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Bioassay and Antibiotic Activity of Jamaican Actinomycetes Isolates

Michael P. Piechoski[1*], Diana R. Cundell[2], Anne H. Bower[2] and John R. Porter[3]

The growth in antibiotic resistance has resulted in a constant need for novel antimicrobials. To slow down the acquisition of resistance many pharmaceutical companies are now focusing on the development of novel, narrow-spectrum therapeutics with specificity, potency and decreased side effects. Antibiotic production is a feature of several soil microbes and may represent a survival mechanism whereby organisms can eliminate competition and colonize a niche. Since many of the original antibiotics were harvested from tropical soil *Actinomycete* species, we collected soil samples from Jamaica as the island's soil microbiology is rich and poorly understood. From sixteen sites twenty- nine colonies of *Actinomyces* and *Bacillus* spp. were obtained and twenty isolates were identified to have antibiotic activity against a panel of clinically important human bacterial pathogens. Eight were effective against one or two of the test bank of microbes and *Actinomyces* were selected for further study. Antimicrobial activity was assessed in methylene chloride:methanol (1:1) extracts of 1 L broth cultures using a 96-well microdilution assay against *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis* (ATCC 14468; model for *M. tuberculosis*), *Pseudomonas aeruginosa* (ATCC 27853), methicillin-resistant *Staphylococcus aureus* (ATCC 43300), *Candida albicans* (ATCC 90028), and *Cryptococcus albidus* (ATCC 34140; model for *C. neoformans*). Three of the five isolates produced activity which was highly effective against *M. smegmatis* with growth inhibition comparable to 3.2 µg/ml of the antibiotic isoniazid). Two isolates inhibited the yeast *C. albicans* to degree similar to amphotericin B at a concentration of 0.25 µg/mL. All three isolates tested for cytotoxicity using the brine shrimp assay were found to be non-lethal. The bacterial source of these compounds with antimicrobial activity were identified commercially as *Actinomyces* using PCR analysis, but had no homology to known species by BLAST analysis. Given their activity against *M. smegmatis* these isolates may have potential for future drug development.

INTRODUCTION

Originally serendipitously discovered in 1928 by Alexander Fleming, antibiotics are now an established part of our culture, being prescribed for an estimated 50-70% of all visits for soft tissue (Hersch *et al*, 2008) and acute respiratory infections (Grijalva *et al*, 2009). This growth in antibiotic usage has been paralleled, however, by the ability of bacteria to resist being killed by these agents. There has been, as a result, a steady decline in the number of effective antibiotics each year (Levy 1998). The Campaign for Rational Antibiotic Use spearheaded by the Centers for Disease Control (Weissman and Besser , 2004) reduced antibiotic usage in most primary care, emergency room and pediatric settings though agents causing soft tissue and respiratory infections still remain recalcitrant (Grijalva *et al*, 2009). Indeed in the case of community-acquired methicillin-resistant *Staphylococcus* aureus (CA-MRSA) the emergence of this antibiotic-resistant bacterium has almost doubled the medical burden for the treatment of soft tissue infections in the United States (Hersh *et al*, 2008). Similar trends are also being seen with other antibiotic-resistant "superbugs," including *Mycobacterium* tuberculosis and *Escherichia* coli (Levy, 1998). Microbial resistance is a multifactorial issue which stems from a combination of selective pressure from usage as well as poor patient compliance due to side effects from gastrointestinal flora disturbance (Levy, 1998). Broad spectrum antibiotics are particularly of issue since when resistance is acquired against them it typically involves many species of bacteria (Levy, 1998). To avoid the generation of new "superbugs" and to manage the presently rising resistant strains pharmaceutical companies will need to develop narrow spectrum therapeutics in which the limited specificity may decrease the likelihood of resistance, have fewer side effects and improve patient compliance.

