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Antimicrobial Producing Bacteria Isolated From Petroleum-Laced Hypersaline Soil

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ANTIMICROBIAL PRODUCING BACTERIA ISOLATED FROM PETROLEUM-LACED HYPERSALINE SOIL

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PREFACE

This thesis is written in the style required by The American Society for Microbiology for the Journal of Bacteriology, to which a portion will be submitted for publication.

ABSTRACT

Given the alternative functions of "antibiotics" as communication molecules or participants in metabolism, it seems probable that production might be influenced by factors such as nutrient availability, interactions with neighboring microbes, and/or colony or community structure and maturity. With this in mind, the present study aimed to broaden the scope of the search for novel antibiotics by experimenting with the following parameters: source of bacterial isolation, growth and assay media, and culturing techniques. Bacteria for this study were isolated from two categories of soil (petroleum-contaminated or uncontaminated) to compare diversity and antimicrobial activity. Compared to the uncontaminated soil, isolates of the petroleum-contaminated soil were as diverse and antimicrobial activity was as frequent. Antimicrobial assays were done on three different types of agar, including the standard Mueller-Hinton and two types of medium typically used for fungal growth, Yeast Peptone Dextrose (YPD) and Yeast Mold (YM). Compared to results on Mueller-Hinton, much more antimicrobial activity was seen when using YPD and YM. Finally, spent media assays were performed with pure and mixed cultures to determine if exposure to a target pathogen affects the production of antimicrobial substances by soil isolates. Those bacteria with activity against *Pseudomonas aeruginosa* in perpendicular streak tests were grown as mixed cultures with *P. aeruginosa*. In the case of *Bacillus amyloliquefaciens* and *Pseudomonas marginalis*, discs impregnated with concentrated spent media from these mixed cultures resulted in significant dose-dependent inhibition of *P. aeruginosa*. The same assay using pure cultures showed no inhibition.

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INTRODUCTION

Given the alternative functions of "antibiotics" as communication molecules or participants in metabolism[1-2], it is probable that production of antibiotics might be influenced by nutrient availability, interactions with neighboring microbes, or colony or community structure and maturity. With this in mind, this study aimed to broaden the scope of the search for novel antibiotics by experimenting with the following parameters: source of bacterial isolation, growth and assay media, and culturing techniques. Bacteria for this study were isolated from two categories of soil (petroleum-contaminated or uncontaminated) to compare microbial diversity and antimicrobial activity. Antimicrobial assays were done on three different types of agar, including the standard Mueller-Hinton and two complex media types typically used for fungal growth. Finally, spent media assays were performed with pure and mixed cultures to determine if exposure to a target pathogen affects the production of antimicrobial substances by soil isolates.

The Need for Novel Antibiotics

According to Dr. Margaret Chan, Director-General of the World Health Organization, in her March 2012 address, "If current trends continue unabated, the future is easy to predict. Some experts say we are moving back to the pre-antibiotic era. No. This will be a post-antibiotic era. In terms of new replacement antibiotics, the pipeline is virtually dry, especially for Gram-negative bacteria. The cupboard is nearly bare." This dwindling arsenal is due to the rise in prevalence of antibiotic-resistant pathogens. While many bacteria are resistant to one antibiotic or one class of antibiotics, it is the existence

of multi-drug resistant (MDR) pathogens that raises concern. Many clinicians are forced to fall back on older, more expensive, or toxic antibiotics that might require longer periods of treatment, while other clinicians find they have no effective treatment. Such occurrences in today's world of modern medicine are terrifying.

The prevalence of MDR infections, especially in clinical settings, is extraordinary. Klevens et al. estimated that, in 2002, approximately 1.7 million hospital acquired infections (HAIs) occurred in the United States; this indicates that 5% of hospitalized patients acquired an infection during their stay. Of these, 198,987 resulted in death, primarily from pneumonia, bloodstream infections, urinary tract infections, and surgical infections.[3] The Annual Summary of Data issued by the National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention for 2006–2007 reports statistics about pathogens responsible for HAIs in 463 hospitals, as well as the prevalence of resistance in these microbes. Ten pathogens accounted for 84% of these infections: coagulase-negative staphylococci (15%), *Staphylococcus aureus* (15%), *Enterococcus* species (12%), *Candida* species (11%), *Escherichia coli* (10%), *Pseudomonas aeruginosa* (8%), *Klebsiella pneumoniae* (6%), *Enterobacter* species (5%), *Acinetobacter baumannii* (3%), and *Klebsiella oxytoca (*2%). Of the HAIs, 16% were MDR pathogens: methicillin-resistant *S. aureus* (8%), vancomycin-resistant *E. faecium*, (4%), carbapenem-resistant *P. aeruginosa* (2%), extended-spectrum cephalosporin-resistant *K. pneumoniae* (1%), extended-spectrum cephalosporin-resistant *E. coli* (<1% each), and carbapenem-resistant *A. baumannii*, *K. pneumoniae*, *K. oxytoca*, and *E. coli*, (<1% each).[4]

The prevalence of MDR infections has many causes, most of which are well understood but not well managed, despite the publication of several guidelines.[5-7] The World Health Organization (WHO) cites the following factors as driving antibiotic resistance:

- Inadequate national commitment to a comprehensive and coordinated response, ill-defined accountability, and insufficient engagement of communities
- Weak or absent surveillance and monitoring systems
- Inadequate systems to ensure quality and uninterrupted supply of medicines
- Inappropriate and irrational use of medicines, including in animal husbandry
- Poor infection prevention and control practice
- Depleted arsenals of diagnostics, medicines, and vaccines as well as insufficient research and development of new products.

As the last point indicates, the problem of MDR infections is exacerbated outside of clinical settings by a lack of interest by pharmaceutical corporations in the development of novel antibiotics. The estimated average cost of developing a single drug is $359 million.[8] Drug development averages 12 years for a candidate to make it from laboratory testing to testing in humans, and only one in five candidates will ultimately receive FDA approval.[8] Given the trend in development of antibiotic resistance, antibiotics are generally effective for ten years or less before a newer derivation, or entirely new antibiotic, becomes preferable. These factors make development of antidepressants and the like financially preferable, because they can be taken for longer periods (the better part of a lifetime versus 7-14 days in the case of antibiotics) and resistance is not a factor.

The dearth of antibiotic development that has continued has been hindered by a lack of understanding of the true nature of antibiotics. The function of antibiotics in an environmental setting has long been clouded by the anthropocentric view that, like humans, bacteria use these small molecules to kill other bacteria. Unlike bacteria, though, humans use antibiotics in extremely high concentrations. Due to lower amounts of nutrients in the environment compared to the high amounts in culturing media, bacteria are thought to produce antibiotics at very low concentrations environmentally, though measurement *in situ* is difficult.[1]

The difference between therapeutic and environmental concentrations is crucial in understanding the natural role of antibiotics. This is due to the phenomenon of hormetic concentration-responses.[1] The term "hormesis" in the context of antibiotics describes the biphasic dose response, wherein high concentrations result in inhibition of growth, but low concentrations result in an enhanced ability to survive.[9] Mlot suggests these small molecules might play crucial roles in microbial metabolism.[2] Alternatively, Davies asserts that, in light of the low concentrations produced in typical environmental settings, the natural role of these small molecules referred to as antibiotics is probably modulation of cellular transcription patterns, not antibiosis.[1] These alternative functions of antibiotics have been slow to be elucidated, given the difficulty of applying laboratory models in assessing microbial community structure and interaction *in situ*.

Given that production of antibiotics, for whatever purpose, requires the expenditure of energy by cells, it would follow that they are not produced unless required. It seems probable that this requirement might be dictated by factors such as nutrient availability, interactions with neighboring microbes, or colony or community

structure and maturity. The requirements, in turn, are dictated by the natural function of the molecule. So, complementary to metagenomics, it seems that studies surrounding production of small molecules with the ability to inhibit microbial growth at high concentrations should focus on what incites production, not just what genes are responsible.

This concept is important when it comes to identifying novel antibiotics. Given that the natural function of molecules with antibiotic properties is not necessarily antibiosis and the conditions that incite their production may be complex, it is necessary to diversify methods for identifying novel antibiotics. This study aims to do so by varying three parameters: source of bacterial isolation, growth and assay media, and culturing techniques.

The ESKAPE Pathogens

The ESKAPE bacteria are a group of pathogens gaining much notoriety. Members of the group are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. Their ubiquity both within the human body and clinical settings, resilience, and ability to rapidly acquire resistance to antibiotics makes them formidable adversaries.[10] Because they are among the major causes of nosocomial infections, these opportunistic pathogens deserve a closer look.

