* strains and presented antimicrobial activity against Gram-negative species and *M. tuberculosis*, marking the beginning of screening *Actinomycetes* (5). Systematic screening of soil for novel *Actinomycetes* unveiled many more potent compounds in this class, including neomycin, gentamicin, and amikacin (kanamycin derivative). The broad-spectrum of targets grants these compounds to be active against life-threatening infections, such as pneumonia in cystic fibrosis patients and as a second line of treatment against tuberculosis (34). Unfortunately, this class of compounds has been associated with ototoxicity (damage to inner hair cells) and nephrotoxicity (damage to renal nephrons), causing mild to irreversible impacts. These effects limit their utilization and showcase the importance of choosing the correct dosage during clinical applications (34–36).

Tetracyclines:

Tetracyclines are identified by the presence of a linear four cyclic structure as their core with varying side chains. Like the aminoglycosides, tetracyclines target the 30S subunit of the ribosome. Specifically, it blocks the docking of aminoacyl-tRNA into the A-site and interferes the codon-anticodon recognition, stalling the elongation process. The binding of tetracyclines to

the ribosomes is reversible, making the drugs bacteriostatic rather than bactericidal (30, 37). Tetracyclines must traverse the cellular membrane since they are active against the intracellular target. They are known to be chelators of divalent cation, especially Mg^{2+} , forming a complex in the extracellular space. The complex passes through the outer membrane of Gram-negative via their porins, OmpF and OmpC. The complex dissociates once in the periplasm, and tetracyclines' natural lipophilicity and neutral charge allow them to move across the inner membrane. The latter method is also known to be used to move across the membranes of Gram-positive targets (38–40).

Chlortetracycline (Aureomycin) and oxytetracycline (Terramycin) are the first tetracyclines discovered in the 1940s, screened from the broths of *S. aureofaciens* and *S. rimosus*, respectively (41). Since discovery, they have been widely used against both Gram-positive and Gram-negative species, intracellular infections like chlamydia, and as a malaria prophylaxis. It also is the first antibiotics to exhibit non-clinical utility, such as an animal growth-promoting food additive and acne treatment (41, 42). Rapid acquisition of resistance against the tetracyclines accelerated the process of finding the semisynthetic second-generation compounds like doxycycline and minocycline. Unfortunately, the pathogens also quickly acquired resistance against this generation, which led to the development of fully synthetic third-generation compounds, the glycylcyclines. This generation was specifically designed to overcome the most common resistance mechanisms against tetracyclines in hopes of bringing back the clinical usage of older generations (43, 44)

Macrolides:

Erythromycin, the first commercialized macrolide, was discovered to be produced by *Saccharopolyspora erythraea* from soil sample collected in the Philippines in the 1950s (42).

This class of compounds is characterized by the presence of 14-, 15-, or 16-carbon macrocyclic lactone with various sugars attached. (45). Macrolides act as a bacteriostatic drug by binding to the nascent peptide exit tunnel (NPET) in the 50S subunit of a ribosome and obstructing the elongation of a forming polypeptide (46). In addition to causing premature dissociation of polypeptides, macrolides bind to specific amino acid sequences, known as macrolide arrest motifs, as their target and directly prevent formation of peptide bonds, indicating a degree of protein/species specificity (47, 48).

Like many other natural classes of antibiotics, macrolides have also been chemically modified to improve their activity and spectrum since the discovery of erythromycin (46). Unlike the natural first generation, the second generation, such as azithromycin, was developed through modifications to erythromycin to give better acid stability and a broader spectrum of activity against Gram-negative species. However, the rise of resistance diminished the efficacy of both generations, which led to synthesis of ketolides, the third generation. This recent generation is also able to target pathogens resistant to earlier generations (49, 50).

1.1.3 Mechanisms of antibiotic resistance (ABR)

As both clinical and environmental microbes encounter diverse classes of antibiotics, they develop equally diverse mechanisms of resistance to evade self-toxicity and counteract the attack of antibiotic. The mechanisms can be classified into two major groups: intrinsic vs. acquired. Intrinsic resistance refers to the innate resistance of bacteria through their structures and produced proteins, whereas acquired resistance refers to gaining of mobile genetic elements (MGEs) that contain resistance mechanism via horizontal gene transfer (HGT) (51, 52). They could be further divided into common mechanisms shared among both groups, based on the exact mechanism of how pathogens fight off the effects of the drugs. It is important to note that a

single pathogen may possess multiple mechanisms simultaneously, encompassing both intrinsic and acquired mechanisms.

Decreased permeability and efflux pumps:

One of the best examples of resistance through decreased permeability is the outer membrane of Gram-negative bacteria, which are inherently harder to be penetrated by larger compounds due to the presence of different structures like lipopolysaccharide. They are also naturally resistant to hydrophobic compounds (53). However, antibiotics are still able to pass through this natural barrier through chelating ions and using porins as their transportation methods. For example, tetracycline have been shown to utilize outer membrane porins. Decreasing membrane permeability through altering the number of embedded porins or replacing to more selective proteins has shown to cause resistance against many antibiotics, including tetracyclines and aminoglycosides (54, 55).

Efflux pumps are specialized transport systems that are expressed by both Gram-positive and Gram-negative species as a stress response to export toxic molecules out of the cells. Their substrates are not limited to antibiotics but include heavy metals, secondary metabolites, pollutants, and many more (56). Efflux pumps involved in ABR are referred as multidrug efflux pumps and can be both intrinsic and acquired. There are currently six families of efflux pumps identified, and exposure to antibiotics can lead to overexpression of the innate systems and/or acquisition of MGEs containing these systems (57). How these pumps present resistance in different species has been extensively studied over the decades (58–60). The spread of the efflux pumps through gaining *tet* genes is a great example of multiple pathogens becoming resistant against a whole class of antibiotics, tetracyclines (61). Combined with reduced permeability to the compounds, these mechanisms confer one of the fastest and most efficient resistance in cells.

Antibiotic inactivation:

There are two major pathways to achieve antibiotic inactivation: 1) modification of the entered drug and 2) direct deactivation of the drug. The modification involves production of modifying enzymes by the targeted cells that add chemical functional groups to the drugs, which in return blocks the binding of drugs to their targets (62). Resistance against aminoglycosides was observed since the 1970s, and the family of aminoglycoside modifying enzymes is the most common cause of widespread dissemination of resistance against aminoglycosides among pathogens (63). This family of proteins contains three classes of enzymes, each responsible for incorporating different functional groups to the drug: 1) acetyltransferases, 2) nucleotidyltransferases, and 3) phosphotransferases. The names present which functional groups they are in charge of adding to the aminoglycosides (64). With the rapid evolution of these enzymes (with more than a hundred variants identified) and the ability of their genes to be loaded on MGEs, aminoglycosides are rendered almost ineffective in clinical settings. This highlights the importance of discovering novel ways to revive this class of antibiotics (65).

β -lactams, which include penicillin, methicillin, and ampicillin, are among the one of the first antibiotics to be produced and utilized at a commercial level. It is not surprising that resistance against β -lactams is one of the first resistance mechanisms observed. β -lactamases are the enzymes responsible for β -lactam resistance, particularly in Gram-negative species. They function by hydrolyzing the core β -lactam ring of the drugs, directly inactivating their activity (12, 66). The first clinical β -lactamase, AmpC β -lactamase, was identified in *E. coli*, and thousands of variants were identified since then (67). These enzymes were previously classified based on their functional properties but are currently grouped based on their amino acid sequence, leading to four major groups: A, B, C, and D (68). The emergence of β -lactamases

exhibiting resistance against third-generation cephalosporins and carbapenems, the extended-spectrum β -lactamases and carbapenemases, has become a significant concern for the public. They drastically reduced the options for last-resort antibiotics for complicated cases (69).

Target modification:

Like the resistance mechanism of drug inactivation, target modification confers resistance by hindering the interaction between the antibiotics and their targets. However, this mechanism involves chemical modification of the targets or acquisition of replacements, rather than affecting the antibiotics (70). Resistance against macrolides provides a prime example of direct chemical modification of the target. As discussed, macrolides are known to target protein synthesis, specifically the 50S subunit of a ribosome and have been used widely against pneumonia-causing pathogens resistant to β -lactams (71). First case of macrolide resistance was reported in *Staphylococci*, in the 1950s, which presented methylation on the 23S rRNA by rRNA methyltransferase (72). Scientists found that this enzyme was encoded by the *erm* genes (erythromycin ribosomal methyltransferase) and often is carried in plasmids which pathogens could obtain through HGT. This mechanism remains the top cause of macrolide resistance (73).

It is possible that bacteria carry multiple copies of genes encoding the target of the antibiotics. The genes may be either natural copies of the species or attained through transformation, conjugation, or transduction (70). Mutations to any one of the natural copies of the genes lead to high resistance against the antibiotics. For example, fluoroquinolones are known to target DNA gyrase and topoisomerase IV responsible for regulating DNA coiling. Each enzyme consists of two subunits encoded by *gyrA* and *gyrB* or *parC* and *parE*, respectively. Mutations leading to amino acid substitution in any of these genes lead to resistance against fluoroquinolones (74, 75). Cells can also produce alternative target that are resistant to

the drugs that often acquired through MGEs. A well-studied example is resistance to trimethoprim and sulfonamides. These classes of drugs are each known to target enzymes in sequential steps of the bacterial folic acid pathway: the DHFR and the DHPS, respectively (76). In addition to genetic mutations in chromosomally produced enzymes, species can obtain plasmid-encoded enzymes. These alternative enzymes take over the functions of naturally produced enzymes, circumventing the effects of the antibiotics (77, 78).

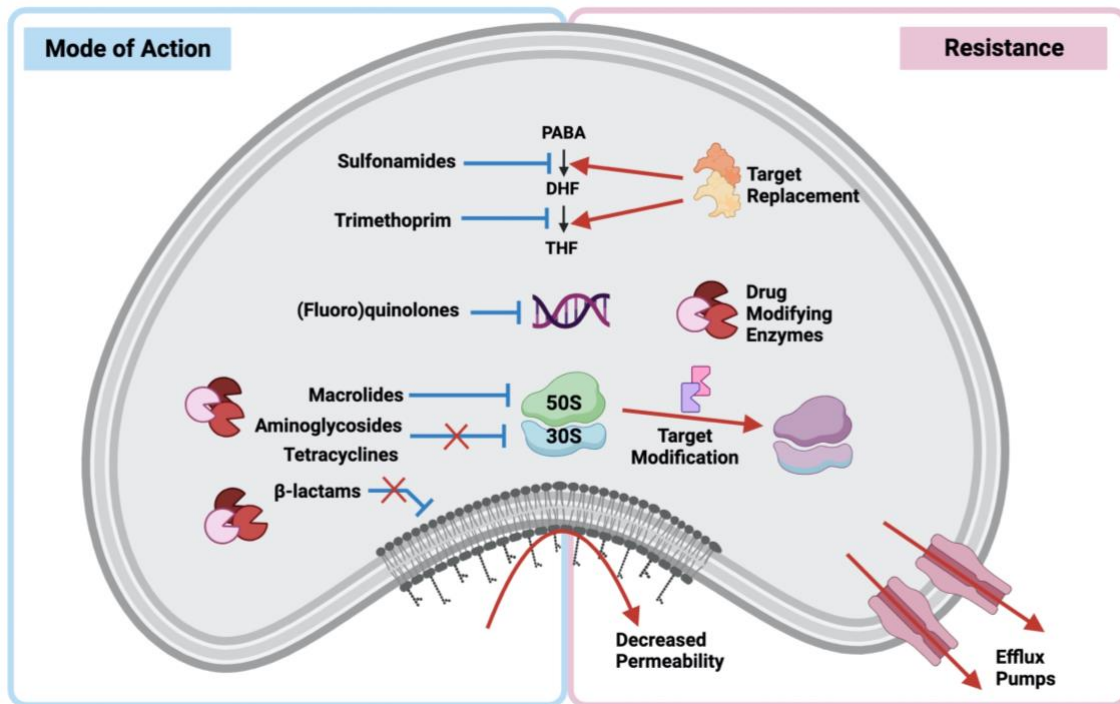


Figure 1.1 A schematic summary of different modes of action (blue lines) of all classes of antibiotics discussed and resistance mechanisms (red lines) against them. This schematic was created with BioRender.com.

1.1.4 Antibiotic resistance is a serious global threat

Because many antibiotics are either directly from or derived from natural products of microbes, it is natural that microbes have resistance against them for survival. However, misuse and overuse of antibiotics by the public in various sectors, such as human and veterinary

medicine, food production, agriculture, and animal husbandry, have placed significant additional selective pressure on microbes, drastically accelerating the rate of resistance acquisition (79).

Due to exposure to multiple classes of antibiotics, pathogens are not only resistant to single drug but are multidrug resistant (MDR) and extensively drug resistant (XDR), becoming superbugs.

The World Health Organization (WHO) has recognized ABR as a global threat that demands an immediate attention (80). A recent study by Antimicrobials Resistance Collaborators (2022) has shown that there were 4.95 million deaths associated with ABR infections globally, of which 1.27 million deaths were directly caused by antibiotic resistant bacteria (ARB) in 2019 alone (81). If the trend continues, we not only may face the danger of returning to a time when infections were the leading cause of death but also risk undermining our medical advances. Compromised therapeutic effects of the antibiotics are not the only negative effects of the ABR. It also imposes a financial burden on both the individual and national levels. Patients with infected with ARB are charged higher hospital costs, often by multiple magnitudes, due to prolonged hospital stays and requirement for additional treatments. Furthermore, extended hospital stays of individuals result in a loss of economic productivity and increased healthcare costs at the national level. This vicious cycle exacerbates the financial burden on both individuals and communities with a possibility of leading to poverty (81–83).

1.2 Cholera and its causative pathogen, *V. cholerae*

1.2.1 Cholera

Cholera is an acute diarrheal disease caused by Gram-negative pathogen, *V. cholerae*, that still affects millions of people (84). The WHO estimates that there are 1.3-4 million cases with 21,000-143,000 deaths annually. Unfortunately, it was estimated that the official cases may only represent 5-10% of actual cases occurring globally. Such low reporting may be because of

the prevalence of the disease in developing countries, where a sufficient systematic surveillance system might lack, and/or affected countries under-reporting to avoid tarnishing their global reputation (85, 86). Cholera has always been endemic in India, especially in the Ganges Delta. However, the development of transportation and global travels have facilitated their dissemination globally, leading the disease to become pandemic (87). There have been seven pandemics of cholera with the seventh pandemic originated from Indonesia in the 1960s still ongoing. In addition to the pandemic, many countries from Asia, Latin America, and Africa remain both endemic and epidemic (88). When infected with cholera, patients may show symptoms of profuse watery diarrhea, characterized as rice-water, vomiting, and high fever. Rapid loss of water and ions due to this watery diarrhea cause severe dehydration, leading to death within hours if not treated promptly (89).

Prevention and Control:

Cholera is transmitted through ingestion of pathogen-contaminated water and food and could be easily prevented and controlled by having access to clean water, sanitation, and hygiene (WASH) facilities (90). However, the reality of population at risk is quite different, with the disease predominantly affecting developing countries and regions affected by natural disasters and wars with refugees, where they may be minimal to no availability of WASH centres (91). This shows the importance of global organizations intervening to assist affected countries in implementing surveillance and control strategies. The Global Task Force on Cholera Control (GTFCC) with the WHO has been organized to set a global strategy to reduce and eliminate cholera by 90% in 20 countries by 2030: “Ending cholera: A global roadmap to 2030” (92). The report highlights the important steps in building a framework to eliminate cholera: 1) rapid detection and control of outbreaks, 2) multi-sectoral interventions for prevention, and 3)

successful implementations of the founded strategy through active participation and partnerships (93). The multi-sectoral interventions particularly play an important role since high-risk countries need more than just WASH facilities for the successful prevention and control of cholera. They also require the distribution of oral cholera vaccines, improvements in health care systems for accurate diagnosis and treatment, the establishment of effective surveillance systems, and general education of the population on the threat of the disease and the importance of the WASH (94–96).