Several current antibiotics, particularly the aminoglycoside and macrolide group, originate from tropical bacteria of the Actinomycete *Streptomyces* group (Drug Discovery, 2005). These microbes also produce the currently poorly understood bacteriocins, which are broad spectrum peptides with efficacy against diverse gram positive bacteria (Jack *et al*, 1995). *Streptomyces* spp. produce several antibiotics, probably as survival mechanisms to colonize a niche and eliminate the variety of competitors that live in tropical soils (Drug Discovery, 2005). *Actinomyces* are a large and diverse group and those originating from tropical locations have been shown to comprise novel species (Janso and Carter, 2010). In addition, since a single soil sample from a tropical forest can yield thousands of bacterial species (Hawksworth and Colwell, 1992), which are in a constant battle for survival, it is likely that antibiotic-producing microbes can be harvested from this source.

[1] Science Department, South Philadelphia High School, Philadelphia, PA [2]School of Science and Health, Philadelphia University, Philadelphia, PA [3]Department of Biological Sciences, University of the Sciences, Philadelphia, PA

[*]To whom correspondence should be addressed: mppiechoski@philasd.org

Jamaica provided a perfect location from which to isolate potential antibiotic-secreting microbes, as it possesses a tropical maritime climate conducive to bacterial proliferation and diversity (Borneman and Triplett, 1997). This means a year round temperature of 77-88˚F with annual precipitation of 3,500 mm (Portland Parish 30 year average statistics; Ahmed and Brown, 2005). In addition to a tropical climate, Jamaica possesses a diversity of soils that range from erosion-resistant, fertile upland plateaus to lowland alluvial loam, sand and gravel soils. This variety in soil construct has been previously shown in tropical climates to be associated with tremendous microbial diversity and unique isolates, particularly in *Actinomycete* species (Torsvik *et al*, 1990; Reiners *et al*, 1994; Juo and Franzluebbers, 2003) and would thus provide a variety of locations from which to recover novel species.

MATERIALS AND METHODS

Collection of Jamaican soil samples: Soil samples were collected from sixteen sites in Port Antonio, Portland Parish, Jamaica following standard sampling procedures and obtaining measurements including soil moisture and temperature (Agricultural Analytical Services, 2011). The sixteen sites consisted of five historical locations i.e. associated with heavy foot traffic and thus physically disturbed (sites 1, 2, 4, 6 and 15), four ecologically disturbed locations (forest transformed to agricultural fields; sites 7, 8, 9 and 11) and six natural locations (coral reef, swamp and tropical forest; sites 3, 5, 10, 12, 13, 14 and 16). Details of the sites are shown in Table 1. At each location, soil samples were collected using a 2.5 cm soil core at a depth of 7.5 cm from five locations within a 1^2 m plot. All samples from a given location were homogenized, sealed and mailed following the quarantine protocol outlined by USDA-APHIS.

Isolation of *Actinomycete/ Bacillus* species: *Actinomycete/Bacillus* species were isolated by the method previously described by Boisvert-Bertrand *et al*, 2005. This involved plating suspended soil samples diluted in sterile saline onto nutrient agar containing 5% bovine serum albumin (albumin-agar). Agar plates were then incubated for one week, and white, leathery colonies characteristic of these species were isolated by subculturing onto fresh albumin agar. Further characterization of their ability to produce antibiotics was then made using the embedded agar assay.

