The Classification of Fungal Derivatives with Clinical Potential

Marjot Foundation Final Report

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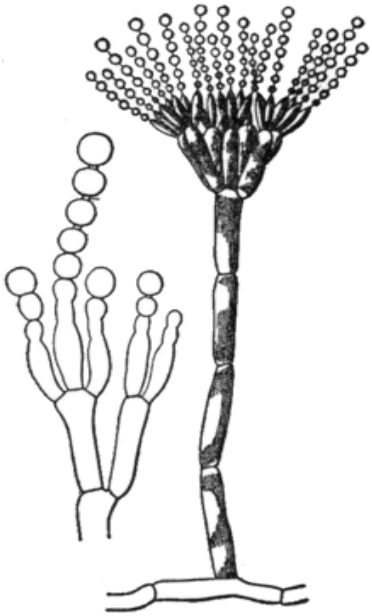

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Introduction

The word “antibiotic” was first used as a noun in 1941 to describe a small molecule synthesized by a microorganism that antagonizes the growth of other microbes. An antibiotic is a substance that either inhibits the growth and replication of a bacterium or kills it. Antibiotics target bacterial infections and can be either bacteriostatic or bacteriocidal. Bacteriostatic compounds prevent the bacterial growth (i.e. keep bacteria in a

stationary phase of growth) while bacteriocidal compounds kill the bacteria (Pankey, 2004). More than 350 agents so far have reached the world market as antimicrobials. They include natural products, semi-synthetic antibiotics and synthetic chemicals (Aminov, 2010).

From 1945 to 1955, the development of penicillin, which is produced by a fungus, along with streptomycin, chloramphenicol, and tetracycline, which are produced by soil bacteria, commenced the "Golden Age of Antibiotics." This era lasted from the 1950s to the 1970s. Paul Ehrlich's theory of a "magic bullet" that could selectively target disease-causing microbes without harming the host was the foundation of early antibiotic discovery. Ehrlich argued that chemical compounds could be synthesized that would "be able to exert their full action exclusively on the parasite harbored within the organism" (Silber, 2016). From this assertion, Ehrlich developed a large-scale, systematic screening program in 1904 to discover a drug against syphilis, a disease that was endemic and almost incurable at that time. Ehrlich's systematic screening approach became the cornerstone of drug search strategies in the pharmaceutical industry and resulted in thousands of drugs being identified and translated into clinical practice. His screening approach also catalyzed the discovery of sulfa drugs, for example, sulfonamidochrysoidine (KI-730, Prontosil), which was synthesized by Bayer chemists Josef Klarer and Fritz Mietzsch and tested by Gerhard Domagk for antibacterial activity. It is well known that the most consequential antibiotic discovery was made by Alexander Fleming. His discovery of penicillin from the fungi *Penicillium notatum* on September 3, 1928 led to the discovery of hundreds of beta-lactam antibiotics. In 1940, a team of scientists from Oxford, led by Howard Florey and Ernest Chain, published a paper describing the purification of penicillin for clinical purposes. Their findings led to the mass production and distribution of penicillin in 1945. Fleming's screening method involving inhibition zones in lawns of pathogenic bacteria on the surface of agar-medium plates required fewer resources than pre existing screening models. This screening method revolutionized the drug discovery process. Fleming was also among the first who cautioned about the potential resistance to penicillin if administered improperly. The discovery of the first three antimicrobials, Salvarsan, Prontosil and penicillin, established the paradigm for future drug discovery research (Silber, 2016).

Since the golden era of antibiotic discovery, there have been no major discoveries involving novel antibiotic classes. Due to the declining discovery rate, the current approach for the development of antibiotics has focused on the modification of existing compounds (Aminov, 2010). For decades, antibiotics have extended global life expectancies by treating bacterial infections. In 1920, the average life expectancy in the U.S. was 56.4 years; now, however, the average U.S. lifespan is nearly 80 years. Even in developing countries, antibiotics have decreased the morbidity and mortality rates heightened by food-borne or other poverty-related infections (Mackenzie, 2015).

The widespread use of antibiotics has placed evolutionary selection pressure on bacterial communities and has resulted in an abundance of microorganisms with antibiotic resistance. The number of effective therapeutic treatments against life-threatening bacterial infections has fallen dramatically due to the emergence of multidrug-resistant (MDR) pathogens. Infectious diseases have recently been listed as the second leading cause of death worldwide (Silber, 2016). Decades after patients were first treated with antibiotics, bacterial infections are once again deadly threats. The crisis of antibiotic resistance is attributed to the overuse and misuse of antibiotic agents, coupled with the exhaustion of terrestrial sources of microorganisms able to synthesize novel, effective antibiotic compounds. Another reason for the lack of drug development by the pharmaceutical industry is the decreased economic incentives as well as the painstaking regulation requirements placed on drug-developing companies (Munita, 2016). Currently, drug discovery pipelines are almost empty, with few new antibiotic candidates in development or approval (Silber, 2016). The Centers for Disease Control and Prevention has recently published an extensive list of bacterial pathogens whose threats are responsible for placing a significant clinical and financial burden on the American healthcare system and patients (Munita, 2016).

In order to comprehend the threat of antibiotic resistance, it is important to understand the molecular and biochemical mechanisms of bacterial resistance. Bacteria are endowed with remarkable genetic plasticity that enables them to respond to a variety of environmental threats, including the existence of nearby

antibacterial molecules. Bacteria sharing an ecological niche with antimicrobial-producing microorganisms have evolved mechanisms to tolerate the harmful antibiotic molecules synthesized by competing microorganisms. These adaptive mechanisms are considered "antibiotic resistance." From an evolutionary perspective, bacteria utilize two genetic strategies to adapt to an antibiotic "attack." First, mutations can occur in genes associated with the mechanism of action of the compound. Second, bacteria can acquire foreign DNA coding for resistance through horizontal gene transfer (HGT) processes (Munita, 2016).

For mutational resistance, a subset of bacterial cells from a susceptible population must develop mutations in genes that are affected by the activity of the antibiotic. These mutations in preserve cell survival in the presence of the antimicrobial molecule. Once a resistant mutant emerges, the antibiotic eliminates the susceptible members of the population, leaving resistant bacteria to predominate. Mutational changes that promote resistance are often costly to cell homeostasis (i.e. decreased fitness) and are only maintained if exposed to the antibiotic. Mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms: modifying the antimicrobial target (decreasing the affinity for the drug, see below), decreasing the drug uptake or activating efflux mechanisms to extrude the threatening molecule or changing metabolic pathways via modulation of regulatory networks (Munita, 2016).

Horizontal gene transfer allows a bacterium to acquire foreign DNA, and it is one of the central drivers of bacterial evolution. Most clinically significant antimicrobial agents are derived from molecules naturally found in the terrestrial (soil) environment. Bacteria sharing the environment with these harmful molecules possess innate genetic determinants of resistance, called environmental resistomes. Each environmental resistome is a prolific source of antibiotic resistance genes in bacterial pathogens. Horizontal gene transfer has disseminated resistance to many popular antibiotics. There are three main strategies used to acquire antibiotic resistance: transformation (incorporation of naked DNA that codes for resistance), transduction (resistance genes injected via phages) and conjugation (resistance passes between bacteria via bacterial "sex"). Transformation is the least complex strategy of HGT, though only a handful of clinically relevant bacterial species are able to "naturally" incorporate naked DNA to acquire resistance. Resistance occurring in a hospital environment is often caused by conjugation. This method of gene transfer involves cell-to-cell contact and occurs in the gastrointestinal tract of humans being treated by antibiotics. Conjugation uses mobile genetic elements (MGEs) as vehicles to share genetic information, although direct chromosome-to-chromosome transfer has been characterized. Examples of relevant MGEs are plasmids and transposons which play a significant role in the development of antibiotic resistance among bacterial pathogens (Munita, 2016).

Different types of bacteria utilize biochemical pathways the counter antibiotics. For example, fluoroquinolone (FQ) resistance occurs due to three distinct biochemical pathways that can coexist in the same bacteria simultaneously. There can be mutations in genes encoding the target site of FQs (DNA gyrase and topoisomerase IV) or over-expression of efflux pumps that extrude the drug from the cell. Other bacterial species have evolved a preference for certain mechanisms of resistance over others. For example, the predominant mechanism of resistance to β-lactam antibiotics in gram-negative bacteria is the production of β-lactamases, whereas resistance to these compounds in gram-positive organisms is mostly achieved by modifications of their target site, the penicillin-binding proteins (PBPs). This phenomenon is likely due to differences in the cell envelope between gram-negative and gram-positive. In the gram-negative bacteria, the presence of an outer membrane permits cell "control" of the entry of molecules to the periplasmic space. Most β-lactam antibiotics require specific porins to reach the PBPs in the inner membrane. Therefore, the bacterial cell controls the access of these molecules to the periplasmic space allowing the production of β-lactamases in sufficient concentrations to tip the kinetics in favor of the destruction of the antibiotic molecule. Gram-positive bacteria lack this advantage (Munita, 2016).

Over the past fifty years, microorganisms have been prolific producers of thousands of antibacterial molecules (Silber, 2016). Over 50% of all new compounds approved as drugs were based on a natural product. Natural products are synthesized by living organisms as part of their secondary metabolism. These organic compounds are "secondary" because, in general, they are not required for life-sustaining primary processes such as respiration or synthesis of biological macromolecules such as DNA, proteins, lipids, and carbohydrates.