Enterococcus is a genus of Gram-positive cocci that normally inhabits the intestine of most animals. They are common transient inhabitants of the oral cavity and vaginal tract of humans and are easily found in the environment, presumably because of

fecal contamination. They can persist in the environment for weeks because of their ability to tolerate a wide range of temperatures, pH, and oxygen levels. Clinically, they are a leading cause of nosocomial bacteremia, surgical and catheter infection, endocarditis, and urinary tract infection.[11-12] Enterococci cause up to 30% of nosocomial endocarditis and 15-20% of nosocomial urinary tract infections.[13] These conditions are caused by two species, *E. faecalis* and *E. faecium*. While *E. faecalis* tends to be more virulent, causing 80% of enterococcal infections, *E. faecium* is more prone to acquired antibiotic resistance. Much of the virulence of *E. faecalis* is attributed to production of cytolysin, extracellular superoxide, and pheromone-responsive plasmid transfer; *E. faecium* lacks these virulence factors. Both are intrinsically resistant to many antibiotics, narrowing the effective treatments. Though rare in *E. faecalis*, resistance to ampicillin and vancomycin is becoming increasingly common in *E faecium*.[11] Vancomycin-resistant enterococci (VRE) are now considered endemic in many hospitals, because of the astounding increase in epidemics over the past decade.[12] NHSN data show that 56.5% of *E. faecium* HAIs are vancomycin resistant and 71.0% are ampicillin resistant; 4.7% *of E. faecalis* infections are vancomycin resistant, and 4.1% are ampicillin resistant.[4] Nearly all infections by MDR enterococci are preceded by regimes of antibiotics, often broad spectrum, that have little or no effect on enterococci, but alter the presence of protective normal flora. MDR enterococcal infections, some of which are resistant to all standard treatments, now occur worldwide.[11]

Staphylococcus aureus, perhaps the most infamous of the ESKAPE group of bacteria, is a Gram-positive coccus that transiently inhabits the nose of 30% of the population. *S. aureus* has a wide variety of virulence factors including enterotoxins,

exfoliative toxins, superantigens, Panton-Valentine leukocidin, adhesins, and proteases, which occur in varying combinations depending on the strain.[14] When introduced to other body sites, *S. aureus* can cause many types of infection, ranging in severity. The mild end of the spectrum includes folliculitis, cellulitis, scalded skin syndrome, and impetigo. Toward the middle of the spectrum are infective endocarditis and necrotizing pneumonia, and at the extreme end are osteomyelitis, necrotizing fasciitis, toxic shock syndrome, and sepsis. Symptoms range from abscesses requiring minor medical treatment, to tissue death and massive lesions often resulting in amputation or death.[14] Adding to the severity of these infections is the uncanny ability of *S. aureus* to rapidly acquire resistance.[14] Through a number of mechanisms, *S. aureus* has acquired resistance to penicillin, streptomycin, tetracycline, methicillin, cephalothin, gentamicin, cefotaxime, linezolid, and vancomycin. All of these resistances, with the exception of vancomycin, developed within four years of the date of approved drug use by the FDA.[15] NHSN statistics show 49.2% of *S. aureus* HAIs associated with cases of surgical site infection are oxacillin resistant.[4] 60% to 70% of all *S. aureus* hospital strains are now MDR.[14] While hospital acquired methicillin-resistant *S. aureus* (HA-MRSA) infections are worrisome, the emergence of community acquired MRSA (CA-MRSA) is cause for even more trepidation.[16]

Klebsiella pneumoniae is a Gram-negative bacillus that normally inhabits the human intestine, skin, and pharynx in low numbers.[17] Environmentally, *K. pneumoniae* is present in soil and water. It causes pneumonia, urinary tract infections, bacteremia, osteomyelitis, wound infections, and meningitis.[18] Virulence factors include capsular serotype, hypermucoviscosity phenotype, lipopolysaccharide, siderophores, and pili.[19]

Mortality rates for *K. pneumoniae* infections are near 50% and up to 100% in cases of alcoholism or bacteremia, even with antimicrobial therapy.[17] 14.8% of *K. pneumoniae* HAIs associated with surgical site infection are resistant to ceftriaxone or ceftazidime, and 5.2% are imipenem, meropenem, or ertapenem resistant.[4] Carbapenem-resistant strains are also becoming a major problem in neonatal units. Extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* strains have been reported worldwide.[19] Panresistance, that is, resistance to all antibiotics, has been also reported.[17]

Acinetobacter baumannii is a Gram-negative coccobacillus. It naturally inhabits water and soil but has been isolated from food and insects[20] and is known to colonize irrigating solutions and intravenous solutions within hospitals.[21] It is a significant cause of ventilator-associated pneumonia, urinary tract infections, and bacteremia. Nosocomial infections of the skin and soft tissue, cerebrospinal fluid, and peritoneal fluid also occur.[22] Most recently, *A. baumannii* infections have been associated with combat wounds acquired in Operation Iraqi Freedom.[23] It is becoming increasingly common in hospitals, where it often becomes endemic for long periods of time after outbreaks.[20] Many of these outbreaks are traced to widespread environmental contamination.[21] The pathogenicity of *A. baumannii* is still being elucidated, but the ability to form a biofilm, ability to adhere to eukaryotic cells, iron acquisition, and use of a cytotoxic outer membrane protein contribute to its virulence.[24] The ability to survive dry, iron-deficient conditions, a polysaccharide capsule that prevents complement activation and might delay phagocytosis, and pili also contribute.[20] Mortality and morbidity of *A. baumannii* infections are difficult to surmise because most patients have other preexisting infections.[25] *A. baumannii* has numerous intrinsic as well as acquired resistance

mechanisms. Resistance to all generations of cephalosporins, macrolides, and penicillins is common.[26] Of the *A. baumannii* HAIs associated with cases of surgical site infection, 30.6% are imipenem or meropenem resistant.[4] For many strains, only polymyxins or carbapenems are effective, while still others are resistant to all antimicrobial agents.[26]

Pseudomonas aeruginosa is a rather ubiquitous Gram-negative bacillus. Environmentally, it can be found in soil, water, plants, and animals. It can be found as part of the normal flora of humans on the skin (particularly moist areas) but also in the gastrointestinal tract and nasopharyngeal cavities of a small percent of individuals.[27] *P. aeruginosa* is most notorious for causing pneumonia, urinary tract infections, surgical site infections, and bloodstream infections.[28] Exotoxin A, exoenzyme S, many proteases, siderophores, and a pseudocapsule of alginate all contribute to its virulence. It is capable of acquiring resistance by most known mechanisms.[29] Of the HAIs associated with surgical site infections caused by *P. aeruginosa*, 15.9% are resistant to fluoroquinolones, 11.8% to imipenem or meropenem, 7.9% to piperacillin or piperacillin-tazobactam, 7.3% to ceftazidime, 5.7% to cefepime, and 2.0% to amikacin.[4] Resistance to colistin is less common but rising, due to its use as salvage therapy in cases of MDR infections.[30] Infections by pandrug-resistant *P. aeruginosa* strains have been reported.[31] All of the ESKAPE pathogens are dangerous, but recent research suggests that *P. aeruginosa* is especially dangerous, accounting for 8% of all nosocomial infections.[4] It is the number one cause of death in patients with cystic fibrosis, the second most common cause of nosocomial pneumonia, and has the highest mortality rate among HAIs.[28]

Enterobacter is another genus of Gram-negative bacilli that commonly inhabit soil and water. The two clinically relevant species are *Enterobacter cloacae* and *E.*

aerogenes, both of which occur in human feces. *E. cloacae* is also part of the normal flora of human skin and the intestinal tracts of both humans and animals.[32] Similar to the other ESKAPE members, *Enterobacter* spp. cause a wide variety of infections when in inappropriate body sites. These infections include but are not limited to respiratory tract infections, skin and soft-tissue infections, urinary tract infections, endocarditis, septic arthritis, osteomyelitis, central nervous system infections, and ophthalmic infections.[32] Though *Enterobacter* spp. virulence is not fully understood, it is thought that the ability to adhere to and invade eukaryotic cells, aerobactin production, and serum resistance contribute to its pathogenicity.[33] Strains resistant to β-lactams, aminoglycosides, fluoroquinolones, sulfonamides, and, most recently, carbapenems have been observed. While pan-resistant *Enterobacter* infections are not yet a problem, MDR infections have made it crucial to identify the infection before administering antibiotics.[34]

The extent of antibiotic resistance seen in the ESKAPE pathogens emphasizes the urgency of continued antibiotic development. Because of the threat they pose, these ESKAPE bacteria, except for *Acinetobacter baumannii,* were used as the target organisms for this research. In addition, *Saccharomyces cerevisiae* was used as a target organism, in order to gauge antimicrobial activity against fungi. Where complexity of procedures required limiting the number of target organisms, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were chosen due to the enormity of the threat they pose.

Objectives

This study used variations on conventional methods to qualitatively characterize the antimicrobial activity of bacterial soil isolates. Bacteria for this study were isolated

from two categories of soil to determine if the source of isolation affected the variety of bacteria or their antimicrobial abilities. In addition to the standard Mueller-Hinton agar, two complex fungal media were used for bacterial growth and antimicrobial-production assays to determine if available nutrients affected the range of antimicrobial activity of the soil isolates. Finally, mixed-culture techniques were used to investigate the effect of interspecies interaction on antimicrobial production in comparison to antimicrobial production seen with pure cultures.