Preparation of embedded agar plates and assessment of antibiotic activity:
Eight commercially obtained live, human bacterial pathogenic strains were used to assess the ability of the soil isolates to produce antibiotics (Ward's Natural Science, Rochester, NY). The panel consisted of four Gram positive bacteria, namely *Bacillus thuringensis, B. subtilis*, *S.* aureus and *S. epidermidis* as well as four Gram negative organisms *Serratia marsescens, Enterobacter cloacae, Klebsiella pneumoniae* and *E.* coli. Embedded agar was prepared separately for each bacterial pathogen tested, by allowing autoclave sterilized agar (120˚C, 2 atmospheres for 30 minutes) to cool until just warm to the touch, adding a 10 ml suspension of the bacterium in nutrient broth (Ward's Natural Science, Rochester, NY) per 90 ml of cooled agar and pouring the agar into sterile 60 x 15 mm Petri dishes (Ward's Natural Science, Rochester, NY). Six Jamaican soil bacterial colonies were then overlaid onto the surface of each embedded agar plate and the plates incubated until visible growth could be observed (24-48 hours at 37˚C). Antibiotic production was defined as the inhibition of embedded bacterial growth by at least a 3-millimeter ring around the soil bacterial isolate and isolates inhibiting either a single test pathogen or only familial pathogens, namely *B. thuringensis* and *B.subtilis* or *S. aureus* and *S. epidermidis* were retained for further study. Isolates were enumerated according to the Jamaican location from which they were obtained and the order recovered. For example 2B was from Folly Ruin and the second *Actinomyces/ Bacillus* bacterial colony isolated. An example of inhibition of bacterial growth is shown in Figure 1.

Figure 1: Jamaican Soil Isolate demonstrating antibiotic activity: Antibiotic activity was assessed by overlaying a single soil isolate on embedded agar containing one of a panel of eight human bacterial pathogens. Shown is the dramatic inhibition of *Staphylococcus aureus* growth (cleared area) by the isolate 2C (colony indicated by the arrow).

Identification of antibiotic-producing *Bacillus* species: All bacterial isolates able to inhibit the growth of one or more of the pathogens tested, were Gram stained and examined by light microscopy. *Bacillus* species (sporulating bacilli by light microscopy) were then typed using a commercial miniaturized API® 50CHB test system (Biomerieux-Vitek, Hazelwood, MO)(Schraft *et al*, 1996). Isolates that were able to be identified using this test system were not studied further, as unlike *Actinomycetes* which tend to occupy specific niches and appear unique in different habitats (Janso and Carter, 2010), *Bacilli* have a more widespread distribution (Berkley *et al,* 2002) and their activity is likely to have been already documented.

Fractionation of the biological activity: Preparation of the activity was performed according to the method previously described by Zgoda and Porter (2001). To produce sufficient

samples for initial purification, 100 µl of each isolate was cultured in nutrient broth for 3-4 days at 37˚C until the mixture had become turbid. Individual 1L cultures were prepared for each isolate with putative narrow spectrum antibiotic activity, spun down (150 rpm) and medium and cells collected separately. The cell and media fractions of the broth materials were then freeze-dried separately. Extraction of the organic compounds was made by repeated filtration and evaporation using a 1:1 solution of methanol/methylene chloride. The hydrophilic fraction for both media and cell pellets was also retained for further testing. Following final evaporation the dried product was weighed, dissolved in minimal methanol/ methylene chloride or water and adjusted to a final concentration of 500 mg of crude extract per ml of solution.

Bioactivity assay

Organic and aqueous extracts obtained from the cells and growth media (50 µg/ml) were assessed for bioactivity using a 96-well microdilution assay (Zgoda and Porter, 2001). These fractions were assessed separately as the partitioning of the antimicrobial moiety was not known and it could therefore be polar or non-polar. Six reference strains were used; the bacteria *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis* (ATCC 14468; model for *M. tuberculosis*), *Pseudomonas aeruginosa* (ATCC 27853) and methicillin-resistant *Staphylococcus aureus* (ATCC 43300)(MRSA) and the yeasts *Candida albicans* (ATCC 90028),and *Cryptococcus albidus* (ATCC 34140; model for *C. neoformans*). Briefly, the assay involved comparing the ability of a dilution (final concentration of 50 µg/ml) of the Jamaican soil *Actinomycete* extract to reduce the spectrophotometric turbidity of the microbial culture compared to a standard curve of known antibiotic. On each plate a standard MIC (minimum inhibitory concentration) curve of growth inhibition was prepared for each test microbe using serial dilutions of a known antibiotic: ampicillin for *E.* coli, gentamycin for *P. aeruginosa*, isoniazid for *M. smegmatis,* vancomycin for MRSA, amphotericin B for *C. albicans,* and ketoconazole for *C. albidus.* The antibiotic growth inhibition curves were then used to estimate the efficacy of the test fractions. Fractions inhibiting the growth of the microbe by > 20% (corresponding to <2 doubling dilutions of the MIC of the antimicrobial positive control) were retained for further investigation.