In spite of this, secondary metabolites are often important to the survival of the organism and function as a "chemical arsenal that allows them to compete with each other" (McPhail, 2010). These compounds are chemically and structurally complex and are excellent sources of new medicine. Organisms at hydrothermal vents, fissures in the seafloor that extrude geothermally-heated water, exhibit unique forms of primary and secondary metabolism and represent untapped potential sources of novel natural products with new biological mechanisms of action.

More than half of all antibiotics are produced by actinomycetes, 10% to 15% by non-filamentous bacteria and 20% by filamentous fungi. Many believe that only a minuscule portion of microbial bioactive compounds have been discovered since drug discovery efforts focus on specific taxonomic groups or environments. Marine ecosystems are severely understudied even though marine microorganisms synthesize structurally unique bioactive compounds that are distinct from those produced by terrestrials fungi (Silber, 2016).

Marine fungi form an ecological, not taxonomic group. The members of this group include obligate and facultative marine fungi. Obligate marine fungi grow and sporulate exclusively in sea water. The spores that they produce germinate in sea water. Facultative marine fungi live in freshwater or a terrestrial milieu. They possess physiological adaptations that allow them to grow and sporulate in the marine environment. 800 species of obligate marine fungi have been characterized and reported thus far. The species mostly belong to ascomycetes, anamorphs, and basidiomycetes.

Fungi have been reported from a number of extreme marine environments. These environments are characterized by elevated hydrostatic pressure, extreme temperature (low temperature deep-sea and sea-ice (2 to 4° C) and high temperatures near hydrothermal vents (400°C), hypersaline water bodies and hypoxic (oxygen deficient) conditions. Deep-sea hydrostatic pressure increases by 1 bar with every 10 m depth (Raghukumar, 2008). Several pharmaceutical companies have attempted to bioprospect in marine extreme environments. These companies focus on extremophilic bacteria for their biodiversity, thermostable and cold-tolerant enzymes, novel secondary metabolites, metal-tolerant enzymes, stress proteins and bioremediation potentials. A variety of commercial products including the enzyme Taq polymerase and Vent polymerase have been obtained from extremophilic microorganisms. Fungi from extreme marine environments have not yet been tapped for any of these commercial applications.

Since the discovery of cephalosporin in 1948, marine fungi have been considered a promising source of bioactive compounds. The initial antimicrobial compound isolated from a deep-sea fungus in the genus *Aspergillus* was gliotoxin, which can inhibit the growth of the gram-positive bacteria *S. aureus* and *B. subtilis*. This fungus was isolated from the mud of the Seto Inland Sea in Japan. Following this finding, several other antibacterial molecules from deep-sea fungi were isolated and characterized. Overall, the compounds isolated from deep-sea fungi are still a minority compared to molecules produced by marine fungi isolated from surface waters. Also, general awareness regarding the presence of fungi in the deep-sea is very meager. Zhang et al. isolated 13 novel fungal species from deep-sea sediments in the South China Sea. Many of the isolates were able to synthesize antimicrobial compounds against pathogenic bacteria and fungi, such as *Micrococcus luteus*, *Pseudoaltermonas piscida, Aspergerillus versicolor*, and *A. sydowii*. Prenylxanthones are a significant group of secondary metabolites possessing a wide range of biological and pharmacological activities. Four new antifungal and antibacterial prenylxanthones, emerixanthones, were identified from the deep-sea fungus *Emericella* sp. SCSIO 05240, isolated in the South China Sea (3258 m). These molecules inhibited *Escherichia coli* (ATCC 29922), *Klebsiella pneumoniae* (ATCC 13883), *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *Acinetobacter baumannii* (ATCC 19606), and *Aeromonas hydrophila* (ATCC 7966). Another compound belonging to the xanthone class, engyodontiumone, was purified from *Engyodontium album* DFFSCS021, a deep-sea fungus collected from the South China Sea (3739 m). This molecule exhibited inhibitory activity against *E. coli* and *B. subtilis*. Fifteen new depsidone-based analogs, spiromastixones A–O have been isolated from a fungus in the genus *Spiromastix* that was collected from the South Atlantic Ocean (2869 m). These compounds showed an antimicrobial activity towards *S. aureus*, *B. thuringiensis*, and *B. subtilis* (Tortorella, 2018). Roth et al. (1964) isolated marine fungi from oceanic waters of the Atlantic Ocean

from a depth of 4450 m. Lorenz and Molitoris (1997) demonstrated the growth of yeasts at 400 bar pressure. Barotolerant fungi were isolated from calcareous sediments obtained from 965 m depth in the Arabian Sea (Raghukumar and Raghukumar 1998). Takami (1999) isolated *Penicillium lagena* and *Rhodotorula mucilagenosa* from the Mariana Trench at about 11,500 m depth in the Pacific Ocean. A number of culturable fungi have been recovered from deep-sea sediments from 5000 m depth of the Central Indian Basin by incubating the sediments under hydrostatic pressure of 200-300 bar. Despite the demonstrated ability to culture some of these fungal species, the biotechnological capacities of these barotolerat fungi regarding culture in commercial fermenters are yet to be assessed (Raghukumar, 2008).

Deep-sea hydrothermal vents are among the most extreme and dynamic environments on Earth. In these locations, islands of highly dense and biologically diverse communities exist in the immediate vicinity of hydrothermal vent flows. These communities are comprised of organisms with distinct metabolisms based on chemosynthesis and growth rates. These organisms are biologically distinct from terrestrial fungi and are only comparable to those previously isolated from shallow water tropical environments. The geological setting and geochemical nature of deep-sea vents significantly impact the biogeography of vent organisms, chemosynthesis, and the known biological and metabolic diversity of Eukarya, Bacteria, and Archaea. As of late, deep-sea hydrothermal vents have been considered hot spots for natural product investigations. In order to harness these natural products, additional research must be conducted to determine how to collect hydrothermally-derived organisms in a manner that preserves the stability and longevity of vent sites and to determine how to culture natural product-producing deep vent organisms in the laboratory. Recently, cost-effective technologies and advanced molecular techniques have been developed to accelerate natural product discoveries from these microbial diversity hotspots (Thornburg, 2010). Hydrothermal environments possess a high microbial biodiversity, high productivity, and therefore high levels of interaction among species. The result of this is a heightened production of secondary metabolites or bioactive chemical compounds with an ecological role. These secondary metabolites are essentially “raw antibiotics.”

The Guaymas Basin is a hydrothermal environment located in the Gulf of California (Sea of Cortez). It is situated between Baja California and mainland Mexico. It is a young marginal rift characterized by active seafloor spreading and the rapid deposition of organic-rich, diatomaceous sediments from the overlying waters. The two (northern and southern) axial troughs of Guaymas Basin are bounded by extensive systems of axial-parallel fault lines on either side. Active hydrothermalism is characteristic of the southern trough, where the hydrothermal sediments, mounds and chimneys form a complex hydrothermal landscape. The diversity of seafloor features reflects a variety of geochemical and temperature settings. The hydrothermal reactions in this environment are driven by underlying thermodynamic disequilibria and they generate and mobilize volatile hydrocarbons that percolate to the sediment surface. This subsurface system produces complex pathways that ultimately reach the sediment surface, where they are evident in hydrothermal edifices such as mineral deposits, venting orifices emitting hot hydrothermal fluids, and hydrothermally active sediments. The unique component of the Guaymas Basin is its abundance of microbial mats dominated by *Riftia* and *Beggiatoa* colonies (Teske, 2016).

The Sonora Margin, located in the Guaymas Basin, is characterized by abundant cold seeps and white microbial mats surrounded by polychaete and gastropod beds. Elevated microbial density has been observed in the underlying sediments. Two groups of Bacteria have been identified at the Sonora Margin: *Desulfosarcina/Desulfococcus* and a *Desulfobulbus*-related group. These bacteria exhibited distinct niche distributions and association specificities depending on sediment surface communities and geochemical parameters (Vigneron, 2013).

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, coliform bacterium belonging to the genus *Escherichia. E. coli* is commonly found in the lower intestine of warm-blooded organisms (e.g. humans). The majority of *E. coli* strains are harmless but some serotypes can cause a variety of infections such as cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), traveler's diarrhea, neonatal meningitis and pneumonia. Neonatal meningitis caused by *E. coli* has a mortality rate of 8% and most survivors are left with neurological or developmental abnormalities. *E. coli* respiratory tract infections are often a byproduct of a UTI

caused by *E. coli. E. coli* pneumonia usually manifests as a bronchopneumonia of the lower lobes and may be complicated by empyema. More than 90% of all uncomplicated UTIs (e.g. urethritis, cystitis, symptomatic cystitis, pyelonephritis, acute prostatitis, prostatic abscess) are caused by *E. coli*. Other miscellaneous *E. coli*-caused infections include septic arthritis, endophthalmitis, suppurative thyroiditis, sinusitis, osteomyelitis, endocarditis and skin and soft-tissue infections (Madappa, 2017). Also, *E. coli* is a frequent cause of life-threatening bloodstream infections. Currently, more than one-third of all *E. coli* strains have acquired resistance to major antibiotic classes, including fluoroquinolones and extended-spectrum (Collignon, 2009).

Staphylococcus aureus (*S. aureus*) is a gram-positive, round-shaped bacterium that is a member of the genus *Staphylococcus*. It is occasionally harmless and found in the upper human respiratory tract and on the skin. *S. aureus* is an opportunistic pathogen and can cause skin infections (i.e. abscesses, respiratory infections, sinusitis, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome). Furthermore, *S. aureus* can cause life-threatening infections such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis. *S. aureus* is one of the five most common causes of hospital-acquired infections or post-surgery infections. Each year in the U.S., 500,000 patients contract a staphylococcal infections and 50,000 of those patients die on average due to the infection. The most well known antibiotic resistance strain of *S. aureus* is methicillin-resistant *Staphylococcus aureus* (MRSA) which often causes hospital outbreaks and can be epidemic. There is no existing vaccine for *S. aureus* (Foster, 1996).