MATERIALS AND METHODS

Acquisition of Soil Bacteria

Soil samples were obtained from 16 sites. Nine of these sites were considered uncontaminated soils: three different playa lakes in milo stubble, one dry creek bed, one spring-fed creek bed, one active cattle pen, one inactive cattle pen, one horse pasture, and one ensilage pit. These soil samples were thought to include organic content considered conducive to microbial growth. Samples from seven other sites were classified as petroleum-contaminated because they were near oil storage tanks. Soil samples for these sites were taken from within the man-made containment dikes surrounding the oil tank batteries. These dikes were designed to prevent the spread of contamination from the batteries, as in the case of salt water overflow or leakage of crude oil during transfer. These petroleum-contaminated soils appeared to have little organic content thought to promote microbial growth, and the aroma indicated harsh chemical components associated with petroleum. In addition to these seven petroleum-contaminated soil samples, a contaminated water sample was also taken from one site because of obvious petroleum content, for a total of 17 samples. Appendix A summarizes the sites associated with soil isolates' identification numbers.

All samples were collected in sterile 50-mL screw-top plastic vials. Uncontaminated soil samples were taken from between 15 and 20 cm below the surface to prevent inclusion of microbes present directly in the humus. Petroleum-contaminated soil samples were taken from directly below the surface to ensure recent contamination. The water sample was taken from the surface using a sterile syringe.

Spread Plates and Preliminary Colony Selection

Contrary to the typical use of Yeast Peptone Dextrose (YPD) agar for growth of fungal microbes, YPD was used for selection and growth of soil bacteria. Various conditions were used for spread plating, because of the unknown response of bacteria to such a medium and the unknown microbial density in these unusual soil samples, particularly those that were petroleum-contaminated.

For all locations, 0.5 g of soil were diluted 1:100, 1:1000, and 1:10,000 using sterile deionized water. For each dilution, 150 µL were applied to regular nutrient-concentration YPD (10.0 g yeast extract, 20.0 g peptone, 20.0 g dextrose, and 15.0 g agar) and to low nutrient (1.0 g of yeast extract, 2.0 g peptone, and 2.0 g dextrose with 15.0 g agar) YPD agar. A flamed L-rod was used to spread the dilute soil evenly over the agar. After the liquid dried, one plate of each soil dilution and media concentration was incubated at 30°C and another at room temperature. This resulted in 12 plates for each of the 17 sites.

After 24 hours incubation, colonies were selected from the spread plates. In an effort to obtain a variety of bacteria, colonies were selected based on uniqueness, as determined by colony color, shape, and margins. Thirty-two colonies were selected from petroleum-contaminated soil and 27 from uncontaminated soil. Selected colonies were taken from plates using a sterile inoculating loop and streaked for isolation on YPD agar. After 24 hours incubation at 30°C, isolated colonies were selected from each plate and used to inoculate 5 mL YPD broth. Inoculated broth was incubated 24 hours at 30°C.

Limiting Isolates

To reduce the number of isolates and minimize the number of duplicates, a preliminary test was performed on each isolate. Each broth-grown isolate was subjected to a perpendicular streak test against the target organisms *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterobacter aerogenes*, and *Saccharomyces cerevisiae*. For this, a single streak of the soil isolate was made across a plate of YPD agar, and the plate was incubated at 30°C for 24 hours. Then, each target organism was streaked perpendicularly to the soil isolate, with the streak beginning about 1 mm from the soil isolate streak. After 24 hours incubation at 30°C, zones of inhibition were documented as indication of antimicrobial activity by the soil isolate (Figure 1).

Soil isolates with antimicrobial activity were preserved at -80°C in 15% glycerol. The results of this preliminary perpendicular streak test were not reliable indicators of antimicrobial activity because bacterial cultures were not yet confirmed as pure; this test was used to reduce the number of isolates that would continue the testing process.

Isolation and Species Identification of Soil Isolates

The 28 isolates with the greatest activity in the preliminary perpendicular streak test (as quantified by activity against the greatest number of pathogens or size of inhibitory zones) or activity against *S. aureus* were selected for identification. For each of the isolates, isolation streaks were performed three consecutive times on YPD agar, with 24 hours incubation at 30°C between each isolation streak. Isolated colonies were inoculated into 5 mL YPD broth, incubated at 30°C for 24 hours, and preserved at -80°C

in 15% glycerol. The same broth cultures were also used as inoculum for the final YPD isolation plates that were sent to Microbial Identification Inc. (MIDI) Labs. There, the first 500bp (from the 5′ end) of the 16S rRNA gene were sequenced using Applied Biosystem's MicroSeq® 500. The sequences were compared to the MIDI Labs and GenBank databases for identification on 18 May 2012. One of the samples was not pure, and so was excluded from further investigation. In this way, soil isolates were identified to the species level. Full sequences of the 500bp analysis can be viewed in Appendix B.

Perpendicular Streak Tests

Perpendicular streak tests against the same six target organisms (*P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. faecalis*, *E. aerogenes*, and *S. cerevisiae*) were performed on YPD agar with the remaining 27 pure culture soil isolates. A single streak of the isolate was made across a plate of YPD agar, and the plate was incubated at 30°C for 24 hours. Then, each target organism was streaked perpendicularly to the soil isolate, with the beginning end of the streak abutting the soil isolate. After 24 hours incubation at 30°C, zones of target organism inhibition were documented as indication of antimicrobial activity by the soil isolate (Figure 1).

For isolates with differing results on the preliminary perpendicular streak test, Gram stains were performed on both original and isolated samples, to determine whether the differences were due to mixed cultures caused by incomplete isolation in preliminary testing. Then, the same perpendicular streak test was performed on yeast mold (YM) and Mueller-Hinton (MH) agars, to compare the effect of growth media on antimicrobial activity.

In addition, all soil isolates with activity against *S. cerevisiae* on YPD agar, were subjected to a perpendicular streak test against *Candida albicans* on YPD and YM agars. Inoculation, incubation, and interpretation were identical to the original perpendicular streak test.

Media pH Investigation

To determine if the inhibitory effects of the soil isolates were influenced by a change in media acidity, the pH was monitored for all three media types using a Denver Instrument UltraBASIC Benchtop pH Meter. Before each set of measurements, the meter was calibrated to within a pH of ±0.02 using 4.0 and 10.0 standards. YPD, YM, and MH broths were autoclaved at 121°C for 15 minutes and allowed to cool before measuring the initial pH. All three types of broth were inoculated with the soil isolates, with 5 mL broth and 50 µL inoculum. These were incubated at 30°C for 24 hours, and the final pH was measured. Target organisms then underwent the same investigation. The final pH measurements of the soil isolates were compared to the preferred pH ranges as well as the measured pH of the target organisms. As a control, 5 mL of each broth type without inoculum were also incubated 24 hours at 30°C, and the pH was measured.

To determine if the inhibitory effects were influenced by the dextrose content (the fermentation products of which might include organic acids), agars were made using the individual components of YPD: 10.0 g yeast extract and 15.0 g agar, 20.0 g peptone and 15.0 g agar, and 20.0 g dextrose and 15.0 g agar. These agars were used to repeat the perpendicular streak test using the same six target organisms (Figure 1). Growth on agar

containing only dextrose was extremely limited, preventing a reliable perpendicular streak test.

Spent Media Disc Assays

Cultures of soil isolates and bacterial target organisms were prepared using 50 µL inoculum in 5 mL YPD broth, incubated 24 hours at 30°C. Soil isolate cultures were vortexed, and the resuspended liquid was transferred to centrifuge tubes. These were centrifuged at 4000 rpm for 10 minutes, and the liquid was decanted into clean centrifuge tubes. This process was repeated two more times, with the final liquid being decanted into culture tubes. The spent media was syringe filtered, using a 0.2 µm filter, into clean culture tubes. From these, 25 µL of spent media from each soil isolate were applied to each of six sterile paper discs. Discs were allowed to dry for two hours. Meanwhile, the bacterial target organisms (*P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. faecalis*, and *E. aerogenes*) were resuspended by vortexing, poured into centrifuge tubes, and centrifuged at 4000 rpm for 10 minutes. The broth was decanted, and the pellets of cells were resuspended in 0.85% NaCl solution. These were standardized to a 0.5 McFarland standard by using a spectrophotometer and applied to MH agar by using sterile swabs in a three-way streak. After the target organism lawn had dried, a dry disc from the spent media of each soil isolate was applied to a quadrant of the plate. As a control, the sixth disc of each soil isolate was applied to MH agar that was not inoculated, to ensure that all bacteria cells were properly removed from the spent media. Plates were incubated 24 hours at 30°C, and the sizes of inhibitory zones around the discs were recorded. Control discs were inspected for bacterial growth. The same assay was performed with the spent

media from soil isolate cultures after 48 and 72 hours incubation, to determine if longer incubation would result in a higher concentration of molecules with antimicrobial activity produced by the soil isolate. The same assay was performed again using spent media from soil isolate cultures after 72-hour incubation, but discs were applied while still damp; that is, without the two-hour drying period.