Brine shrimp assay

Using a standard microwell lethality assay (Solis *et al*, 1993) the concentration of each test fraction required to kill 50% of a sample of brine shrimp (LD_{50}) was compared with podophyllotoxin (0.05-50 µg/ml; LD_{50} of 2-3 µg/ml). Ten living *Artemia salina* brine shrimp, (defined as moving and with extended appendages), were placed in a microwell dish containing final concentrations of the extracts (0.05-50 µg/ml) in brine shrimp growth medium or brine shrimp growth medium alone. Numbers of living shrimp after a 48 hour incubation period were assessed on three separate occasions in duplicate and fractions with LD_{50} of < 3 µg/ml were retained for further study.

Commercial Identification of Actinomyces species producing bioactivity of interest: *Actinomyces* isolates inhibiting a single test pathogen or only familial pathogens, were commercially identified by Environmental Microbiology Laboratory, Inc. (San Bruno, CA).

RESULTS

Jamaican soil isolate recovery: Twenty- nine *Actinomyces*/*Bacillus* isolates were obtained from fifteen of the sixteen Jamaican soil sites sampled. One site (16 Blue Hole) was under water from which no isolates were recovered. Seven sites sampled yielded soil isolates possessing antibiotic activity fitting the criteria of either a) inhibiting a single pathogen or b) inhibiting only familial pathogens (Table 2). Humidity levels at these sites were slightly higher than the remaining eight sampling sites (mean ± SEM of 79.3 + 3.7% and 75.9 + 7.8%, respectively) but this was not statistically significant. No significant differences were found between the air and soil temperatures between the seven sites yielding putative narrow spectrum antibiotic activity and the remaining eight sites from which antibiotic producing microbes were isolated (air temperatures of 31.3 + 2.5 and 30.9 + 3.2˚C and soil temperatures of 25.8 + 2.6 and 25.1 + 1.6˚C respectively).

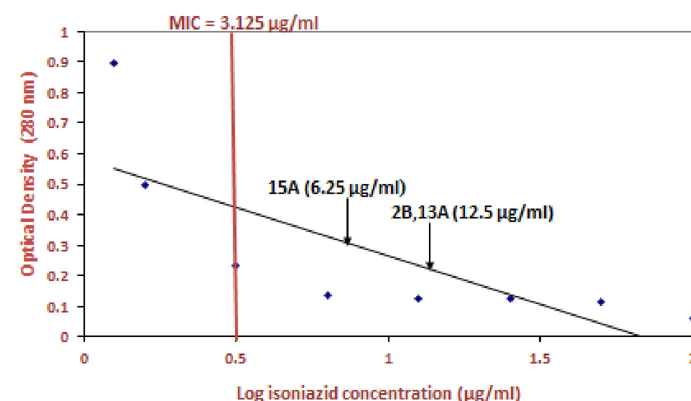

Figure 2: Minimum inhibitory concentration (MIC) of three Jamaican soil isolate extracts against *Mycobacterium tuberculosis* in comparison to isoniazid Extracts from Jamaican soil isolates 2B (organic and aqueous medium fraction), 13A (organic extracts of medium and cells) and 15A (aqueous extract of cells and organic extract of medium), were found to possess antibiotic activity against *Mycobacterium tuberculosis.* The MIC of the antibiotic substance extracted was either one (15A; 6.25µg/ml) or two (2B and 13A 12.5 µg/ml) doubling dilutions from that of isoniazid (3.125 µg/ml). Samples were tested in duplicate on three separate occasions.

Recovery of antibiotic-producing isolates selected for further study

Eight isolates possessing the potential to produce putative narrow spectrum antibiotics were recovered, of these, three (11B , 12A and 15B) were identified as *Bacillus* species by Gram stain and API testing. We therefore focused on the five potentially unknown remaining five *Actinomyces* isolates (2B, 3A, 6A, 13A and 15A).

Bioactivity Assay

Sample extracts (50 µg/ml) from none of the five *Actinomyces* isolates tested possessed antimicrobial activity against *E. coli*, *P. aeruginosa* , methicillin-resistant *Staphylococcus aureus* or *C. albidus*. Activity comparable to human physiological concentrations of commercial antimicrobials was found in 50 µg/ml extracts of isolates 2B, 13A and 15A against *M. smegmatis* and from isolates 2B and 13A against *C. albicans* (Figures 2 and 3). Several extracts from 2B (organic and aqueous medium fractions) and 13A (organic extracts of medium and cells) possessed activity similar to a concentration of isoniazid of 12.5 µg/ml (MIC value for isoniazid 3.125 µg/ml; Figure 2). An aqueous extract of 15A cells and organic extract of 15A growth medium both possessed activity similar to 6.125 µg/ml isoniazid (Figure 2). Activity against *C. albicans* was recovered from the media of 2B (organic and aqueous extracts) and 13A (organic extract of medium) and was similar to 0.63 µg/ml amphotericin-B (MIC amphoteracin B of 0.25 µg/ml; Figure 3).

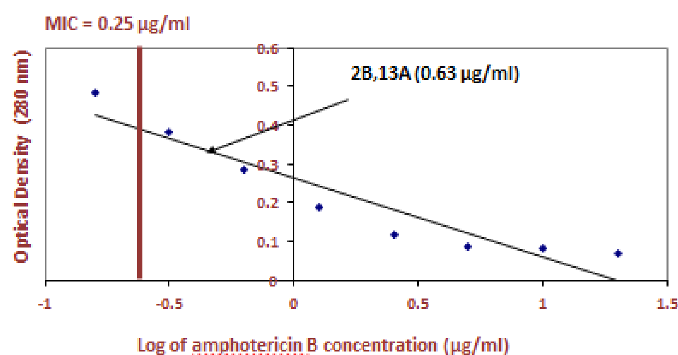

Figure 3 Minimum inhibitory concentration (MIC) of two Jamaican soil isolate extracts against *Candida albicans* in comparison to amphotericin B Extracts from Jamaican soil isolates 2B (organic and aqueous medium fraction) and 13A (organic extracts of medium), were found to possess antibiotic activity against *Candida albicans.* The MIC of the antifungal substance extracted from both these two isolates was within two doubling dilutions (0.63 µg/ml) of that of amphotericin B (0.25 µg/ml). Samples were tested in duplicate on three separate occasions

Brine shrimp assay

Organic extracts of 2B, 13A and 15A were further tested to evaluate their potential cell toxicity over the range of 0.05-50 µg/ml (Figure 4). Exposure of brine shrimp to extracts from isolates 2B and 13A at the highest concentration tested (50 µg/ml) resulted in viabilities of the brine shrimp of 85.0 $\pm$ 9.6 and 90.0 $\pm$ 5.8%, respectively. The extract from 15A produced slightly lower viabilities at this concentration (70.0 $\pm$ 11.5%) but still did not approach an LD_{50} value (Figure 4). Using the same dose response curve for podophyllotoxin produced an LD_{50} for the toxin of 3µg/ml (Figure 4).

DISCUSSION

Since many novel and powerful antimicrobial agents have been isolated from species of tropical terrestrial *Actinomyces* our study aimed to see whether novel species could be identified in diverse samples of Jamaican soil. Samples were obtained from physically and environmentally disturbed locations as well as those of natural importance and from the twenty nine isolates collected, three *Actinomyces* soil isolates were identified as generating putative narrow spectrum antimicrobial activity against *M. smegmatis* and/or *C. albicans*. Two of these isolates came from sites identified as physically disturbed (Folly Ruin and Sunset Cove), which were frequent sites for heavy foot traffic from visitors, and the third came from the site of a 1920s cholera epidemic burial (Bonnie View). Actinomycetes are known to thrive in warm, humid soils that are high in humus and often impart an "earthy" smell to these locations (Juo and Franzluebbers, 2003). Although these microbes are among the dominant species in temperate forest and arable soils, many are intolerant of higher temperatures (Juo and Franzluebbers, 2003). Those that can tolerate tropical or semi-tropical conditions need to evolve a selective advantage over the many other species competing with them for nutrients and space (Hawksworth and Colwell, 1992). It is therefore of interest that many antibiotics currently in use have come from tropically-derived actinomycetes, particularly *Streptomyces* (Juo and Franzluebbers, 2003).

Streptomyces was originally found to be capable of generating the antibiotic streptomycin, with strong efficacy against *M. tuberculosis* (Wood, 1995) and subsequently tetracycline and dapsone as well as antifungal compounds, including amphotericin-B (Marinelli, 2009). Indeed, of the usable, non-toxic antibiotics discovered to date, 55% of them originated in *Streptomyces* species and a further 11% from other *Actinomyces* species (Marinelli, 2009). Much focus in biotechnology is been on the *Actinomyces*, so our study seems especially pertinent given the likelihood of identifying novel microbes. A major problem for many of the antibiotics discovered has been their toxicity towards humans: Of an estimated 12,000 active compounds discovered since the 1940s, only 160 were clinically usable (1%, Marinelli, 2009). Since our extracts preliminarily appear non-toxic, at least to brine shrimp, indicating potential value as leads for testing for their therapeutic capabilities.

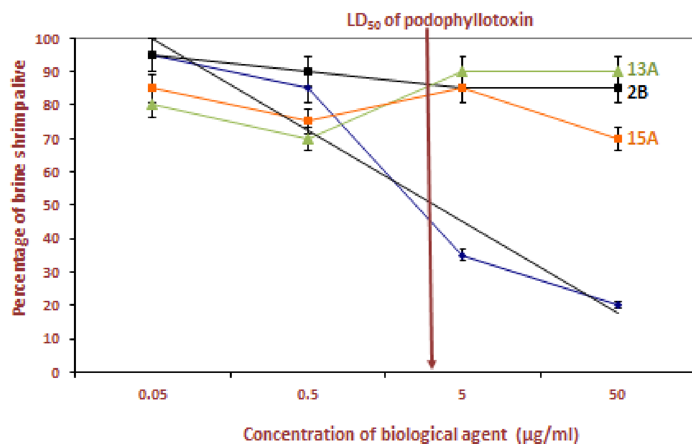

Figure 4 Brine shrimp cytotoxicity of Jamaican soil isolate extracts in comparison with podophyllotoxin Brine shrimp were exposed to between 0.05 and 50 µg/ml of organic extracts of Jamaican soil isolates 2B, 13A and 15A for up to 48 hours. None of the samples produced ≥ 50% death of the brine shrimp at any concentration tested. In contrast, the LD_{50} of podophyllotoxin (dose response curve symbol of blue diamonds) was 3µg/ml, as indicated by the arrow and as expected from previous studies. Samples were tested in duplicate on three separate occasions.

