Isolating and Testing Antibiotic-Producing Bacteria from Marine and Terrestrial Samples from St. Petersburg, FL

By

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CERTIFICATE OF APPROVAL

Honors Thesis

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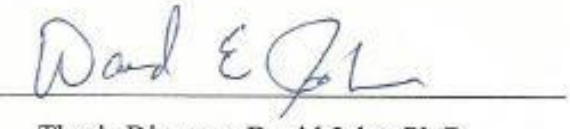

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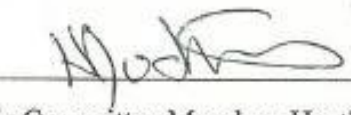

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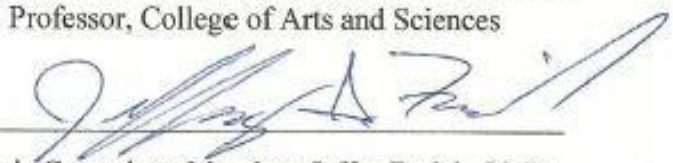

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Abstract

Many current antibacterial agents on the market are derived from compounds naturally produced by bacteria. This experiment was conducted to isolate and assess possible antibacterial strains, namely *Actinomycetes,* from 50 mL of marine and terrestrial samples collected from nine locations around the St. Petersburg area. The agar plates were prepared with Jensen's AMM agar and inoculated with bacterial samples which had been diluted by factor 1:100 and heated. Three plates were initially assigned to a sample to test against three laboratory bacteria by the overlay method. Then isolates were selected and inoculated onto TSA and BHI agar plates to test against ten common pathogenic bacteria, or relatives of pathogenic bacteria, by lateral streaking. Overlays were also done for some isolates on AMM agar, and a test was performed to measure inhibition over time.

The strains yielded from the terrestrial samples appeared to have far greater effectiveness against the test bacteria than those from the marine samples. One terrestrial isolate in particular showed effectiveness against all ten bacteria, whereas the marine samples showed little to no effectiveness against any of the bacteria with the exception of one. The Gram stains performed for four best terrestrial isolates revealed purple rods, indicating Gram-positive *Bacillus* species but not *Actinomycetes*. The strains were identified via DNA extraction, amplification and sequencing, as *Brevibacillus choshinensis, Brevibacillus laterosporus, Bacillus amyloliquefaciens subsp. plantarum* and *Bacillus amyloliquefaciens*. Extraction of the unknown compounds with dichloromethane and subsequent IR spectroscopy revealed a similar-looking molecule produced by all four isolates, with amine, arene and ketone or ester groups.

Introduction

Antibiotics are chemical compounds, natural or artificial, that inhibit the growth of or kill microbes and are used primarily to treat or prevent infections (Pidcock & Piotrowski, 2013). In their natural forms, they are secondary metabolites typically produced and secreted when resources are scarce as a means to hinder interspecific competition (Madigan et al, 2012). An ideal antibiotic should be effective against a wide range of pathogens when used correctly and be able to kill the pathogens without disrupting the cellular processes of the user (Pidcock & Piotrowski, 2013). Between 1930 and 1962 over twenty classes of antibiotics were discovered and produced, though after this period, only four new classes have been marketed, none of which are truly novel as derivatives of them had been available long before they were approved for widespread use (Gualerzi et al, 2014). One can synthesize or modify antibiotics in a laboratory but most are natural chemicals derived directly from other microbes, and are classified based on structure and mechanism of action (Gualerzi et al, 2014).

Some classes of antibiotics work by destroying the cell wall or plasma membrane (Harvey et al, 2007; Procópio et al, 2012). Beta-lactams, also called penicillins, are produced by the fungus *Penicillium* and target the penicillin-binding enzymes that are involved in peptidoglycan synthesis, thus interfering with the pathogen's cell wall (Gualerzi et al, 2014; Harvey et al, 2007; Pidcock & Piotrowski, 2013). They work best against rapidly proliferating bacteria with a peptidoglycan cell wall and though they are the most widely effective, many bacteria have developed resistance to them (Harvey et al, 2007). Cephalosporins are like penicillins except that they have more counter-resistance to beta-lactamases produced by bacteria and depending on their generation can potentially be used against gram-negative bacteria (Gualerzi et al, 2014; Harvey et al, 2007). Lantibiotics come from *Actinobacteria* and *Firmicutes*

strains and are modified to also sabotage the cell wall by inhibiting the production of its components (Gualerzi et al, 2014).

Other classes work by sabotaging protein synthesis or enzyme activity (Harvey et al, 2007; Procópio et al, 2012). Aminoglycosides inhibit protein synthesis by attacking the ribosome of the pathogen but only work against aerobic bacteria because they target the oxygen-dependent transport system; they are acquired through fermentation of *Bacillus, Micromonospora* and *Streptomyces* (Gualerzi et al, 2014; Harvey et al, 2007). Thiazolylpeptides, which are more specific about their target ribosomal area, come from genera *Micrococcus, Streptomyces* and *Planobispora* (Gualerzi et al, 2014). Fluoroquinolones inhibit DNA replication in bacteria by hindering DNA gyrase activity and inducing lethal DNA cleavage (Gualerzi et al, 2014; Harvey et al, 2007). Macrolides, the first of which was reaped from *Streptomyces erythreus,* inhibit translocation of protein synthesis by binding to the 50S subunit on the ribosome, though unlike all the aforementioned classes, macrolides are bacteriostatic rather than bactericidal (Gualerzi et al, 2014; Harvey et al, 2007). Tetracyclines are also bacteriostatic, binding to the 30S subunit of the bacterial ribosome to block amino acyl-tRNA from the mRNA ribosome complex to hinder protein synthesis (Gualerzi et al, 2014; Harvey et al, 2007).

The demand is always high for new antibiotics, especially antibacterials, with a broad spectrum of activity in the wake of pathogenic bacteria continuing to develop resistance to past and current available drugs due to overexposure and misuse (Cantas et al, 2013; Pidcock & Piotrowski, 2013; Procópio et al, 2012). Resistance has been a problem ever since antimicrobials became widespread and today virtually every known pathogenic or commensal bacterial species displays resistance to at least one clinically applicable antimicrobial (Cantas et al, 2013). This alarming trend owes itself to the evolution of biological processes that render antibiotics

ineffective that arise through mutation and genetic exchange between species, such as production of denaturing enzymes (Cantas et al, 2013; Madigan et al, 2012). Though many of these "superbugs" are most often associated with the human hospital setting—methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci are among the first to come to mind—they are also appearing in agriculture, veterinary medicine and even in natural environments such as soil and water, often accompanying overlap with one another (Cantas et al, 2013). Not only are resistant pathogens usually more virulent, and especially dangerous to the very young, very old and immunocompromised, they are harder and more expensive to treat (Cantas et al, 2013).

Actinomycetes are free-living, spore-producing Gram-positive filamentous bacteria that can be found in soil and are related to the streptomycetes which provide 70-80 percent of clinically useful antibiotics, including aminoglycosides, beta-lactams, tetracyclines and macrolides (Atta, 2011; Harvey et al, 2007; Madigan et al, 2012; Procópio et al, 2012; Ramazani et al, 2013). It is thought that 100,000 new compounds may eventually be discovered with future screening of this genus and other actinobacteria (Ramazani et al, 2013). One type of antibiotic that was recently harvested from a *Streptomycetes* strain is Tunicamycin, a nucleotide antibiotic that was isolated by fermentation of the *S. torulosus* strain and DNA isolation and amplification techniques (Atta, 2011). This experiment focused on finding antibacterial agents.

The purpose of this experiment was to answer the question: *Are there more novel antibiotics available in the marine environment compared to the terrestrial, if any?* As *Actinomycetes* is a producer of many antibiotics on the market, this was the target species for culturing (Atta, 2011; Cantas, 2013; Harvey, 2007; Jensen, 2005; Madigan, 2012; Procópio, 2012; Ramazani, 2013). The species would be isolated from both marine and terrestrial samples

and screened for their spectrum of activity against ten common pathogenic bacteria or relatives thereof. Spectra of activity would be compared between marine and terrestrial isolates and antibiotic stability over time would be tested using the best isolates. Then the 16S ribosome from the best isolates would be analyzed to confirm the species' identities. Identification of compounds was done with IR spectroscopy and a test was conducted to assess the stability of this antibiotic production over time.