To determine if growth in the presence of a target organism encouraged production of antimicrobial substances, as could possibly have been the case in the perpendicular streak tests, the same assay was performed using spent media from mixed cultures. Soil isolates that had positive results against S. *aureus* in the wet disc spent media assay or YPD perpendicular streak test, as well as those that showed activity against *P. aeruginosa* in any of the perpendicular streak tests, were selected for this assay. For mixed cultures, 5 mL YPD broth were inoculated with 50 µL of the soil isolate and 50 µL of the target organism against which inhibition had previously been observed. Discs were applied while still damp to lawns of the target organism that was included in the mixed culture.

Finally, the same spent media disc assay was performed using concentrated spent media from the mixed cultures that included *P. aeruginosa*. Using a vacuum centrifuge, 50, 75, 100, 125, and 150 µL aliquots of the spent media were dehydrated to a few microliters, resuspended to 25 µL in sterile deionized water, and applied to sterile paper discs. As a control, YPD broth was concentrated and applied to discs in the same fashion. Discs were applied to lawns of *P. aeruginosa* while still damp. For those soil isolates with positive results in this assay, the same assay was performed using concentrated spent media from pure cultures.

RESULTS AND DISCUSSION

Species Identification of Soil Isolates

Based on the sequences of the first 500bp (from the 5′ end) of the 16S rRNA gene provided by MIDI Labs, the petroleum-contaminated soil yielded 10 different species from 17 isolates, while the uncontaminated soil yielded 6 species from 10 isolates (Table 1). Appendix B contains full 500bp genetic sequences. Because only a third of the entire 16S rRNA gene was used and because the MIDI database is not all-inclusive, it is possible that the identifications were not entirely accurate.[35] Sequencing the entire 16S rRNA gene and including other databases in the identification process could solve this problem.

The petroleum-contaminated soil had three species of *Bacillus* (*B. amyloliquefaciens*, *B. cereus*, and *B. subtilis*) and three species of *Pseudomonas* (*P. corrugata*, *P. fulva*, and *P. marginalis*), which are both common genera of bacteria in soil.[36] The uncontaminated soil also yielded these genera (*B. amyoliquefaciens*, *P. corrugata*, and *P. fluorescens* – F), though with less species richness. *Burkholderia cepacia* is another common soil inhabitant that was found in both soil types. *B. cepacia* is a Gram-negative bacillus that frequently causes complications for patients with cystic fibrosis.[37]

Enterobacter sakazakii, more recently known as *Cronobacter sakazakii*,[38] was isolated from the petroleum-contaminated soil; it has been known to inhabit crude oil, among other environmental sources.[39] *E. sakazakii* is medically relevant for its role as a rare cause of invasive infection in neonates.[40] Petroleum-contaminated soil also yielded a species of *Enterococcus* equally related to *E. durans* and *E. faecium*. Both are common inhabitants of soil[41] as well as part of the normal intestinal flora of both humans and

animals.[34] To determine if the isolate was *E. durans* or *E. faecium*, a test could be done to determine if acid is produced from metabolism of glycerol or mannitol, for which a positive result would likely indicate *E. faecium*. Another test which could determine the species is growth at 50°C, with growth indicating *E. faecium*.[42] *Klebsiella pneumoniae*, either subspecies *pneumoniae* or *rhinoscleromatus*, was isolated from the uncontaminated soil. The subspecies could be determined by performing a Simmons citrate test, where subspecies *pneumoniae* would have a positive result.[43] While *K. pneumoniae* does inhabit soil and water, it should be noted that both it and *Staphylococcus epidermidis* (found in both soil types) could have been contaminants from handling. As part of human normal flora, they are a constant concern for contamination in the laboratory.

Perpendicular Streak Tests

In the YPD perpendicular streak test, all of the uncontaminated soil isolates showed at least some degree of antimicrobial activity, and only one isolate from petroleum-contaminated soil (*Bacillus cereus*) lacked antimicrobial activity (Table 2). This test also indicated no significant difference in the range of antimicrobial activity within a species between sources, as seen with *Bacillus amyloliquefaciens*, *Burkholderia cepacia*, *Pseudomonas corrugata*, and *Staphylococcus aureus*.

In the perpendicular streak tests, the general trend was an increase in the range of target organism inhibition from MH, to YPD, to YM (Tables 2-4). With MH, inhibition was limited to *Staphylococcus aureus* and *Klebsiella pneumoniae*. Both YPD and YM extended the activity, showing numerous incidences of inhibition against *P. aeruginosa*, *E. faecalis*, *E. aerogenes*, and *S. cerevisia*e in addition to *S. aureus* and *K. pneumoniae*.

Results of the perpendicular streak test against *Candida albicans* showed nearly identical results on YPD and YM, with the only significant difference having been a lack of *C. albicans* inhibition on YPD by one isolate of *Pseudomonas corrugata* (Table 5).

Media pH Investigation

Except in a few instances, pH was not the cause of the differential inhibition seen among the media types (Tables 6-9). *Pseudomonas aeruginosa* showed relative sensitivity to pH changes, and *Klebsiella pneumoniae* and *Enterobacter sakazakii* were prone to altering pH in YM and YPD. The inhibition of *P. aeruginosa*, *S. aureus*, *E. faecalis*, and *E. aerogenes* on YPD and YM by *K. pneumoniae* was probably not due to the production of an antibiotic, but rather a result of pH change caused by *K. pneumoniae*. The inhibition of *P. aeruginosa* on YM by *E. sakazakii* might or might not have been due to production of an antibiotic. Because the same inhibition was seen on YPD where pH was not responsible, further investigation is required to determine definitively whether or not *E. sakazakii* produces a substance that inhibits the growth of *P. aeruginosa*.

Based on the single-component-agar perpendicular streak tests, it appeared that yeast extract had slightly more effect on inhibitory activity than did peptones (Tables 10-11); however, the most inhibition was seen using complete YPD agar (Table 2). In fact, *Staphylococcus epidermidis*, *Enterobacter sakazakii*, *Pseudomonas marginalis*, and *Klebsiella pneumoniae* seemed to require the dextrose component to encourage their full range of inhibitory activity. There were exceptions however: *Bacillus cereus* and *B.*

subtilis had more inhibitory activity in the absence of dextrose, and *B. amyloliquefaciens* and *P. fluorescens* –F had equal ranges of inhibition with or without dextrose.

Spent Media Disc Assays

The pure culture spent media dry disc assays indicated no inhibition of any target organisms (Table 12); however, when the discs were left damp, *Staphylococcus aureus* was inhibited, to varying degrees, by spent media of the following isolates: *Pseudomonas fluorescens-F*, *P. corrugata*, *Bacillus cereus*, *B. subtilis*, *Enterobacter sakazakii*, and *Klebsiella pneumoniae* (Table 13). It is possible that, when allowed to dry, the antimicrobial substances in the spent media were not able to diffuse from the discs. Using an Oxford cup instead of the sterile discs might resolve this issue.[44] When the same spent media damp disc assay was performed from *S. aureus* mixed culture spent media, only one isolate of *P. corrugata* showed inhibition against *S. aureus* (Table 14). In instances where inhibition was seen in pure cultures but not mixed cultures, it is possible that in mixed cultures an antimicrobial substance was not produced, was used up, or that *S. aureus* effectively neutralized the substance.

While no inhibition against *P. aeruginosa* was seen in the pure culture spent media dry or damp disc assays (Tables 12-13), there was inhibition by three soil isolate species in the mixed culture version of the assay: *Bacillus amyloliquefaciens*, *Burholderia cepacia*, and *Pseudomonas marginalis* (Table 15). This might indicate that the soil bacteria produced substances in both situations but at inhibitory levels only in the mixed culture, or that the substances were produced only in response to being grown in the mixed culture. Either way, the dose-dependent response seen from the spent media of

B. amyloliquefaciens and *P. marginalis* in the concentrated spent media assay (Table 16 and Figure 2) suggested the substances produced by these species had antimicrobial effects against *P. aeruginosa*.

The mixed culture version of the spent media damp disc assay showed increased inhibition when using *Pseudomonas aeruginosa* but decreased inhibition when using *Staphylococcus aureus*, in comparison to the pure culture version of these assays. This phenomenon has many possible explanations. One possibility is that, in the case of *S. aureus*, the molecules produced in pure cultures that caused antibiosis were not produced in the mixed cultures. This would suggest that the molecules were not produced for the purpose of protection against *S. aureus*. It would also imply that the molecules produced in pure culture that were inhibitory against *P. aeruginosa* were for the purpose of protection and were upregulated in its presence. Another possibility is that antibiotic production by the soil isolates occurred in both pure and mixed cultures for both target organisms, but that *S. aureus* was more effective at neutralizing the substances than was *P. aeruginosa*. Further testing would be necessary to determine the exact cause of the differential effectiveness of using mixed cultures to encourage antibiotic production.