Three of our isolates were able to release a compound or compounds that were effective in killing the *M. tuberculosis* surrogate *M. smegmatis* and *Candida albicans* (Figures 2 and 3). Although a crude 50 µg/ml extract was used, for two of the *Actinomyces* tested (2 and 13) this extract was comparable in killing strength against *M. smegmatis* to four times the MIC of isoniazid and for the third extract twice the MIC (Figure 2). For *C. albicans*, extracts of two isolates (2 and 13) were comparable in killing strength to twice the MIC amphoteracin B (Figure 3). Both of these pathogens are important global causes of morbidity and mortality and with known resistant strains (Global Tuberculosis Control, 2011; White *et al*, 1998). Drug resistance is a major concern in the worldwide treatment of tuberculosis, particularly given the long time frame (6-9 months) a patient needs to be on antibiotics to eradicate the infection hence the consequent selective pressure selects for drug-resistant strains. Two such tuberculosis strains exist, multi-drug resistant (MDR-TB), which now account for approximately 400,000 cases of the disease worldwide, and extensively drug resistant (XDR-TB), which have been reported in 58 countries (Mlambo *et al,* 2008). More worrying are recent genotyping studies that suggest between 63 and 75% of MDR-TB strains can progress to becoming XDR-TB (Mlambo *et al*, 2008). Selective pressure has also increased the incidence of anti-fungal resistant strains of *C. albicans* (White *et al*, 1998). Initially isolated from immunocompromised AIDS patients in the 1980s, *C. albicans* strains resistant to azole drugs, particularly fluconazole, can now be community-acquired and have even been isolated from patients with vulvovaginal candidiasis ("yeast infection"; White *et al*, 1998).

The data presented indicate the potential benefits for the isolation and identification of *Actinomyces* from Jamaican soil but the methodologies used have several limitations. The first is our narrow panel of screening organisms used in the embedded agar assay. It is possible if our assay had included a larger panel of bacteria that we might have seen more isolates able to generate antimicrobial activity. A second issue is that only one isolate of each pathogen was used in the test and it is known that pathogens are highly variable in their susceptibility to antimicrobial agents especially when environmental strains are compared with those isolated from human hosts (Gillespie *et al*, 2002; Alavandi and Anathan, 2003). Since our pathogens were obtained from a supplier (Ward Biologicals) who serves a variety of customers, including high schools, the likelihood is that these are strains with lower virulence. This limitation is underlined by the finding that one *Actinomyces* isolate released antibacterial agents effective against *S. aureus* and *S. epidermidis* in the embedded agar assay (Table 2; isolate 2) but that this was not borne out by efficacy of the crude extract against MRSA in the 96-well microdilution assay. In addition, growth of the microbes in the 96-well assay may also be a factor since bacteria particularly are known vary in their antibiotic susceptibility depending on their phase of growth and age (Brown *et al*, 1990). Finally, some antimicrobial agents may be produced or diffuse more slowly into agar than allowed for by the 24-48 hour incubation period for the embedded agar assay.

Given these limitations as well as the need for new directed therapeutics to combat both tuberculosis and *Candida* our next step is to purify and characterize the active compound or compounds in each extract to ensure that, although the *Actinomyces* species appear unique, the antimicrobials themselves have not already been identified. Our next step will now be to separate the extracts further and given their solubility organic solvents gas liquid chromatography (GLC) is the most optimal approach. GLC would allow the separation of individual moieties in the organic extract and would also provide a measure of the number of components present. Comparison of the compounds present in organic extracts of the *Actinomyces* culture medium with those from culture medium alone would allow the elimination of culture reagents and allow us to test the individual moieties for biological activity using the 96-well microdilution assay. We also plan to resample the original Jamaican locations as well as extending our testing to include more areas of disturbed habitat since these may provide additional novel species and the potential for as yet undiscovered narrow spectrum therapeutics.

One major issue with many current antimicrobials is that they are broad spectrum and thus have a major side effect of removing susceptible beneficial commensal microbes as well as pathogens, increasing the chances of development of resistance. The usage of selectively toxic antimicrobials would be a great step, since they would be effective in the treatment of important human pathogens and would be anticipated to have lesser side effects, which in itself would improve patient compliance. It is also hoped by the authors that as new narrow spectrum therapeutic agents are discovered and developed this will lead to

a more responsible patient prescribing regimen to begin to limit the acquisition of microbial resistance.

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