Materials and Methods

Part 1: Finding Candidates

A single 50-mL soil or sediment sample was collected from one of nine locations within the St. Petersburg area: under a shrub in front of the Science and Technology Building (STG) on USFSP campus; from under a tree at Williams Park; from under a tree near the gift shop at Weedon Island Nature Preserve; from under a tree at Vinoy Park; from under a tree in front of Harbor Hall on campus, formerly the Dalí Museum; from Dr. John's backyard in Northwood; the beach at Bay Pines Park; Redington Beach; and the beach behind Davis Hall also on USFSP campus. Marine sediment was collected from under water at the littoral zone of a given beach. Soil samples were collected from under plants because it was thought that with the increased competition with fungi and other microbes by the roots of these plants would mean an increased likelihood of finding potent antibiotic bacteria (Madigan et al, 2012).

If a sample was moist it would first be poured into a weight boat and left out to dry overnight. The medium used to grow the bacteria was AMM agar based on the recipe outlined by

Jensen: 500 mL of sterile water mixed with 9 g of agar, 5 g of starch, 2 g of yeast extract, 1 g of peptone and 50 µg/mL of nystatin to kill fungal specimens (Jensen et al, 2005). If the sample came from a marine environment, 5 g of Instant Ocean™ was added to the concoction; for terrestrial samples, salt was omitted (Jensen et al, 2005). Samples were also prepared based on the dilute/heat method outlined by Jensen: marine samples were diluted with sterile sea water, terrestrial samples with 0.85% sterile saline, followed by heating in a water bath of 55°C for 6 minutes (Jensen et al, 2005). A dilution factor of 1:3 was used for the initial campus beach sample, but later it was decided that to avoid excessive growth and obtain more isolated colonies, a higher dilution factor of 1:10 and then 1:100 was needed instead (Jensen et al, 2005). After a sample was heated and cooled, 75 µL was pipetted to inoculate three AMM plates (Jensen et al, 2005). The plates were allowed to incubate for about 24 hours at approximately 30°C. Heating was performed to select for *Actinomycetes* and other heat-resistant Gram-positive bacteria and to reduce the presence of vegetative Proteobacteria (Jensen et al, 2005; Madigan et al, 2012).

To identify inhibitory isolates, the colonies for these plates were subjected to a preliminary overlay test with three test bacteria, one per plate: *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*. For each overlay, 5 mL of molten agar was cooled to 50°C. Prior to this the bacterial cultures were prepared in 5 mL of LB nutrient broth. Each tube was inoculated with 750 µL of culture before being poured quickly and evenly over their respective plate before the agar solidified again. The plates were incubated for 24 hours at 37°C.

Each plate was checked for colonies with clearings—zones of inhibition—around them, evidence for antibiotic activity. These colonies with zones of inhibition around them larger than 2 mm in diameter were numbered and a primary isolation was conducted for each one using isolation streaks on AMM agar. After the primary isolations were incubated at 30°C for 24

hours, up to two colonies from each plate were assigned letters A or B and each of these were transferred to new AMM agar plates for a secondary isolation. These plates were also incubated at 30°C for 24 hours.

Two methods of antibiotic testing were employed: lateral streaking and bacterial overlaying. For the former test, a colony was selected from each secondary isolation to create a center streak on a tryptic soy agar plate and brain-heart infusion plate, both of which to be incubated at 30°C for 24 hours. BD Diagnostic Systems Bacto™ Tryptic Soy Broth was used to create the TSA plates, the recipe for which consisted of 17 g of pancreatic digest of casein, 3 g of papaic digest of soybean, 2.5 g of dextrose, 5 g of sodium chloride and 2.5 g of dipotassium phosphate—15 g of mixture combined with 500 mL of sterile water per plate. BD Diagnostic BBL™ Brain Heart Infusion was used to create the BHI plates, the recipe for which was 6 g of brain-heart infusion from solid, 6 g of peptic digest of animal tissue, 5 g of sodium chloride, 3 g of dextrose, 14.5 g of pancreatic digest of gelatin and 2.5 g of disodium phosphate—18.5 g of mixture combined with 500 mL of sterile water per plate.

The two types of plates were each assigned five different test bacteria from laboratory plate cultures: for TSA, *E. coli*, *S. aureus*, *Bacillus cereus*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*; for BHI, *E. faecalis*, *Proteus vulgaris*, *Lactococcus lactis*, *Streptococcus pyogenes* and *Mycobacterium smegmatis*. The total test spectrum used for testing involved 6 Gram-positive species, 3 Gram-negative species and one acid-fast species (Madigan et al, 2012). A sample of cells from each type of bacteria was streaked laterally from the putative antibiotic producer, and every plate was incubated for 24 hours at 37°C.

For the bacterial overlaying test, each overlay was prepared in a similar way as a preliminary overlay. Four test bacteria—*E. coli, S. aureus, E. faecalis* and *E. aerogenes*—were

cultured in nutrient broth and 750 µL of each culture was inoculated into a tube of molten agar after allowing the water bath to cool to 50°C. Prior to this each isolate was prepared on a fresh AMM plate using a zigzag streaking method, and allowed to incubate for 24 hours at 37°C. The contents of the tubes were poured evenly and quickly into each designated plate, and after the agar was allowed to dry over the growth, they were incubated for 24 hours at 37°C.

Part 2: Testing Antibiotic Stability over Time

Two isolates were selected to test antibacterial stability over time, the ones with the broadest and third-broadest spectra of activity respectively. For each isolate, 6 AMM agar plates were inoculated with a center streak with 6 test "pathogenic" bacteria streaked laterally to the isolate: *E. faecalis, E. coli, M. smegmatis, B. cereus, S. aureus* and *P. aeruginosa*. One pair was incubated at 37°C for 14 days, the second pair for 7 days and the third pair for a single day. At the end of each incubation period, the distance between the isolate and "pathogen" streaks, the inhibitory distance, was measured and an average was taken for each "pathogen" species. Three statistical analyses were performed on JMP™ software to find any relationships between isolate, test pathogen or incubation time, and the average inhibitory distance.

Part 3: DNA and Chemical Analysis

Gram stains were performed prior to DNA extraction for the 16S ribosome to observe the morphology and Gram reaction of the top four candidates, selected based on number of test species they proved effective against. To extract DNA, a sample colony from each of these isolates was incubated in a 1.5-mL microcentrifuge tube for 30 minutes at 37°C with 180 µl Enzymatic Lysis Buffer (20 mM tris-Cl, 2 mM EDTA, 1.2% Triton X-100 and 20 mg/ml

lysozyme). They were incubated again for 30 minutes at 56°C with the addition of 25 µl ThermoFisher™ Proteinase K and 200 µl Qiagen™ Buffer AL.

200 µl ethanol was added and the solution was transferred to an Econospin™ silica membrane column. The DNA was washed by centrifuging with 500 µl Wash Buffers 1 (4 M guanidine Cl, 20 mM tris-Cl, 38% ethanol, pH 6.6) and 2 (10 mM tris-Cl, 80% ethanol, pH 7.5), and eluted in 200 µl Elution Buffer (10 mM tris-Cl, 1 mM EDTA). The DNA was then amplified through PCR, each reaction consisting of 25 µl ThermoFisher™ 2X PCR mixture, 2 µl each of 27F forward primer AGAGTTTGATCMTGGCTCAG and 1492R reverse primer GGTTACCTTGTTACGACTT, 5 µl of the DNA template and 16 µl of sterile water (Weisburg et al, 1991). PCR reaction conditions were as follows: initial denaturation of 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 60 seconds, and a final extension stage at 72°C for 10 minutes.

Next the DNA was purified with DNA binding buffer and Wash Buffer 2, and cloned using the Promega™ easy cloning kit: 10 µl ligase reaction of 2X T4 ligase buffer, T4 DNA ligase and T-vector. The DNA was then inoculated with competent *E. coli* cells to be incubated on plates containing ampicillin after treatment with 600 µl of SOC. White colonies that grew on the plates contained the plasmid that coded for ampicillin resistance and the DNA. From these plates two colonies were selected for each antibiotic producer and amplified in a PCR solution containing 25 µl of 2X PCR mix, 1 µl of M13 forward primer and M13 reverse primer, and 22 µl of sterile water. The conditions set for the Thermocycler™ for this second PCR reaction differed slightly from the first PCR reaction: initial denaturation of 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds, 57°C for 45 seconds, and 72°C for 60 seconds, and a final extension stage at

72°C for 10 minutes. Finally the DNA samples were ran on an electrophoresis agarose gel and sent to MWG Eurofins for sequencing.