The chemical nature of the antimicrobial substance may also have affected all versions of the spent media disc assays. If the active molecule possessed a polarity not complementary to medium, it may have been attracted to the cells during centrifugation and pulled out of the medium or attracted to the paper disc and not allowed to diffuse. Also, if the compound was volatile, it may have evaporated out of the disc. A low molecular concentration of the antimicrobial substance would exacerbate these issues.

The antibiosis seen by *P. corrugata* and *P. marginalis* is typical. Pseudomonads, especially the fluorescent varieties, are known for production of a wide range of antifungals[45-46], antibiotics[47-51], and organic volatiles[52]. Most testing with Pseudomonads has centered on their use as biocontrol agents in agriculture, and testing against human pathogens has been limited. While the antibiotic activity seen might be an extension in the spectrum of an already-documented molecule, it is also possible that there was a novel antibiotic involved. To determine this, the active compound would need to be isolated, identified, and tested in its pure form. This seems a worthy endeavor, considering the possible implications in clinical settings.

The antibiosis seen by *B. amyloliquefaciens* is also typical. Its antifungal[44] and antibiotic[53] properties are well documented. Again, most studies surrounding antimicrobial production by *B. amyloliquefaciens* center on biocontrol in agriculture. One study by Nastro et al., though, produced similar results to this study, in demonstrating the direct inhibition of *P. aeruginosa* by growth of *B. amyloliquefaciens* on Tryptone Soy Agar, but not Plate Count Agar.[54] While it is not clear whether it was the same molecule being produced in both studies, the repeatability of the phenomenon of *P. aeruginos*a inhibition by *B. amyloliquefaciens* makes the isolation and identification of the active compound a worthy endeavor.

CONCLUSION

The number of species isolated from petroleum-contaminated soil indicates that searching for a diverse community of bacteria is practical in petroleum-contaminated soil. This type of soil is also a good source of antimicrobial producing bacteria, as is indicated by the YPD perpendicular streak test. These results, combined with the absolute ubiquity of microbes, warrants study of other less orthodox soils as well.

Given the extent of antimicrobial activity seen on YPD and YM, it can be concluded that complex media such as YPD and YM are viable alternatives to MH for this type of screening, though the effects of pH should be closely monitored. While the use of complex media makes it difficult to determine exactly which ingredient is inciting the antimicrobial activity, the variety of components caters to a wide range of bacteria. This comprehensive approach is appropriate for early stages of screening and might be preferable as a complement to metagenomic studies.

The increased inhibition of *Pseudomonas aeruginosa* by *Bacillus amyloliquefaciens* and *Pseudomonas marginalis* in the mixed culture spent media assay versus the pure culture version merits further investigation of the mixed culture approach. The dose-dependent response seen with the concentrated spent media of *B. amyloliquefaciens* and *P. marginalis*, along with their notoriety as antimicrobial-producers, warrants isolation and identification of the compound(s) that inhibited *P. aeruginosa* in this study. Given that all three bacteria that had success in the mixed culture spent media assay (*B. amyloliquefaciens*, *P. marginalis*, and *P. corrugata*) are used as biocontrol agents, it would also seem prudent to test other agricultural biocontrol agents against human pathogens.

Diversification of the methods used to identify new antibiotics produced by environmental bacteria seems a valuable enterprise. The bacteria isolated from petroleum-laced hypersaline soil showed great diversity and extensive antimicrobial activity. The increased antimicrobial activity seen when using the YPD and YM encourages use of other complex media, with the caution that pH might become an issue with certain isolates and pathogens. Culturing methods that encourage microbial interaction, such as the mixed culturing used with the spent media disc assay, should be employed to encourage production of molecules with unknown natural functions which, at high concentrations, cause antibiosis. Finally, concentrated spent media assays should be used in order to identify antibiotic molecules that may be naturally produced at subinhibitory levels.

Ultimately, though, it must be acknowledged that antibiotics are not a cure-all. While antibiotics will likely always be necessary in treating mild to moderate infections and in use for combination therapies, the inevitability of antibiotic resistance, especially with certain pathogens, necessitates the exploration of other solutions. One of the most obvious solutions is to prevent infection in the first place, by continual improvement of sanitation and infection containment methods, especially within hospitals. Use of ultraviolet radiation and novel sanitation chemicals is helpful.[55-56] Upon infection, there is an increasing number of alternatives to simple antibiotic therapy. Immunotherapy, or treatment of disease by inducing, enhancing, or suppressing an immune response, is an alternative that shows promise in treating many robust infections, such as MRSA .[57] Another option is gene therapy, which can be used to genetically reprogram cells to protect themselves, as with the use in HIV/AIDs and Hepatitis B virus infections.[58-59] It

is important to employ these complementary treatments, rather than exclusively targeting the pathogens with antibiotics.

Antibiotic resistance is controllable, and yet frighteningly unavoidable. Even with complementary treatments, we must maintain the antibiotic pipeline in order to sustain the benefits we currently receive from them. The fate of mankind as we know it is dependent on continued discovery and development of novel antibiotics.

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Table 1. Identification of soil isolates based on the first 500 bp at the 5′ end of the genetic sequence for the 16S rRNA. Isolates 14AS and 14AR were separated during isolation, but were derived from the same original culture. For all other cultures that were originally mixed, a single colony type was selected and isolated. Isolate 17D was a mixed culture and, so, was eliminated from further testing.

Petroleum-Contaminated Soil		Uncontaminated Soil	
Isolate ID	Species	Isolate ID	Species
11B	*Bacillus amyloliquefaciens*	9B	*Bacillus amyloliquefaciens*
12B	*Bacillus amyloliquefaciens*	9A	*Bacillus amyloliquefaciens*
14B	*Bacillus amyloliquefaciens*	6C	*Burkholderia cepacia*
14AR	*Bacillus amyloliquefaciens*	3B	*Klebsiella pneumoniae*
15C	*Bacillus amyloliquefaciens*	5C	*Klebsiella pneumoniae*
13A	*Bacillus cereus*	4B	*Pseudomonas corrugata*
17B	*Bacillus subtilis*	4A	*Pseudomonas fluorescens-F*
10I	*Burkholderia cepacia*	1A	*Staphylococcus epidermidis*
16B	*Enterobacter sakazakii*	1C	*Staphylococcus epidermidis*
10B	*Enterococcus durans/faecium*	3C	*Staphylococcus epidermidis*
10C	*Pseudomonas corrugata*		
16A	*Pseudomonas corrugata*		
14AS	*Pseudomonas fulva*		
16C	*Pseudomonas marginalis*		
10D	*Staphylococcus epidermidis*		
10J	*Staphylococcus epidermidis*		
10K	*Staphylococcus epidermidis*		
17D	mixed		

Table 2. Perpendicular streak test on YPD agar. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm. Cells shaded gray correspond to results of soil isolates originating from uncontaminated soil. Isolates are arranged in alphabetical order of their species ID, which can be found in Table 1.

Isolate ID	*P.a.	S.a.	K.p.	E.f.	E.a.	S.c.
11B	+	+		minor	minor	
12B	minor	+		minor	minor	
14B	+	+		minor	minor	
14AR	+	+		minor		
15C	minor	+			minor	
9B	+	+		+	+	
9A	+	+		minor		
13A						
17B		+				
10I	minor	+		minor	minor	+
6C	minor	+		minor		+
16B	+	minor	+	minor	+	
10B	minor		+	minor		
3B	+	+	+	+	+	
5C	+	+	+	+	+	
10C	+	+	+	minor		minor
16A		+				+
4B	+	+	+			
4A	minor	+	+			
14AS	+	+	+			
16C	minor	minor	+			
10D	+	+	+	+	+	
10J	+	+	+	+	+	
10K	+	+	+	+	+	
1A	+	+	+	+	+	
1C	+	+	+	+	+	
3C	+	+	+	+	+	

**P.a. = Pseudomonas aeruginosa, S.a.= Staphylococcus aureus, K.p. = Klebsiella pneumoniae, E.f. = Enterococcus faecalis, E.a. = Enterobacter aerogenes, S.c. = Saccharomyces cerevisiae*

Table 3. Perpendicular streak test on YM agar. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm. Cells shaded gray correspond to results of soil isolates originating from uncontaminated soil. Isolates are arranged in order of their species ID, which can be found in Table 1. Refer to Table 2 for an explanation of target organism abbreviations.