Pseudomonas aeruginosa (P. aeruginosa) is a gram-negative, rod-shaped bacterium that can cause human infections. *P. aeruginosa* is a multidrug resistant pathogen that has considerable medical importance due to its advanced antibiotic resistance mechanisms and its association with deadly illnesses. *P. aeruginosa* is especially dangerous for immunocompromised patients. *P. aeruginosa* causes a variety of infections, including respiratory tract (e.g. pneumonia), bloodstream (e.g. bacteremia), heart (e.g. endocarditis), ear (e.g. otitis externa and media), eye (e.g. bacterial keratitis, endophthalmitis), bone and joint (e.g. osteomyelitis), skin (e.g. ecthyma gangrenosum) and urinary tract. *P. aeruginosa* is particularly dangerous for its ability to develop resistance quicker than any other pathogen. In fact, *P. aeruginosa* is able to acquire resistance to an antibiotic during the course of treating an infection. This pathogen has acquired resistance to almost all antibiotic agents in circulation (Friedrich, 2017).

This study will contribute to the ongoing scientific effort towards the recovery of novel fungal taxa from unique ecological niches such as Guaymas Basin hydrothermal sites. A fungal collection comprised of hydrothermally-derived filamentous fungi and yeasts will be screened against pathogenic and drug-resistant strains of *E. coli, S. aureus* and *P. aeruginosa.* Ultra performance liquid chromatography tandem mass spectrometry will be used to classify and identify the clinically significant fungal derivatives. Overall, this study will hopefully contribute to the search for substitutional antibiotics to cope with the crisis of antibiotic resistance.

Abstract

This research responds to the threat of antibiotic resistance and the concurrent depletion of sources of terrestrial microorganisms able to synthesize novel antimicrobial molecules. This crisis has necessitated efforts, such as bioprospecting in geochemically extreme environments, to augment the existing arsenal of antibiotics. The morphological and phylogenetic diversity of fungal communities colonizing Guaymas Basin hydrothermal vent sediment were explored through culture-dependent methods and analyses of the ribosomal RNA internal transcribed spacer and ribosomal RNA 26S subunit sequences. A fungal collection was established via inoculation, culture and isolation methods. Through modern molecular protocols (DNA extraction, precipitation, amplification), the fungal isolates were taxonomically affiliated with *Cadophora malorum, Xylaria feejeensis, Engyodontium album, Cladosporium lycopodium, Cladosporium halotolerans, Ramularia glennii, Aspergillus niger, Penicillium rubens, Rhodotorula mucilaginosa, Dioszegia xingshanensis, Aureobasidium pullulans* and *Torulaspora delbrueckii.* The inhibitory activities of these isolates against pathogenic strains of *Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC-35556, and a drug-resistant strain of *Pseudomonas aeruginosa* ATCCMP-23 were explored through variations of the Kirby-Bauer

antimicrobial susceptibility testing (AST) method. The natural products of *X. feejeensis* and *C. lycopodium* were able to kill *S. aureus,* while the derivatives of *C. halotolerans, R. glennii* and *T. delbrueckii* were able to kill both *S. aureus* and *E. coli.* None of the fungal isolates could inhibit the growth of the drug-resistant strain of *P. aeruginosa.* Overall, the clinically relevant fungi identified in this study have augmented the finite source of known microorganisms able to synthesize antibacterial molecules.

Research Objectives

The basic question underscoring this study was if deep-sea fungi can withstand laboratory culture at 1 atm (versus 200 atm) and 23°C (versus 2°C or 156–319°C depending on vent proximity). Once the culturability of the fungi was confirmed, the central question was if hydrothermal vent-derived fungi from Guaymas Basin sediments can synthesize compounds effective at inhibiting the growth of pathogenic, drug-resistant bacterial pathogens. This research aimed to discover and harness the natural products from clinically unstudied, barophilic fungi that could function as biological templates for alternatives to mainstream antibacterial agents that have been rendered ineffective by antibiotic resistance. Furthermore, this study was designed to investigate the fungal diversity present at Guaymas Basin hydrothermal vents, and to augment the fungal collection established in 2017-2018 through the use of additional culture media.

Research Hypotheses

Since there is no existing report of the diversity nor the antibacterial abilities of Guaymas Basin-derived fungi, it was difficult to form species-specific hypotheses. A general hypotheses was that at least one member of the fungal collection would exhibit antagonistic activity towards either *E. coli, S. aureus* or *P. aeruginosa.*

The working hypothesis underscoring this research is that significant environmental variations between terrestrial and deep-sea hydrothermal environments may promote the synthesis of chemically distinct metabolites with distinct modes of action and cellular targets. It was further hypothesized that the bacterial pathogens would be more susceptible to the molecules produced by hydrothermal vent-derived fungi due to the pathogens' unfamiliarity with the molecules.

Methodology

All work not done in a fume hood was done on a lab bench sterilized with a 10% bleach solution. All measuring performed with 10, 100, 500, or 1000µl Eppendorf Research pipetters. All plates and tubes were labeled according to experimental sample code. All tools were UV crosslinked before use. All bacterial plates and fungal plates whose growth was transferred to new plates were parafilmed and disposed in an autoclave bag. The metal inoculation loop was always flamed between samples, and isolates using a Bunsen Burner.

Sample Collection

Approximately 12 sediment and water samples were collected from off-axis and hydrothermal sites at Guaymas Basin, Mexico. Samples were collected on the R/V Atlantis in December 2016 using the ROV Alvin. Sample access was granted by Dr. Andreas Teske.

Potato Dextrose Agar Recipe

Potato Dextrose Agar (PDA-) without antibiotics was prepared using a 3% sea salt recipe with a 1:5 dilution to simulate the low nutrient conditions of deep-sea sediments. 7.8 g of Remel Potato Dextrose, 12 g of Bacto Agar and 30g of Sigma Sea Salt were combined with 1 liter of Milli-Q double-distilled water (ddH_2O). This was repeated for the production of Fungal Potato Dextrose Agar (PDA+) media (with antibiotics), to which 0.5 g of Fisher BioReagents Chloramphenicol as well as 0.2 g of Fisher BioReagents Penicillin-G were added after autoclaving and cooling to 60°C. Both media were labeled, stirred on a magnetic stir plate, shaken for 30 seconds, and placed into a Tuttnauer Autoclave 3870 with their caps partially unscrewed. They were sterilized for 60 minutes at 121□. Approximately 25 mL of liquid PDA- or PDA+ were transferred to each

Fisherbrand Sterile Polystyrene Petri Dishes using an Eppendorf Easypet 3 and a 25-mL pipette. Once solidified, the poured plates were stored at 20□. This recipe was additionally diluted 1/10 and 1/100.

Maltose Extract Agar Recipe

Fungal Maltose Extract Agar (MEA-) without antibiotics was made following a 3% sea salt recipe with a 1:5 dilution. 30 g of Sigma-Aldrich Malt Extract, 15 g of Bacto Agar, 1 g of Sigma-Aldrich mycological Peptone, and 30 g of Sigma Sea Salt were measured. All ingredients were added to a flask containing 1 liter of Milli-Q ddH_2O and a magnet. This recipe was repeated for the production of MEA with antibiotics (MEA+) as above. Autoclaving, plate pouring, and media storing procedures were repeated as for PDA, and the plates were stored at 20□. This recipe was additionally diluted 1/10 and 1/100.

Czapek Dox Agar Recipe

Czapek Dox Agar (CDA-) (with 3% sea salt and without antibiotics) was made by combining 30 g of Sucrose, 2 g of Sodium nitrate, 1 g of Dipotassium phosphate, 0.5 g of Magnesium sulphate, 0.5 g of Potassium chloride, 0.01 g of Ferrous sulphate, 15 g of Bacto Agar, 30 g of sea salt and 1 liter of Milli-Q ddH_2O. The mixture was agitated using a heating plate set to 235□ and 700 rpm. Autoclaving, plate pouring, and media storing procedures were repeated as for PDA, and the plates were stored at 20□.

Sabouraud Dextrose Agar Recipe

Sabouraud Dextrose Agar (SDA-) (with 3% sea salt and without antibiotics) was made by combining 10 g of mycological peptone, 40 g of Dextrose, 15 g of Bacto Agar, 30 g of sea salts and 1 liter of Milli-Q ddH_2O. The mixture was agitated using a heating plate set to 275□ and 800 rpm. Autoclaving, plate pouring, and media storing procedures were repeated as for PDA, and the plates were stored at 20□.

Potato Glucose Agar Recipe

Potato Glucose Agar (PGA-) (with 3% sea salt and without antibiotics) was made by combining 39 g of Remel Potato Glucose Agar, 10 g of Bacto Agar, 30 g of sea salts and 1 liter of Milli-Q ddH_2O. Autoclaving, plate pouring, and media storing procedures were repeated as for PDA, and the plates were stored at 20□.

Minimal Media Recipe

50 g of Guaymas Basin sediment was autoclaved for 30 minutes at 121□. Minimal media (MM) was made by combining 15 g of sea salts, 10 g of agar, 30 g of sterilized sediment and 1 liter of Milli-Q ddH_2O. Autoclaving, plate pouring, and media storing procedures were repeated as for PDA, and the plates were stored at 20□.