Following genetic identification, the following chemical analysis was performed to identify the exact antibiotic compounds. A sample colony from each of the four isolates was mixed for 5 minutes in a 1.5-mL microcentrifuge tube filled with dichloromethane. Dichloromethane is a moderately polar solvent chosen to pull the presumably polar compound out of the colony (Pandey & Alegria, 2014). Without taking up the pellet, the dichloromethane solution was collected into a second tube and a few drops of it were applied to a set of lenses to be run for an IR spectrum using a Shimadzu™ FTIR-8400S infrared spectrophotometer. The device was "zeroed" for the presence of dichloromethane through a background scan prior to taking each measurement. IR spectroscopy was employed to identify each compound by its functional groups, each of which vibrate differently when it absorbs photons of infrared light within the necessary range and thus produce different lengths and shapes of absorption bands on the spectrum (McMurry, 2012; Pandey & Alegria, 2014).

Results and Observations

In general, the colonies (see Fig. 1.1) produced by the samples from STG and Vinoy were thick, dry and opaque creamy white to yellow with irregular edges; the colonies produced by the samples from Bay Pines and Northwood were also thick with irregular edges but were moist, colorless and translucent. Some bacteria colonies from other specimens had a more filamentous growth.

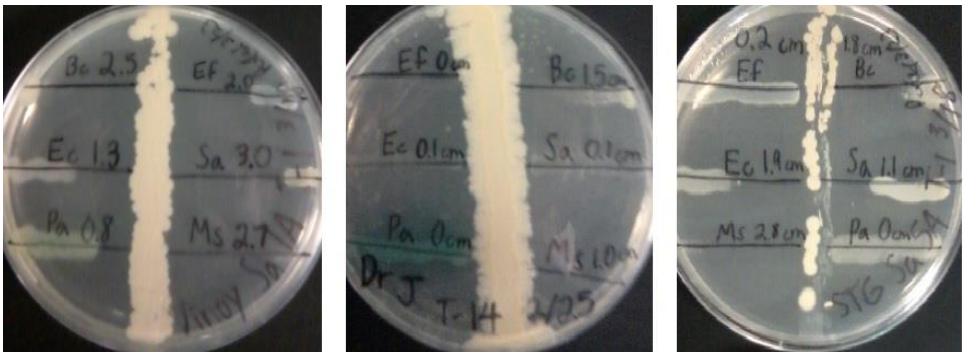


Fig. 1.1: Time stability test plates—Vinoy *Sa* 1A (left), Northwood 1A (center) and STG *Sa* 5A (right).

Table 1.1: Preliminary overlay results indicating numbers of isolates obtained from respective sample sites

				# of colonies with zones of inhibition	
Location	**Dilution factor**	**Agar medium**	*E. coli*	*E. faecalis*	*S. aureus*
STG	1 to 100	AMM, no salt	2	5	6
Weedon	1 to 100	AMM, no salt	2	0	6
Williams	1 to 100	AMM, no salt	1	3	0
Campus beach	1 to 3	AMM, w/ salt	0	0	0
Old Dalí	1 to 100	AMM, no salt	0	3	5
Redington Beach	1 to 100	AMM, w/salt	0	0	0
Vinoy	1 to 100	AMM, no salt	3	4	4
Northwood	1 to 100	AMM, no salt	1	0	0
Bay Pines	1 to 100	AMM, w/ salt	1	0	0

Those bacteria isolated from marine sediment—from Bay Pines, Redington or the USFSP campus beach—showed little to no inhibition against any test bacteria from the preliminary overlay test onward (Table 1.1). The majority of inhibitory isolates came from terrestrial soil samples. Of the 46 isolates observed from the preliminary overlays, 31 were tested further with only one isolate as marine. 9 of the candidates demonstrated no inhibition against any bacteria, 11 showed inhibition against only one test bacteria, and 11 more showed a spectrum of activity against 2 or more bacteria. Only 4 candidates seemed potentially useful, "useful" defined as effective against 5 or more species.

Overall, it appeared that these isolates were inhibitory towards both Gram-positive and Gram-negative bacteria. Interestingly, *P. aeuginosa* appeared vulnerable to the greatest number of isolates, followed by *L. lactis, S. pyogenes, B. cereus, E. coli, E. faecalis, S. aureus, M. smegmatis,* and *P. vulgaris* and *E. aerogenes* as vulnerable to the least number of isolates (see Tables 1.2 and 1.3). But statistical analysis (see Graph 1.1) showed no significant difference between the Gram reaction of a test bacterium and the number of isolates that could inhibit it (One-way ANOVA: F-ratio = 0.6186; p-value = 0.5657).

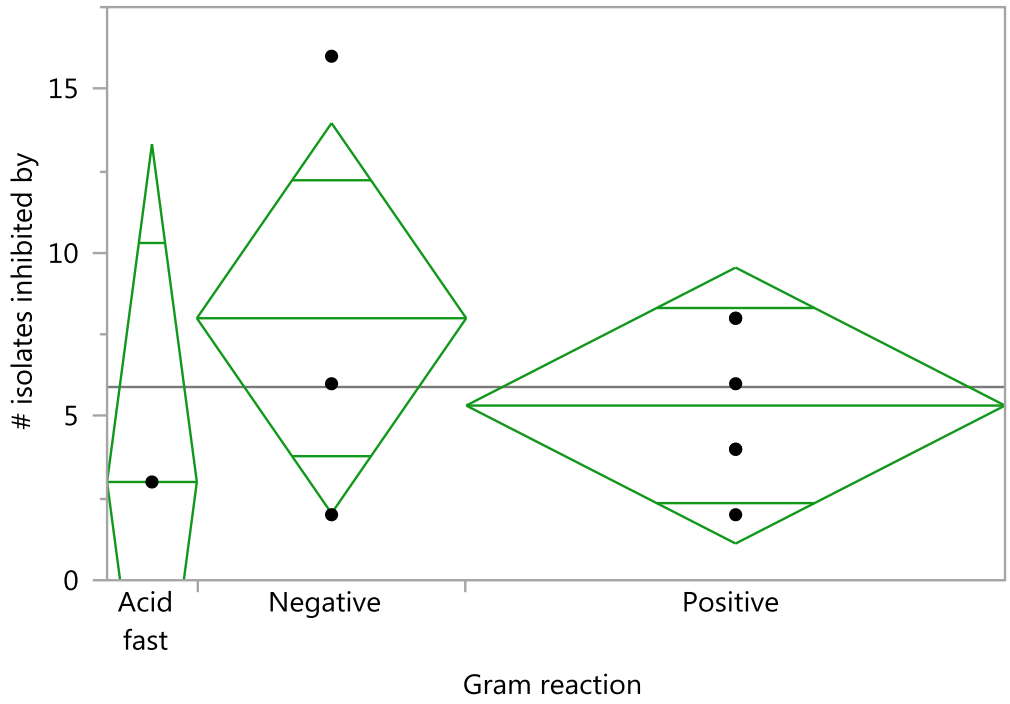
Table 1.2: List of test bacteria, their Gram reaction and the number of candidate isolates that showed inhibition against them based on combined overlay and lateral streak results

Species	Gram reaction	# isolates that inhibit it
P. aeruginosa	negative	16
L. lactis	positive	8
S. pyogenes	positive	8
B. cereus	positive	6
E. coli	negative	6
E. faecalis	positive	4
S. aureus	positive	4
M. smegmatis	acid-fast	3
P. vulgaris	negative	2
E. aerogenes	positive	2

Table 1.3: Combined lateral and secondary overlay streak results indicating isolate and bacteria inhibited