Isolate ID	*P.a.*	*S.a.*	*K.p.*	*E.f.*	*E.a.*	*S.c.*
11B	minor	+	minor	+	+	+
12B		+	+	+	+	+
14B	minor	+	+	+	+	+
14AR	+	+	+	+	+	+
15C		+	+	+	+	+
9B	+	+	+	+	+	+
9A	+	+	+	+	+	+
13A						
17B		+	+	+	+	+
10I	+	+	+	+	+	+
6C	+	+	+	+	+	+
16B	+			minor		
10B			minor			
3B	+	minor	+	+	+	
5C	+	minor	+	+	+	
10C	+	+	+	+	+	
16A	+			+	+	+
4B	+	+	+	+	+	
4A		+	+			+
14AS	+	+	+	+	+	
16C	+	+	+	+	+	
10D	+	+	+	+	+	
10J	+	+	+	+	+	
10K	+	+	+	+	+	
1A	+	+	+	+	+	
1C	+	+	+	+	+	
3C	+	+	+	+	+	

Table 4. Perpendicular streak test on MH agar. A (+) indicates inhibition ≥5 mm. Cells shaded gray correspond to results of soil isolates originating from uncontaminated soil. No results exist for *Saccharomyces cerevisiae* because it does not grow well on MH agar. Isolates are arranged in order of their species ID, which can be found in Table 1. Refer to Table 2 for an explanation of target organism abbreviations.

Isolate ID	*P.a.*	*S.a.*	*K.p.*	*E.f.*	*E.a.*
11B		+			
12B		+			
14B		+			
14AR		+			
15C		+			
9B		+			
9A		+			
13A		+			
17B		+			
10I					
6C					
16B					
10B					
3B					
5C					
10C		+	+		
16A		+			
4B		+	+		
4A		+			
14AS		+			
16C		+			
10D					
10J					
10K					
1A					
1C					
3C					

Table 5. Perpendicular streak test against *Candida albicans* performed on YPD and YM agars. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm. Cells shaded gray correspond to results of soil isolates originating from uncontaminated soil. Isolates are arranged in order of their species ID, which can be found in Table 1.

Isolate ID	YPD	YM
11B	+	+
12B	+	+
14B	+	+
14AR	+	+
15C	+	+
9B	+	+
9A	+	+
17B	+	+
10I	+	minor
6C	+	+
16A		+
10C	+	+
4B		+
4A	+	+

Table 6. Comparison of media pH without inoculation.

	Media pH		
	YPD	YM	MH
After autoclaving	6.52	6.62	7.65
After 24 hours refrigeration	6.65	6.65	7.69
After 24 hours incubation	6.58	6.52	7.60

Table 7. Investigation of pH using YPD broth. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm when the respective agar formulation was used in the perpendicular streak test. Gray highlighted boxes indicate the possibility of inhibition seen in perpendicular streak tests being due to a change in pH caused by the soil isolate. Sources for pH ranges are listed as 60-66 in the Literature Cited, respective to their appearance in this table. Refer to Table 2 for an explanation of target organism abbreviations.

		P.a.	*S.a.*	*K.p.*	*E.f.*	*E.a.*	*S.c.*
	pH Range	>4.5	4.5-9.3	5.0-9.0	4.5-10.0	4.4-9.0	2.6-8.5
Isolate ID	Measured pH	7.61	5.05	4.58	4.48	5.19	5.33
11B	6.03	+	+		minor	minor	
12B	6.02	minor	+		minor	minor	
14B	6.11	+	+		minor	minor	
14AR	6.08	+	+		minor		
15C	5.88	minor	+			minor	
9B	5.98	+	+		+	+	
9A	5.86	+	+		minor		
13A	5.05						
17B	5.87		+				
10I	5.62	minor	+		minor	minor	+
6C	5.55	minor	+		minor		+
16B	4.74	+	minor	+	minor	+	
10B	7.13	minor		+	minor		
3B	4.59	+	+	+	+	+	
5C	4.59	+	+	+	+	+	
10C	7.13		+				+
16A	6.12	+	+	+	minor		minor
4B	6.14	+	+	+			
4A	6.66	minor	+	+			
14AS	7.21	+	+	+			
16C	7.30	minor	minor	+			
10D	7.05	+	+	+	+	+	
10J	5.02	+	+	+	+	+	

Table 7 Continued.

		P.a.	*S.a.*	*K.p.*	*E.f.*	*E.a.*	*S.c.*
	pH Range	>4.5	4.5-9.3	5.0-9.0	4.5-10.0	4.4-9.0	2.6-8.5
Isolate ID	Measured pH	7.61	5.05	4.58	4.48	5.19	5.33
10K	5.01	+	+	+	+	+	
1A	5.04	+	+	+	+	+	
1C	5.04	+	+	+	+	+	
3C	5.01	+	+	+	+	+	

Table 8. Investigation of pH using YM broth. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm when the respective agar formulation was used in the perpendicular streak test. Gray highlighted boxes indicate the possibility of inhibition seen in perpendicular streak tests being due to a change in pH caused by the soil isolate. Sources for pH ranges are listed as 60-66 in the Literature Cited, respective to their appearance in this table. Refer to Table 2 for an explanation of target organism abbreviations.

		P.a.	*S.a.*	*K.p.*	*E.f.*	*E.a.*	*S.c*
	pH Range	>4.5	4.5-9.3	5.0-9.0	4.5-10.0	4.4-9.0	2.6-8.5
Isolate ID	Measured pH	7.31	4.92	4.42	4.13	4.7	5.07
11B	5.95	minor	+	minor	+	+	+
12B	5.89		+	+	+	+	+
14B	5.93	minor	+	+	+	+	+
14AR	5.90	+	+	+	+	+	+
15C	5.56	+	+	+	+	+	+
9B	5.80	+	+	+	+	+	+
9A	5.59		+	+	+	+	+
13A	4.94						
17B	6.03		+	+	+	+	+
10I	4.92	+	+	+	+	+	+
6C	4.73	+	+	+	+	+	+
16B	4.53	+			minor		
10B	6.45			minor			
3B	4.40	+	minor	+	+	+	
5C	4.41	+	minor	+	+	+	
10C	6.02	+	+	+	+	+	
16A	6.59	+			+	+	+
4B	6.15	+	+	+	+	+	
4A	6.55		+	+			+
14AS	5.46	+	+	+	+	+	
16C	6.59	+	+	+	+	+	
10D	6.52	+	+	+	+	+	
10J	4.74	+	+	+	+	+	
10K	4.76	+	+	+	+	+	

Table 8 Continued.

		P.a.	*S.a.*	*K.p.*	*E.f.*	*E.a.*	*S.c*
	pH Range	>4.5	4.5-9.3	5.0-9.0	4.5-10.0	4.4-9.0	2.6-8.5
Isolate ID	Measured pH	7.31	4.92	4.42	4.13	4.7	5.07
1A	4.85	+	+	+	+	+	
1C	4.77	+	+	+	+	+	
3C	4.80	+	+	+	+	+	

Table 9. Investigation of pH using MH broth. A (+) indicates inhibition ≥5 mm when the respective agar formulation was used in the perpendicular streak test. No results are shown for *Saccharomyces cerevisiae* because it does not grow well on MH. Sources for pH ranges are listed as 60-66 in the Literature Cited, respective to their appearance in this table. Refer to Table 2 for an explanation of target organism abbreviations.

		P.a.	*S.a.*	*K.p.*	*E.f.*	*E.a.*
	pH Range	>4.5	4.5-9.3	5.0-9.0	4.5-10.0	4.4-9.0
Isolate ID	Measured pH	7.99	6.51	6.4	6.91	7.07
11B	6.88		+			
12B	7.26		+			
14B	7.22		+			
14AR	7.18		+			
15C	7.22		+			
9B	7.29		+			
9A	6.88		+			
13A	5.88		+			
17B	7.32		+			
10I	7.63					
6C	7.69					
16B	7.19					
10B	7.88					
3B	6.75					
5C	6.71					
10C	7.84		+	+		
16A	7.82		+			
4B	7.77		+	+		
4A	7.77		+			
14AS	7.87		+			
16C	7.97		+			
10D	7.85					
10J	7.04					
10K	6.98					
1A	6.94					

Table 9 Continued.

		P.a.	*S.a.*	*K.p.*	*E.f.*	*E.a.*
	pH Range	>4.5	4.5-9.3	5.0-9.0	4.5-10.0	4.4-9.0
Isolate ID	Measured pH	7.99	6.51	6.4	6.91	7.07
1C	6.99					
3C	6.97					

Table 10. Perpendicular streak test on yeast extract agar. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm. No results are shown for *Saccharomyces cerevisiae* because it does not grow well on yeast extract agar. Refer to Table 2 for an explanation of target organism abbreviations.