Inoculation

A 50-mL falcon tube containing sample P8-3 5-10 AH was removed from the 20□ refrigerator and vortexed for one minute using a Vortex-Genie 2 (MoBio Laboratories Inc.). Two 50-mL falcon tubes were sterilized using a Spectrolinker XL-1000 UV crosslinker. A 1X PBS dilution was made in an AirClean 600 PCR Workstation fume hood by adding 5 mL of BP399-500 Phosphate Buffered Saline 10X Solution to 45 mL of Fisher BioReagents BP2470-1 Sterile Water in a sterile 50-mL falcon tube. A small portion of P8-3 5-10 AH was dispensed into a sterile 50-mL falcon tube along with 35 mL of the PBS 1X Solution. The falcon tube was vortexed for 30 seconds. The sample-PBS mixture was used to inoculated 5 plates per media type (30 plates per sample). A sterile Fisherbrand Serological Disposable Rake was used to spread the solution around the plate until every part was covered. All six plates were parafilmed and then stored in the 23□ incubator. This entire inoculation process was repeated with the sample from Alvin Push Core 4868-12, Alvin Push Core 4871-10 Supernatant, Gravity Core P10-4 55-60, Alvin Push Core 4867-14, Gravity Core P5-3 55-60, Alvin Push Core 4862-4, and P3_4 55-60. The original sample containers were returned to a 20□ refrigerator along with leftover sample-PBS slurry. After inoculation, plates were put directly into a 23□ incubator.

Samples from Alvin Push Core 4868-12, Alvin Push Core 4872-13 Supernatant, Dark Silicate Crusts from Dive 4864, White Silicate Crusts from Dive 4864, and Barytes from Alvin Dive 4872 were crushed in a sterile portable rock crusher until they reached a relatively fine consistency. The rock crusher was rinsed with Milli-Q water and ethanol between samples. After being crushed, sediment from each sample was completely transferred to a crosslinked 50-mL falcon tube. The inoculation procedure above was directly followed.

Transferring of Fungal or Yeast Growth from Original Sample Plates

15 mL of 70% ethanol solution were transferred to a sterile 50-mL beaker. For every plate with visible growth, a sterile inoculation loop was used to scoop up as much of the chosen colony as possible and streak the new plate in the same "lawn" manner. The cells were streaked on a plate of the same medium as the original plates (preferred medium). The newly transferred and original plates were parafilmed and stored to the 23□ incubator. Transferred plates were incubated until there was enough growth to make a secondary transfer.

General Procedure

All 12 environmental samples were inoculated onto MEA+, MEA-, PDA+, PDA-, CDA-, SDA-, PGA- and MM-. Plates with unique fungal growth were transferred to secondary plates, following the methods detailed above until plates with only one morphotype were obtained.

Light Microscopy to Identify Questionable Growth (lactophenol blue --- cotton blue)

For plates with microbial growth that did not match the typical pattern of yeasts or filamentous fungi (ambiguous morphology), light microscopy was used to visualize the growth and determine if it was in fact bacterial. Cells from each plate with the questionable growth were collected with a sterile loop and smeared onto a microscope slide. 3 µl of Lactophenol Cotton Blue were dispensed onto the smear as a mounting solution. A coverslip was placed on the top and the slide was incubated in a dark environment (drawer) for ten minutes. Then, the slide was focused under a Zeiss AxioScope microscope. The growth was focused initially under 10X and then the magnification was increased (63X - 100X) until individual cells could be viewed and the morphology could be analyzed. In some cases, DAPI staining was used to identify nuclei of yeasts. Plates that were determined to be bacterial were parafilmed and disposed of in an autoclave bag. All microscope slides and coverslips were disposed of in a hazardous glass bin.

Isolation of Unique Fungal Growth

If there were visually differing growths (different colony morphology or color) on a single plate, a sterile inoculation loop was used to collect cells from each of distinct growth and streak them onto separate plates in an "isolation" streaking pattern. Cells were transferred from the most isolated colony to a new Petri dish of the same medium using a sterile loop. Plates were monitored for 72 hours to ensure growth visually matched the source plate and that there were no additional contaminant colonies. This process was repeated until the streaked fungal growth had a uniform physical appearance with no differences in color or colony morphology. There were approximately 8 transfers made for each of the 57 unique isolates.

Cryopreservation

For each of the 57 morphotypes, cells were archived via cryopreservation. There were two distinct procedures depending on whether the fungal growth was a filamentous fungi or yeast (distinction based on growth morphology and cellular characteristics). For pure yeast cultures, a sterile inoculation loop was used to collect some of the cells and transfer them directly into a Copan cryotube containing beads and glycerol. The tube was vortexed using a Fisher Scientific Pulsing Vortex Mixer for 30 seconds and left on the lab bench for 30 minutes undisturbed. Approximately 400 µl of the glycerol liquid were removed from the tube. The cryotube with beads was stored in a -80□ freezer.

For filamentous fungi, approximately 400 µl of glycerol in new Copan cryobank cryotubes were transferred to the surface of the Petri dish with the filamentous growth, targeting the colonies with the densest fungal growth. A sterile inoculation loop was used scrape the growth under the glycerol and detach the spores while not spreading the liquid all over the plate. This was continued until the spores were transferred into the liquid phase. The glycerol and spores were transferred back into the original cryotube. The tube was vortexed for 30 seconds. The glycerol was removed as above from the tube and discarded. The tube was left on the bench for 30 minutes undisturbed and was then stored in a -80□ freezer.

DNA Extraction

Prior to DNA extraction, cells from each of the 57 morphotypes were streaked onto two plates in a "lawn" pattern. Using a sterile inoculation loop, the growth from each of the plates was transferred to separate 1.5 mL eppendorf tubes. Each tube had ~¼ to ~½ pea-sized pellet of cells. The growth (hyphae and spores) from the first of the two tubes for all 57 isolates was used for the first round of DNA extractions. 200 µl of ddH_2O were added to each tube. Using a Fisher Scientific Dry Bath Incubator pre-heated to 100□, each tube was submerged in the hot water for 5 minutes. Each tube was immediately transferred and submerged in a container of ice for 5 minutes. Then, each tube was transferred to a Fisher Scientific Isotemp Centrifuge 5810 R and spun at 10,000 rpm for 5 minutes. The supernatant of each tube was transferred to a sterile eppendorf tube. The supernatant was suspended in 200 µl of ddH_2O. Using a Qubit DNA Quantification protocol (described below), the quantity of DNA in each tube was assessed. If the was more than 1 ng of DNA per µl of ddH_2O, the tube was stored in a 20□ refrigerator. If the concentration was lesser than 1 ng/µl, the second 1.5 mL eppendorf tube of growth was used to extract DNA using a more comprehensive protocol.

50 mL of 2X Lysis Buffer and CTAB (10%) were preheated in a Thermo Scientific Circulating Water Bath at 70□ for ~1 hour. 800 µl of the 2X Lysis Buffer were added to the sample's 50-mL falcon tube containing the garnet. The falcon tube was left in the water bath for 5 minutes, then vortexed. The lysate for each sample was aliquoted into two 1.5 mL clear Eppendorf tubes with approximately ~400-500 µl of lysate each. At this point, all of the samples in that round were put into the Fisher Scientific Isotemp Centrifuge 5810 R and spun at 2,000 rpm for 2 minutes. The samples were then vortexed for 5 seconds, put into a Epicentre floating rack, and incubated for 5 minutes in a 70□ water bath. Each sample was again vortexed for 5 seconds and then submerged in the water bath for 5 minutes. 63 µl of 5M NaCL and 50 µl of 10% CTAB were measured and dispensed into each centrifuge tube. All the tubes were put into a floating rack and incubated for 15 minutes in the water bath. 500 µl of Chloroform were dispensed by a laboratory supervisor into each centrifuge tube, and the tube was vortexed for 30 seconds. All of the tubes were returned to the centrifuge in the same configuration, and spun for 10 minutes at the maximum rpm. The aqueous layer was transferred to a fresh 1.5 mL eppendorf tube. 300 µl of isopropanol were measured and dispensed into each centrifuge tube. All of the tubes were incubated for 30 minutes at -20□. They were returned to the 4□ centrifuge for 10 minutes, and spun at 14,000 rpm. DNA pellets were rinsed with 100 µl of 70% ethanol and then tubes were placed on a piece of paper towel to air dry for 5-10 minutes. 100 µl of PCR water were dispensed into each tube once fully air dried. The pipette was used to fully suspend the DNA and then to combine the solution with the yield from the other tube for that given sample. This process was repeated for all 20 samples.

Polymerase Chain Reaction (PCR)

For yeasts, the D1 and D2 region of the 26S subunit of the ribosomal RNA gene was amplified using the NL1 and NL4 primers. For filamentous fungi, the ribosomal RNA ITS region was amplified using ITS1F and ITS4 primers.