Location	Preliminary bacteria	Colony	Sub-colony	Bacteria inhibited
STG	E.c.	1	A	*L. lactis, S. pyogenes*
			B	*L. lactis, S. pyogenes*
	E.f.	1	C	*L. lactis, S. pyogenes*
		3	A	*L. lactis, S. pyogenes, E. coli, P. aeruginosa*
		4	A	*L. lactis, S. pyogenes, E. coli, P. aeruginosa*
	S.a.	2	A	none
		5	A	*L. lactis, S. pyogenes, M. smegmatis, E. faecalis, P. vulgaris, E. coli, S. aureus, B. cereus, P. aeruginosa, E. aerogenes*
		6	A	*P. aeruginosa*
Weedon	E.c.	1	A	none
	E.f.	3	A	*B. cereus*
	S.a.	1	A	none
		2	A	*P. aeruginosa, M. smegmatis*
		3	A	*B. cereus*
		4	A	*B. cereus*
		5	A	*P. aeruginosa*
		6	A	*P. aeruginosa, L. lactis*
Williams	E.c.	1	A	none
			B	none
Harbor Hall	E.f.	1	A	*P. aeruginosa*
			B	none
		2	A	none
			B	none
	S.a.	2	A	*P. aeruginosa*
Vinoy	E. f.	1	A	*P. aeruginosa*
		2	A	*P. aeruginosa*
	S. a.	1	A	*E. coli, S. aureus, B. cereus, P. aeruginosa, E. aerogenes, L. lactis, S. pyogenes, M. smegmatis, E. faecalis*
			B	none
		2	A	*P. aeruginosa*
			B	*P. aeruginosa*
Northwood	S.a	1	A	*S. pyogenes, E. faecalis, P. vulgaris, E. coli, S. aureus, B. cereus, P. aeruginosa*
Bay Pines	S.a.	1	A	*P. vulgaris, S. aureus, E. coli, E. faecalis, P. aeruginosa*

Graph 1.1: One-way ANOVA comparing number of isolates that inhibit test bacteria by Gram reaction of test bacteria



Based on spectrum of activity, the top 4 isolates selected for genetic and chemical analysis, in order of broadest to narrowest spectrum, were STG *S.a.* 5A, Vinoy *S.a.* 1A, Northwood *S.a.* 1A and Bay Pines *S.a.* 1A.

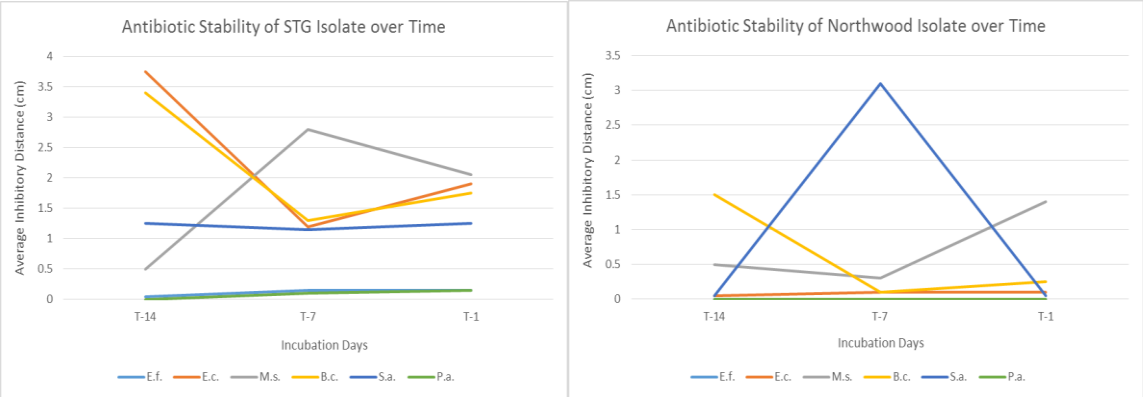
Testing for antibiotic stability over time (see Table 1.4 and Graphs 1.2 and 1.3) revealed a trend in which both isolates showed far less inhibition when incubated for one day or 14 days, with maximum effectiveness observed for those plates incubated for 7 days. However, statistical analysis (see Graphs 1.4, 1.5 and 1.6) of the data gave no significant correlation between time and average inhibitory distance (Linear regression: $R^2 = 0.004021$; p-value = 0.7133). No significant difference was found between test pathogen and average inhibitory distance either (One-way ANOVA: F-ratio = 2.3705; p-value = 0.0631). Still, a significant difference was found between isolate and average inhibitory distance (One-way ANOVA: F-ratio = 6.5404; p-value = 0.0152). Indeed even at one day or 14 days, STG *S.a.* 5A showed more inhibition than

Northwood *S.a.* 1A as evidenced by the longer distances between the center isolate streak and the lateral test streaks.

Table 1.4: Antibiotic stability results indicating isolate, number of days incubated (T-n), the inhibitory distance observed per plate and the average between plates

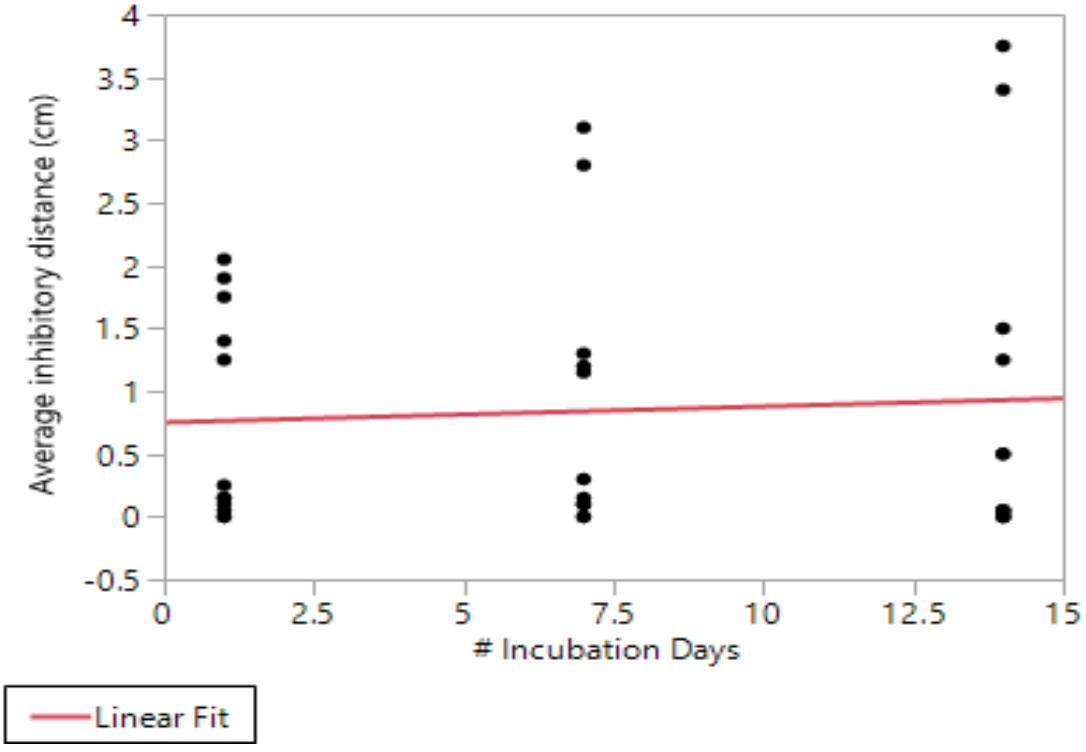
T-14	*Bacterium*	*Distances (cm)*	**T-7**	*Bacterium*	*Distances (cm)*	**T-1**	*Bacterium*	*Distances (cm)*
STG	E.f.	0.1 and 0	*STG*	E.f.	0.1 and 0.2	*STG*	E.f.	0.2 and 0.1
	E.c.	3.5 and 4.0		E.c.	1.9 and 1.5		E.c.	1.9 and 1.9
	M.s.	3.4 and 3.4		M.s.	2.5 and 3.1		M.s.	2.8 and 2.3
	B.c.	3.4 and 3.4		B.c.	0.9 and 1.7		B.c.	1.8 and 1.7
	S.a.	2.4 and 0.1		S.a.	1.9 and 0.4		S.a.	1.1 and 1.4
	P.a.	0 and 0		P.a.	0 and 0.2		P.a.	0.2 and 0.1
Northwood	E.f.	0 and 0	*Northwood*	E.f.	0	*Northwood*	E.f.	0 and 0
	E.c.	0 and 0.1		E.c.	0.1		E.c.	0 and 0.2
	M.s.	0 and 1.0		M.s.	0.3		M.s.	1.8 and 1.0
	B.c.	1.5 and 1.5		B.c.	0.1		B.c.	0.3 and 0.2
	S.a.	0.1 and 0		S.a.	3.1		S.a.	0 and 0.1
	P.a.	0 and 0		P.a.	0		P.a.	0 and 0

			AVERAGES			
STG						
Distance (cm)	E.f.	E.c.	M.s.	B.c.	S.a.	P.a.
T-14	0.05	3.75	0.5	3.4	1.25	0
T-7	0.15	1.2	2.8	1.3	1.15	0.1
T-1	0.15	1.9	2.05	1.75	1.25	0.15
Northwood						
Distance (cm)	E.f.	E.c.	M.s.	B.c.	S.a.	P.a.
T-14	0	0.05	0.5	1.5	0.05	0
T-7	0	0.1	0.3	0.1	3.1	0
T-1	0	0.1	1.4	0.25	0.05	0

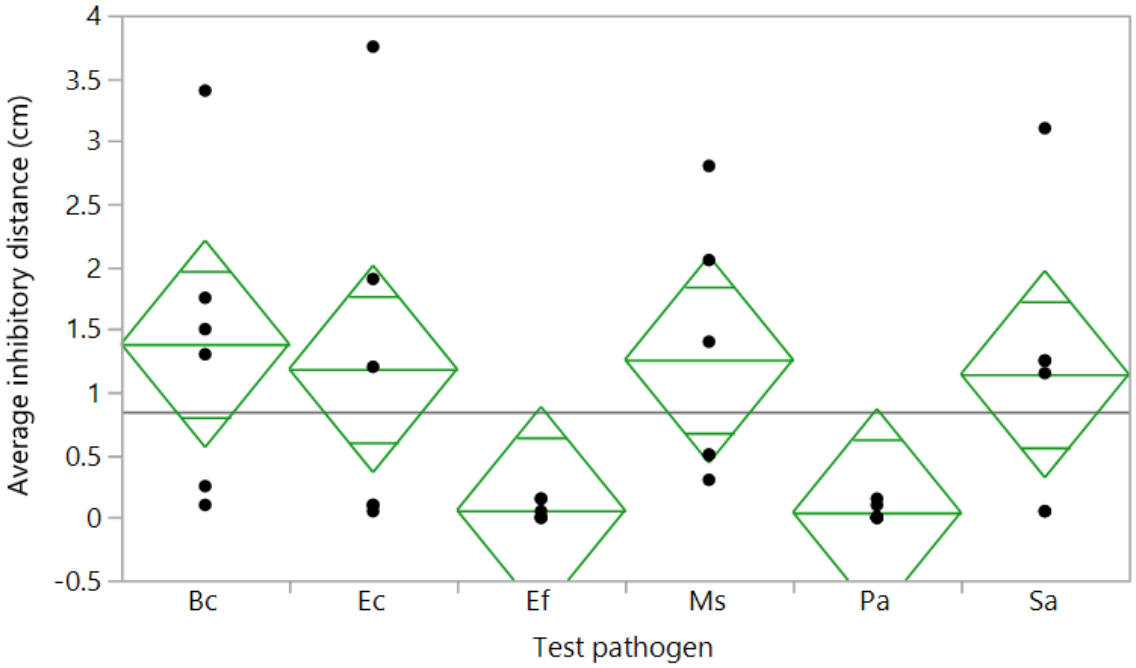


Graph 1.2 and 1.3: Antibiotic stability of STG (left) and Northwood (right), comparing average inhibitory distance per "pathogen" vs. length of incubation

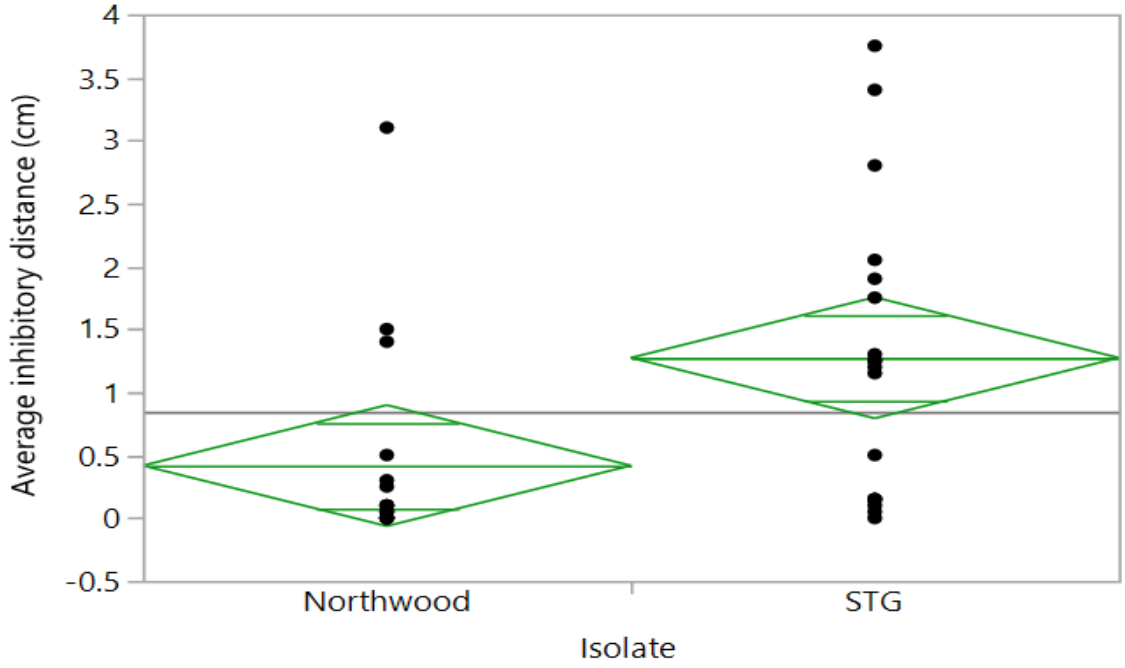
Graph 1.4: Linear regression comparing average inhibitory distance to number of incubation days



Graph 1.5: One-way ANOVA comparing average inhibitory distance to test pathogen



Graph 1.6: One-way ANOVA comparing average inhibitory distance to isolate



Initial Gram stains appeared to yield negative reactions (see Figure 1.2) due to the pink color of the cells under the microscope (Madigan et al, 2012). However, later Gram stains performed with fresh cultures refuted this and gave positive Gram reactions as evidenced by purple-colored cells (see Figure 1.3) (Madigan et al, 2012). In any case, every Gram stain revealed a bacillus morphology or each of the four isolates, arranged as single cells (see Figure 1.4). Because *Actinomycetes* has a filamentous appearance by contrast, it was ruled out immediately (Atta, 2011; Harvey et al, 2007; Madigan et al, 2012; Procópio et al, 2012; Ramazani et al, 2013).

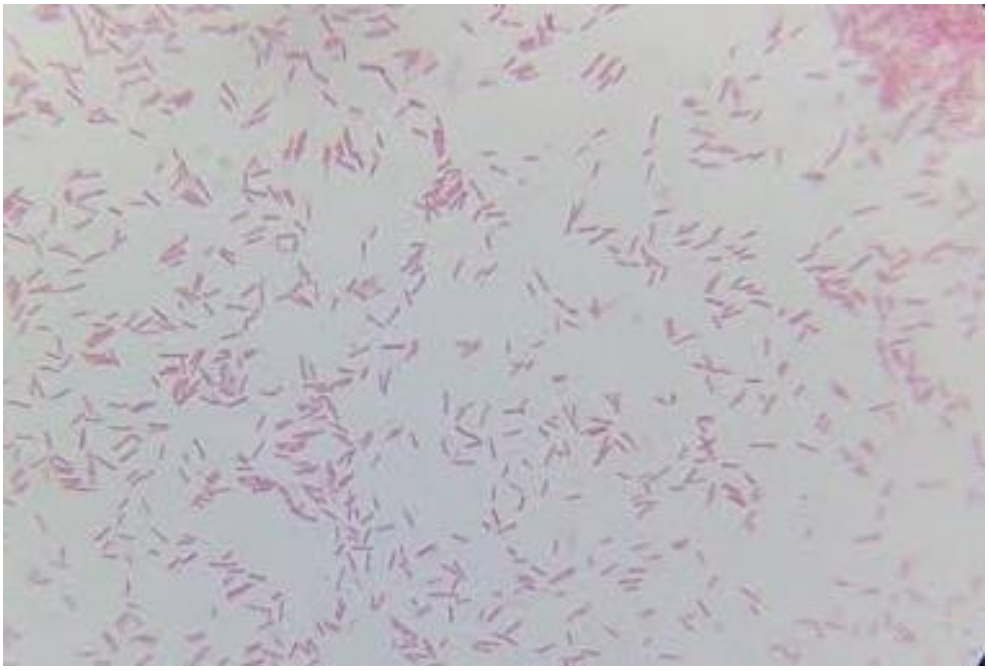


Fig. 1.2: False-negative Gram stain of STG *S.a.* 5A, at 1000x.

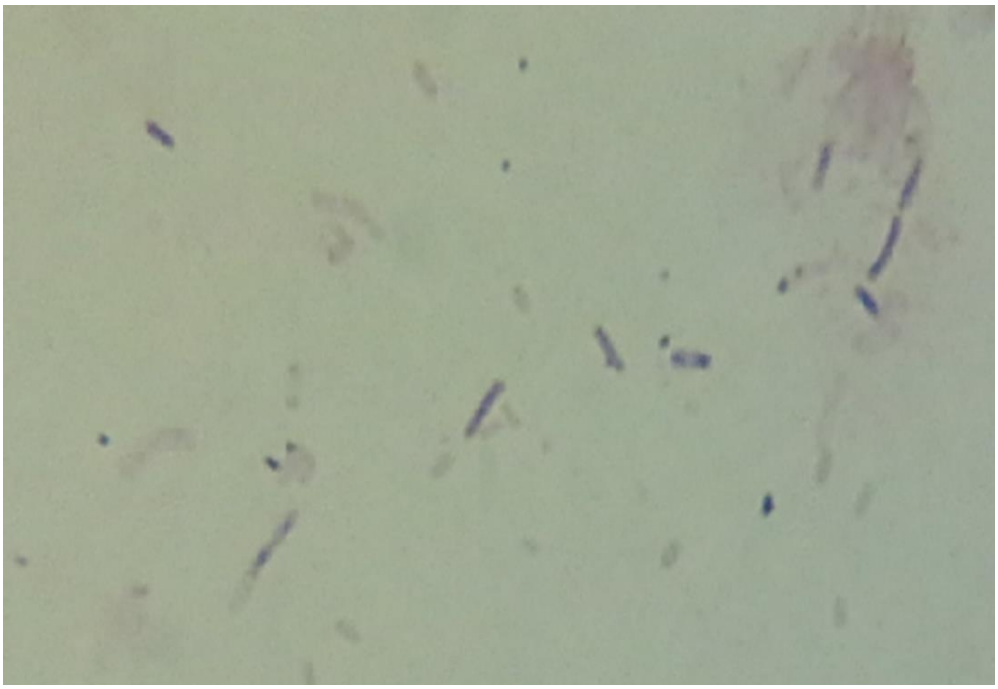


Fig. 1.3: Positive Gram stain of STG *S.a.* 5A, one of the major antibiotic producers among the isolates tested, at 1000x.

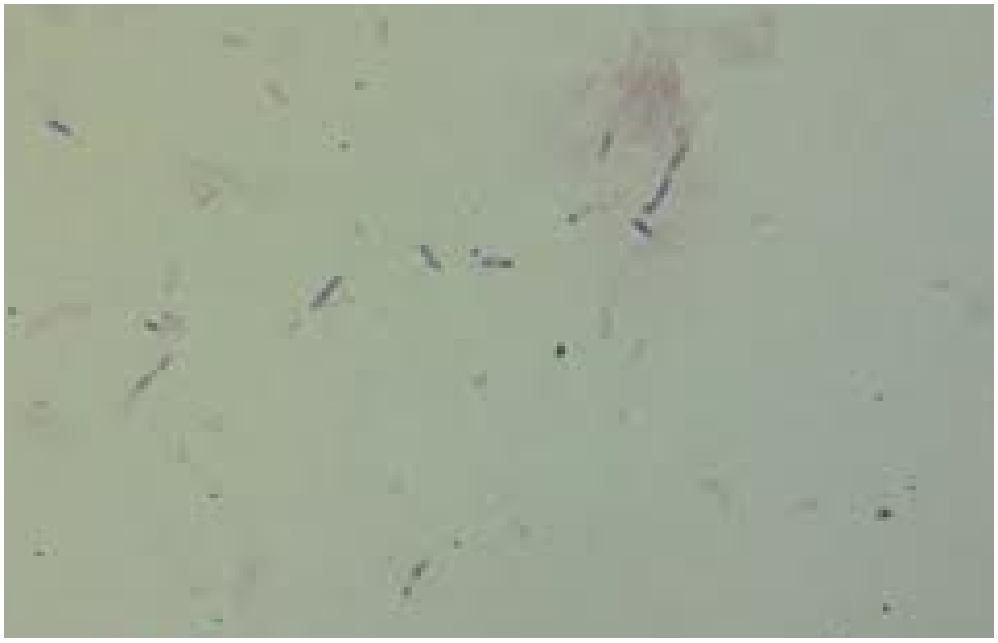


Fig. 1.4: Positive Gram stain of Northwood *S.a.* 1A, at 1000x.

Table 1.5: Genetic analysis of top four isolates reflecting organism with BLAST score and similarity

Location	Overlay	Isolate	# of bacteria effective against	Identity based on sequencing	BLAST score	Similarity (%)
STG	*S.a.*	5A	10	*Brevibacillus choshinensis*	1142	99
Vinoy	*S.a.*	1A	9	*Brevibacillus laterosporus*	1676	99
Northwood	*S.a.*	1A	7	*Bacillus amyloliquefaciens subsp. plantarum*	1703	99
Bay Pines	*S.a.*	1A	5	*Bacillus amyloliquefaciens*	1700	99

DNA analysis of the top 4 isolates (see Table 1.5) combined with the morphology given by the Gram stains revealed 4 completely different bacterial species than the targeted *Actinomycetes*. STG *S.a* 5A and Vinoy *S.a.* 1A were identified as *Brevibacillus choshinensis* and *Brevibacillus laterosporus*. Bay Pines and Northwood were identified as *Bacillus amyloliquefaciens* and *Bacillus amyloliquefaciens subsp. plantarum*. Top BLAST similarity for all of them was 99% with up to 2 mismatches.

IR spectrum analysis (see Figures 1.5 and 1.6) revealed a molecule that appeared in common with all four isolates. All the spectra have stretching peaks at ~1400 cm^{-1}, indicating the presence of aromatic rings (McMurry, 2012; Pandey & Alegria, 2014). Peaks at ~1300 and 1600 cm^{-1} suggest ketone and/or ester groups, and the twin peaks at ~3000 and ~3100 cm^{-1} that imply that amine groups are present (McMurry, 2012; Pandey & Alegria, 2014).

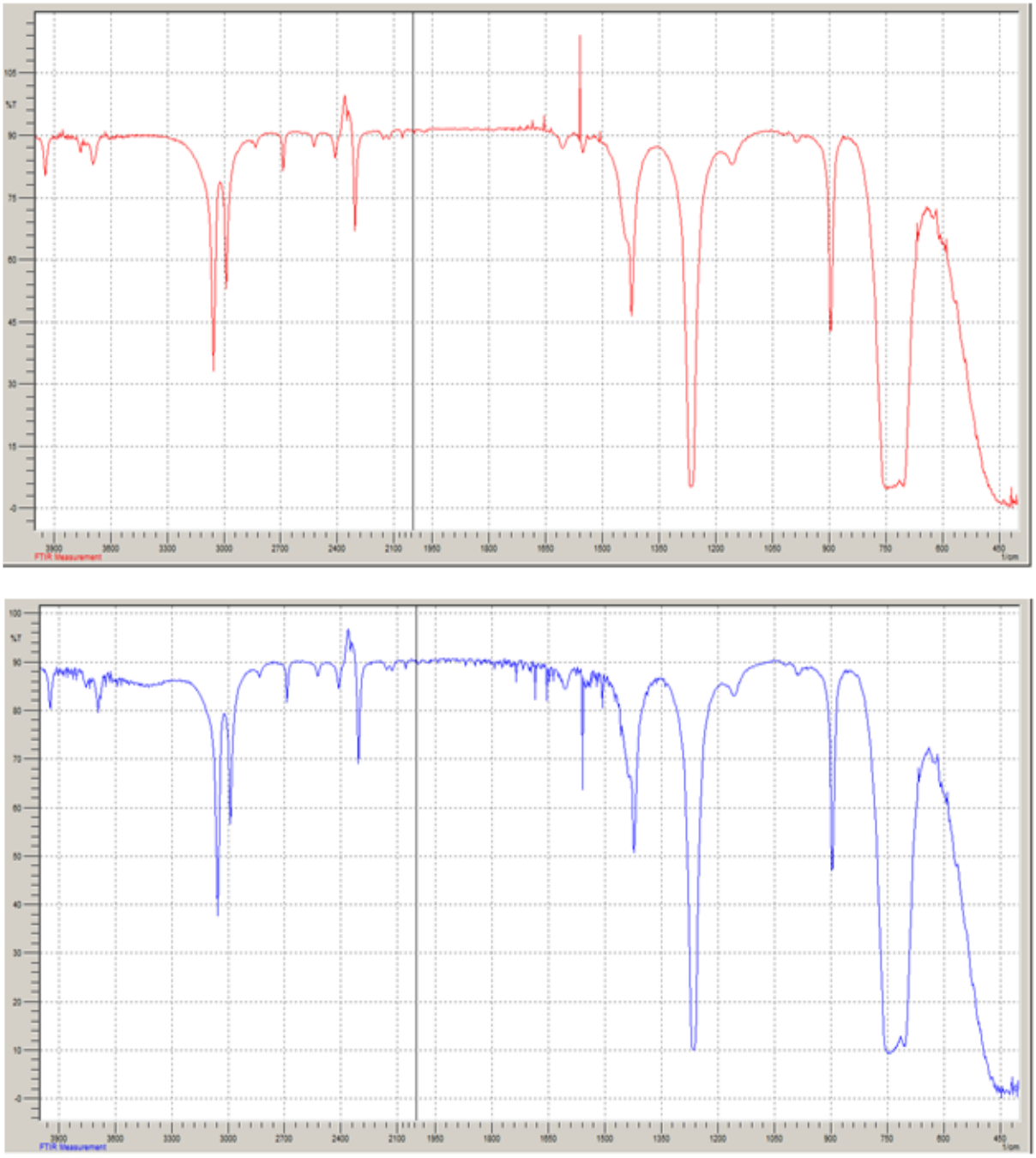


Fig. 1.5: IR spectra for STG *S.a.* 5A (red, top) and Northwood 1A (blue, bottom).

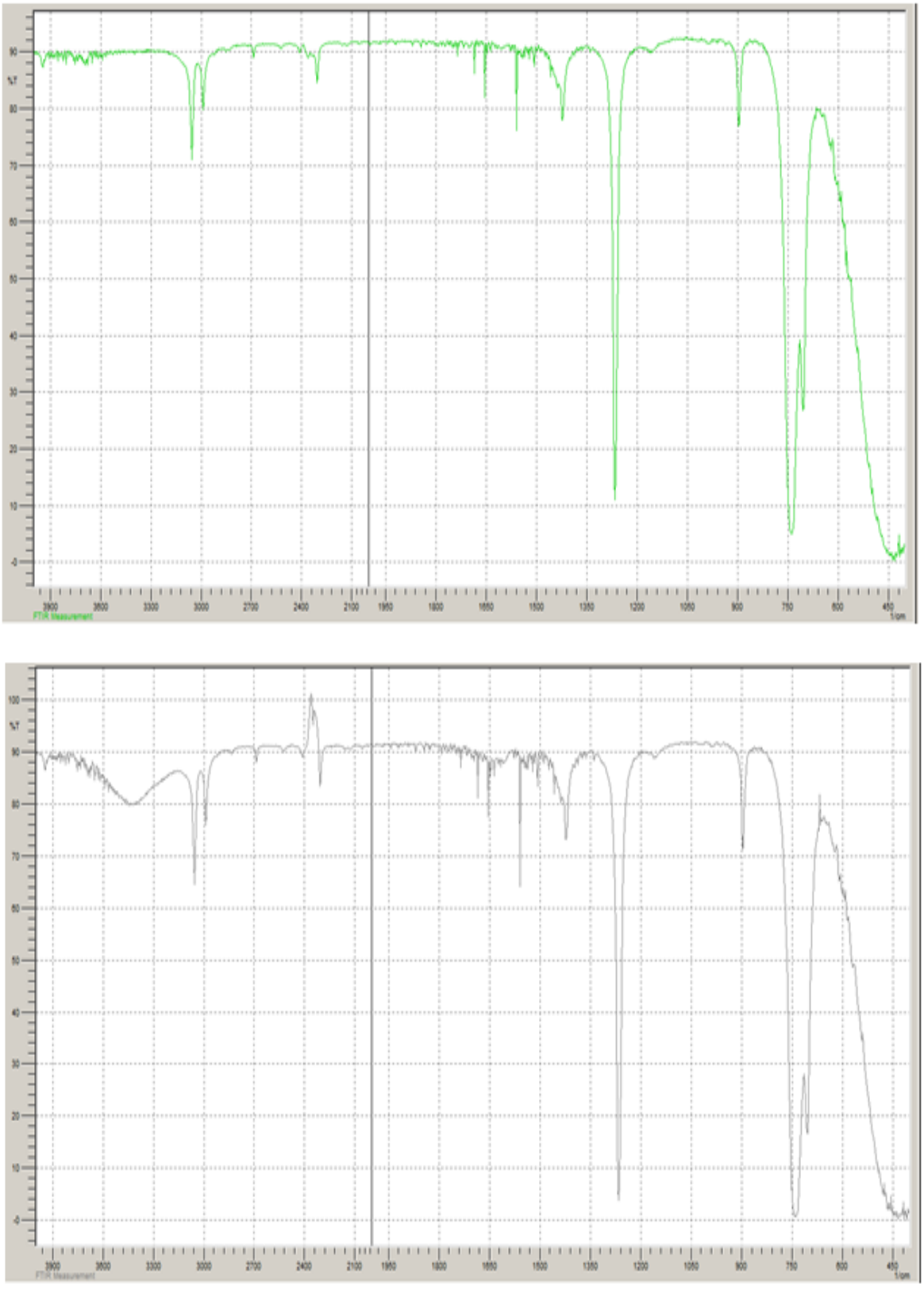


Fig. 1.6: IR spectra of Vinoy *S.a.* 1A (green, top) and Bay Pines 1A (black, bottom).

Discussion

This project was meant to discover novel antibacterial compounds from marine-dwelling *Actinomycetes* and compare it with the potency of terrestrial species. Of the 46 isolates cultured and 31 tested, less than half of them showed a spectrum of activity against 2 or more bacteria; only 4 isolates gave a potentially useful spectrum against at least 5 bacteria. Marine bacteria are more difficult to culture in a laboratory because of their generally low nutrition requirements and heavy dilution in their natural environment (Madigan et al, 2012). Only the specimen from Bay Pines seemed to possess a relatively broad spectrum of activity of the three marine samples cultured. The other three best were terrestrial, with STG *S.a.* 5A having the broadest spectrum overall. The terrestrial samples might have had a broader spectrum because they are more concentrated, have higher nutritional requirements, are generally more adapted to harsh conditions and share a closer proximity with these bacteria and other competitive microbes than marine bacteria, and so are more likely to evolve potent antibiotic processes fairly quickly (Madigan et al, 2012).

Statistical analysis of the results for the stability test did not support a significant relationship between time and effectiveness in itself. However, given that a significant difference was found between effectiveness and type of isolate, it would not be unreasonable to propose that an antibacterial compound with a broader spectrum of activity would retain more inhibitory ability over time than one of a narrower spectrum. The isolate from Vinoy was also tested but was not included in the results due to failure to maintain a viable culture at the allotted incubation times; the isolate from Bay Pines was not tested at all. A replication of the test with these new parameters is under consideration. Other future work includes finding the minimum

inhibitory concentrations and testing for toxicity—how much a user of a given drug can take to produce the desired effects without it becoming toxic to the user.

While *Actinomycetes* was the original genus sought, four totally different species were cultured instead based on the morphology given by the Gram stains and DNA testing. Based on sequencing of the partial 16S ribosomes all of the isolates were Gram-positive *Bacillus* and *Brevibacillus* strains, with 99% similarity and up to 2 mismatches. *Brevibacillus*, particularly the *B. laterosporus* species, is similar to *Bacillus* in that both are rod-shaped, endospore-forming and found in virtually any environment, but *Brevibacillus* has a canoe-shaped parasporal attached to one side of its spore (Madigan et al, 2012; Ruiu, 2013). It has been shown to have toxic principles against many invertebrates including insects, mollusks and nematodes, as well as a broad antibiotic spectrum against bacteria and phytopathogenic fungi; it is also treated as a beneficial probiotic for mammals and birds (Ruiu, 2013). The antibiotic properties demonstrated vary in some strains, from chitinases that kill fungi to laterosporamine that has been shown to kill both Gram-positive and –negative bacteria (Ruiu, 2013). As previously mentioned, the two *Bacillus* species are likely to produce lantibiotics (Gualerzi et al, 2014; Madigan et al, 2012).

Analysis of IR spectra revealed the production of a similar compound between them; at the very least the compounds were identical between STG and Northwood, and between Vinoy and Bay Pines. Further isolation and testing is necessary for confirmation—such as mass or NMR spectroscopy, and liquid chromatography—but these compounds are may be along the lines of bacitracin (see Figure 1.7), a pre-existing non-ribosomal dodecapeptide that inhibits cell wall synthesis in Gram-positive bacteria and is commonly used to treat eye and skin infections (Baruzzi et al, 2011; McMurry, 2012). It would be recommended that IR spectra be run for these

isolates again with a control for comparison that is an isolate from a non-antibacterial *Bacillus* species.

Some possible errors and biases must be taken into account. A possible bias may have existed for terrestrial bacteria from the start since only a third of the samples collected were unambiguously marine in origin. In addition, the samples were all taken from sites that experience fairly intensive human activity or are sprayed with chemicals, and as such bacteria from these places may behave differently from bacteria from more remote natural locations. More Gram-positive bacteria were used for testing than Gram-negative, and only one acid-fast species was included.

Although fresh cultures of test bacteria were consistently maintained, laboratory bacteria tend to be attenuated and might be more sensitive to antibiotics than wild-types (Madigan et al, 2012). This could explain, for instance, why *P. aeruginosa* appeared sensitive to so many isolates despite this species' reputation for being one of the most antibiotic-resistant (Madigan et al, 2012). If *Actinomycetes* were to be pursued, another culture technique modified from the method outlined here may be necessary to find the desired species.

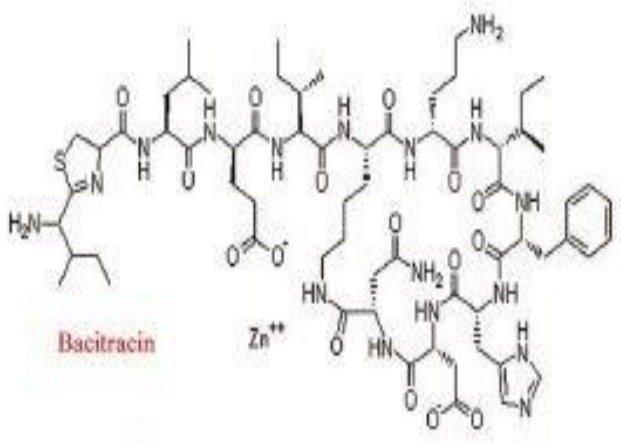


Fig. 1.7: Structure of bacitracin (Baruzzi et al, 2011).

Conclusions

Continuing development of novel antibiotics, and discovery of producers, is critical to protect the public against the ongoing evolution of drug-resistant strains. While antibiotics are secondary metabolites that would be generated after a period when resources are exhausted, time alone does not seem to be an important factor in compound stability and effectiveness (Madigan et al, 2012). While little was found in the context of this experiment for marine novel producers, never mind for *Actinomycetes,* a few terrestrial producers with broad spectra of activity were discovered instead, some of whom are species that are already receiving more attention for their production of antibiotics. It is still tentative but as it stands it is unlikely that the compounds isolated are totally novel.

Bibliography

Atta, H.M. "Biochemical studies on antibiotic production from *Streptomyces* sp.: Taxonomy, fermentation, isolation and biological properties." *Journal of Saudi Chemical Society,* Nov. 2011. Web. *EBSCOhost: University of South Florida Libraries.* Accessed 14 Nov. 2014.

Baruzzi, F., L. Quintieri, M. Morea and L. Caputo. "Antimicrobial compounds produced by *Bacillus* ssp. and applications in food." *Science against microbial pathogens: communicating current research and technological advances,* 2011, pg. 1102-1111. Web. *Google Scholar.* Accessed 14 Jun. 2015.

Cantas, L., Syed Q. A. Shah, L. M. Cavaco, C. M. Manaia, F. Walsh, M. Popowska, H. Garelick, H. Bürgmann and H. Sørum. "A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota." *Frontiers in Microbiology*, May 2013, vol. 4, pg. 1-14. Web. *PMC.* Accessed 22 Jun. 2015.

Gualerzi, C.O., L. Brandi, A. Fabbretti and C.L. Pon, eds. *Antibiotics: Targets, Mechanisms and Resistance.* Weinheim: Wiley-VCH, 2014. Web. *Wiley Online Library.* Accessed 14 Nov. 2014.

Harvey, R.A., P.C. Champe and B.D. Fisher. *Lippincott's Illustrated Reviews: Microbiology.* 2nd ed. Philadelphia: Lippincott Williams & Wilkins, 2007. Print.

Jensen, P.R., E. Gontang, C. Mafnas, T.J. Mincer and W. Fenical. "Culturable marine *actinomycete* diversity from tropical Pacific Ocean sediments." *Environmental Microbiology*, Jul. 2005, vol. 7(7), pg. 1039-1048. Web. *EBSCOhost: University of South Florida Libraries.* Accessed 14 Nov. 2014.

Madigan, M. T, J. M. Martinko, D. A. Stahl and D. P. Clark. *Brock Biology of Microorganisms*, 13th ed. San Francisco: Benjamin Cummings, Pearson Education Inc., 2012. Print.

McMurry, John. *Organic Chemistry*, 8th ed. Canada: Brooks/Cole, Cengage Learning, 2012. Print.

Pandey, Madhu and Henry Alegria. *Organic Chemistry Lab: University of South Florida St. Petersburg.* St. Petersburg, FL: McGraw-Hill, 2014. Print.

Pidcock, K.A. and N.A. Piotrowski. "Antibiotics." *Magill's Medical Guide (Online Edition).* N.p., 2013. Web. *EBSCOhost: University of South Florida Libraries.* Accessed 14 Nov. 2014.

Procópio, R.E. de Lima, I.R. da Silva, M.K. Martins, J.L. de Azevedo and J. M. de Araújo. "Antibiotics produced by *Streptomyces*." *The Brazilian Journal of Infectious Diseases,* Oct. 2012, vol. 16(5), pg. 466-471. Web. *EBSCOhost: University of South Florida Libraries.* Accessed 14 Nov. 2014.

Ramazani, A., S. Moradi, R. Sorouri, S. Javani and M. Garshasbi. "Screening for antibacterial activity of *Streptomyces* species isolated from Zanjan province, Iran." *International Journal of Pharmaceutical, Chemical and Biological Sciences,* Apr.-Jun. 2013, vol. 3(2), pg. 342-349. Web. *International Journal of Pharmaceutical, Chemical and Biological Sciences.* Accessed 15 Nov. 2014.

Ruiu, Luca. "*Brevibacillus laterosporus,* a Pathogen of Invertebrates and a Broad-Spectrum Antibacterial Species." *Insects,* Sept. 1, 2013, vol. 4(3), pg. 476-492. Web. *EBSCOhost: University of South Florida Libraries.* Accessed 15 Mar. 2015.

Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (January 1991). "16S ribosomal DNA amplification for phylogenetic study." *Journal of Bacteriology,* Jan. 1991, vol. 173(2), pg. 697-703. Web. *American Society for Microbiology*. Accessed 22 Jun. 2015.