1.2.2 V. cholerae in their natural reservoirs

V. cholerae is a rod-shaped, Gram-negative bacterium with strains that cause cholera outbreaks (84). There are over 200 serotypes of *V. cholerae*, distinguished based on their O-antigen composition of the lipopolysaccharide on the outer membrane of the cells (97).

Different aquatic environment function as natural reservoirs of *V. cholerae*: fresh, estuarine, and marine waters. Therefore, coastal lines are often hot spots for endemic regions and outbreaks (97, 98). When living in the environment, *V. cholerae* can be found as either planktonic cells or biofilms attached to abiotic and biotic surfaces to increase their chance of surviving unfavorable conditions (99).

To study physiological and genetic characteristics of a species, *in-vitro* experiments are often carried out through culturing it under laboratory conditions. However, there were difficulties in culturing *V. cholerae* from their natural habitats, even during the endemic seasons (100). It was soon revealed that many pathogenic species, such as *Escherichia coli*, *Legionella pneumophila*, and *Shigella* spp., that survive both human intestines and natural environment often enter a dormant “viable but

nonculturable (VBNC)” state when living in the environment (101, 102). *V. cholerae* is no exception (103). When faced with unfavorable conditions (which include sudden changes in temperature, pH, osmotic pressure, nutrient starvation, and many more), cells undergo reversible changes in cell morphology, metabolic pathways, and gene expressions, and enter the VBNC state (104, 105). *V. cholerae* primarily exhibits change in morphology, going from rod-shaped to coccoid with upregulation of genes directly involved in survival of the cells (106). Staying in VBNC state also provides protection to *V. cholerae* against acid and other factors while passaging through the host digestive system until they reach the intestinal cells where they become actively dividing and virulent again (107, 108).

Biofilms are three-dimensional, multicellular micro-community encased by an extracellular matrix that many bacteria, including *V. cholerae*, create to protect themselves from adverse conditions and predation (108). They are frequently found on both abiotic and biotic solid surfaces where adhesion can be easily achieved. The biofilms of *V. cholerae* can be found on the bottom of ships, egg masses of Chironomid, body surfaces of zoo- and phytoplankton, or free-floating in water (109–111). *V. cholerae* particularly prefers surfaces with chitin, one of the most abundant polysaccharides in an aquatic environment. This may be due to their ability to degrade and utilize chitin as their main carbon source, providing a solution to nutrient scarcity in an aquatic environment (112, 113). Creating a biofilm matrix requires a complex, multi-step process. It starts with the founder cells identifying and adhering to an appropriate surface through their flagella and type IV pili, the mannose-sensitive haemagglutinin (114). Forming a mature,

three-dimensional biofilm from the cells' initial attachment requires a few important structures and proteins: the *Vibrio* polysaccharide (VPS) and the matrix proteins (114). The VPS is secreted exopolysaccharides that functions as a glue and an envelope to the actively dividing and colonizing cells within the matrix. The rugose morphology of biofilms that facilitate resistance to multiple stresses is provided by the VPS, and they are also known to be required for survival and successful colonization in hosts (115, 116). While the VPS is essential for maturation of biofilms, the matrix proteins (RbmA, RbmC, and Bap1) are essential for maintaining the matured structure. RbmA plays a role of additional glue along with the VPS, inducing cell-to-cell adhesion and organizing the inner structure (117, 118). RbmC and Bap1 are homologs that activate adhesion between the formed matrix and the surface. Their activities allow the cells within the biofilm to grow vertically and expand three-dimensionally (119). The VBNC cells are also present in biofilms, emphasizing the essentiality of both forms of viable *V. cholerae* to survive and effectively transition between two distinct environments: the host vs. natural reservoirs (120).

1.2.3 V. cholerae and their pathogenicity

The serotype O1 is particularly famous as pandemic-causing agents: the classical vs. the El Tor. The classical O1 has been identified to be the cause of the first six pandemics, whereas the El Tor is responsible for the current seventh pandemic. There are less important cholera-causing serotypes indicating active horizontal gene transfer between the strains (87, 121). As most strains of *V. cholerae* are non-pathogenic, the pathogenic strains have been developed

through acquisition of key virulence factors that derive infection symptoms and host colonization: the toxin-coregulated pilus (TCP) and the cholera toxin (CT) (122, 123).

The TCP is a type IV pilus responsible for the colonization of ingested *V. cholerae* in the host intestine. It is found on pathogenicity island I, which contains the *tcp* operon responsible for the expression of the pilus. This island also contains integrases and transposases to excise itself from the chromosome and form into a plasmid. This suggests its potential origin from a phage and indicates its aptitude for the HGT (124, 125). The TCP has a few critical roles in host invasion: 1) secretion of colonization factor, 2) formation of micro-colony via interaction between ingested bacteria, and 3) direct involvement in attachment to the host epithelial cells (126, 127). In addition to all its important roles, the TCP also acts as a receptor for CTX ϕ , *V. cholerae*-specific bacteriophage, that carries the genes for the CT (128). This highlights the importance of having both virulence factors for environmental strains to gain pathogenicity.

The CT is the enterotoxin that causes the rice-water diarrhea of cholera. When infected with CTX ϕ , its DNA that includes the genes for the CT (*ctxAB*) integrates itself into the chromosome of the cells, turning them pathogenic (129). The CT comprises two subunits: the active A subunit (CTA) and the binding B subunit (CTB). When the pathogen is ingested into the host intestine, the CT is secreted out of the cells through type II secretion system (130). The secreted toxin is then endocytosed by binding ganglioside GM1 (host target), where the subunits dissociate with the CTA becoming active. The activated CTA influences a series of enzymatic activities, including permanent activation of the adenylate cyclase and accumulation of the cAMP in the host cells (131). Increased level of cAMP activates numerous ion/electrolyte channels,

including the cystic fibrosis transmembrane receptor (CFTR). This induces the extrusion of chloride ions and inhibits the absorption of sodium ions resulting in excessive movement of water into the intestinal lumen, the rice-water diarrhea (132, 133). The diarrhea containing highly infective *V. cholerae* cells can be attributed as the source of outbreaks when a nearby water source becomes contaminated and is ingested by the community.

1.2.4 Treatment and ABR of V. cholerae

Cholera treatments:

Majority of cholera patients suffer mild to moderate diarrheal symptoms. However, about 10% of patients show extreme diarrhea accompanied by vomiting which leads to severe dehydration and rapid deterioration of the patient's condition (134). In these cases, patients need to be quickly rehydrated by replacing lost fluids and electrolytes, and an adequate hydration level must be maintained throughout the duration of the symptoms (135). Oral rehydration solution (ORS) is the go-to hydrating solution used during cholera treatment. It contains glucose, sodium, chloride, potassium, and citrate to induce glucose-based absorption of lost electrolytes (136, 137). It is administered via intravenous line in cases of severe dehydration whereas direct oral intake is encouraged for patients showing mild-to-moderate symptoms with consciousness (138). Administration of antibiotics is recommended for patients presenting severe symptoms, as they help lessen the duration of the symptoms and reduce the shedding of highly infective *V. cholerae* (135). Tetracycline and doxycycline are the first line of antibiotics used in severe cholera cases. Erythromycin, ampicillin, and chloramphenicol are used as alternatives whereas Co-trimoxazole is recommended for children. Ciprofloxacin is used if all mentioned antibiotics fail due to resistance (138, 139).

ABR in *V. cholerae*:

Active usage of antibiotics as both treatment and prophylaxis has added to selective pressure against *V. cholerae*. The alarming rate of pathogenic *V. cholerae* strains becoming MDR worldwide is concerning, especially considering that frequently used antibiotics are rapidly becoming ineffectual (140, 141).

MGEs are the primary source of resistance that confer protection against different classes of antibiotics to the cells. Plasmids are circular extrachromosomal DNA that could be transferred between different strains and species through conjugation. They are notorious for carrying genes responsible for MDR and represent one of the key mechanisms by which *V. cholerae* acquires resistance. These plasmids often carry multiple resistance genes, encompassing all antibiotic classes (142, 143). For example, Wong *et al.* (2015) showed that newly identified pVC1447, an IncA/C plasmid isolated from a cholera patient in China, carries resistance genes against chloramphenicol, tetracycline, erythromycin, aminoglycosides, co-trimoxazole, and cephalosporins. The list includes all antibiotics used to treat cholera (144).

Another important MGE is the integration conjugative elements (ICEs). They share a common property with a plasmid that it could be transferred via conjugation; however, unlike plasmids, they require to be integrated into the chromosome of the affected individual before expression (145). SXT constin is the first ICE identified in *V. cholerae* serotype O139. It is always found integrated in the 5' end of *prfC*, a gene encoding a protein for translation termination (146). Its name comes from its MDR characteristic against four antibiotics: trimethoprim, sulfamethoxazole, chloramphenicol, and streptomycin (147). It also possesses all the necessary genes for self-excision and conjugation, making them highly transferrable. In fact, SXT-derivates are present in nearly all clinical strains from Asia (148, 149). The activation of

stress response is the one of the factors that promotes self-transmission of STX, highly signifying how selective pressure from misuse and overuse of antibiotics accelerates the propagation of antibiotic resistance among different *V. cholerae* strains (150).

1.3 Exploiting resources to combat MDR *V. cholerae*

The Pathogen Box

The Pathogen Box, organized by the Medicine for Malaria Venture (MMV), is a library of 400 chemical compounds that presented antimicrobial activity against neglected tropical diseases like kinetoplastids, cryptosporidiosis, and tuberculosis (151). The MMV also provides detailed information on cytotoxicity and pharmacokinetics of each compound. These factors are important when deciding to move forward with clinical developments (152). Due to its accessibility, the library has been screened against multiple pathogens, both eukaryotic and bacterial, including *S. aureus* (bacterial pathogen), *Toxoplasma gondii* (protozoan parasite), and *Cryptococcus neoformans* (fungus) (153–155). My lab noted this library to be a great candidate to screen against *V. cholerae* to discover new compounds with high antibacterial activity.

The wastewater treatment plants:

The wastewater treatment plants (WWTPs) are essential in communities due to the negative impacts of the release of insufficiently treated water back into the environment. Because effluents from all levels of sectors flow into the WWTPs, they have become a hub for antibiotic resistance genes and pathogens (156). With the advancement in nanoscience, nanoparticles (NPs) have emerged as an area of interest in discovering green, non-antibiotic agents capable of effectively killing pathogens present in wastewater (157, 158). As *V. cholerae* has the ability to survive environmental water reservoirs before being ingested by hosts, they are also frequently found in sewage systems, posing a possibility of causing unforeseeable outbreaks. Therefore,

effective removal of the pathogen during water treatment processes plays a vital role in outbreak prevention and control, reducing the likelihood of encountering the pathogen in water systems. (139, 159). This research, collaborated with Dr. Xiao He, aims to study the antibacterial activity of newly synthesized NP dispersed on a cellulose aerogel.

1.4 Thesis Objectives

The discovery of antibiotics is one of the best scientific breakthroughs in human history. Not only have they helped us combat infectious diseases, but they have also significantly increased our life expectancy by decades through the development of invasive treatments such as chemotherapy, surgeries, and organ transplants. However, the public currently faces a serious health risk at a global level due to the increasing rate of environmental and pathogenic bacteria acquiring antibiotic resistance through various routes.

Cholera is a water-borne disease spread through fecal-oral transmission, primarily caused by ingestion of contaminated water or food. It induces serious acute diarrhea, leading to severe dehydration and potential fatality if not promptly treated. While less prevalent in developed countries, it is one of the diseases strongly addressed by the WHO to be strategically reduced by 90% within the next decade, “Ending Cholera- A global roadmap to 2030”. Cholera is caused by Gram-negative bacterium *V. cholerae*, making antibiotics crucial in managing symptoms and treating the infection, particularly in children under the age of 5. Unfortunately, *V. cholerae* has progressively developed resistance to multiple drugs over the decades, rendering it MDR and significantly more challenging to treat.

Since cholera is commonly spread via contaminated water, wastewater treatment plays a vital role in preventing outbreaks in high-risk areas. Because of the presence and richness of various chemical compounds and complex microbial communities, WWTPs have become a hot

spot for antibiotic resistance acquisition. Consequently, the field of wastewater treatment has piqued substantial interest in searching for “green” and efficient processes for producing higher-quality effluents before releasing them back into the environment.

This thesis aims to explore alternative methods for treating *V. cholerae* in both clinical and environmental settings. The first objective involves characterizing two chemical compounds from the Pathogen Box, originally found to be effective against neglected tropical diseases, that exhibited notable antibacterial activity against *V. cholera*. The goal of this study is to identify their potential mechanisms of action. The second objective involves studying the antibacterial efficacy of newly synthesized biopolymer, cellulose aerogel, crosslinked with metallic NPs. Overall, the research aids in gaining valuable insights into the development of potential novel approaches to combat various pathogens, primarily on *V. cholerae*.

Chapter Two: Identification of small molecule inhibitors of the pathogen box against *Vibrio cholerae*

2.1 Introduction

Most antibiotics that we currently use are molecules naturally produced by microbes to survive in complex microbial communities (160). Therefore, it is only natural for these microbes to be resistant to different antibiotics to out-compete their opponents and neighbors (79). However, antibiotic resistance has become a serious public threat due to an increased rate of microbes gaining resistance against not one, but multiple antibiotics. It was reported that approximately 700,000 people die annually due to complications with antibiotic-resistant infections (161). In the United States alone, two million patients suffer from multi-drug resistant (MDR) infections annually, incurring a cost of \$20 billion to the health care system (161). In June 2019, the Public Health Agency of Canada announced that there is a 1-in-16 chance of developing a superbug (a bacterium resistant to most commonly available antibiotics) infection while being hospitalized (162). There is also an increase in the number of Canadians carrying bacteria resistant to carbapenem, one of the last line of defense antibiotics (162). The problem of antibiotic resistance requires immediate attention to prevent further damage to the world's population and economy (161).

V. cholerae is a rod-shaped, Gram-negative bacterium that causes a serious diarrheal disease, cholera (86, 99, 160, 163). After being ingested through contaminated food or water, *V. cholerae* colonizes the small intestine of the infected host (163). Its secreted CT causes severe rice-water diarrhea due to the uncontrolled movement of water into the small intestine (163). There have been seven pandemics of cholera with the first pandemic recorded in 1817 and the seventh pandemic still ongoing in the areas of Africa and Asia (164, 165). Pandemic-causing *V. cholerae* strains all belong to the O1 serogroup, which is further divided into two biotypes: the Classical strains that caused the first six pandemics and the El Tor strains that caused the current pandemic and replaced the Classical strains (86).

Even though cholera can be mitigated through proper hygiene and access to clean water, it remains one of the major health problems in developing countries around tropical regions (166). It has been estimated that 1.3 to 4 million people suffer from cholera annually with tens of thousands of deaths (86, 166). Cholera is mostly treated by administering patients with ORS (166). However, in severe cases, antibiotics such as tetracyclines and quinolones are administered together to lessen the symptoms, especially in children aged 5 and under (163). Although antibiotics are not the first line of treatment for cholera, the use of antibiotics is critical for countries with endemic cholera and ongoing epidemics, especially in the countries with limited access to clean water and ORS supplies (163).