Isolate ID	*P.a.*	*S.a.*	*K.p.*	*E.f.*	*E.a.*
11B		+	minor	minor	minor
12B		+	minor	minor	minor
14B		+	minor	minor	minor
14AR		+	minor	minor	minor
15C		+	minor	minor	minor
9B		+	minor	minor	minor
9A		+	+	minor	minor
13A	+	+	+	+	+
17B		+	+	+	+
10I		+			
6C		+			
16B					
10B		+			
3B					
5C					
10C		+			
16A		+			
4B		+			
4A		+	+	minor	minor
14AS		+			
16C					
10D		+			
10J					
10K					
1A					
1C					
3C					

Table 11. Perpendicular streak test on peptones agar. "Minor" indicates a zone of inhibition <5 mm, while a (+) indicates inhibition ≥5 mm. No results are shown for *Saccharomyces cerevisiae* because it does not grow well on peptones agar. Refer to Table 2 for an explanation of target organism abbreviations.

Isolate ID	*P.a.*	*S.a.*	*K.p.*	*E.f.*	*E.a.*
11B		+	+	minor	minor
12B		+	+	minor	minor
14B		+	+	minor	minor
14AR		+	+	+	minor
15C		+	+	+	minor
9B		+	+	minor	minor
9A		+	+	+	minor
13A		+	+	+	+
17B		+	+	+	minor
10I					
6C		minor			
16B					
10B		+			
3B					
5C					
10C		minor			
16A		minor			
4B		+			
4A		+			
14AS					
16C					
10D					
10J					
10K					
1A					
1C					
3C					

Table 12. Pure culture spent media dry disc assay. A (-) indicates no inhibition against any of the bacterial pathogens.

	Incubation Time (hrs)		
Isolate ID	24	48	72
11B	-	-	-
12B	-	-	-
14AR	-	-	-
9B	-	-	-
10I	-	-	-
6C	-	-	-
14AS	-	-	-
4A	-	-	-
4B	-	-	-
16A	-	-	-
13A	-	-	-
17B	-	-	-
16B	-	-	-
10B	-	-	-
3B	-	-	-
5C	-	-	-
16C	-	-	-
10D	-	-	-
1C	-	-	-

Table 13. Pure culture spent media damp disc assay. Numbers indicate the diameter of inhibitory zones, including the discs, in millimeters. Discs are 6 mm in diameter. A (-) indicates no inhibition. Refer to Table 2 for an explanation of target organism abbreviations.

Isolate ID	*P.a.*	*S.a.*	*K.p.*	*E.f.*	*E.a.*
11B	-	-	-	-	-
12B	-	-	-	-	-
14AR	-	-	-	-	-
9B	-	-	-	-	-
10I	-	-	-	-	-
6C	-	-	-	-	-
14AS	-	-	-	-	-
4A	-	11	-	-	-
4B	-	11	-	-	-
16A	-	11	-	-	-
13A	-	12	-	-	-
17B	-	11	-	-	-
16B	-	11	-	-	-
10B	-	-	-	-	-
3B	-	11	-	-	-
5C	-	12	-	-	-
16C	-	-	-	-	-
10D	-	-	-	-	-
1C	-	-	-	-	-

Table 14. Mixed culture spent media damp disc assay. Within a column, cultures were grown with and tested against the target organism listed in the top row. "NA" indicates that the soil isolate was not used with the target organism for the assay. Numbers indicate the diameter of inhibitory zones, including the discs, in millimeters. Discs are 6 mm in diameter. A (-) indicates no inhibition.

Isolate ID	*P. aeruginosa*	*S. aureus*
11B	7	-
12B	7	-
14AR	7.5	-
9B	8	-
10I	7	NA
6C	6.5	NA
14AS	-	-
4A	-	-
4B	-	9
13A	NA	-
17B	NA	-
16A	-	-
16B	-	NA
3B	-	NA
5C	-	NA
16C	7.5	-
1C	-	NA
10D	-	NA

Table 15. *Pseudomonas aeruginosa* mixed culture concentrated spent media damp disc assay. Cultures were grown with and tested against *P. aeruginosa*. Numbers indicate the diameter of inhibitory zones, including the discs, in millimeters. Discs are 6 mm in diameter. A (-) indicates no inhibition.

	Volume (in µL) Concentrated to 25µL					
Isolate ID	**25**	**50**	**75**	**100**	**125**	**150**
11B	7	11	12.5	13	15.5	17
12B	7	11	12.5	13.5	16	19
14AR	7.5	10	12.5	14	14	19
9B	8	9	11	12.5	14	17
10I	7	-	-	-	-	-
6C	6.5	-	-	-	-	-
14AS	-	-	-	-	-	-
4A	-	-	-	-	-	-
4B	-	-	-	-	-	-
16A	-	-	-	-	-	-
16B	-	-	-	-	-	-
3B	-	-	-	-	-	-
5C	-	-	-	-	-	-
16C	7.5	9	14	15	16	17.5
10D	-	-	-	-	-	-
1C	-	-	-	-	-	-
YPD	-	-	6.5	7	7	7.5

Table 16. *Pseudomonas aeruginosa* pure culture concentrated spent media damp disc assay. Cultures were tested against *P. aeruginosa*. A (-) indicates no inhibition.

	Volume (in µL) Concentrated to 25µL					
Isolate ID	**25**	**50**	**75**	**100**	**125**	**150**
11B	-	-	-	-	-	-
12B	-	-	-	-	-	-
14AR	-	-	-	-	-	-
9B	-	-	-	-	-	-
16C	-	-	-	-	-	-

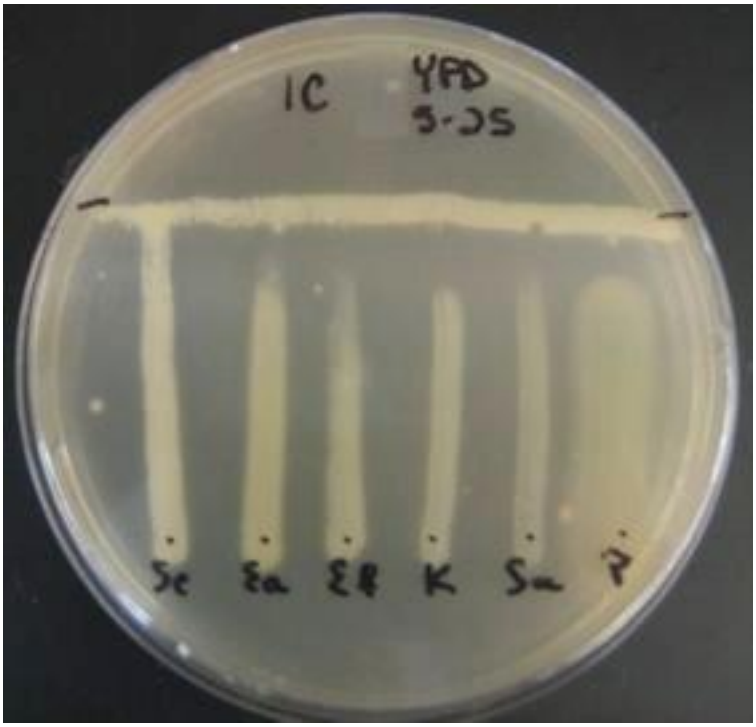
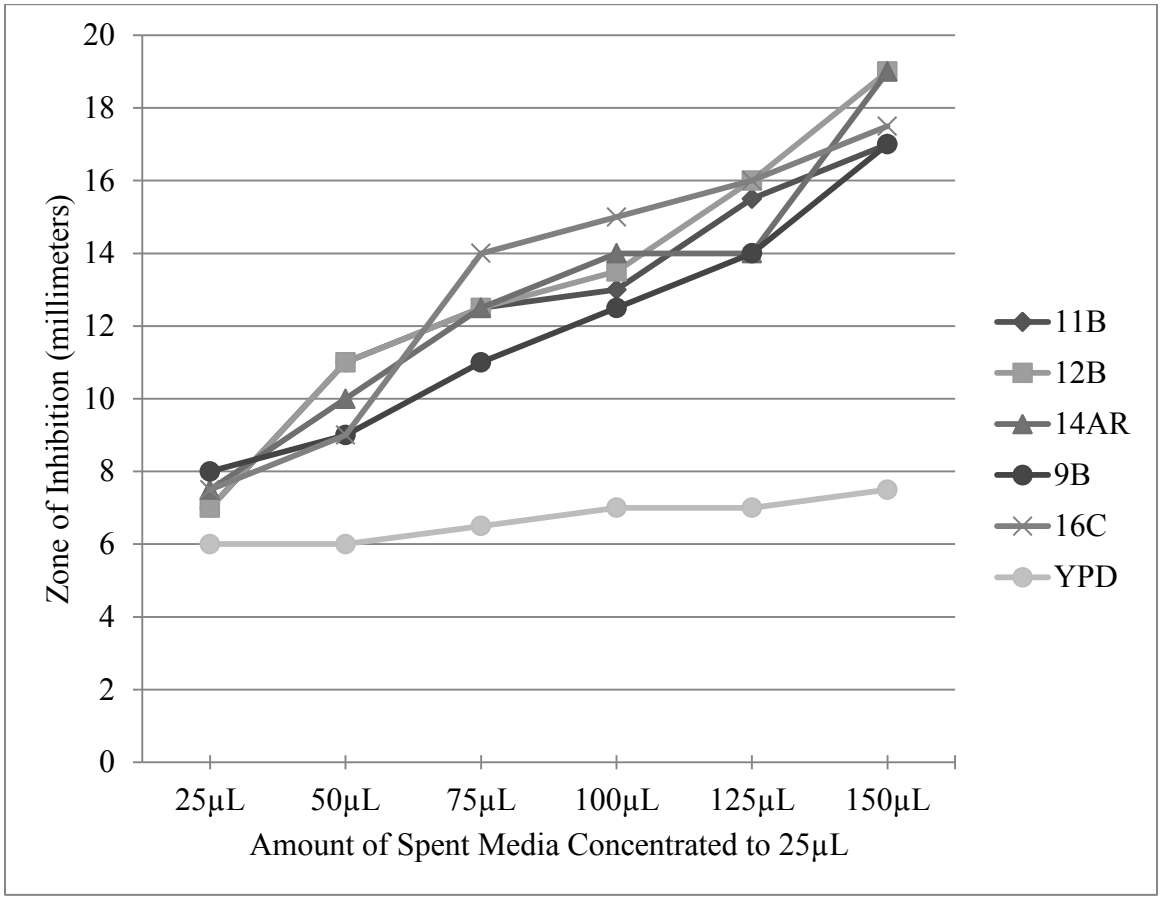