Primer Sequence for D1/D2 (yeast):

NL1: GCA TAT CA TAA GCG GAG GAA AAG / NL4: GGT CCG TGT TTC AAG ACG G

Primer Sequence for ITS1F/ ITS4 (filamentous fungi):

IB1F: CTT GGT CAT TTA GAG GAA GTA A / IB4: TCC TCC GCT TAT TGA TAT GC

For each PCR reaction, a master mix was prepared that had each of the ingredients multiplied by how many samples were being analyzed and aliquoted into PCR tubes. All of the pre-PCR work was done in an Airclean 600 non-DNA hood and all ingredients were kept on ice before returning them to a -20□ freezer. For each sample in a PCR run, 30 µl of PCR master mix were dispensed into a 1.5 mL centrifuge tube, consisting of 10 µl of Promega 5X Green GoTaq Flexi Buffer, 5 µl of Promega MgCL2 (25 mM) and 1 µl of Promega dNTP Mix (10 mM) and PCR water. For yeasts or filamentous fungi, different primers were added to the mix. For fungi, 1 µl of IB1F (10 mM) and 1 µl of IB4 (10 mM) were added to the tube, and for yeasts, 1 µl of NL1 (10 mM) and 1 µl of NL4 (10 mM) were dispensed into the tube. Finally, 0.5 µl of Promega GoTaq Flexi DNA Polymerase were measured and added to the master tube. Each PCR reaction also included a positive and

negative control. All tubes were vortexed for 10 seconds. The volume of mastermix was divided by the number of tubes (samples and + / - control). The 0.5 milliliter Eppendorf tubes were transferred to an Airclean PCR Workstation where the tubes containing each suspended DNA were defrosting. 1 µl of suspended DNA was dispensed into the corresponding PCR 0.5 mL tube. Each tube with DNA and master mix were spun in an Eppendorf Minispin Plus centrifuge for 30 seconds at 1,000 rpm. The tubes were then transported to an Eppendorf Vapo Protect PCR machine and the following amplification conditions were programmed: 95□ for 25 minutes, 35 cycles of 95□ for 60 seconds, 52□ for 90 seconds, 72□ for 2 minutes with a final extension of 72□ for 5 minutes. All of the tubes were placed together with their tab pressed down, and facing the same way.

1X TBE Buffer was made by combining 900 mL of Milli-Q water with 100 mL of 10X TBE. 50 mL of 1X TBE Buffer were added to a 100-mL Erlenmeyer flask along with 0.5 g of Agarose. The flask was gently swished and microwaved for one minute. The flask was removed and cooled for a few minutes. 2 µl of SYBR Safe DNA Gel Stain were dispensed into the flask and the liquid was mixed. A 12-prong comb was placed into a Thermo Scientific Owl EasyCast Gel Electrophoresis System, and the liquid gel mixture was then poured in. After 30 minutes, the liquid solidified into a gel. At that point, the comb was removed and the gasket component of the system holding the gel was rotated. Once the PCR reaction finished, enough 1X TBE Buffer was poured into the gel box to fully submerge the gel. 5 µl of PCR product were distributed into each well created by the comb. 5 µl of DNA ladder were dispensed into the middle well. The cover was put on the Easy Cast, its plugs were connected to the Fisher Scientific FB300 power source, and the power was turned to 100 volts for 30 minutes. After 30 minutes, the gel was carried to a Gel Logic 212 Pro machine. The E-gel Analysis software was used to view and photograph the gel under White Light, and then UV lightening. There were sixteen rounds of PCRs that were conducted on 57 different samples, yielding 56 samples whose marker gene successfully amplified.

Purification of the PCR Products for Sequencing

For each tube, a 0.7 volume was added in isopropanol. The tubes were then stored in the freezer for 90 minutes. They were transferred to the centrifuge (pre-cooled to 4□) and spun at the maximum rpm for 30 minutes. The isopropanol was then poured off, and 100 µl of 70% ethanol were added. The tubes were returned to the centrifuge (pre-cooled to 4□) and spun at the maximum rpm for 5 minutes. The ethanol was pipetted off and the tubes were spread out to airdry for 20 minutes. Once there was no visible liquid in the tubes, the pellet was resuspended in 20 µl of ddH_2O.

DNA Quantification

The DNA for the 56 samples was quantified using a Qubit Assay Kit. 1 µl of amplified DNA was measured and dispensed into the corresponding 0.5 milliliter clear Eppendorf PCR tube for each sample. The volume of required Molecular Probes Qubit dsDNA HS Buffer was multiplied by the number of DNA tubes being quantified, plus the two additional Standard tubes. Two Assay Tubes (0.5 milliliter clear Eppendorf PCR tubes) for the standards were set up and 190 µl of the Buffer were measured into both the (A) tube and the (B) tube. 10 µl of Qubit DNA HS Reagent (A) were measured into one of the Standard tubes using an Eppendorf Research Pipette, and 10 µl of Qubit DNA HS Reagent (B) were measured into the other tube. For each sample, 195 µl of Buffer were added along with 5 µl of purified DNA. 1 µl of dye was dispensed into each tube. The tubes were vortexed for 3 seconds, and incubated in a dark, room-temperature environment for 2 minutes. First, the (A) tube and the (B) tube were placed in a Qubit 2.0 Invitrogen fluorescence by Life Technologies to establish a baseline value. Then, all user Assay tubes were inserted into the fluorometer one at a time and the DNA values recorded. If there was sufficient DNA, then the sample was sent to Genewiz for sequencing.

Dilution

Depending on the quantity of DNA, the tubes were diluted to conform to Genewiz's DNA format: 20 ng of DNA in 10 µl of PCR H_2O and 5 µl of primer at 5 micromolar.

Interpreting Sequence Data using BLAST Outputs

Once the partial gene sequences were received from Genewiz in the form of a .txt file, they were cross referenced against all known fungal and yeast species in the U.S. National Library of Medicine's archives (GenBank NCBI non-redundant database). The collection was dereplicated to 12 isolates, based on phylogenetic analyses (one representative from each phylogenetic branch was selected).

Re-Culture of Representative Isolates

The cryopreserved tubes of the 12 representative isolates were removed from the -80□ freezer. The cryotubes were briefly vortexed and thawed. A pair of sterile tweezers were dipped in 70% ethanol, flamed and used to remove one bead from the cryotube and transfer it to a plate of the specific isolate's preferred culture medium. A sterile inoculation was used to rub the bead around the plate. For each of the 12 isolates, three plates were prepared. All 36 plates were incubated at 23□ until there was dense growth covering the entirety of the plate. Once there was dense growth, the plate was stored in a 20□ environment to slow the growth.

Brain Heart Infusion Agar and Broth Recipe

Brain Heart Infusion (BHI) media plates and broth were both made. 15 g of Bacto Agar and 37 g of Bacto Brain Heart Infusion powder were combined with 1 liter of Milli-Q ddH_2O. This procedure was repeated again without the addition of Bacto Agar. Each container was put on a Fisher Scientific Isotemp Table Top stirring hotplate at 300□ and 500 rpm for an hour and a half until all the particles were completely dissolved. The autoclave setting and plate pouring procedure were repeated as described above. Once solidified, the plates were stored in a 20□ refrigerator.

Mueller Hinton Agar Recipe

38 g of Sigma Aldrich Mueller Hinton Agar and 2 liters of Milli-Q ddH_2O were added to a 2-liter bottle and stirred with a magnet on a Fisher Scientific Isotemp Table Top stirring hotplate at 350 rpm and 300□ for 90 minutes (until the particles were completely dissolved). The media was autoclaved for 60 minutes at 121□.

Preliminary Antimicrobial Susceptibility Testing

All work with pathogens was performed within a BSL2-certified Thermo Scientific Herasafe Class II fume hood. Dr. Virginia Edgcomb retrieved an archived sample of each pathogen (*Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC-35556, and a drug-resistant strain of *Pseudomonas aeruginosa* ATCCMP-23) culture from the -80°C freezer and inoculated one Brain Heart Infusion (BHI) agar plate with each culture. After growing each culture at 37°C overnight, a preparation of a suspension of each pathogen was prepared in liquid BHI medium at 0.5 McFarland or 0.1 optical density (determined on a spectrophotometer and equating to $1x10^8$ cells/mL) and diluted 1/100 into sterilized Mueller Hinton agar (pre-cooled to 60°C). Each container had 720 mL of Mueller Hinton agar in addition to the suspended pathogenic cells. The inoculated Mueller Hinton agar for each of the three pathogens was then poured into sterile, 6-well Petri dishes. Once the plates were solidified, the back end of a sterile Pasteur pipette was used to poke one hole in each of the 4 of the 6 wells. A sterile needle was used to spear the agar plug and dispose of it in a 100% bleach solution. This was repeated with all of the pathogen plates. New, sterile Pasteur pipettes were used to poke ~30 holes in each of the plates providing fungal growth (generating ~90 plugs per isolate). Sterile needles were used to fit each fungal plug into the four holes in the pathogen plates. In the other 2 wells, the plug was placed hyphae-down on the pathogen. This was repeated until all of the fungal plugs were inserted into the corresponding bacterial plates. The exteriors of the plates were wiped with a 10% bleach solution and stored in a 37□ incubator for 24 hours. At 24 hours, the plates were observed to determine which fungal plugs affected the pathogenic growth.

Secondary Antimicrobial Susceptibility Testing

The 9 fungal isolates that passed the preliminary screening moved on to a secondary screening. Two plates per isolate were streaked in a "lawn" fashion and incubated at 23□. Once grown, the plates were stored

in a 20□ refrigerator. Dr. Edgcomb prepared a liquid culture of *E. coli* ATCC-25922, *S. aureus* ATCC-35556, and *P. aeruginosa* ATCCMP-23 from the cells that were frozen at -80□. The 40 mL of liquid culture were stored in a 50 mL falcon tube. 2,150 mL of Mueller Hinton agar were prepared according to the recipe above. Each Mueller Hinton plate was inoculated with the liquid pathogen culture using a spiral plater and a sterile rake. 27 plates were prepared per pathogen. Using the back end of a sterile Pasteur pipette, ~50 holes per plate sourcing fungal growth were made. Sterile needles were used to place 5 fungal plugs (hyphae-down) on each pathogen plate. This was repeated with all 81 plates. The plates were packaged in ziploc bags and incubated at 37□ for 48 hours. At 24 hours, the plates were photographed and their inhibition zones were measured in mm using a ruler. At 48, the plates were photographed and measured again. All screening materials (including the plates) were disposed of in accordance to BSL-2 guidelines.

Results

Figure 1. The Relative Inhibitory Activity of Five Deep-Sea Fungal Isolates against *S. aureus* ATCC-35556 and *E. coli* ATCC-25922 after 24 hours.

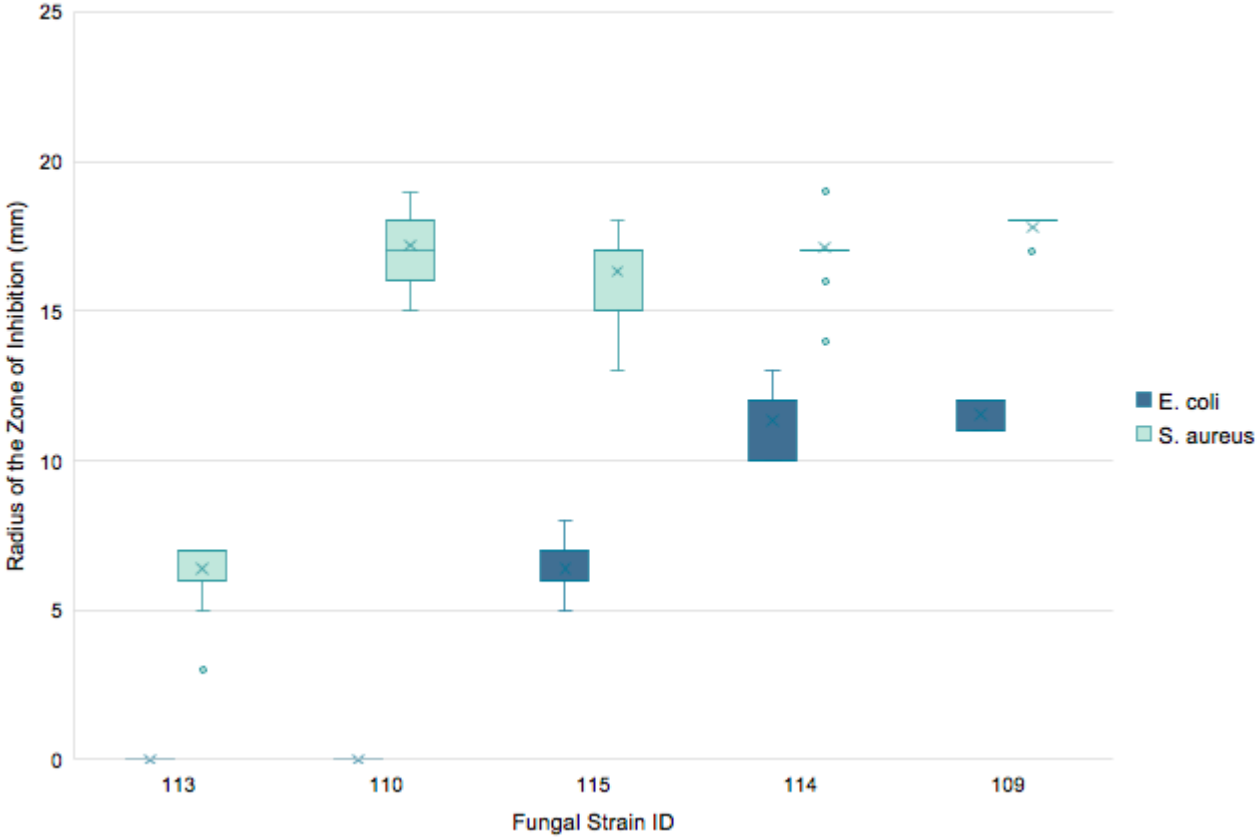


Figure 2. The Relative Inhibitory Activity of Five Deep-Sea Fungal Isolates against *S. aureus* ATCC-35556 and *E. coli* ATCC-25922 after 48 hours.

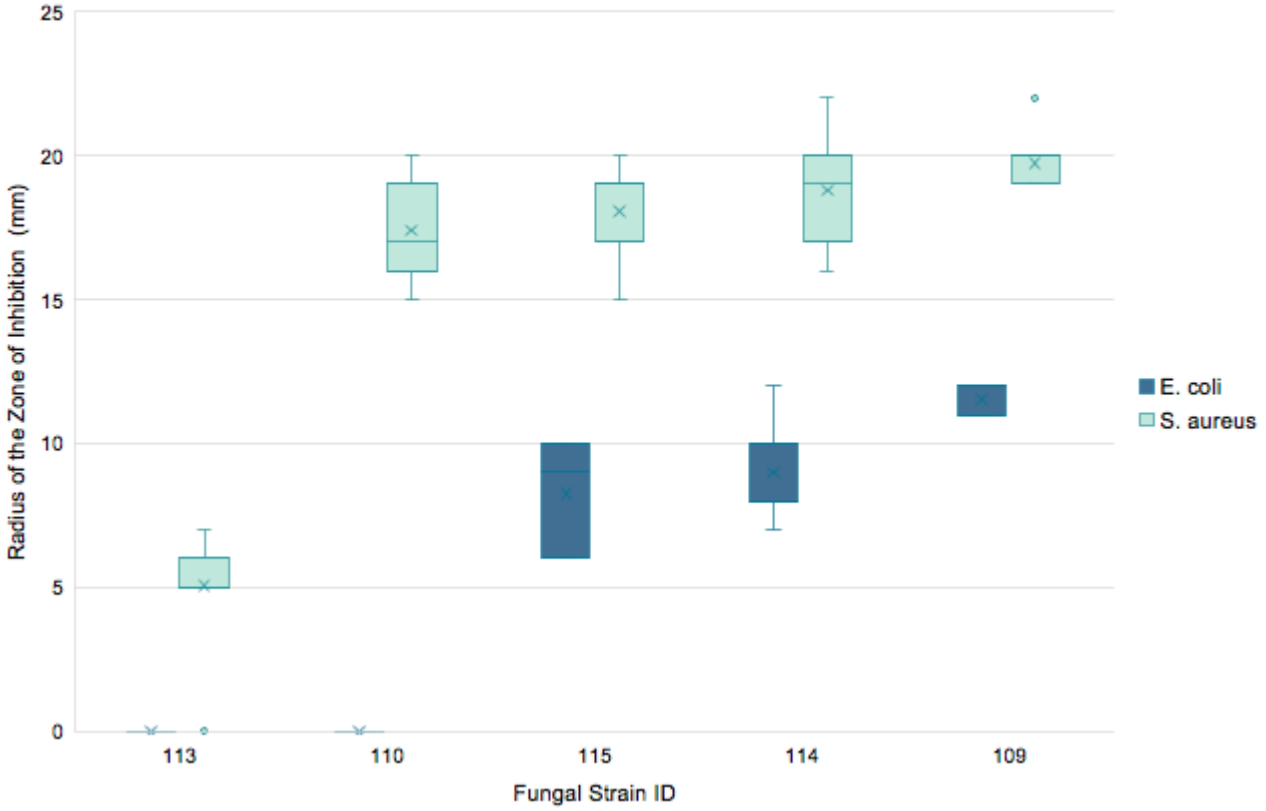
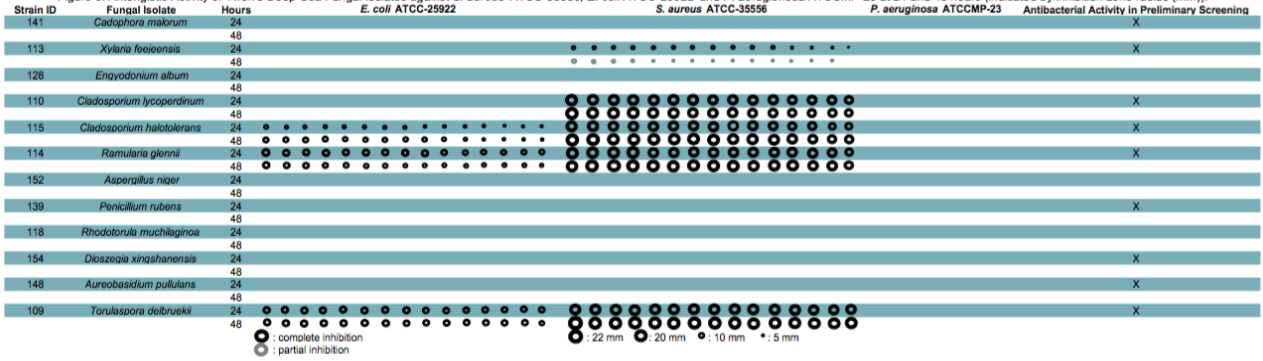


Figure 3. Antongistic Activity of Twelve Deep-Sea Fungal Isolates against *S. aureus* ATCC-35556, *E. coli ATCC-25922* and P. aerugionosa ATCCMP-23 at 24 and 48 hours (indicated by inhibition zone radius (mm)).



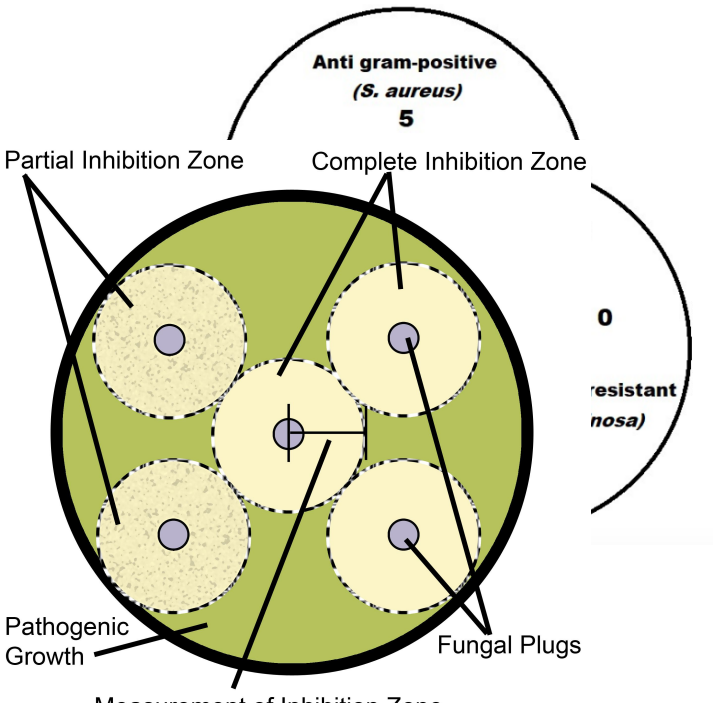


Figure 4. Venn diagram summarizing the spectrum of activity of the 12 antimicrobial producing deep-sea fungal isolates.

Figure 7. The experimental design for the antimicrobial susceptibility testing (AST). The visible differences between a complete inhibition within the observable zone (suggesting the synthesis of bactericidal compounds) and a partial inhibition within the observable zone (suggesting the synthesis of bacteriostatic compounds). The radius of the zone of inhibition indicates the effectiveness of the antibacterial compounds; the larger the diameter, the greater the sensitivity of the bacterium to the antibiotic.

Discussion

Antimicrobial Susceptibility Testing Results

Twelve genetically distinct fungal isolates underwent a preliminary screening for antagonistic activity against *E. coli, S. aureus,* and *P. aeruginosa.* Of the twelve screened, nine (75% of collection) were sent to a secondary screening with increased replication. The inhibition zone radii are displayed in Figure 3. The derivatives of *X. feejeensis* and *C. lycopodium* were able to kill *S. aureus,* while those of *C. halotolerans, R. glennii* and *T. delbrueckii* were able to kill both *S. aureus* and *E. coli.* None of the fungal isolates could inhibit the growth of the drug-resistant strain of *P. aeruginosa* in the secondary screening. None of the members of the Guaymas Basin fungal collection screened in this study have previously been studied in the context of antibiotic development.

The most prolific producers of effective antibacterial molecules were *R. glennii* and *T. delbrueckii.* Both fungi were able to inhibit *E. coli* and *S. aureus.* The average inhibition zone of *R. glennii* against *E. coli* was 11.3 mm at 24 hours and 9 mm after 48 hours. The average inhibition zone of *R. glennii* against *S. aureus* was 17.3 mm at 24 hours and 18.8 mm after 48 hours. The average inhibition zone of *T. delbrueckii* against *E. coli* was 11.53 mm at 24 hours and 11.3 mm at 48 hours. The average inhibition zone of *T. delbrueckii* against *S. aureus* was 17.8 mm at 24 hours and 19.7 mm at 48 hours. The relative inhibitory activity of the fungi that produce effective antibacterial molecules is displayed in Figure 1 and Figure 2.

Of the inhibitions measured, 87% were complete within the observable zone, suggesting the synthesis of bactericidal compounds (able to kill the pathogenic cells). All of the inhibitions of *S. aureus* caused by *X. feejeensis* (12% of total inhibitions) were partial, suggesting the synthesis of bacteriostatic compounds (able to inhibit the growth of the pathogen without killing all of the cells). Both types of compounds are promising in the context of antibiotic development. *S. aureus* exhibited intermediate sensitivity to the natural products of *X. feejeensis.*

Out of the three bacterial pathogens screened against, *S. aureus* exhibited the greatest sensitivity to the compounds produced by the members of the Guaymas Basin fungal collection. The fungal isolates were much more effective inhibitors of *S. aureus* than *E. coli*. Two fungi that were able to inhibit *S. aureus* could not inhibit *E. coli.* Furthermore, in the case of the three fungi that inhibited both *S. aureus* and *E. coli,* the inhibition zones associated with *E. coli* consistently had a smaller radius than those associated with *S. aureus.* The average inhibition of *E. coli* by the three isolates that inhibited both *E. coli* and *S. aureus* was 8.8 mm smaller than that of *S. aureus.*

The radius of the zone of inhibition indicates the effectiveness of the antibacterial compounds; the larger the diameter, the greater the sensitivity of the bacterium to the antibiotic. The zone sizes are typically compared to a standardized chart to determine if the bacterium is sensitive, resistant, or shows intermediate sensitivity to the molecules. At this point in the study, it is impossible to compare the size of the inhibition zones to those caused by mainstream antibiotic agents since the fungal plugs used in the screening have a complex array of molecules. After fractionation of a crude extract and isolation of pure compounds, the inhibition zones will be compared to the standardized charts.

Exhibitor's Interpretation of Results

The large percentage of the Guaymas Basin fungal collection that exhibited antibacterial activity was likely attributed to the fungal isolates' hydrothermal vent origins. Deep-sea, hydrothermal environments are ideal bioprospecting locations due to their high levels of microbial productivity. These locations are populated with islands of dense and biologically distinct fungal communities occurring in the direct vicinity of venting orifices. These communities are composed of evolutionarily unique fungi exhibiting unusual metabolisms (based upon chemosynthesis and growth rates). The unmatched cellular density of hydrothermal environments promotes high levels of interaction among species and a subsequent increased production of defensive secondary metabolites (natural antibiotics). On average, the cellular density in the direct vicinity of active hydrothermalism is 10^8 cells per cm^3 in comparison to the 10^6 cells per cm^3 density that characterizes "dense" terrestrial environments.

The geological and geochemical nature of hydrothermal locations promotes a high level of biological and metabolic diversity among Eukarya. Screening phylogenetically unique organisms from extreme ecosystems is a rational approach to discovering novel chemotypes and serotypes with potentially medically relevant biological activities. The microbial diversity of the Guaymas Basin represents a largely untapped reservoir of genetic and metabolic heterogeneity that could offer novel chemistry to ongoing drug discovery efforts. Since the Guaymas Basin hydrothermal site is situated 2,000 m below the sea surface, the fungi inhabiting the vents have likely been separated from their terrestrial subspecies for thousands, if not millions, of years. During this period of geographic separation, the fungi would have been constantly adapting to their environment. It is possible that the separate evolution of deep-sea fungi and terrestrial bacteria could mean that terrestrial pathogens may not be able to defend against the molecules synthesized by deep-sea fungi.

Vent microorganisms have proven to possess unusual physiological adaptations necessary for survival in such an extreme environment, meaning that these species are evolutionarily and ecologically distinct from terrestrial ones. Hydrothermal vents can support densely populated ecosystems, where faunal density and biomass are comparatively greater than the surrounding seafloor. The various metabolisms of the organisms at vents are unique in the way that they utilize metals in their energy-producing chemical reactions. Due to this, the byproducts are surely different molecules than what can be found in the terrestrial biome here on land (Raghukumar, 2008).

Recent literature has described the deep-sea as a new frontier in natural product discovery. Locations colonized by chemosynthetic microorganisms have provided a new paradigm for primary production in the absence of sunlight. The rate of discovery of novel natural products from marine microbes peaked in the late 1990s and has declined over the turn of the century. However, increasing numbers of novel, antibacterial marine natural products are once again being reported: 961 new compounds in 2007 versus 779 new compounds in 2006 (Raghukumar, 2008). This dramatic increase (24%) is attributed to a renewed interest in marine microorganisms. In general, there are few studies detailing the production and screening of the derivatives of hydrothermally-derived fungi. It is hoped that the results of this study will encourage other scientists to further investigate the Guaymas Basin's antibiotic potential and to screen vent-derived fungi against antibiotic-resistant pathogens.

Inhibition of S. aureus vs E. coli

The fungal isolates in this experiment were more successful in inhibiting the growth of *S. aureus* than that of *E. coli.* An explanation for this could be that *E. coli* is a gram negative bacterium while *S. aureus* is a gram positive bacterium. The differences in the cell wall structure for these two types of bacteria would affect their susceptibility to compounds synthesized by the fungal isolates. As shown in Figure 9, the cell wall of the gram positive *S. aureus* is a cytoplasmic lipid membrane with a rigid peptidoglycan layer made of cross-linked peptidoglycan. The wall also possesses a volume of periplasm which is smaller than that in gram-negative bacteria. The cell wall of *E. coli* on the other hand has an inner cytoplasmic cell membrane, a peptidoglycan layer that is substantially thicker than that of gram-positive bacteria, an outer membrane that contains lipopolysaccharides, porins integrated into the membrane which are very selective about admitting foreign

compounds, and an area of periplasm between the outer membrane and the cytoplasmic membrane that is thicker than in gram-positive bacteria. These differences in cell wall structure, specifically the fact that the cell wall of *E. coli* is thicker with more protective features, may explain why the antimicrobial compounds produced by the fungal isolates were usually more effective against *S. aureus.* The pathogen's cell structure is important regarding protection from antimicrobial peptides (AMP) because the initial site of AMP interaction is at the bacterial cell surface. The cell wall is the first line of defense to minimize potential damage to the bacterial cell. Since the cell wall of *E. coli* is thicker and has a different composition, it appears to be more difficult for the compounds produced by these fungi to penetrate it and kill the cell either by compromising the integrity of the cell membrane or by gaining entry into the cell where they can interfere with key cellular processes such as DNA replication, transcription, or protein synthesis (Kathryn L., 2014). Since many of the compounds synthesized are likely penicillins, their cellular target would be the cellular membrane.

Sources of Error and Limitations

Although care was taken to minimize the potential sources of error in this experiment, there were some experimental challenges. The main potential for error originates from the possibility of contamination. Contamination can be introduced into cultures from airborne fungal spores that interfere with growth of fungi from the designated sample. This can happen when plates are exposed during the inoculation or isolation process. Although possible, this is highly unlikely for a couple of reasons. First, there were two Petri dishes that were intentionally left uncovered on the lab bench during isolation procedures, and their fungal growth was visually distinct from that obtained from these samples. Plates exposed during the isolation process tended to only recover growth of a black mold. Those plates were discarded. Second, there are close genetic relatives of these isolates that have been identified from coastal samples as well as deep-sea environments. Care was taken to ensure the sterility of the lab bench as well as the instruments being used for the culture-based methods.

Kirby-Bauer AST occasionally indicates *in vitro* susceptibility to certain antibacterial compounds, despite a lack of therapeutic efficacy in actual practice. Similarly, certain antibacterial compounds can be problematic to test with the Kirby-Bauer method due to the physicochemical properties of the molecules. Some compounds, such as vancomycin and macrolides such as clarithromycin, have higher molecular weights and therefore diffuse slowly through agar. This means that it may take longer than the typical 24 to 48 hours for an inhibition to occur. Furthermore, AST results can be heavily influenced by the position of the plates relative to a light source. To mitigate this potential source of error, all plates were measured from the bottom using transmitted light to ensure an accurate measurement of zone radius.

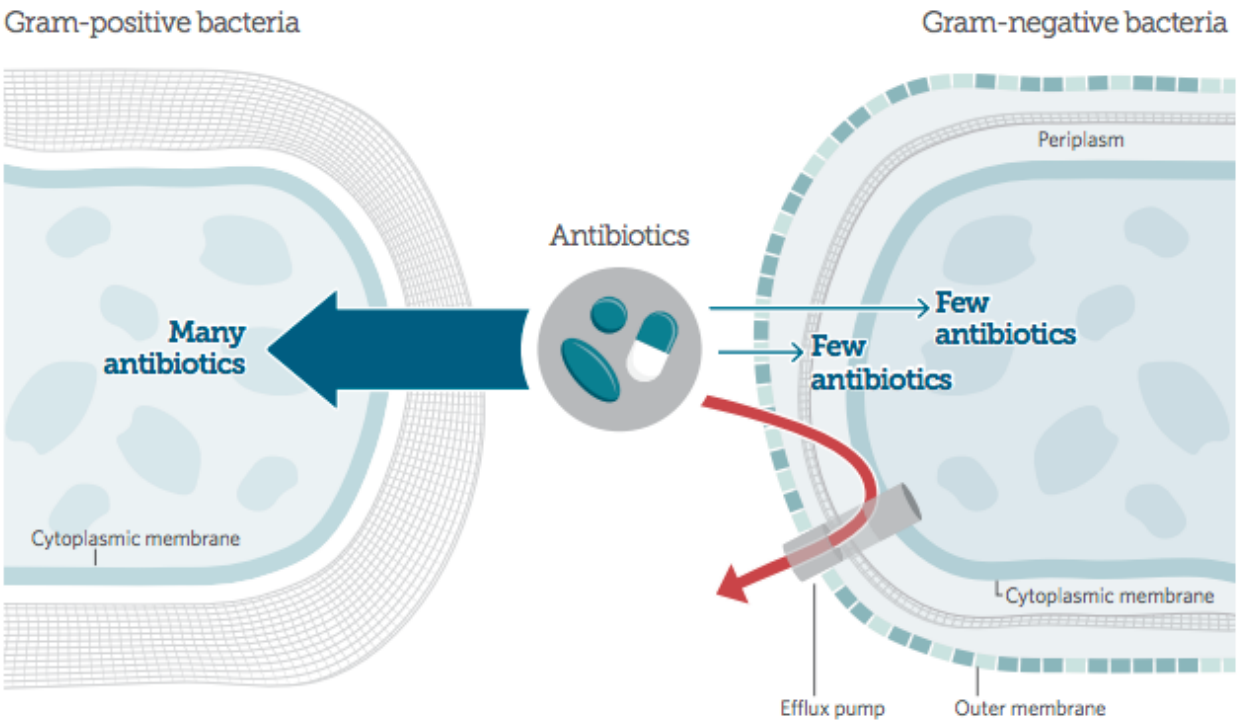


Figure 9. Comparison of the exterior cellular structure of gram-positive (*S. aureus)* versus gram-negative bacteria (*E. coli*).

Conclusion

The fungi in this experiment displayed promising culturability in a laboratory setting. The genetic and morphological variations in the fungal collection illustrate the breadth of fungal diversity present in unconventional deep-sea habitats, in particular the Guaymas Basin. The AST results revealed the clinical potential of the natural products of the Guaymas Basin fungal collection. Of the twelve fungi screened, nine (75% of collection) were sent to a secondary screening with increased replication. In the second screening, five fungal isolates (42% of collection) exhibited potent antibacterial activity. The natural products of *X. feejeensis* and *C. lycopodium* were able to kill *S. aureus,* while those of *C. halotolerans, R. glennii* and *T. delbrueckii* were able to kill both *S. aureus* and *E. coli.* None of the members of the Guaymas Basin fungal collection screened in this study have previously been studied in the context of antibiotic development. Due to this, it is possible that their natural products are novel. The hypotheses established prior to experimentation were overwhelmingly supported by the results of this study. The isolates screened were more effective at inhibiting the growth of *S. aureus* than *E. coli*. This disparity in effectiveness may be attributed to variations in the cellular structure of gram-negative versus. gram-positive bacteria. Also, the use of seven additional types of culture media augmented the Guaymas Basin fungal collection established in 2017-2018 with eleven additional species. The extended Guaymas Basin fungal collection supplements the pool of microorganisms available for natural product screening. This study is the first step towards the development of a novel antibiotic drug.

Social and Scientific Relevance

This study is the first step towards the development of an antibiotic drug. Currently, there is no single antibiotic to which all strains of *E. coli, S. aureus,* and *Pseudomonas aeruginosa* are sensitive. Some of the infections caused by these pathogens are: UTIs, dermatitis, bacteremia, soft tissue infections, cholangitis, pneumonia, neonatal meningitis, cholecystitis, endocarditis, meningitis, toxic shock syndrome and scalded skin syndrome. The pathogens screened against in this experiment have acquired resistance to almost all antibiotics previously used to treat them, including: ampicillin, tetracycline, fluoroquinolone, (benzyl) penicillin, methicillin, vancomycin, and all beta-lactam antibiotics. The loss of effective antibiotic compounds will not only have negative consequences for the treatment of infectious bacterial diseases, but also for the management

of common infectious complications experienced by immunocompromised patients who undergo medical procedures (e.g. surgery, chemotherapy, dialysis). This study revealed five fungal isolates possessing the ability to inhibit the growth of this strain of *S. aureus* and three that could also inhibit the growth of this strain of *E. coli.* These results offer promise regarding alternatives to mainstream antibiotics that target these pathogens, as well as promise that microbiota from extreme marine environments may provide novel antimicrobial compounds to fill the current absence. It is also important to raise awareness of environments possessing a high bioprospecting potential, so that other scientists will investigate them.

Future Work

This specific project will be continued through July in anticipation of independent publication in the Microbiology Society's *Journal of Medical Microbiology.* In the next four months, the natural products will be analyzed using ultra-performance liquid chromatography–tandem mass spectrometry, thus determining if each metabolite is a Penicillin, a Cephalosporin, a Teteracycline, a Macrolide, or an Aminoglycoside. There is also the possibility that some compounds will be novel, in that case, there will be more specialized methods to determine the specific molecule. The compounds will also undergo fractionation and isolation. Finally, the minimum inhibitory concentration for the antimicrobial molecules will be determined. The extended Guaymas Basin fungal collection will also be screened against *Borrelia burgdorferi* ATCC-35211 as well.

A secondary study will screen bacterial pathogens that have acquired resistance to penicillins (amoxicillin, ampicillin, piperacillin, nafcillin, oxacillin, dicloxacillin, cloxacillin, ticarcillin) against the deep-sea subspecies of *P. chrysogenum* as a way to counter antibiotic resistance to penicillins while not sacrificing the unsurpassed medicinal capacities of these compounds. Significant environmental differences suggest the microbial synthesis of chemically unique metabolites likely possess distinct modes of action and cellular targets. Due to this, terrestrial pathogens should exhibit varying degrees of sensitivity to the compounds synthesized by marine and terrestrial serotypes of the same fungus due to the potential chemical dissimilarity of their natural products. It is hoped that these chemical differences would mean that resistance to the natural products terrestrial *P. chrysogenum*, which is increasingly common, would not also equate to resistance to the products of marine *P. chrysogenum.* On the other hand, it is also hoped that, since they are both technically members of the same species, the derivatives of the marine subspecies of *P. chrysogenum* would be as potent and medically relevant as penicillin and similar compounds produced by terrestrial *P. chrysogenum.* Since many terrestrial species of fungi, some who have proven to be prolific producers of antibiotics, possess marine subspecies, having their marine subspecies as possible sources of novel clinically promising chemotypes would increase society's pool of available antibiotics.

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Supplemental Photos