As discussed above, bacteria with acquired antibiotic resistance pose a global threat, and *V. cholerae* is not an exception. *V. cholerae*'s resistance to antibiotics was first described in the 1960s, which was due to spontaneous mutations in the drug targets. More recently, MDR *V. cholerae* is on the rise due to lateral gene transfer, particularly through the movement of ICEs, leading to a rise of various strains of MDR *V. cholerae* among clinical and environmental isolates (160, 167). It is clear that there is an ever-increasing need to identify more efficient antibiotics against this notorious bacterium.

Pathogen Box is a library of 400 drug-like compounds that have been shown to target neglected tropical diseases like kinetoplastid infections as well as malaria and tuberculosis (168). These compounds, with known cytotoxicity and pharmacokinetics, serve as an important resource for drug development against a broad range of pathogens (152, 169). In this study, we have screened the library and identified two small molecules, MMV687807 and MMV675968, that individually target different biological processes to either kill or inhibit *V. cholerae* growth. We report that both compounds cause significant transcriptomic changes to a clinical isolate of

V. cholerae, strain C6706. We also show that a mutation in *vcI408*, a gene encoding for a negative regulator of an efflux pump, confers resistance to MMV687807. Furthermore, we present strong evidence that MMV675968 could be targeting the dihydrofolate reductase of *V. cholerae* with a MIC 14-fold lower than trimethoprim (an analog of the compound). These findings may not only facilitate effective treatment of cholera disease but also lead to discovery of effective compounds against other MDR-related infections.

2.2 Materials and Methods

2.2.1 Growth conditions

Unless otherwise stated, *V. cholerae* El Tor strain C6706 was aerobically grown at 37°C in lysogeny broth (LB; wt/vol 1% tryptone, 0.5% yeast extract, 0.5% NaCl) shaking at 225rpm or on LB-agar plates with indicated concentrations of compound.

2.2.2 Pathogen Box screening against *V. cholerae*

The Pathogen Box molecules were diluted to a concentration of 1 mM in dimethyl sulfoxide (DMSO) and used at 10µM in LB for screening. Then, 200µL of *V. cholerae* cell culture with OD₆₀₀ ~1 was spread on 15-cm diameter LB-agar plates as bacterial lawns and left to dry. Also, 5µL of each Pathogen Box molecule at 10µM concentration was spotted on the plate using a replicator and the plate was incubated at 37°C overnight. Six Pathogen Box molecules out of 400 tested molecules resulted in a zone of clearing of *V. cholerae* after overnight growth on solid LB-agar. These six molecules were re-tested for inhibition of *V. cholerae* growth on LB-agar plates (Table 2.1).

2.2.3 Growth curve with Pathogen Box compounds

C6706 was grown overnight and diluted to OD₆₀₀ ~0.05 in 200µL of fresh LB containing indicated concentrations of each compound and DMSO (0.2%) in 96-well plate. The growth was observed every 30 min for 8 h at 37°C by measuring OD₆₀₀. For strains containing plasmid, 0.2% arabinose was used for expression and 0.4% glucose for repression.

2.2.4 CFU/mL counting with Pathogen Box compounds

V. cholerae was grown either overnight for 18h or to mid-logarithmic stage (OD₆₀₀ of 0.5) and then varying concentrations of a Pathogen Box molecule or equivalent percentage of DMSO control (0.05% maximum) were added. The growth was monitored over time by measuring the OD₆₀₀. At each time point, a 10-fold serial dilution of cells was plated on LB-agar and grown overnight at 37°C. Colonies were counted to quantify colony forming units per mL (CFU/mL). This method was carried out by Burkinshaw, B. and Lam, L.

2.2.5 MIC assay

The MIC was determined following the Clinical and Laboratory Standards Institute microdilution assay using the cation-adjusted Mueller-Hinton broth (CAMHB) (170). *V. cholerae* was grown on solid LB-agar at 37°C overnight. The colonies were resuspended in CAMHB to a 0.5 McFarland standard. The 100µL of adjusted culture was further diluted with serially diluted concentrations of MMV675968 in CAMHB in a round bottom 96-well plate to a final concentration of $\sim 5 \times 10^5$ CFU/ml. The plate was grown at 37°C for 18h. The lowest concentration with no visible growth was recorded and averaged over three biological replicates to identify the MIC.

2.2.6 RNA purification from V. cholerae C6706

For RNA transcriptome analysis, C6706 was grown to an OD₆₀₀ of 0.4. Cells were then incubated with sub-inhibitory concentrations of MMV687807 (2.5µM), MMV675968 (10nM), or DMSO control (0.2%) in triplicate. After 30 min of growth with each concentration of compound or DMSO, 700µL of cells were harvested for RNA extraction. For RNA extraction, 700µL of cells were incubated with 100µL of 8x lysis buffer (0.8% SDS and 16 mM EDTA) and an equal volume of acidic phenol and incubated at 65°C for 5 min, followed by incubation on ice for 10 min. After phase separation by centrifugation for 2 min at 13,000 × g, the aqueous phase was isolated and combined with an equal volume of anhydrous ethanol. RNA was purified using the Direct-zol RNA miniprep kit (Zymo Research) with an in-column DNase I digest to remove contaminating DNA. After quantification of RNA, the rRNA was removed with the RiboZero kit from Illumina. This method was carried out by Burkinshaw, B. and Lam, L.

2.2.7 RNA-seq transcriptome analysis

The extracted RNA samples were sequenced at McGill University and Génome Québec Innovation Centre using the HiSeq4000 Illumina sequencer. The resulting reads were 50-nucleotide single-end sequences. Using FastQC (171), each of the raw reads were checked for their per base sequence quality and overrepresented sequences for any adaptor sequence recognition. Following parameters were used with Trimmomatic on Galaxy platform to improve the quality of the sequences including removing any of the recognized adapters: (i) initial ILLUMINACLIP with customized adapter sequences, (ii) filtering out poor quality sequences using pre-set parameters of sliding window, leading, and trailing functions, and (iii) heading cropping sequences improving per base sequence content.

The quality checked sequences for each sample were mapped against the genomic sequence of *V. cholerae* O1 biovar El Tor str. N16961 which were calculated for expression levels and normalized using the Geneious software version 10.1.3. DESeq2 was used to calculate the differential expression between DMSO control and each of the compound-treated samples. For MMV687807-treated reads, genes with differential expression \log_2 value greater than 2 were considered upregulated whereas genes with differential expression \log_2 value less than -2 were considered downregulated. On the other hand, genes from MMV675968-treated samples had \log_2 value greater than 1 and less than -1 as its cut-offs. Both sets of genes were considered statistically significant if the adjusted p -value was <0.05 . The biological pathways of upregulated or downregulated genes were identified using KEGG pathway mapper and UniProtKB.

2.2.8 Light microscopy acquisition

C6706 was grown to an OD_{600} of 0.6 before the addition of each indicated compound. At each time point, cells were concentrated to an $OD_{600} \sim 5$, resuspended in 0.5X PBS, and spotted on 1% agarose-0.5X PBS pads. All the phase-contrast images were obtained using a Nikon Ti-E inverted microscope with a 1 Perfect Focus System 2 (PFS) and a CFI Plan Apochromat Lambda 100X oil objective lens. Three separate fields for each treatment in individual replicate were obtained. Fiji software was used for image manipulation to have equal contrast between the compared images. Each panel is a representative image from three biological replicates.

2.2.9 Biofilm assay

C6706 was grown overnight and subcultured to reach OD_{600} of 0.6. Subculture was mixed 1:1 in a 96-well plate with LB containing indicated concentration of MMV675968. Cells were grown at room temperature for 20h without shaking. Floating cells and LB were removed, and the biofilm was washed with deionized water twice then airdried. Biofilm was stained with

200 μ L of 0.1% crystal violet for 10 min. Excess crystal violet was removed, and the wells were washed twice with deionized water then airdried. Crystal violet was resuspended with DMSO and measured at OD₅₉₅.

2.2.10 Spontaneous repressor mutants

Overnight culture of wildtype C6706 was adjusted to OD₆₀₀ = 1. 200 μ L of the adjusted culture was spread as bacterial lawn on LB-agar plates containing 3 μ M or 5 μ M MMV687807 or 25nM or 50nM MMV675968. Plates were then incubated at 37°C overnight. Resistance of colonies that grew was further confirmed by growing collected colonies on new LB-agar plates with appropriate concentrations of each compound. This method was carried out by Burkinshaw, B. and Lam, L.

2.2.11 Whole genome sequencing (WGS) and analysis

Total DNA was extracted with DNeasy blood and tissue kit (Qiagen) from an overnight culture grown from a single colony of wildtype or resistant mutant strains. DNA was quantified using Qubit 4.0. NEBNext Ultra II DNA Library Prep Kit for Illumina was used for library preparation. High through-put genome sequencing was carried out at the McGill University and Génome Québec Innovation Centre ($n = 6$) on Illumina HiSeq4000 platforms, generating 100bp single-end reads, resulting in a mean of 1,625-fold coverage. Short reads of wildtype C6706 were assembled with Unicycler version 0.4.4 and annotated with Prokka v1.12 with default parameters. A reference-based alignment for each of the resistant mutant strain was generated by mapping Illumina-generated short reads of resistant mutants to *de novo* assembly of wildtype C6706 from above using Snippy v3.2-dev. The predicted mutations were further confirmed to be causing resistance by growing transposon mutants of the genes in indicated concentrations of

each compound following the growth curve assay. This method was carried out by Burkinshaw, B. and Lam, L.

2.2.12 Diagrams

Schematic of the screening (Figure 2.1A) and the summarizing cell model (Figure 2.5) were created with BioRender.com.

2.2.13 Data availability

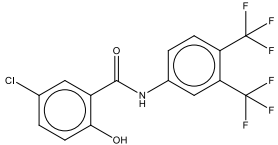
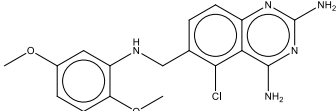
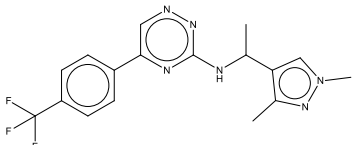
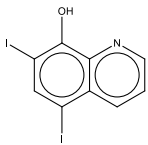
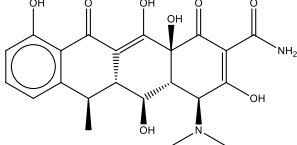
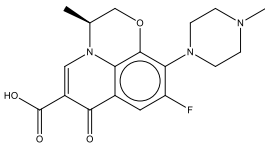
The raw sequence reads of RNA-seq have been deposited to NCBI's Gene Expression Omnibus (GEO) under the accession number GSE185596. The raw sequence reads of whole-genome sequencing have been deposited to Sequence Read Archive (SRA) with the accession number PRJNA771235.

2.3 Results

2.3.1 Discovery of *Vibrio cholerae*-inhibiting compounds by screening the Pathogen Box

V. cholerae El Tor strain C6706, referred as C6706 hereafter, is a seventh-pandemic strain isolated from Peru in 1961 (172, 173). To identify inhibitors of C6706, we screened 400 compounds of the Pathogen Box at 10 μ M concentration (Figure 2.1A). While most compounds showed no growth inhibition, six compounds exhibited a zone of clearing, indicating inhibition of growth (Table 2.1). Out of the six compounds identified, MMV687807 and MMV675968 showed the most potent inhibiting activities, suppressing growth by 50% or more after 8h at concentrations of 1.25 μ M and 10nM, respectively (Figure 2.1B,C). We further focused on these two compounds as they showed the greatest inhibition but are also yet to be commercially used.

Table 2.1 Pathogen Box compounds that inhibit growth of *V. cholerae* at 10 μ M

Identification	Common Name	Formula/Structure*	Commercial Use	Pathogen Box Target*	HepG2 IC20 (μ M)*	Mode of Action
MMV687807	NA	<chem>C15H8NO2CIF6</chem> 	NA	<i>Mycobacterium tuberculosis</i>	0.658	unknown
MMV675968	NA	<chem>C17H18N5O2Cl</chem> 	NA	<i>Cryptosporidium parvum</i>	3.44	Inhibition of DHFR
MMV019993	NA	<chem>C17H17N6F3</chem> 	NA	<i>Plasmodium falciparum</i>	39.3	unknown
MMV002817	Iodoquinol	<chem>C9H5NOI2</chem> 	amebicide	<i>Brugia pahangi</i>	2.53	Chelation of ferrous ions
MMV000011	Doxycycline	<chem>C22H24N2O8</chem> 	antibiotic	broad range spectrum	18.2	Inhibition of protein synthesis
MMV687798	Levofloxacin	<chem>C18H20N3O4F</chem> 	antibiotic	broad range spectrum	>80	Inhibition of DNA gyrase and topoisomerase IV

* Data provided by the MMV

To gain a deeper understanding of growth inhibition after treatment with different concentrations of the compounds, we employed serial dilution plating and enumeration of CFU/mL at numerous time points. At 5 μ M, MMV687807 showed bactericidal activity since it brought the CFU/mL counts below the limit of detection (Figure 2.1D). In contrast, MMV675968 showed a more modest reduction in surviving colonies, suggesting a predominantly bacteriostatic activity at the concentrations tested (Figure 2.1E). We further

examined the activities of these compounds by treating non-dividing cells with these compounds. Neither compound exhibited antibacterial activities when cells were in the stationary phase compared to cells treated during exponential phase (Figure 2.2). This suggests that both compounds target a system involved in active growth and cell division.

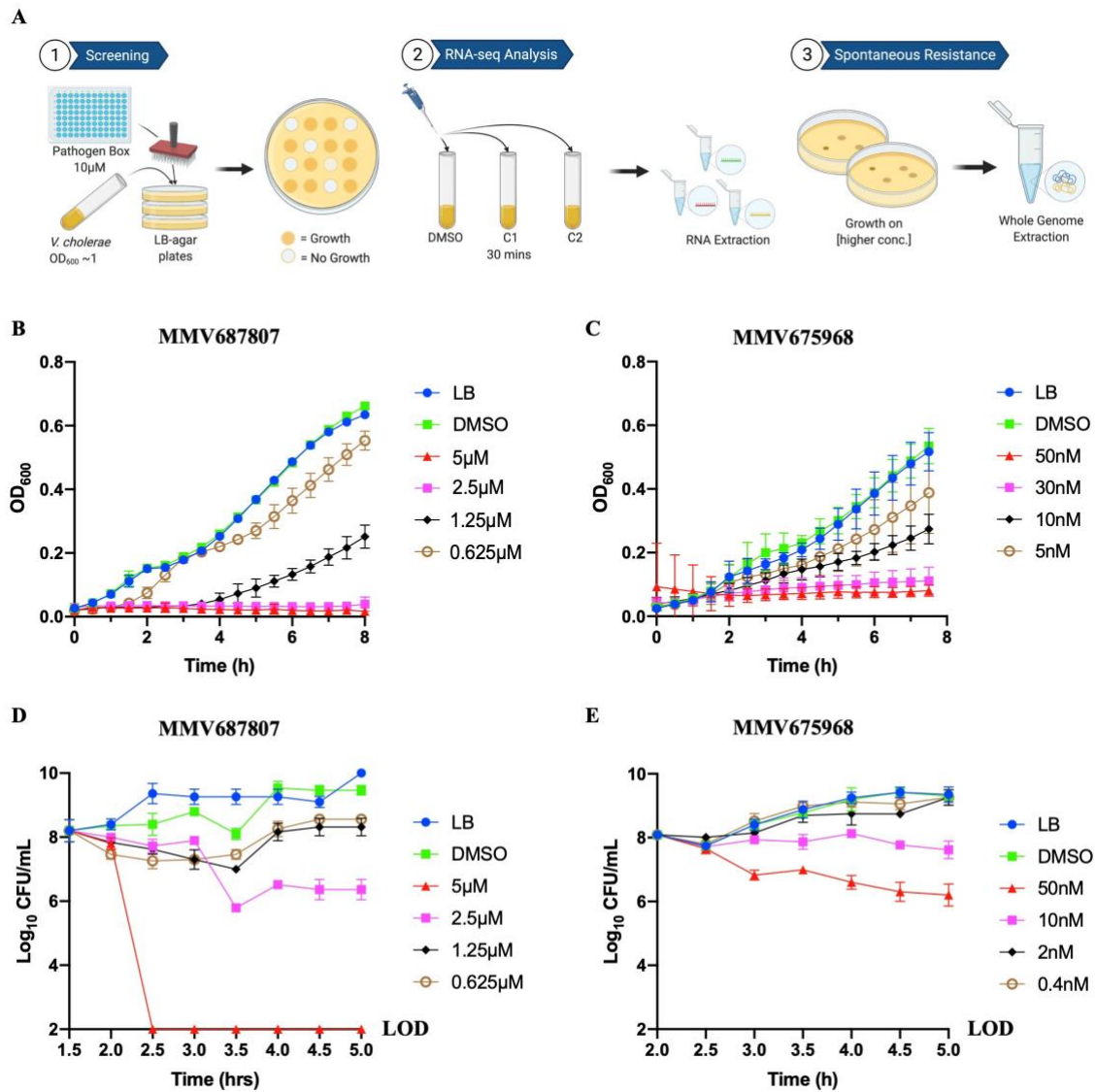


Figure 2.1 *V. cholerae* C6706 is either killed or inhibited by MMV687807 and MMV675968, respectively. A) Schematic of screening Pathogen Box compounds against C6706. C1 shows

MMV687807, and C2 indicates MMV675968. OD600 growth curve measurements of C6706 cells inhibited with varying concentrations of B) MMV687807 or C) MMV675968. Legend shows the concentration of the compound used. Log₁₀ CFU/mL of C6706 after growing with varying concentrations of D) MMV687807 or E) MMV675968. Cells were grown to an OD600 of 0.4 before the addition of each compound or DMSO. 10-fold serial dilution of cells was plated on LB-agar for counting colonies at each time point. LOD, limit of detection. Error bars indicate the mean ± standard deviation of three biological replicates.

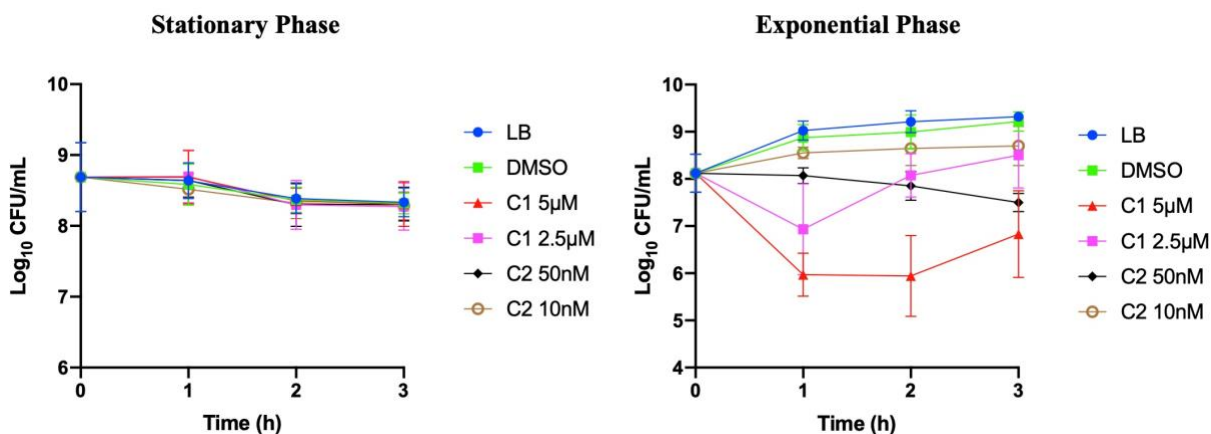


Figure 2.2 Both compounds target systems required for active growth of the cells. Log₁₀ CFU/mL was calculated for viable cells after treatment of indicated concentrations of each compound during the stationary (overnight growth for 18h) or exponential (OD600 ~0.5) phase. C1: MMV687806 and C2: MMV675968. Error bars indicate the mean ± standard deviation of three biological replicates. This experiment was carried out by Burkinshaw, B.

2.3.2 Both compounds modulate multiple biological functions

To gain insight into the molecular mechanisms of inhibition, we performed RNA-seq analyses of C6706 exposed to sub-inhibitory concentrations of each compound, 2.5µM MMV687807 and 10nM MMV675968, then grouped differentially expressed genes based on

biological functions. To visualize the analysis, volcano plots were generated for each treatment (Figure 2.3)

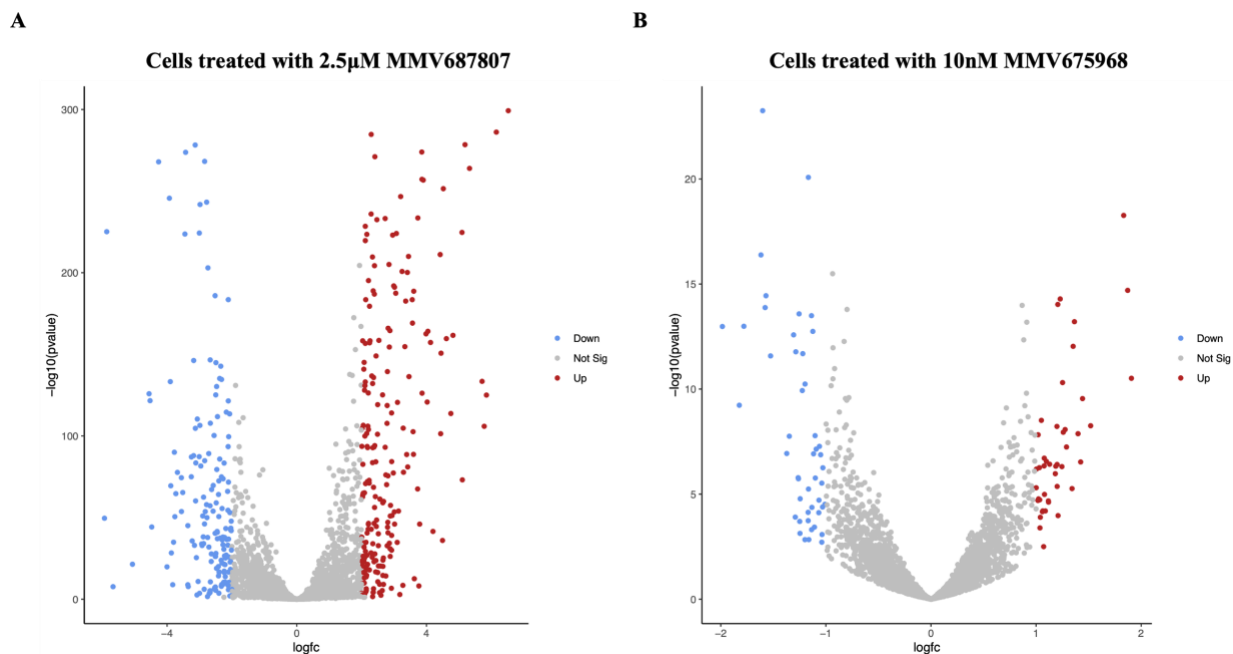


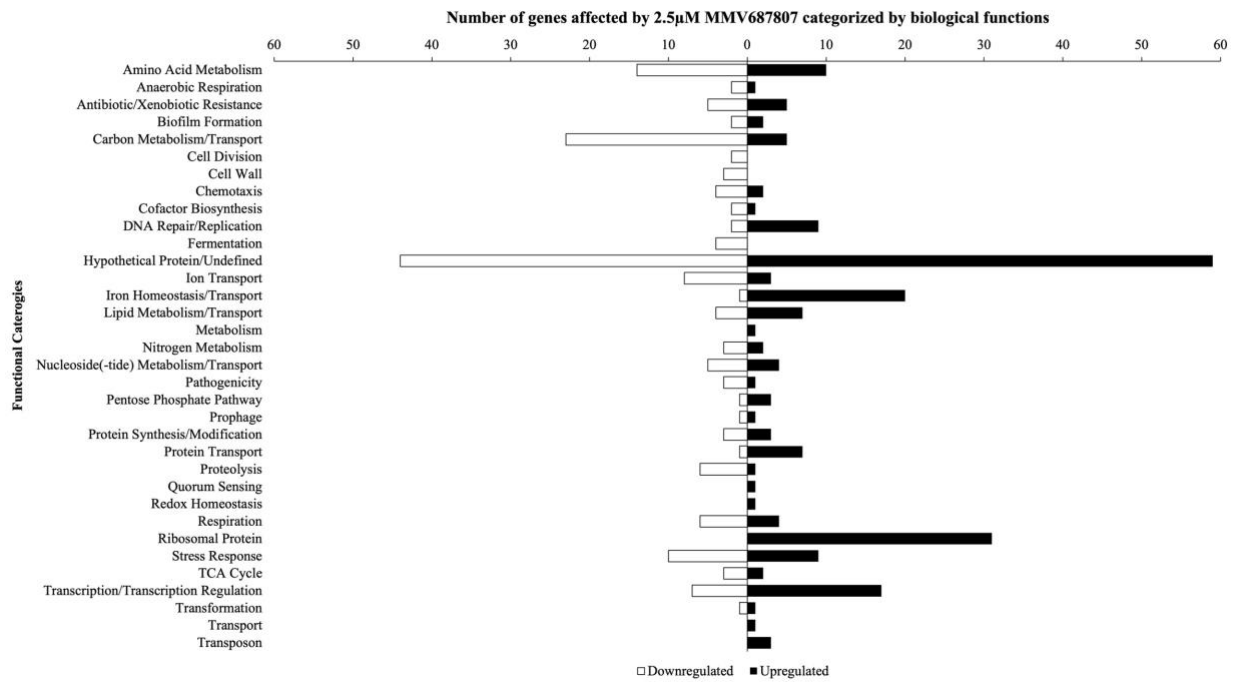
Figure 2.3 Volcano plots of all differentially expressed genes for cells treated with sub-inhibitory concentrations of A) MMV687807 and B) MMV675968. Blue dots represent significantly down-regulated genes, red dots represent up-regulated genes, and grey dots represent non-significant genes. Colored dots represent significant differentially expressed genes selected at adjusted p -value < 0.05 , \log_2 fold change ≥ 2 for MMV687807, and \log_2 fold change ≥ 1 for MMV675968. Logfc, \log_2 fold change.

When cells were exposed to 2.5µM MMV687807, 387 genes were identified with adjusted p -value < 0.05 and differential expression values greater than or equal to 4-fold ($\log_2 \geq 2$ or $\log_2 \leq -2$). Each of the identified genes was classified into their biological functions, with 103 genes falling under hypothetical proteins with unknown functions. The most downregulated group was genes involved in carbon metabolism/transport. A few of the most downregulated

genes, *vc1820*, *vc1826*, and *vc1821*, are involved in the uptake of fructose. Iron homeostasis/transport showed the most upregulated number of genes along with ribosomal proteins (Figure 2.4A). These include *vca0676* and *vc1184*, involved in the formation of iron-sulfur clusters, and *vc0364* and *vc0475*, which have iron storage and siderophore activities. This analysis revealed that MMV687807 has a very broad effect on the cells. It is possible that these transcriptomic changes are an indirect result of the compound targeting an essential system of the cell or due to the result of targeting multiple cellular pathways.

Cells exposed to 10nM MMV675968 exhibited 91 genes that are differentially expressed with values greater than or equal to 2-fold ($\log_2 \geq 1$ or $\log_2 \leq -1$) and adjusted *p*-value < 0.05 . When these genes were classified into biological functions; 26 genes were classified as hypothetical proteins with unidentified functions (Figure 2.4B) The category of biofilm formation showed the most upregulated genes. However, when biofilm formation was quantified using crystal violet assay, there was no significant difference between the DMSO control and different concentrations of MMV675968 (Figure 2.5). Additionally, genes involved in exopolysaccharide formation, like *vc0937* and *vc0934*, were also upregulated. Similar to MMV687807, genes involved in transport of carbon or small molecules showed most downregulation (Figure 2.4B). We additionally found a few stress response genes that had significant upregulation but with modest and less than 2-fold changes in expression. These included genes involved in stringent response (*rpoZ*) and envelope stress response (*rpoE* and *rseABC*). Specifically, we also discovered that a gene involved in SOS response (*uvrA*) was upregulated as well. This suggests that the target of MMV675968 could be involved in pathways maintaining proper membrane structure and DNA repair.

A



B

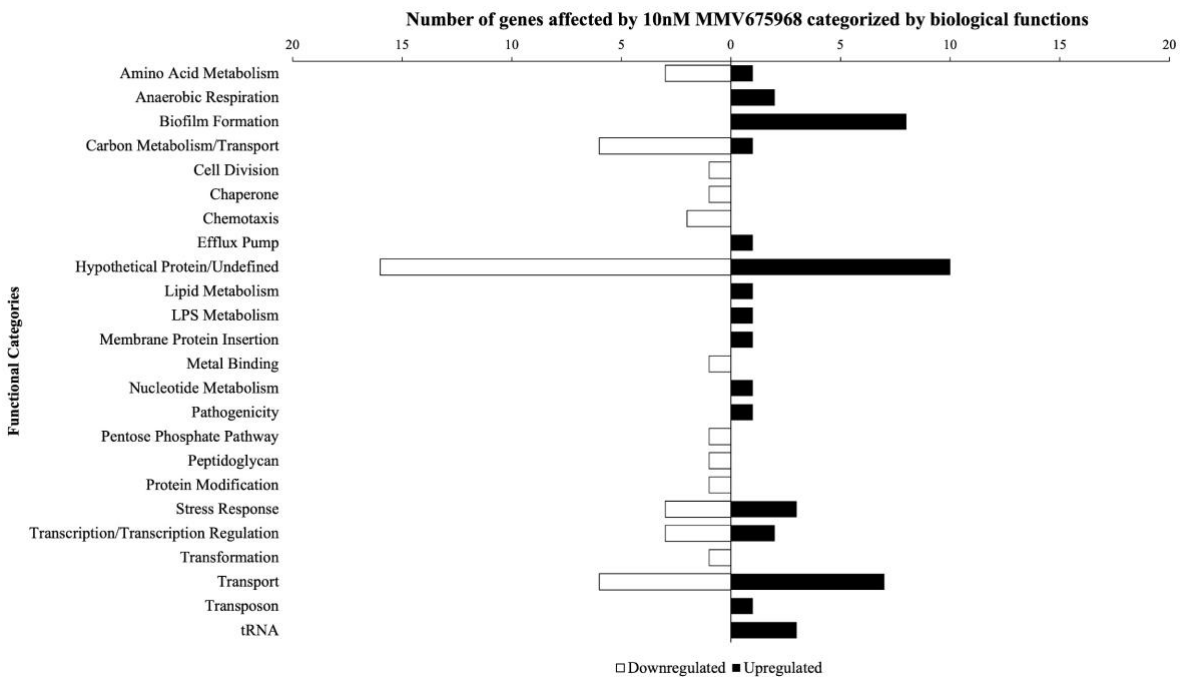


Figure 2.4 Classification by functional categories of the genes that are significantly upregulated or downregulated when treated with sub-inhibitory concentrations of A) MMV687807 (n = 387) and B) MMV675968 (n = 91). Each set of RNA-seq data was compared

to the cells exposed to the DMSO control (0.2%). Differentially expressed genes were identified using DESeq2 and filtered for adjusted p -value < 0.05 . Genes with differential expression values were selected at $\log_2 \geq 2$ or $\log_2 \leq -2$ for MMV687807 and $\log_2 \geq 1$ or $\log_2 \leq -1$ for MMV675968. Functional categories of the genes were identified through KEGG pathway mapper and UniProtKB.

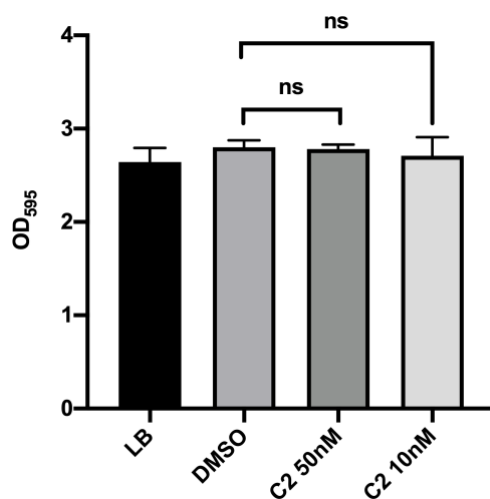


Figure 2.5 Biofilm formation is not affected by exposure to MMV675968. C6706 grown overnight was subcultured to reach OD₆₀₀ ~ 0.6. The subculture was then mixed 1:1 with LB containing indicated concentrations of MMV675968 and grown at room temperature for 20h without shaking. Formed biofilm was stained with crystal violet, suspended in DMSO, and measured at OD₅₉₅. Error bars indicate mean \pm standard deviation of three biological replicates. One-way ANOVA with Sidak's multiple comparison used between DMSO and treatments; ns: not significant.

Next, we compared the RNA-seq analyses between the compounds in order to see if there were genes and/or pathways that are similarly affected by both compounds. Nineteen genes were

differentially expressed under both treatment conditions (Table 2.2). The genes belong to multiple functional categories including stress response, carbon metabolism and transport, and hypothetical proteins. Notably, a number of genes that were downregulated in the presence of MMV687807 showed upregulation in the presence of MMV675968 suggesting that these two compounds have different modes of action when inhibiting the growth of C6706.

Table 2.2 A list of differentially expressed genes shared by treatments with MMV687807 or MMV675968.

Genes	Pathway	2.5μM MMV687807 Differential Expression Log₂ Ratio	10nM MMV675968 Differential Expression Log₂ Ratio
<i>VC1825</i>	Transcription/Transcription Regulation	-4.52447346	-1.16247612
<i>VC1823</i>	Carbon Metabolism/Transport	-4.002474029	-1.036580223
<i>VCA0933</i>	Stress Response	-3.867387751	1.182871545
<i>VC0783</i>	Hypothetical Protein/Undefined	-3.558289189	1.040377877
<i>VCA0224</i>	Hypothetical Protein/Undefined	-3.078572653	-1.246415311
<i>VC0833</i>	Biofilm Formation	-2.998387378	1.208995516
<i>VC1490</i>	Cell Division	-2.932643648	-1.984886502
<i>VC1824</i>	Carbon Metabolism/Transport	-2.873412541	-1.244772036
<i>VCA0364</i>	Hypothetical Protein/Undefined	-2.699914322	-1.168529012
<i>VCA0439</i>	Stress Response	-2.638387464	-1.117379373
<i>VC0282</i>	Chemotaxis	-2.484549796	-1.263702804
<i>VCA0867</i>	Stress Response	-2.466022503	-1.224602877
<i>VC1780</i>	Hypothetical Protein/Undefined	-2.401863053	-1.169008527
<i>VCA1008</i>	Pathogenicity	-2.397081107	1.031372885
<i>VCA0630</i>	Amino Acid Metabolism	-2.252574049	-1.049751938
<i>VC2371</i>	Hypothetical Protein/Undefined	-2.204657024	1.050279712

VC2357	Hypothetical Protein/Undefined	-2.197206944	-1.780489895
VC1153	Transformation	-2.042134608	-1.372329097
VCA0025	Ion Transport	-2.021105448	-1.136989815

2.3.3 Whole genome sequencing of spontaneous resistance mutants

To gain further insight into the molecular targets of these compounds, we obtained spontaneous resistance mutants by plating C6706 cells on LB plates containing each compound. Spontaneous resistance mutants were purified on a new LB plate with appropriate concentrations of each compound and then subjected to WGS analysis using the Illumina HiSeq4000 platform.

Three spontaneous mutants resistant to MMV687807 at 3 μ M or 5 μ M were selected. WGS revealed multiple mutations in *vca0792* and *vca0791*, encoding subunits of transposase OrfAB; however, these were found in all sequenced mutants suggesting that they reflect the genome background differences between our C6706 strain and the reference *V. cholerae* isolate N16961. More promisingly, *vc1408* showed a frameshift mutation in one of the resistant mutants. This gene encodes an HTH transcriptional regulator VceR, a negative regulator of the efflux pump VceCAB (174). On the other hand, two resistant mutants were selected from cells grown at 25nM MMV675968. Both mutants showed a mutation in *vca0767*, a gene responsible for another HTH transcriptional regulator with an unknown target.

To confirm that the mutation in these identified genes leads to resistance against these two compounds, we transformed WT C6706 and transposon mutants of each gene with an arabinose-inducible plasmid containing each of our genes of interest (*vc1408* and *vca0767*). When *vc1408* was induced in the VC1408::Tn mutant, the cells lost resistance to MMV687807 compared with the cells expressing an empty plasmid (Figure 2.6A). Although the strains induced with *vca0767* show significantly reduced protection from MMV675968, this is likely

due to toxicity of *vca0767* overexpression as both wild type and transposon mutant overexpressing *vca0767* presented significantly reduced growth in the untreated control (Figure 2.6B). On the other hand, we can confirm that disruption of *vc1408* instigates resistance. This is likely through de-repression of the VceCAB efflux pump (174, 175).

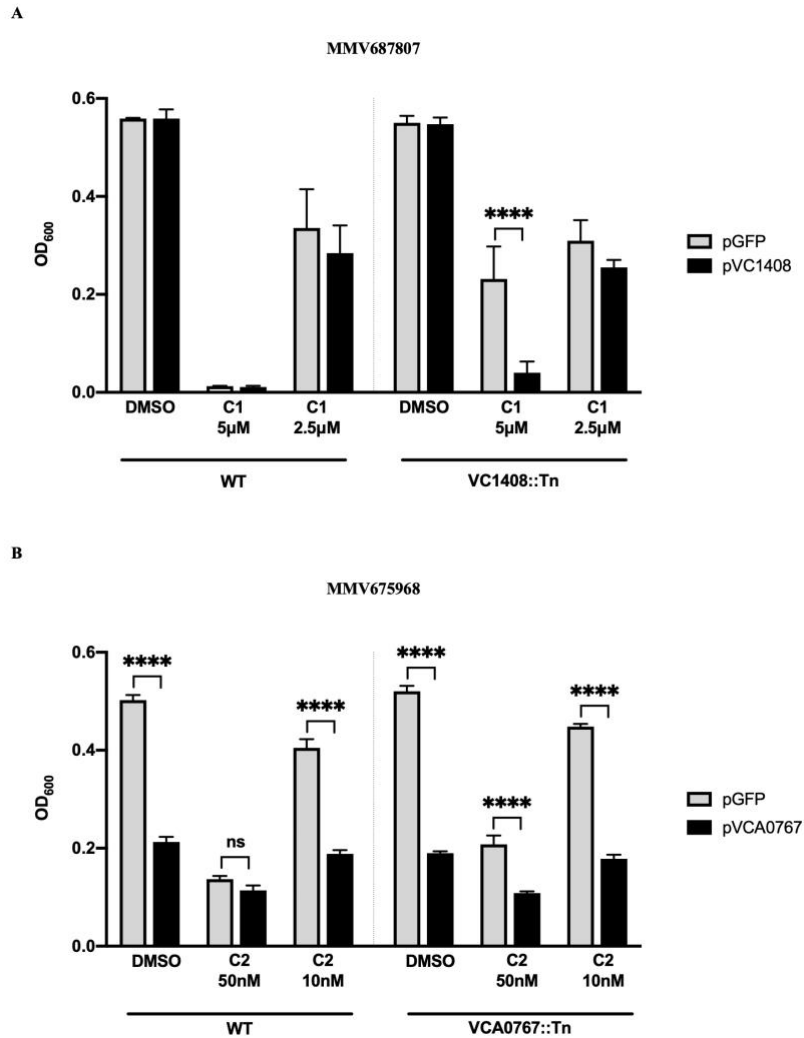


Figure 2.6 Effects of transposon mutations of genes affected in spontaneous resistance mutants on the growth of C6706 during treatment with each compound. A) Effect of mutation of VC1408 on the survival of C6706 treated with MMV687807 (C1). B) Effect of mutation of VCA0767 on the survival of C6706 treated with MMV675968 (C2). C6706 was transformed with an arabinose-inducible plasmid containing each of the indicated genes.

Transformants were grown overnight and diluted to OD₆₀₀ ~0.05 in fresh LB with the indicated concentrations of each compound and 0.2% arabinose. Each bar represents OD₆₀₀ after an 8-h growth in a 96-well plate. I did comparisons between the strains expressing GFP and gene of interest using one-way ANOVA with Sidak's multiple comparison, ****, $p < 0.0001$, ns, not significant. Error bars indicate the mean \pm standard deviation of three biological replicates.

2.3.4 MMV675968 targets the dihydrofolate reductase

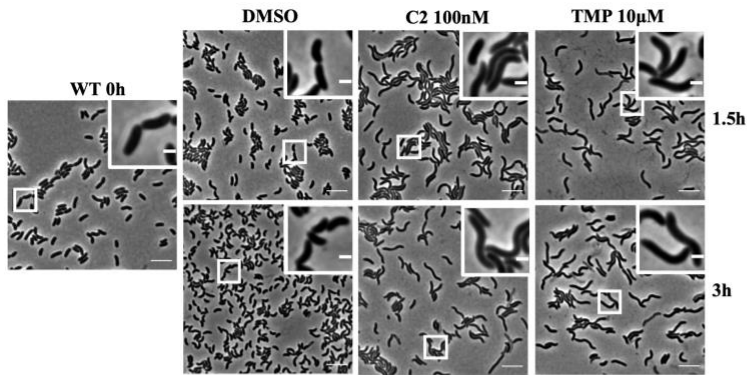
A recent study has shown that MMV675968 targets the DHFR in *Acinetobacter baumannii* (another Gram-negative pathogen with strains resistant to numerous antibiotics) in a manner similar to how trimethoprim targets the DHFR (176). We speculated that a similar mechanism could be causing growth inhibition in C6706. We hypothesized that if MMV675968 targets the DHFR, it will cause C6706 to elongate similar to trimethoprim causing cell elongation (177, 178). As expected, cells exposed to high concentrations of both MMV675968 and trimethoprim showed obvious cell elongation, indicating disrupted cell division (Figure 2.7A).

To further support that MMV675968 could be targeting the DHFR, we transformed C6706 with arabinose-inducible plasmids containing either *sfgfp* (empty plasmid) or trimethoprim resistant *dhfr* (TpR-*dhfr*). Expressing TpR-*dhfr* in C6706 was able to rescue the growth of cells treated with high concentrations of both MMV675968 and trimethoprim, while GFP expression had no impact (Figure 2.7B). Additionally, when plasmid expression was repressed by the addition of glucose, no growth difference was observed between empty plasmid and TpR-*dhfr* containing strains. We also studied whether MMV675968 is able to inhibit growth of C6706 at a lower concentration than trimethoprim using the broth microdilution method (170). Trimethoprim exhibited growth inhibition at a concentration around 2.71 μ M whereas

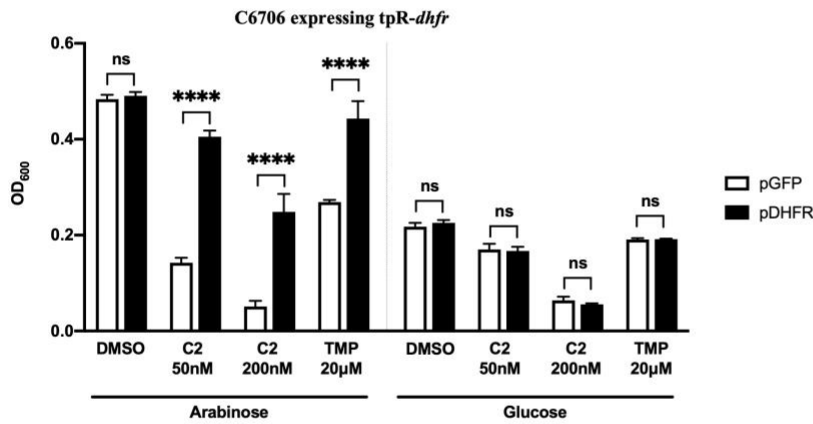
MMV675968 showed inhibition around 198.4nM, a concentration 14-fold lower than trimethoprim (Figure 2.7C).

Finally, we tested whether MMV675968 targets bacteria as broad as trimethoprim. *Pseudomonas aeruginosa* PAO1 and trimethoprim-resistant MDR *E. coli* (B7) did not exhibit growth reduction. However, a Gram-positive bacterium, *Staphylococcus aureus*, treated with 10 μ M MMV675968 showed significant growth inhibition compared to trimethoprim-treated cells. We also observed growth inhibition of *E. coli* MG1655 and non-trimethoprim-resistant MDR *E. coli* (C10) (Figure 2.7D). These results suggest that MMV675968 may be an improved substitute for trimethoprim across multiple species including C6706.

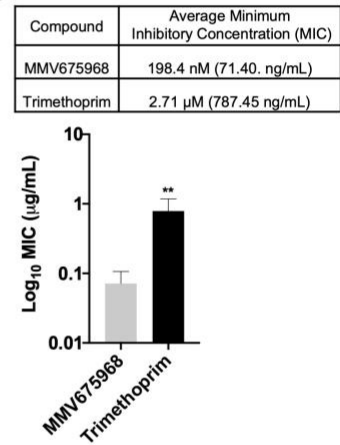
A



B



C



D

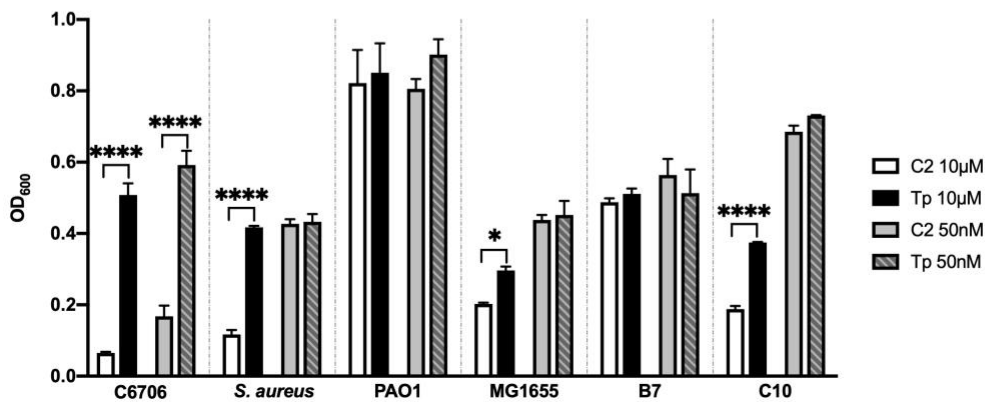


Figure 2.7 MMV675968 targets the DHFR of C6706 with better efficacy. A) Phase contrast images of C6706 exposed to DMSO, MMV675968 (C2), or trimethoprim (TMP). Representative image of 40- × 40-µm field of cells with a magnified 5- × 5-µm inset of a selected region shown. Scale bar is 5 µm for the original field of view and 1 µm for the inset. B) Effect of trimethoprim-

resistant *dhfr* (TpR-*dhfr*) on the survival of C6706 when treated with MMV675968 (C2) or trimethoprim (TMP). C6706 was transformed with an arabinose-inducible plasmid containing TpR-*dhfr*. Transformants were grown overnight and diluted to OD600 ~0.05 in fresh LB with indicated concentrations of each compound and 0.2% arabinose for expression or 0.4% glucose for repression. Each bar represents OD600 after an 8-h growth in a 96-well plate. One-way ANOVA with Sidak's multiple comparison was used to compare the growth of cells expressing TpR-DHFR and GFP under each condition, ****, $p < 0.0001$; ns, not significant. Error bars indicate mean \pm standard deviation of three biological replicates. C) MIC comparison between trimethoprim and MMV675968. C6706 was grown in CAMHB with varying concentrations of each compound overnight. MIC was determined by observing for a concentration that did not present growth to the naked eye. To compare the observed MIC between the two compounds, an unpaired t test was used, **, $p < 0.05$. Representative data from three biological replicates are shown. D) Effect of MMV675968 across multiple species. Each species were grown overnight, normalized to OD600 ~2, and diluted to OD600 ~0.05 in fresh LB with indicated concentrations of MMV675968 (C2) or trimethoprim. (Tp). Each bar represents OD600 after an 8-h growth in 96-well plate. One-way ANOVA with Sidak's multiple comparison was used to compare the treatments between MMV675968 and trimethoprim, *, $p < 0.0332$; ****, $p < 0.0001$. Error bars indicate mean \pm standard deviation of three biological replicates.

2.4 Discussion

V. cholerae is a pathogen that causes the serious diarrheal disease, cholera. Although cholera can be prevented and treated by maintaining good hygiene and having access to clean water, developing countries still suffer from thousands of deaths annually (166). Antibiotics are often administered together with rehydration solution during cholera treatment to lessen the

severity and duration of the symptoms. As *V. cholerae* has become resistant to many of the commonly used antibiotics like trimethoprim-sulfamethoxazole and chloramphenicol, there is an increasing need for new antibiotics against this pathogen (179). In this study, we have screened the Pathogen Box and identified two compounds, MMV687807 and MMV675968, that either kill or inhibit the growth of *V. cholerae* El Tor strain C6706 by at least 50% at concentrations as low as 2.5 μ M and 10nM, respectively.

MMV687807 was first identified as a derivative of IMD-0354, a salicylamide that is in clinical trials for atopic dermatitis (eczema) and is also known to suppress the development and metastasis of colon cancer (168, 180). MMV687807 not only shows comparable activities to IMD-0354 but also targets multiple pathogens like *Mycobacterium tuberculosis* and *Candida albicans* though its mechanism of action is not fully understood (181, 182).

We show that treatment with MMV687807 causes global changes in the transcriptome, causing downregulation of many genes involved in amino acid metabolism and carbon metabolism, whereas genes involved in iron homeostasis were upregulated (Figure 2.4A). A common response of cells exposed to bactericidal antibiotics is a reduced rate of carbon metabolism, which decreases sensitivity to well-known antibiotics like ampicillin (183–185). It has also been shown that disrupting the transcriptional regulator of iron homeostasis increases antibiotic resistance (186). These previous findings could explain the observed changes after the exposure to the compound. These responses allowed the cells to survive during the treatment period. However, this analysis does not give the primary mode of action for the compound because these changes seem to be global across different species exposed to various antibiotics and are secondary to the compound's mode of action.

We show that a transposon mutation in a repressor gene of the efflux pump VceCAB causes C6706 to become resistant to MMV687807 (Figure 2.6A). VceCAB is one of the efflux pumps found in *V. cholerae* that plays an important role in excreting toxic molecules out of the cell (163). Substrates of VceCAB include multiple antibiotics such as chloramphenicol, a proton motive force uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), and salicylate (174, 175). It has been shown that VceR, the product of *vc1408*, normally represses the expression of VceCAB (174, 175). Interestingly, VceR switches between DNA-binding and non-DNA-binding conformations depending on the interaction with its substrates, like CCCP, through competition with dsDNA in a concentration-dependent manner (187). Because disruption of VceR in C6706 led to strong resistance against MMV687807 at varying concentrations, it suggests that de-repressed VceCAB is able to efflux MMV687807.

Although MMV687807 seems to be a promising new option for treating cholera, there are many other aspects, such as cellular toxicity, that need to be considered before it can be further developed into a commercially viable drug. The MMV, the provider of Pathogen Box, provides critical information on drug metabolism and pharmacokinetics of each compound. MMV687807 has been shown to only require 0.658 μ M to reach a concentration of 20% inhibition (IC₂₀) in human hepatoma cells (HepG2) (Table 2.1). This concentration is lower than the effective concentration against *V. cholerae*. Further studies are required to fully understand the target of this compound to improve its utility.

Similarly, MMV675968 is also able to inhibit multiple pathogens like *Cryptosporidium parvum*, *Plasmodium falciparum*, and *A. baumannii* (176, 188, 189). Multiple studies have shown that MMV675968 acts by targeting the DHFR and could be a potential replacement for

trimethoprim (190, 191). We also provide evidence that DHFR may be a target of MMV675968 in *V. cholerae*.

MMV675968 treatment caused a noticeable downregulation of genes involved in carbon metabolism/transport (Figure 2.4B). We speculate that this is similar to how MMV687807 affected the carbon pathways. An interesting observation of this compound's effect is that there was a significant increase in the expression of the genes involved in biofilm formation. Biofilm formation plays an important role in the pathogenicity and the survival of *V. cholerae* when living in a free environment where residual concentration of antibiotics can be commonly found (99). The upregulation of genes involved in biofilm formation may protect against an attacking antibacterial molecule by limiting the rate of compound penetration into the cells. In addition, we have also observed upregulation of genes involved in stress-induced proteolysis (192), sigmaE stress response (192, 193), DNA repair (194). These findings suggest that MMV675968 could be directly or indirectly affecting pathways involved in cell growth and division.

Trimethoprim was discovered in the 1960s as a 2,4-diaminopyrimidine that inhibits a broad-spectrum of bacteria and exhibits minimal mammalian toxicity (27). In the 1980s, the structure of trimethoprim binding to its ligand was extensively studied and dihydrofolate reductase (an essential enzyme that reduces dihydrofolate into tetrahydrofolate which is used in nucleic acid synthesis) was identified as its target (195, 196). When its effectiveness was studied against *V. cholerae*, the MIC against El Tor strains were 1.7 to 2.0 $\mu\text{g}/\text{mL}$ (197). Our MIC test of trimethoprim against C6706 showed a similar range with average MIC of 2.71 μM (787.45ng/mL) (Figure 2.7C). However, *V. cholerae* resistance to trimethoprim has been reported to be through acquiring a resistance gene via mobile genetic materials like ICEs (160, 163). Interestingly, MMV675968, an analog of trimethoprim, works much more effectively

against C6706 than trimethoprim at a MIC 14 times lower in molarity, 198.4nM (71.4ng/mL). Furthermore, MMV675968 exhibits a broad range of inhibiting activities against multiple species including *S. aureus* and MDR *E. coli* (Figure 2.7D). How exactly MMV675968 exhibits inhibition at lower concentrations than trimethoprim is to be further studied. Unlike MMV687807, MMV675968 has greater potential for further development as a new treatment for cholera. MMV data have shown that HepG2 IC₂₀ of this compound is 3.4μM (Table 2.1), much higher than the effective anti-*Vibrio* concentration (50nM). Further study is needed for testing any synergistic effects it may have when used with sulfamethoxazole, an antibiotic commonly used with trimethoprim.

In summary, we have identified two compounds, MMV687807 and MMV675968, that effectively inhibit the growth of *V. cholerae* (Figure 2.8). Future research will be focused on determining the target of MMV687807, structurally characterizing the interaction between MMV675968 and the DHFR, and their efficacy through *in vivo* models. Collectively, these findings will facilitate not only the treatment of cholera but also the discovery of novel compounds against broad MDR infections.

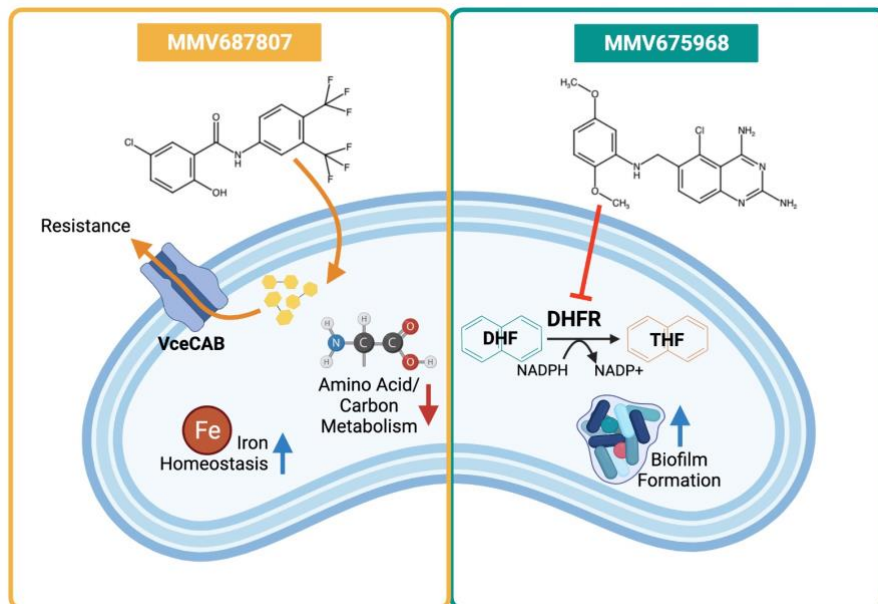


Figure 2.8 A summary model of each compound's effect on *V. cholerae*. MMV687807 causes upregulation of genes involved in iron homeostasis whereas metabolism of amino acids and carbon is downregulated. Efflux pump VceCAB expels the compound leading to resistance. MMV675968 causes genes involved in biofilm formation to be upregulated. The compound inhibits the activity of DHFR, enzyme involved in nucleic acid synthesis, causing growth inhibition of the cells.

**Chapter Three: Antimicrobial effect of Ag/lignin nanoparticle-loaded cellulose aerogel
against *Vibrio cholerae* and other bacteria**

3.1 Introduction

3.1.1 Wastewater and its pollutants

Although approximately 71% of Earth's surface is covered with water, only 0.5% of the total volume is readily accessible for usage. Water is one of the most essential chemical compounds to support lives on our planet. Water is especially important to humans when it comes to quality of life and economical growths. It is used in all aspects imaginable, from simple personal hygiene to industrial and agricultural developments. However, our access to clean water is becoming more and more compromised not only due to climate changes but also due to large quantities of pollutants imprudently being released back into water bodies with or without our knowledge (198). With increasing attention to environmental issues, negative ecological impacts caused by releasing untreated and polluted water back into surface water have raised various concerns during the last few decades. It is not surprising that water sustainability plays a crucial role in achieving the 17 Sustainability Development Goals set by the UN (199).

Wastewater refers to all liquid and solid wastes derived from human utilization carried in water (200). It could be further categorized into four different sources: 1) domestic wastewater from households, 2) stormwater runoff, 3) agricultural wastewater, and 4) industrial wastewater (201, 202). Each source brings in diverse groups of concerning toxic pollutants into the water system, and list includes heavy metals, fertilizers, dyes, organic and inorganic chemicals, radioactive waste, contaminants of emerging concern (CECs), and pathogenic microorganisms (201). Because these pollutants both directly and indirectly impose serious health and environmental risks (especially aquatic ecosystem), many countries require very strict treatment of wastewater through laws and regulations before releasing back into the environment (203, 204).

CECs refer to synthetic organic chemicals often found as ingredients, products, or wastes from pharmaceutical and personal care products (PPCPs), hospitals, industrial manufacturing sites, farms, and more (205). A list of compounds from the group includes antibiotics, endocrine disruptors, flame retardants, pesticides, and artificial sweeteners (206, 207). The effects of exposure to these compounds on human health and the environment are a relatively new area of concern since these were often neglected from treatment regulations and only recently they started to be detected in environmentally relevant concentrations (208). Here are a few examples of the effects of exposure to these compounds: disruptions to reproductive and endocrinal systems of aquatic animals, carcinogenic to both humans and animals, and interference with plant growth (209–211). Out of all, antibiotics are one of the priority groups of CECs to target during wastewater treatment processes. This is due to their toxicity to microorganisms, tendency to accumulate in soil, and potential to increase the presence of antibiotic resistant pathogens (212).

Because of high concentrations of both organic and inorganic compounds, as well as nutrients, wastewater itself and sludge produced after treatment are perfect living environment for many micro-organisms, including clinically important pathogens (213). Pathogens present in wastewater include gastroenteritis-causing bacteria like *V. cholerae* and *Campylobacter jejuni*, infectious viruses like hepatitis-causing viruses, and parasitic organisms like *Cryptosporidium* spp. and *Ascaris lumbricoides*. Without conscious efforts to strictly remove them during treatment processes, they may cause preventable outbreaks in both humans and animals (213–215). The presence of these pathogens alone is a public threat at multiple levels but combined with the presence of many antibiotics at various concentrations in the wastewater environment further jeopardizes the public health. Varying concentrations of antibiotics in wastewater create

selective pressure for pathogens to acquire resistance genes prevalent in the surroundings to survive (216). This phenomenon has led wastewater plants to be one of the leading reservoirs of ARB alongside hospitals, contributing to the global health threat we currently face due to lack of effective frontline antibiotics (217). Serious consequences of releasing inadequately treated wastewater containing antibiotics and ARB into water bodies highlight the importance of wastewater treatment plants (WWTPs) and proper processing of wastewater.

3.1.2 Wastewater Treatment

WWTPs are built and utilized globally for sufficient treatment of influents before discharge or reuse of wastewater. Conventional treatment processes often follow common four steps: preliminary, primary, secondary, and tertiary treatments. Each step uses combinations of physical, chemical, and biological processes to remove diverse pollutants, both organic and inorganic. (201, 218).

Preliminary treatment involves removal of obvious large solids and particles like plastics, garbage, and grit through screening and sedimentation. This step is essential to eliminate debris that could clog and damage machinery units used for subsequent treatment steps. Pre-treated wastewater then enters the primary sedimentation tank (also referred as a clarifier) for primary treatment which aims to remove suspended solids, grease and oil, and any large particles directly interacting with solids. Common practices are a combination of physical and physicochemical processes: flocculation, sedimentation, and flotation. Metal salts and coagulants are often added as a form of enhanced primary treatment to initiate removal of organic carbons and nutrients at an earlier stage. Since the primary treatment removes the majority of suspended solids in the wastewater, the goal of secondary treatment is to remove colloidal and dissolved pollutants like organic particles, nitrogen and phosphorus, and pathogens. Secondary treatment utilizes

microorganisms like bacteria and algae, a biological process, for the removal of organic particles through natural metabolic processes of these organisms before being sent to secondary clarifier for further removal via sedimentation. A few examples of the process are activated sludge bioreactors, stabilization ponds, and high-rate algal ponds. Tertiary treatment, also called advanced wastewater treatment, refers to any additional processes beyond secondary treatments to achieve desired water quality followed by disinfection steps like chlorination or UV treatment. The treatment can be a mix of physical, chemical, and biological treatments and highly depend on the intended usage of the treated water (201, 202, 213, 219–221).

3.1.2.1 Antibiotic resistant bacteria in wastewater and their removal

WWTPs have become hotspots for regional and global dissemination of ARB due to the gathering of influents from human communities and animal husbandry, which contain under-metabolized antibiotics (222). A few important factors that contribute to the accumulation of environmental ARB through WWTPs are: 1) presence of antibiotics in wastewater influents from various sources like hospitals and animal agriculture industries causing selective pressure, 2) release of untreated or poorly treated wastewater into surface waters, and 3) nutrient-rich environment of the WWTPs enhancing HGT between different species (223, 224). If inadequately treated wastewater with ARB contaminates the drinking water systems, it could lead to major outbreaks.

Removing bacterial pathogens from the wastewater can be achieved using either natural systems or conventional high-energy systems. Natural systems refer to treatments that solely depend on natural processes performed by the environment, such as the sunlight or weather-dependent temperature changes, and various organisms living in the system. These systems require minimal energy and machinery usage (225). They are often used in smaller rural

communities with greater land availability; constructed wetlands and stabilizing ponds are common examples. Conventional systems are commonly employed in more developed communities with higher population and include treatments like activated sludge bioreactors and membrane filtration. Although they may achieve similar quality effluents to natural systems with shorter retention times, conventional systems demand high energy consumption, relying on electrical systems to operate, and are also associated with high installation and operational costs (226). These treatments are often not sufficient for the ample removal of ARB and require additional disinfection steps, negatively impacting the treatment costs. Scientists and industries are always in search of energy-sustainable and cost-efficient treatments with better end quality.

Recently, utilizing polysaccharide-based biopolymers in wastewater treatments has gained a high level of interest. This is mainly because of their highly efficient adsorption properties, low-cost production, and negligible generation of toxic by-products after usage (227–229). Along with studies on biopolymers, metallic NPs are also being extensively studied as a new technology for wastewater treatments due to their catalytic properties in degrading various levels of pollutants, like inorganic compounds and dyes, and being antibacterial. Being easily crosslinked onto varying biopolymers is also to their benefit (230–232). In this chapter, Ag/lignin nanoparticle-loaded cellulose aerogel (Ag/L@Cellulose aerogel) produced by He, X. have been studied for its antibacterial properties against different bacteria spanning both Gram-positive and Gram-negative species (233).

3.2 Materials and Methods

3.2.1 Synthesis of the Ag/L@Cellulose aerogel

The aerogels were synthesized by He, X. Lignin was chosen as both reducing and stabilizing agent for formation of Ag NPs, as in a previous study (234). 5mL of lignin solution

(20 mg/mL) was mixed with 5mL of silver ion solution (63mg AgNO₃ + 4mL deionized water + 1 mL 5M ammonium hydroxide) via stirring at 300 rpm for 6 hours at room temperature. Produced Ag/Lignin (Ag/L) NPs were dried through freeze drying. Cellulose solution was prepared by dissolving 2g cellulose into 40mL NaOH/urea solution via constant stirring at 300 rpm and freeze-thawing to obtain transparency. Each of 10mg, 20mg, or 30mg dried Ag/L NPs was dissolved in 10mL cellulose solution along with 600µL epichlorohydrin (EPH) as a crosslinking agent. The solutions were then each transferred to a vial for gelation at room temperature for 12 hours. Each synthesized hydrogel was freeze-dried to produce aerogels. Aerogels containing different concentrations of Ag/L NPs are referred as follows: L@Cellulose aerogel (lignin only), 1Ag/L@Cellulose aerogel (10mg), 2Ag/L@Cellulose aerogel (20mg), and 3Ag/L@Cellulose aerogel (30mg).

3.2.2 Antibacterial assay with the Ag/L@Cellose aerogels

This assay was modified from a previous literature from He's lab to fit our conditions (232). Species from both Gram-negative and Gram-positive bacteria were used: *E. coli* BL21 DE3, *V. cholerae*, and *P. aeruginosa* are Gram-negative, and *S. aureus* and *Bacillus subtilis* are from Gram-positive. Cells were exposed to L@Cellulose aerogel, 1Ag/L@Cellulose aerogel, 2Ag/L@Cellulose aerogel, or 3Ag/L@Cellulose aerogel to test their antibacterial activities. Overnight cultures of each species were sub-cultured in fresh LB with a 1:50 ratio and grown at 37°C, shaking at 225 rpm until they reached OD₆₀₀ ~0.5 (mid-log phase). 5mL of each subculture was taken for centrifugation at 5000 rpm for 20 minutes in a 50mL conical tube. Supernatant was removed, and the pellet was resuspended in 5 mL of sterilized distilled water. 100µL of the suspension was taken for plating as starting point (0h), the negative control. 50mg of each aerogel was added to the suspensions. The suspensions were incubated shaking at 37°C and 225

rpm for 24 hours. At the end of incubation, bacteria that survived were counted as CFUs using either spotting assay from 96-well serial dilutions or standard plating.

3.3 Results

3.3.1 Synthesis of the Ag/L@Cellulose aerogels

Naturally, lignin contains many functional groups, i.e. phenolic hydroxyls and methoxy groups, that have the ability to reduce silver ions into its neutral form (233, 235). Using the silver ions from the AgNO_3 solution and mixing with lignin solution under high pH (addition of NH_4OH), Xiao was able to achieve even adhesion of Ag NPs onto the lignin surface, producing Ag/L NPs. Produced NPs were then crosslinked with cellulose hydrogel, which functions as a polymer to attract water, at various concentrations to complete synthesis of Ag/L@Cellulose aerogels (Figure 3.1).

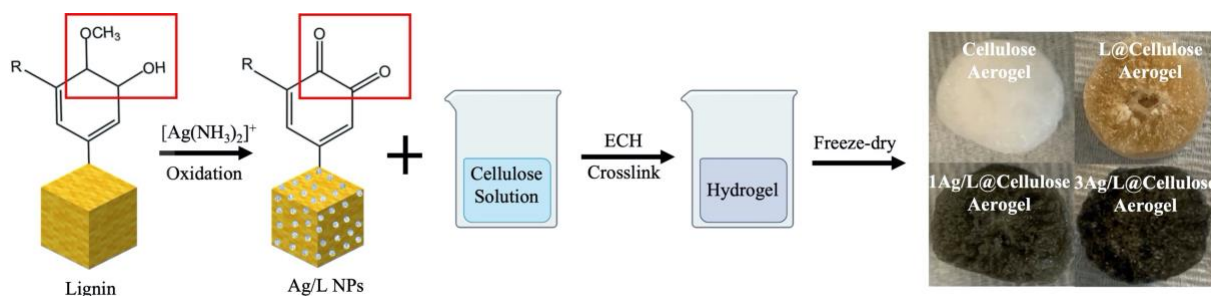


Figure 3.1 Schematic of Ag/L@Cellulose aerogel synthesis. Reduction of Ag^+ ions into Ag NPs via oxidation of phenolic hydroxyls and methoxy groups of the lignin indicated in the red box. Ag/L NPs were crosslinked with cellulose hydrogel through addition of epichlorohydrin (EPH) and freeze-dried to form cellulose aerogels containing indicated amount of Ag/L NPs. L = lignin only, 1Ag/L = 10mg, and 3Ag/L = 30mg. This figure is modified from Xe, H. (2022).

Cellulose aerogels are freeze-dried cellulose hydrogels with all the liquid portion removed, leaving behind empty pores with high specific surface areas for water absorption and catalytic reactions (236). The author observed that the size of the pores within the aerogels increased when Ag/L NPs were added during the crosslinking step (Figure 3-2). He speculated that this may be due to the hydrogen bonds formed between the cellulose and the Ag/L NPs, interfering with the crosslinking between the cellulose chains increasing the space between each other. He also observed that there were no obvious pore size difference between different amounts of Ag/L NPs loaded (Figure 3-2B,C,D).

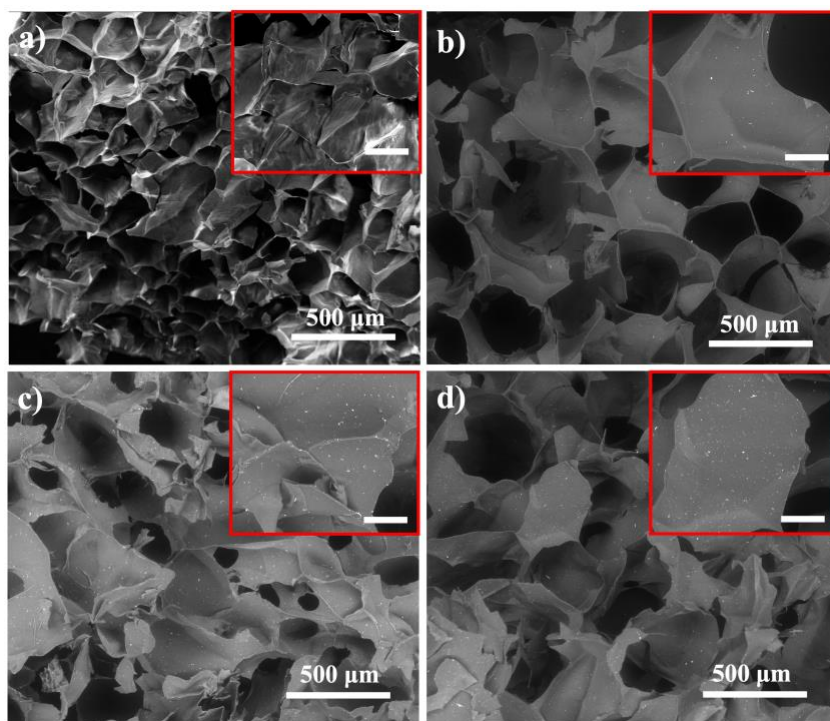


Figure 3.2 SEM images of the constructed aerogels. A) pure cellulose aerogel, B) 1Ag/L@Cellulose aerogel, C) 2Ag/L@Cellulose aerogel, and D) 3Ag/L@Cellulose aerogel. The inset images are magnified images of each aerogel with 100 μ m scale bar. The microscopic images were taken with FEI Quanta 250 FEG. This figure is taken from Xiao, H. (2022).

3.3.2 Enhanced antibacterial activity of Ag/L@Cellulose aerogels with increasing concentration of Ag/L NPs

A previous study published by He's lab has demonstrated that cellulose aerogel crosslinked with copper-benzenedicarboxylate show great antibacterial activities, 90 - 99.9999%, against a broad range of species suspended in sterile deionized water are incubated with synthesized aerogels for 24 hours at 37°C (232). We speculated that the Ag/L@Cellulose aerogels may exhibit similar antibacterial activity; however, new conditions had to be found since different metallic NP has been crosslinked to the cellulose aerogel.

E. coli BL21 DE3 was used as the experimental strain through the modifications. First trial was to see if the conditions used in the previous study are also transferrable to our aerogels. The conditions were incubating 5mL of subcultured *E. coli* resuspended in sterile deionized water with 5mg of each aerogel for 24 hours at 37°C, and no aerogel addition was used as control. I decided to use a 50mL conical tube during incubation, instead of a 15mL conical tube, to increase physical contact between the aerogels and the cells. In addition, I have checked for its killing ability at 2h and 4h time points. No antibacterial activities were observed under these conditions (Figure 3.3A). We hypothesized that this may be due to lack of physical contact between the Ag/L NPs and the cells. I decided to cut the aerogels into bigger size blocks, 50mg, to increase the contact. Although, 2h and 4h incubations still presented no antibacterial activity for all the aerogels, there was complete wipe out of cells for the cultures containing 2Ag/L@Cellulose aerogel and 3Ag/L@Cellulose aerogel that were incubated for 24h (Figure 3.3B). This confirmed our conditions for the following antibacterial experiments: resuspension of cells in sterile deionized water, 50mg blocks of each cellulose aerogel, and incubation at 37°C for 24 hours.

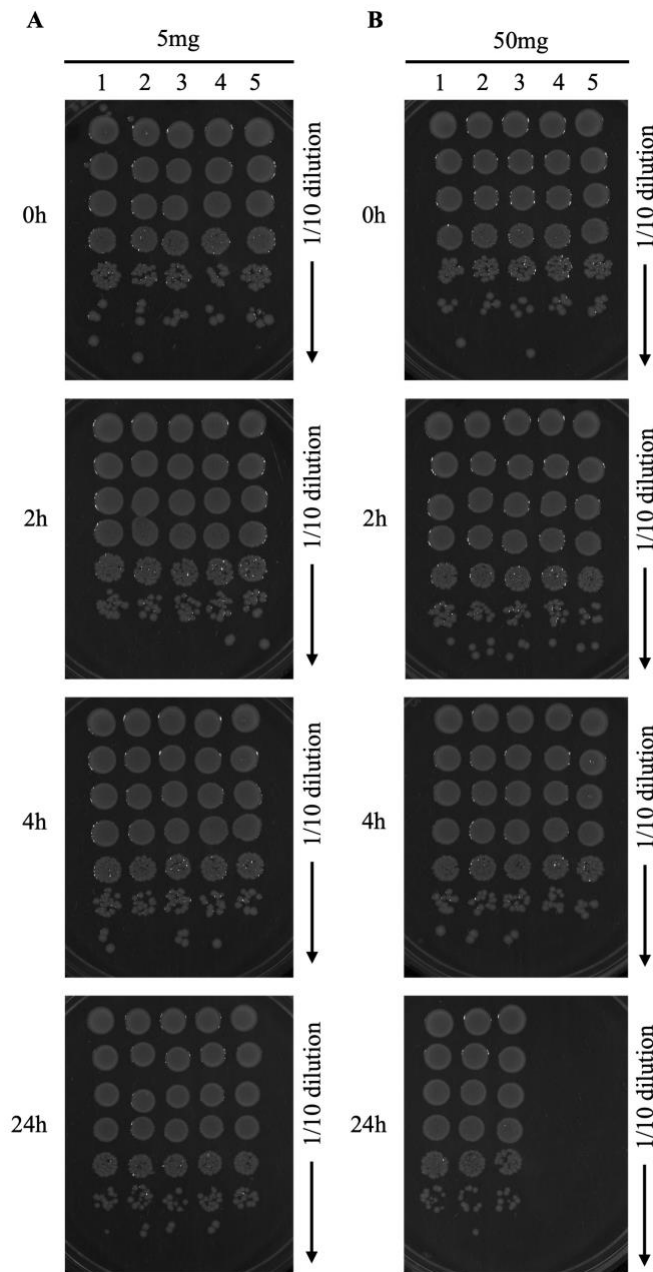


Figure 3.3 Antimicrobial activity of Ag/L@Cellulose aerogels under modified conditions.

Antimicrobial activity of aerogels containing different concentrations of Ag/L NPs cut into different sizes: A) 5mg or B) 50mg. 200 μ L of *E. coli* samples were taken from 5mL treatment cultures in 50mL conical tubes containing cellulose aerogels with varying concentrations of Ag/L NPs at each indicated time point and 10-fold serial diluted in 96-well plate. 5 μ L from each

dilution was taken and spotted on LB plates and grown overnight at 37°C. Treatments in each tube are 1: no aerogel, 2: L@Cellulose aerogel, 3: 1Ag/L@Cellulose aerogel, 4: 2Ag/L@Cellulose aerogel, and 5: 3Ag/L@Cellulose aerogel.

Since the conditions were confirmed, we wanted to scale up the experiment to standard plate counting for better representation of the survival of the cells after being exposed to the aerogels for 24 hours. There were minimal killing activities for cells exposed to L@Cellulose aerogel and 1Ag/L@Cellulose aerogel showing 30.8% and 26.5% killing efficiency, respectively. However, with increasing concentration of Ag/L NPs from 2Ag/L to 3Ag/L, the average number of surviving cells reduced by ~1.5 log, killing efficiency increasing from 99.8% to 99.99% (Figure 3.3A). This indicates sufficient loading of Ag/L NPs is required for cellulose aerogels to acquire successful antibacterial property. Because silver NPs have been studied to show antibacterial property against bacteria other than *E. coli* multiple times, we also speculated that our 3Ag/L@Cellulose aerogel, the most effective against *E. coli*, may show a broad-spectrum killing across varying species (237, 238). Relative to the negligible antimicrobial activity of L@Cellulose aerogel, 3Ag/L@Cellulose aerogel demonstrated high killing efficiency across all species tested (Figure 3.4B). It showed the strongest killing efficiency against *V. cholerae* and *S. aureus* (both at 99.998%), *P. aeruginosa* (99.9%) next, and least yet still strong against *B. subtilis* at 97.4%. These results revealed a promising effectiveness of Ag/L@Cellulose aerogels in wastewater treatment application.

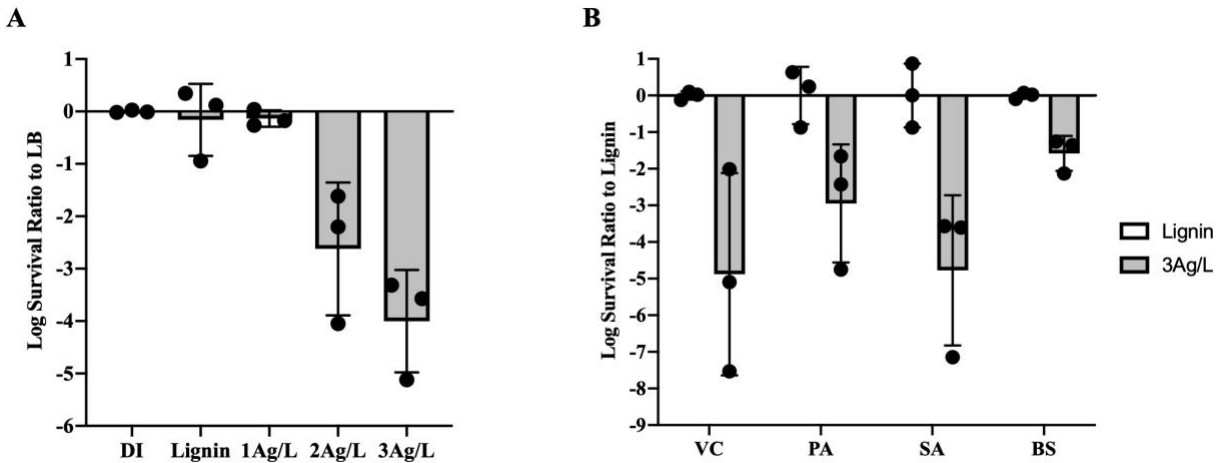


Figure 3.4 Ag/L@Cellulose aerogels exhibit antibacterial property against all tested species

of pathogens. A) Antibacterial activity improves with increasing concentration of Ag/L NPs in cellulose aerogel. *E. coli* CFUs were recovered after being exposed to cellulose aerogels containing indicated concentrations of Ag/L NPs for 24 hours at 37°C with standard plating method (1:10 serial dilution). The relative log survival of cells in each treatment was compared to the survival of cells in DI, deionized water only. B) 3Ag/L@Cellulose aerogel shows antibacterial property against various bacterial species. CFUs of each species were recovered after being incubated 3Ag/L@Cellulose aerogel for 24h at 37°C with standard plating method (1:10 serial dilution). The relative log survival of each species was compared to the survival of cells exposed to lignin, L@Cellulose aerogel only. VC: *V. cholerae*; PA: *P. aeruginosa*; SA: *S. aureus*; and BS: *B. subtilis*. Error bars indicate mean \pm standard deviation of three biological replicates. This figure is modified from Xe, H. (2022).

3.4 Discussion

Adsorption is one of the commonly used processes in wastewater treatment to remove heavy metal ions and organic dyes from industrial effluents due to its high efficiency (229). It

involves the attachment of pollutants from different phases onto solid surfaces with high specific surface area. Activated carbon, silica, and synthetic polymers are a few examples of materials often used in the process. However, exploring biocompatible and biodegradable adsorbents has gained attraction due to their cost-effectiveness and reduced impact on the environment.

Aerogels, especially synthesized with natural polysaccharides like cellulose, have been intensely studied in the past couple of decades due to its cost-efficiency, low density, and high porosity for adsorption (229, 239, 240). In addition, nanotechnology is another field of interest when it comes to developing adsorption processes. Metal NPs have been extensively studied for inducing catalytic reactions in degrading organic pollutants, and often they are crosslinked with polymers for synergistic effects in wastewater treatments (158, 241, 242). In this chapter, Ag/L@Cellulose aerogels (cellulose-based aerogels crosslinked with 10mg, 20mg, or 30mg of Ag/L NPs, synthesized by Xiao, H) have been investigated for their killing ability against varying pathogens.

Studying the cells that survive 24h exposure to the Ag/L@Cellulose aerogels via CFU counting revealed that the antimicrobial activity of the aerogels against *E. coli* improved with increasing amount of Ag/L NPs dispersed on the aerogels from 26.5% to 99.99% killing (Figure 3.3). In addition, 3Ag/L@Cellulose aerogel has shown impressive antibacterial activity against a broad range of species, including both Gram-negative and Gram-positive bacteria (Figure 3.4). This phenomenon, however, is somewhat expected since silver has been used for centuries for treating wounds to prevent infections (243). Although the exact antibacterial mechanism of Ag NPs is yet to be fully understood, it is speculated that it may be similar to that of free silver ions. Silver ions, positively charged, have the tendency to be attracted to the negatively charged bacterial membranes. When in contact, the ions can penetrate the membrane disintegrating the

cellular envelope leading to death by lysis which nanoparticle were also observed to behave similarly. In addition, they can also interact with sulfur or phosphorous containing proteins of the cell interfering enzymes involved in essential cellular processes, including respiration, replication, and growth. This also means that the ions can directly damage the cell's DNA, all leading to death (244–246).

An interesting observation was the antimicrobial activity of lignin only cellulose aerogel with a killing efficiency of 30.8% (Figure 3.3A). This may be due to the functional groups of the lignin that contain a high number of oxygens, like phenolic hydroxyls and methoxy groups (Figure 3.1) (247). The antibacterial activity of lignin has been shown to depend highly on its extraction methods since the final 3D structure of lignin plays a crucial role (248). Exploring if Ag/L NPs made from different lignin sources show similar, if not better killing, efficiency than the NPs used in this chapter is a point of interest. The 1/L@Cellulose aerogel also showed similar efficiency at 26.5%, suggesting that a sufficient amount of NPs is needed for successful bacterial killing, and that killing at this amount is likely due to the natural antibacterial property of lignin.

The findings from this chapter further support the multifunctionality of polysaccharide-based biopolymer linked with metal NPs and their synergistic effect against bacterial pathogens. Future studies can be focused on the reusability of the aerogels synthesized: how many times can we reuse the aerogel before it loses its integrity and antibacterial properties? During the incubation period, the disassembly of the aerogels into smaller pieces was observed. The shaking process potentially negatively impacts its reusability since it leads to loss of physical mass over time, which also could affect the removal process at the end of the treatment if used at a commercial level. Studying their killing efficiency against VBNC cells also would be important

since many environmental pathogens enter this phase when exposed to a harsh aquatic environment (249).

Chapter Four: Conclusion and Future Directions

It is now widely recognized that antibiotic resistance imposes a serious global threat against both public health and economy of the society. Since the first commercialization of penicillin in the 1940s, antibiotics have revolutionized our medicine practice and lifestyle. Through the Golden Age of antibiotics, hundreds of them have been developed and categorized into different classes depending on their structure and molecular activity, with inhibiting property encompassing both Gram-positive and Gram-negative pathogens. However, our dependence, misuse, and overuse of these miraculous compounds have driven rapid emergence of MDR and XDR in critical pathogens such as *E. coli* and *V. cholerae*, rendering them completely useless at times.

V. cholerae is a Gram-negative bacterium that causes cholera, an acute diarrheal disease that affects millions of people every year. Characterized by rice-water diarrhea with signs of severe dehydration in patients, cholera can quickly lead to fatalities within hours if not attended and treated swiftly. Antibiotic treatment is frequently accompanied by ORS for patients presenting with moderate-to-severe rehydration. However, with increasing number of *V. cholerae* strains becoming MDR, we are in desperate need of alternative options to prevent, control, and treat cholera.

In Chapter 2, compounds that exhibit bactericidal or bacteriostatic properties against growth of *V. cholerae* O1 El Tor strain C6706 (C6706) with high efficacy have been identified, MMV687807 and MMV675968. These compounds were effective at concentrations as low as 2.5 μ M and 10nM, respectively. The compounds are part of a library of drug-like compounds that presented antimicrobial activities across various pathogens (both eukaryotic and prokaryotic), accessible as the Pathogen Box from the MMV. C6706 was chosen as the experimental strain since

it is one of pathogenic strains of the seventh pandemic, directly isolated from a patient in Peru. Although unsuccessful in finding the molecular mechanism of MMV687807, studies revealed it is bactericidal against C6706, it leads to significant changes in genetic profile of exposed cells through RNA-seq analysis, and it can induce resistance under sub-inhibitory concentration. The WGS analysis highlighted that mutation to *VCI408* causes C6706 to become intrinsically resistant to MMV687807. *VCI408* is a gene for transcriptional regulator that negatively regulates an efflux system, VceCAB. Mutation to this gene causes expression of the efflux pump to pump out intruding toxic molecule. The resistance mechanism was confirmed through complementation with arabinose-inducible plasmid containing the WT gene. On the other hand, a possible bacteriostatic mechanism of MMV675968 has been identified. It was previously shown that the compound acts as a trimethoprim analog, directly interacting with the DHFR of *A. baumannii*. I hypothesized MMV675968 may act similarly in C6706 and confirmed that it triggers a cellular morphological change resembling those caused by trimethoprim. Furthermore, when WT C6706 was transformed with an arabinose-inducible plasmid containing trimethoprim-resistant DHFR, the cells were protected from the inhibiting activity of MMV675968. The compound also exhibited a broad-spectrum antibacterial activity at higher concentration akin to trimethoprim.

Overall, novel antibacterial compounds against *V. cholerae* were discovered, demonstrating the usefulness of utilizing the Pathogen Box as a starting point for discovering and re-directing drugs against MDR pathogens. Recently, Kanatani *et al.* (2022) have discovered that kinases play a crucial role during the transmission of *Plasmodium falciparum* between different hosts by targeting a specific cellular pathway, the motility (250). This study highly exemplifies the possibility of further exploitation of the Pathogen Box. By screening the library to identify compounds that specifically target a cellular system of interest, unknown functions of

hypothesized proteins or steps in complex cellular pathways can be uncovered to further expand our knowledge of interest.

Although promising, there are still several limitations in our understanding of these compounds before they could be applied in clinical setting, especially with the mechanism of MMV687807 unknown. MMV687807 has been described as salicylanilide-derivate of known compounds, IMD-0354 and niclosamide, and is known for its antimycobacterial activity (168, 251). Since antimycobacterial salicylanilides have been described as potent uncoupling agents, studying if it shows similar activity in bacteria may be a good starting point. If shown to target proton gradient, this will explain its high cytotoxicity against hepatic cells. As for MMV675968, studying its possible synergistic effect with sulfamethoxazole will be of interest, since trimethoprim is often administered with sulfamethoxazole. Although its resistant mechanism has been identified, further lowering the MIC of MMV675968 can be a great selling point to the public. The research can be expanded into studying compound in animals infected with C6706 to understand its active concentration in bodies, pharmacokinetics, and pharmacodynamics.

In Chapter 3, I have collaborated with Xiao He and his lab in understanding the antimicrobial characteristic of newly synthesized cellulose aerogel linked with Ag/lignin NPs. When antibiotics are prescribed and taken by the patients, they are not fully metabolized and are released into the sewage systems via bodily excretion. In addition, inappropriate disposal of the drugs also introduces highly concentrated raw antibiotics into the environment. These antibiotics exposed to the environment tend to be collected in water and into the WWTPs. Effluents gathered in WWTPs not only contain disposed antibiotics but also grow diverse communities of microbes due to the presence of rich nutrients in wastewater. WWTPs have been selected as one of the hotspots for the HGT of antibiotic resistance genes. For such reason, finding effective treatment

processes to remove both the pathogens and the antibiotics are crucial to reduce the rate of ABR dispersal. The Ag/L@Cellulose aerogel was synthesized to study its effectiveness in treating organic dyes, antibiotics, and pathogens that may reside in wastewater. To test the antibacterial activity of the aerogels against various pathogenic species (*V. cholerae*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*), each species was incubated with aerogels containing three different concentrations of Ag/lignin NPs and were counted for surviving CFUs. An increasing bactericidal activity of the aerogel was observed with increasing concentration of Ag/lignin NPs. The 3 Ag/L@Cellulose aerogel presented the highest potency, with a killing efficiency as high as 99.99% against *E. coli*, *S. aureus*, and most importantly *V. cholerae*. This study showcased a possible alternative approach to remove pathogen load effectively from wastewater. Because the synthesized aerogel exhibited both antibiotic degrading and bactericidal properties, commercialized usage of the material can aid us in reducing the HGT of antibiotic resistance in WWTPs.

However, there is much to be learned about the synthesized aerogel before it could be used as conventional wastewater treatment. The conditions used for this thesis cannot represent the environment of the WWTPs. It will be necessary to test the aerogel under conditions that mimic the complex environment to answer important questions like 1) how the aerogel will be mixed in with the wastewater for maximum contact, 2) how efficient the aerogel is at removing pathogens in complex microbial community, 3) is there other classes of antibiotics that could be removed, and more. The study also lacks on characterizing the properties of Ag/Lignin NPs. Silver NPs are known to transform when in contact with environment, which may lead to causing harm to ecosystem and the residing organisms (252, 253). To understand its impact, it is important to study on their physicochemical properties, such as the size, shape, and ionization and dissolution

properties (254). Furthermore, investigating on how much NPs are released from the aerogel during the agitation period and if they could be successfully removed at the end of the treatment will be critical for commercial development of the aerogel.

The best way to control the rate of antibiotic resistance dissemination is to eliminate the exposure of environmental microbes to the antibiotics. However, due to the extensive usage of antibiotics in all sectors of our society, achieving complete elimination is quite unrealistic and practically impossible. As a society, we must make conscious efforts to lessen and strictly control the amount of antibiotics being used and released back into the environment. Without strong and immediate actions, we may face the detrimental consequences of losing our power to combat these notorious pathogens.

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Re: Permission to use Figures



 Xiao He
To:  Sara Kim

Hi Sara,

I confirm that you can use the figures in our published papers in *Cellulose Journal* (Cellulose 29 (17), 9341-9360, 2022)

Please let me know if you have any other concerns.

Have a great weekend!

Best,

Xiao

发件人: Sara Kim
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收件人: Xiao He
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Hello Xiao,

As to our discussion today, I am looking to include modified and original figures from our paper on *Cellulose Journal*. Would you be able to give me permission on this?

Thank you,

Sara