Figure 1. Example of a completed perpendicular streak test. The horizontal line is the soil isolate streak, and the vertical lines are the target organisms. In this example, target organisms (from left to right) *Enterobacter aerogenes*, *Enterococcus faecalis, Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* are considered inhibited. *Saccharomyces cerevisiae* (far left) is uninhibited.

Figure 2. Inhibition of *Pseudomonas aeruginosa* in mixed culture concentrated spent media damp disc assay.



Appendix A. Identification of soil source sites for isolates of both uncontaminated and petroleum-contaminated soils. Isolation sites termed "Oil wells" refer to the soil samples taken from within dikes surrounding oil tank batteries.

Isolate ID	Site of Isolation
1A-1F	Playa lake 1
2A-2B	Spring-fed creek bed
3A-3C	Horse pasture
4A-4C	Inactive cattle pen
5A-5C	Ensilage pit
6A-6B	Dry creek bed
7A-7B	Playa lake 2
8A-8B	Active cattle pen
9A-9C	Playa lake 3
10A-10K	Oil well 1
11A-11C	Oil well 2
12A-12C	Oil well 3
13A-13C	Oil well 4
14A-14B	Oil well 4 water
15A-15C	Oil well 5
16A-16C	Oil well 6
17A-17D	Oil well 7

Appendix B. Sequences of the first 500bp at the 5′ end of the gene for the 16S rRNA of soil isolates and species identification.

IUPAC nucleotide code interpretation[66]:

A = Adenine	R = A or G	B = C or G or T
C = Cytosine	Y = C or T	D = A or G or T
G = Guanine	S = G or C	H = A or C or T
T = Thymine	W = A or T	V = A or C or G
	K = G or T	N = any base
	M = A or C	. or - = gap

1A: *Staphylococcus epidermidis*

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGC

GGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTT

CGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAAT

AGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAG

CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTG

AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG

GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA

CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGG

AAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

1C: *Staphylococcus epidermidis*

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGC

GGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTT

CGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAAT

AGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAG

CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTG

AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG

GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA

CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGG

AAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

3B: *Klebsiella pneumoniae* subspecies *pneumoniae* or *rhinoscleromatus*

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGC

GGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC

TGGAAACGGTAGCTAATACCGCATAAYGTCGCAAGACCAAAGTGGGGGAC

CTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT

GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA

CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA

GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG

TGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCG

ATRAGGTTAATAACCTYRTCGATTGACGTTACCCGCAGAAGAAGCACCGG

CTAACTCCGTGCCAGCAGCCGCGGTA

3C: *Staphylococcus epidermidis*

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGC

GGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTT

CGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAAT

AGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAG

CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTG

AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG

GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA

CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGG

AAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

4A: *Pseudomonas fluorescens* – F

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGG

ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCG

GAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCT

TCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGA

GGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATC

AGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT

GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTG

TGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG

TTACCTAATACGTATCTGTTTTGACGTTACCGACAGAATAAGCACCGGCT
AACTCTGTGCCAGCAGCCGCGGTA

4B: ***Pseudomonas corrugata***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC
ATGCAAGTCGAGCGGTAGAGAGGTGCTTGCACCTCTTGAGAGCGGCGGAC
GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGA
AACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC
GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGG
TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG
TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTA

5C: ***Klebsiella pneumoniae*** **subspecies** ***pneumoniae*** **or** ***rhinoscleromatus***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC
ATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGC
GGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC
TGGAAACGGTAGCTAATACCGCATAAYGTCGCAAGACCAAAGTGGGGGAC
CTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA

CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA

GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG

TGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCG

ATRAGGTTAATAACCTYRTCGATTGACGTTACCCGCAGAAGAAGCACCGG

CTAACTCCGTGCCAGCAGCCGCGGTA

6C: ***Burkholderia cepacia***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACAC

ATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAGTGGCGA

ACGGGTGAGTAATACATCGGAACATGTCCTGTAGTGGGGGATAGCCCGGC

GAAAGCCGGATTAATACCGCATACGATCTACGGATGAAAGCGGGGGACCT

TCGGGCCTCGCGCTATAGGGTTGGCCGATGGCTGATTAGCTAGTTGGTGG

GGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACC

AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT

GGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTG

TGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTT

GGCTCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCT

AACTACGTGCCAGCAGCCGCGGTA

9A: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG

ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTYTGAACCGCATGGTTCAGACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

9B: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG

ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTYTGAACCGCATGGTTCAGACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

10B: ***Enterococcus durans*** **or** ***faecium***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAA

GAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAG

GGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGC

ATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCG

CGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCA

TAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA

AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTC

TGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTC

TGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGT

ATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

10C: ***Pseudomonas corrugata***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAGCGGTAGAGAGGTGCTTGCACCTCTTGAGAGCGGCGGAC

GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGA

AACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGG

TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG

TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG

GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG

AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA

ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTA

10D: ***Staphylococcus epidermidis***

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC
ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGC
GGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTT
CGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAAT
AGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAG
CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGG
AAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

10I: ***Burkholderia cepacia***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACAC
ATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAGTGGCGA
ACGGGTGAGTAATACATCGGAACATGTCCTGTAGTGGGGGATAGCCCGGC
GAAAGCCGGATTAATACCGCATACGATCTACGGATGAAAGCGGGGGACCT
TCGGGCCTCGCGCTATAGGGTTGGCCGATGGCTGATTAGCTAGTTGGTGG
GGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACC

AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT

GGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTG

TGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTT

GGCTCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCT

AACTACGTGCCAGCAGCCGCGGTA

10J: ***Staphylococcus epidermidis***

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGC

GGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTT

CGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAAT

AGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAG

CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTG

AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG

GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA

CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGG

AAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

10K: ***Staphylococcus epidermidis***

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGC

GGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTT

CGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAAT

AGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAG

CTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTG

AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG

GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA

CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGG

AAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

11B: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG

ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

12B: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG

ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTYTGAACCGCATGGTTCAGACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

13A: ***Bacillus cereus***

TGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGC

GGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTC

CGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAA

ATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTA

GCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT

GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG

GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA

ACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGG

GAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCA

GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

14AR: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG

ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

14AS: ***Pseudomonas fulva***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAGCGGATGAAAGGAGCTTGCTCCTGGATTCAGCGGCGGAC

GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGA

AAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG

TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG

TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTA
GATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTA

14B: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC
ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG
GGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACA
TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA
AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

15C: ***Bacillus amyloliquefaciens***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC
ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTYTGAACCGCATGGTTCAGACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA

16A: ***Pseudomonas corrugata***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAGCGGTAGAGAGGTGCTTGCACCTCTTGAGAGCGGCGGAC

GGGTGAGTAAAGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGA

AACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG

TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG

TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG

GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG

AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA

ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA

CTCTGTGCCAGCAGCCGCGGTA

16B: ***Enterobacter sakazakii***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTG

GCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGCGGGGGATAACT

ACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGG

ACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAG

GTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT

GACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG

CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCG

TGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGG

CGATACGGYTAATAACCGTGTCGATTGACGTTACCCGCAGAAGAAGCACC

GGCTAACTCCGTGCCAGCAGCCGCGGTA

16C: ***Pseudomonas marginalis***

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC

ATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGAC

GGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGA

AACGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTC

GGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGG

TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG

TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG

GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG

AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCCATT

ACCTAATACGTGATGGTTTTGACGTTACCGACAGAATAAGCACCGGCTAA

CTCTGTGCCAGCAGCCGCGGTA

17B: ***Bacillus subtilis – subtilis***

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATAC

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGG

ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACA

TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGA

GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG

AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC

GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA

AGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGA

AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA