CHARACTERIZATION OF ANTIBIOTIC-PRODUCING BACTERIUM ISOLATED FROM ANTHILL SEDIMENT WITH ACTIVITY AGAINST ESKAPE PATHOGENS

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ABSTRACT

Healthcare institutions have seen an increase in infections caused by antibiotic-resistant ESKAPE pathogens. Current antibiotics have become less potent against pathogenic bacteria due to their overuse and misuse. In recent years, scientists have revisited local environments in search of novel antibiotic-producing microbes to address the increasing threat of resistance. One species of bacteria was isolated from anthill sediment in coastal North Carolina. This environment was selected for its abiotic properties, including organic substrates, moisture saturation and aeration. Anthill isolate A2, inhibited various Gram-positive and negative ESKAPE pathogens or their surrogates, including *Staphylococcus aureus, Klebsiella pneumoniae,* and *Acinetobacter calcoaceticus,* in cross-streak tests. 16S rRNA sequencing identified isolate A2 as *Pseudomonas koreensis.* Mass spectrometry and small-molecule analysis performed on ethyl-acetate extracts of culture supernatant were used to evaluate bioactivity and identify the probable structure of one potential antimicrobial compound, monolauryl maleate. Discovery of novel antimicrobial compounds to replace overused antibiotics may help reduce the impact of antibiotic-resistant pathogens.

INTRODUCTION

Antibiotics are becoming increasingly ineffective in treating bacterial infections because of misuse and overuse, particularly in healthcare institutions. Antibiotic-resistant bacteria, known as ESKAPE pathogens, are a rising concern in healthcare settings (2). The ESKAPE pathogens – *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter sp.* – have been designated as global threats because they have developed resistance mechanisms and "escape" death by antibiotics.

Discovering and developing novel antibiotics are one solution to combat antibiotic-resistant infections. Unfortunately, there were

only 16 broad-spectrum antimicrobial agents approved and utilized in the U.S. between 1983–1987, and only 5 novel antimicrobial compounds were approved for use from 2003–2007 (2). Soil microbes are a potential source of new antibiotics. When microbes are in close quarters in terrestrial and aquatic environments, they compete for resources and utilize "chemical weapons" to inhibit growth of other microbes. The renewed search for antibiotic-producing microbes has focused on recruiting students at all levels to explore unique environments across the globe (4). Furthermore, new approaches to identifying previously uncultured microbes have shown promise, as demonstrated by the discovery of teixobactin, an antibiotic with a novel mechanism of action against Gram-positive bacteria, with use of the iChip (11).

In this study, bacteria were cultured from anthill soil in southeastern North Carolina and evaluated for their potential to produce antibiotics. Anthills were chosen for investigation because ant activity was shown to alter soil properties where they build their nests, and these soils were shown to contain an abundance of organic matter and cellulosic polysaccharides that could foster novel microbial communities (7). In fact, some ants that "farm" fungi for food have been shown to have mutualistic relationships with actinomycetes that produce antifungal agents to protect their "crops" against fungal parasites (1). However, little is known about antibacterial activities that may be associated with anthills. Antibiotic activities of anthill isolates were characterized with use of inhibition assays against a variety of Gram-positive and negative bacteria, including ESKAPE pathogens. A cell-free extract from one anthill isolate was analyzed to identify the active compound and to determine whether this finding was novel. One previously unidentified compound, monolauryl maleate, appears to possess activity against the Gram-positive pathogen, *S. aureus*.

MATERIALS AND METHODS

ISOLATION OF ANTIBIOTIC-PRODUCING BACTERIA

Soil was collected from the surface of an anthill (red imported fire ant, *Solenopsis invicta*) located in front of a residential area in Shallotte, North Carolina (33°58'36"N 78°31'30"W). 1:100 dilutions of soil in sterile water were plated on actinomycete isolation agar (AIA; HiMedia Laboratories, West Chester, PA) and incubated at 35°C for 3 days. 100 µL of an overnight tryptic soy broth (TSB; Hardy Diagnostics, Santa Maria CA) culture of *S. epidermidis* was transferred to 8 mL of molten TSB soft agar (TSB with 0.7% agar), vortexed, and poured over the AIA culture. After solidification, the plate was incubated at 35°C for 24 h, and then observed for zones of inhibition (ZOI) in the overlay. Isolates showing ZOI were picked with a sterile loop and streaked for isolation on AIA. *S. epidermidis* was used in initial screens because it has little natural resistance and should display ZOI against a variety of antimicrobials. Additionally, as a BSL-1 microbe, it is safer to work with as students are training in the lab and learning these techniques.

CROSS-STREAK TEST

Each isolate was streaked down the center of Mueller-Hinton agar (MHA; Hardy Diagnostics) and incubated at 35°C for 3 d. Bacteria used in this study are listed in Table 1. Some were acquired from Carolina

Biological Supply Company (Burlington, NC) and strain identity was unknown. Strains with known identity (indicated with ATCC number) were purchased from Fisher Scientific (Hampton, NH). Six ESKAPE pathogens or surrogates (Table 1 and Fig. 2) were streaked perpendicular to the isolate, and the plate was incubated at 35°C for an additional 24 h. Isolate A2 was later retested by cross streak against a panel of 10 Gram-positive and negative bacteria, including some ESKAPE pathogens and surrogates (Table 1 and Fig. 3). The inhibition distance was measured in mm.

IDENTIFICATION OF ANTHILL ISOLATES

Each isolate was Gram-stained and observed at 1000x magnification with a Lecia DM750 compound light microscope. Genomic DNA was extracted from each isolate with the use of the Ultra Clean Microbial DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA), according to manufacturer's specifications. The cell disruption step was performed in a Bead Bug homogenizer (Benchmark Scientific, Edison, NJ) for 3 x 60 seconds at 320 rpm with cooling on ice between cycles. The 16S rRNA sequence was amplified with use of primers Eco8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1509R (5'-GGTTACCTTGTTACGACTT-3') (5). Each 50-µL PCR reaction (GeneMate reagents, BioExpress, Kaysville, UT) contained: 1x PCR buffer, 0.025 U/µL Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, and 5.0 µL of genomic DNA. PCR was carried out in an Eppendorf (Hauppauge, NY) Master Cycler under the following conditions: 95°C for 5 min; 30 cycles of 95°C for 1 min, 50°C for 30 s, 72°C for 1.5 min; and then 72°C for 5 min. The 1.5-kb PCR product was confirmed by gel electrophoresis, and then purified with use of the E.Z.N.A Cycle Pure kit (Omega Bio-tek, Norcross, GA), according to manufacturer's specifications, and sequenced by Eurofins Genomics (Louisville, KY). Raw sequences were edited with Finch TV software, v1.4 (Geospiza). A contiguous sequence was created from the forward and reverse sequences from the same isolate with Serial Cloner software, v 2.6 (Serial Basics). Sequences were analyzed with BLAST (National Center for Biotechnology Information) with use of the 16S ribosomal RNA sequences (Bacteria and Archaea) database to identify species with similar sequences

Table 1. Test bacteria used in this study.

Gram-negative	Gram-positive
*Acinetobacter calcoaceticus***	*Bacillus cereus*
Aeromonas hydrophila	*Enterococcus faecalis ATCC 29212***
*Escherichia coli***	*Kocuria rhizophila*
*Klebsiella pneumoniae ATCC 13883**	*Staphylococcus aureus ATCC 25923**
Proteus vulgaris	*Staphylococcus capitis ATCC 35661***
*Pseudomonas aeruginosa ATCC 27853**	*Staphylococcus epidermidis ATCC 12228***
Serratia marcescens	

* ESKAPE pathogen
** ESKAPE surrogate

PREPARATION OF CELL-FREE ETHYL-ACETATE EXTRACT

Isolate A2 was cultured in TSB at 25°C for 7 d, and cells were pelleted by centrifugation for 10 min at 2000 rpm in an Eppendorf 5804R centrifuge. The supernatant was passed through a 0.2 µm filter (Corning, Oneonta, NY). The filtrate was transferred to a separatory funnel and combined with 2 parts ethyl acetate (Alfa Aesar, Haverhill, MA). The aqueous layer was removed, extracted again with 2 parts ethyl acetate, and the two organic extracts were combined. The ethyl acetate was evaporated in a rotary evaporator and the dried cell-free extract was dissolved in 100% methanol (Alfa Aesar).

COLUMN CHROMATOGRAPHY

A Strata C18-E (55µm, 70 A) giga tube column (Phenomenex, Torrance, CA) was conditioned with three washes of 100% HPLC-grade methanol to rinse the column, and then was equilibrated with three washes 20% methanol. 23.0 mg of isolate A2 extract was suspended in 5.0 mL of 20% methanol, then was pipetted onto the column. The crude extract was washed with increasing HPLC-grade methanol gradient with pico-pure filtered H_2O at 20%, 40%, 60%, 80%, and 100% MeOH. A final rinse of 100% acetone removed everything else from the column. The column was placed in a Supelco Visiprep vacuum apparatus for eluting these compounds with vacuum pressure at 8 Hg. The fractionated compounds were dried on a speed vacuum, then were resuspended in 1.0 mL of 100% methanol and tested for antibacterial activity by disk diffusion.

DISK DIFFUSION ASSAY

S. aureus and *E. coli* were freshly cultured on tryptic soy agar (TSA; Hardy Diagnostics) at 35°C for 24 h. Test bacteria were suspended in saline solution (0.9% NaCl) to match a 0.5 McFarland turbidity standard. A sterile swab was used to transfer test bacteria to MHA. Dried extracts of isolate A2 were resuspended in 1.0 mL of 100% MeOH, then 30–40 µL of the mixture was pipetted onto sterile filter disks (Whatman #3, GE Healthcare Life Sciences, Marlborough, MA) in 10 µL increments (10). Dried disks were transferred to the MHA plate, which were then incubated at 35°C for 24 h. Diameters of ZOI were measured in mm.

SMALL-MOLECULE ANALYSIS

Small-molecule analysis of culture supernatants from isolate A2 involved preparation of cell-free ethyl-acetate extracts, as described above. Ultrahigh-performance liquid chromatography, low-resolution mass spectrometry, and small-molecule analysis yielded an S-plot, chromatogram of mass over ionization ratios, and UV absorption of a specific secondary metabolite. These results were analyzed with Progenesis QI software (Nonlinear Dynamics, Durham, NC) to compare known compounds to the secondary metabolites with potential antimicrobial capabilities (3).

ULTRAHIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-LOW RESOLUTION MASS SPECTROMETRY (UPLC-LRMS)

C-18 column chromatography elutions 3, 4, 5, and 6 were analyzed by mass spectrometry to search for similar patterns within the fractions that showed inhibition against S. *aureus*. The 60–100% methanol and 100% acetone washes were dried down on a speed vacuum, and then were suspended in 1 mL of 1:1 acetonitrile: pico-pure water. 30 µL of elutions 3, 4, 5, and 6 were run through

RESULTS

ISOLATION OF ANTIBIOTIC- PRODUCING BACTERIA FROM ANTHILL SEDIMENT

Because anthills have unique characteristics compared to the surrounding soil and ants have been shown to have symbiotic relationships with antibiotic-producing microbes, bacteria were cultured from an anthill in southeastern North Carolina colonized by red imported fire ants to search for antibiotic producers. The culture of anthill sediment on AIA was overlayed with *S. epidermidis* and yielded a few small ZOI (Fig. 1). Potential antibiotic-producers were picked and streaked on AIA. In initial cross-streak tests against ESKAPE species or surrogates, one of the anthill isolates, A2, demonstrated inhibition of five of six test bacteria, but not Gram-positive *E. faecalis*, an ESKAPE surrogate for *E. faecium* (Fig. 2). Overall, isolate A2 yielded the strongest inhibitory characteristics against three Gram-negative ESKAPE bacteria, particularly *E. coli* (surrogate for *Enterobacter sp.*), *K. pneumoniae* and *A. calcoaceticus* (surrogate for *A. baumannii*). Moderate inhibition was shown against Gram-negative *P. aeruginosa* and Gram-positive *S. aureus*. Additional cross-streak tests with A2 showed inhibition of variety of Gram-negative and Gram-positive bacteria, including another species of *Staphylococcus* (Fig. 3). Two Gram-negatives, *S. marcescens*

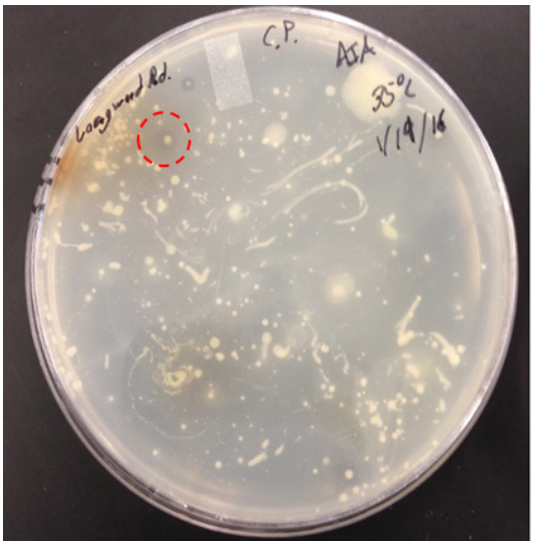

Figure 1. Soft agar overlay of *S. epidermidis* on AIA culture of anthill soil. The red-dashed circle shows the location of the A2 colony showing ZOI.

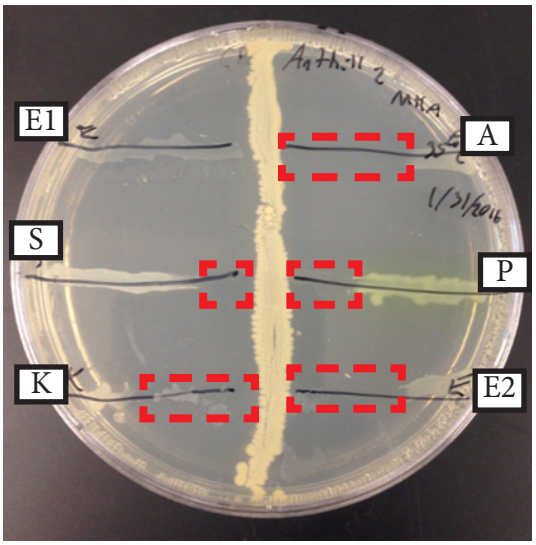

Figure 2. Cross-streak testing with cultured isolate A2 on MHA plates against ESKAPE pathogens and surrogates. Isolate A2 was streaked vertically, and the test bacteria were streaked horizontally. A lack of culture growth from the horizontally streaked test bacteria revealed inhibition (represented by areas boxed by red-dashed lines). E1, *E. faecalis*; S, *S. aureus*; K, *K. pneumoniae*; A, *A. calcoaceticus*; P, *P. aeruginosa*; E2, *E. coli*.

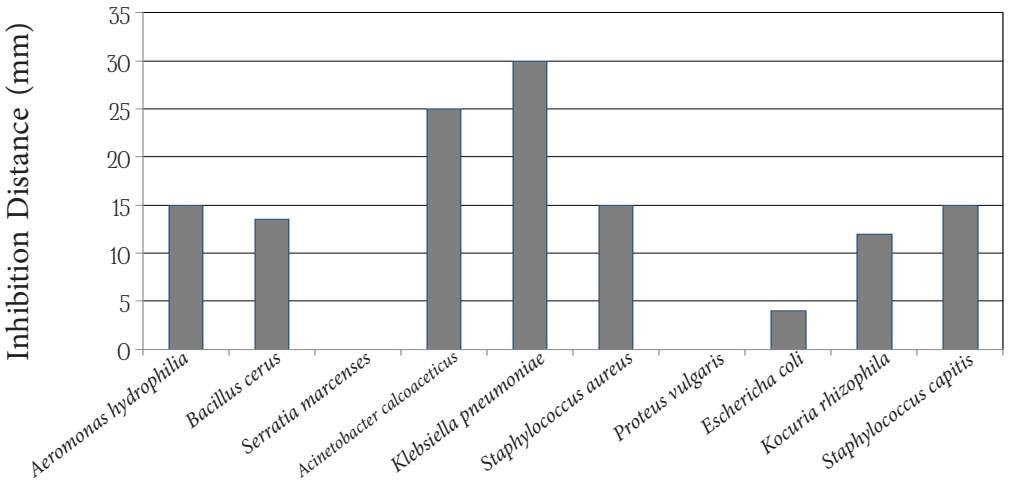

Figure 3. The inhibition distance (mm) displayed by isolate A2 in a cross-streak test against various Gram-positive and negative test bacteria. Bars represent data from a single cross-streak assay. There were no ZOI detected when tested against S. *marcescens* and *P. vulgaris*.

and *P. vulgaris*, were not inhibited.

IDENTIFICATION OF ISOLATE A2

BLAST analysis of the 16S rRNA sequence from the A2 isolate retrieved a sequence with only one mismatch in the 973-base alignment. This species, *P. koreensis*, was previously identified in soils from a Korean farm (8) and a rice paddy (9). Gram staining of A2 revealed a Gram-negative bacillus, as expected for a *Pseudomonas*. Limited knowledge regarding antibacterial activity has been reported for this species, so it is likely the broad-spectrum inhibition seen in cross-streak tests represents a novel finding. To further characterize this activity, an attempt to isolate and identify the antibacterial compound(s) secreted by the A2 isolate was pursued.

ANTIBIOTIC ACTIVITY OF FRACTIONATED CELL-FREE A2 EXTRACTS

Culture supernatants of isolate A2 (putative *P. koreensis*) were extracted with ethyl acetate. These cell-free extracts were tested for antibacterial activity in disk diffusion assays against two of the bacteria that were inhibited in cross-streak tests – Gram-positive S. *aureus* and Gram-negative *E. coli*. The A2 extract produced a 10-mm zone of inhibition against S. *aureus* (Fig. 4, disk A); however, no inhibition was produced against *E. coli* (data not shown). The ability to detect inhibitory activity in cell-free extracts was useful for the preliminary isolation of antibacterial compound(s).

C-18 column chromatography was used to fractionate compounds from 23.0 mg of A2 ethyl-acetate extract suspended in 20% methanol. The largest portion of the extract (9.1 mg) was eluted in the second fraction at 40% methanol, while the smallest portion (0.2 mg) was eluted at 80% methanol (Table 2). Elutions 2-6, were tested by disk diffusion against S. *aureus* to determine which fraction(s) contained the active compound(s). Only 10% of each fractionated sample was loaded onto the sterilized disks (Table 2). Elution 2 produced a small and poorly defined ZOI that could not be measured, while elution 3 and 6 yielded no ZOI (Fig. 4, disks 2, 3 and 6, respectively). Elutions 4 and 5 both produced 10-mm ZOI, similar to that displayed by the original cell-free extract

Table 2. C-18 column chromatography distribution of compounds from the cell-free, ethyl-acetate extract based on polarity with increasing methanol (MeOH) concentrations.

Elutions	[Solvent]	Solvent	Dried Sample Weight (mg)	Amount Loaded onto Disk (mg)
1	20%	MeOH/H_2O	2.0	0.02
2	40%	MeOH/H_2O	9.1	0.91
3	60%	MeOH/H_2O	2.1	0.21
4	80%	MeOH/H_2O	0.2	0.02
5	100%	MeOH/H_2O	0.9	0.09
6	100%	Acetone	0.4	0.09

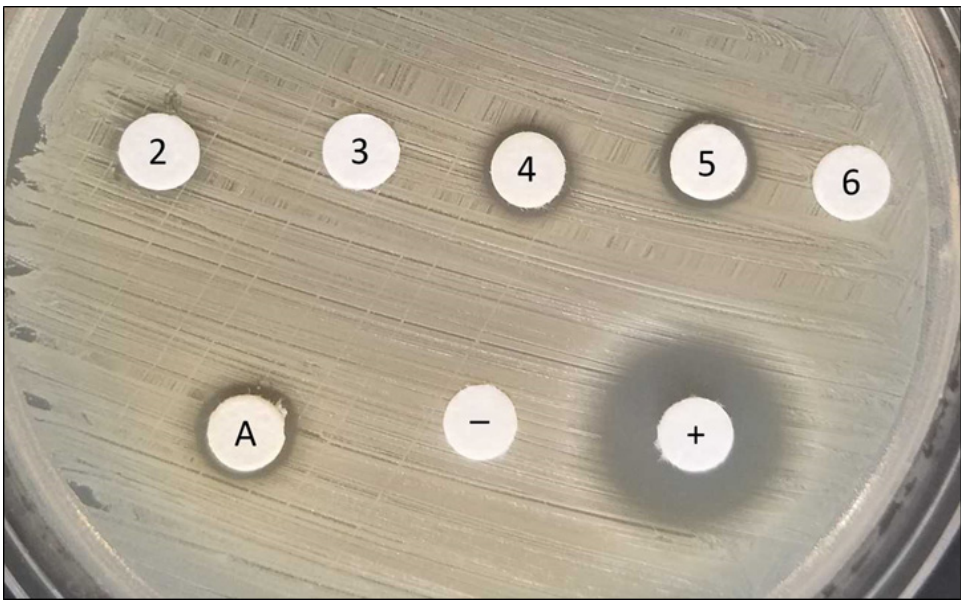

Figure 4. Disk diffusion assay performed with cell-free ethyl-acetate extract of A2 (A) and its C-18 column fractions (2-6) against S. *aureus*. Streptomycin was the positive control (+). Methanol was the negative control (-).

(Fig. 4, disks 4 and 5, respectively).

MASS SPECTROMETRY ANALYSIS OF A2 COLUMN FRACTIONS

C-18 column elutions 3–6 were selected for analysis by mass spectrometry. There were clear differences in number and location of peaks between the four fractions observed on the chromatogram, whereas elutions 4 and 5, which showed antibacterial activity by disk diffusion, had 13 peaks in common (Fig. 5). There was an abundance of one compound at a 276.6 m/z ratio in both active fractions that was absent from the other two non-active fractions (Fig. 5). There was another peak of interest at 294.5 m/z only present in elutions 4 and 5. Although, this may represent an additional active compound, focus was directed at peak 276.6 m/z because of its greater abundance in the extract. Future studies will isolate and assess the antibiotic potential of other peaks.

SMALL-MOLECULE ANALYSIS OF CELL-FREE A2 EXTRACT

Small-molecule analysis was performed on cell-free, ethyl acetate extracts of A2 culture supernatant, which yielded over 1,000 different compounds, as displayed on the S-plot (Fig. 6). The S-plot, where each dot represents a different compound, compared compounds from the A2 cell-free, ethyl-acetate extract and a matched TSB media control. Focusing on the 13 outlier dots in the bottom left quadrant, each represented a compound unique to the A2 culture supernatant. One, if not more, of these 13 compounds may represent the antimicrobial agent produced by isolate A2. The potential active compound identified by mass spectrometry from elutions 4 and 5 was similar to one of the outliers (265.23 m/z) identi-

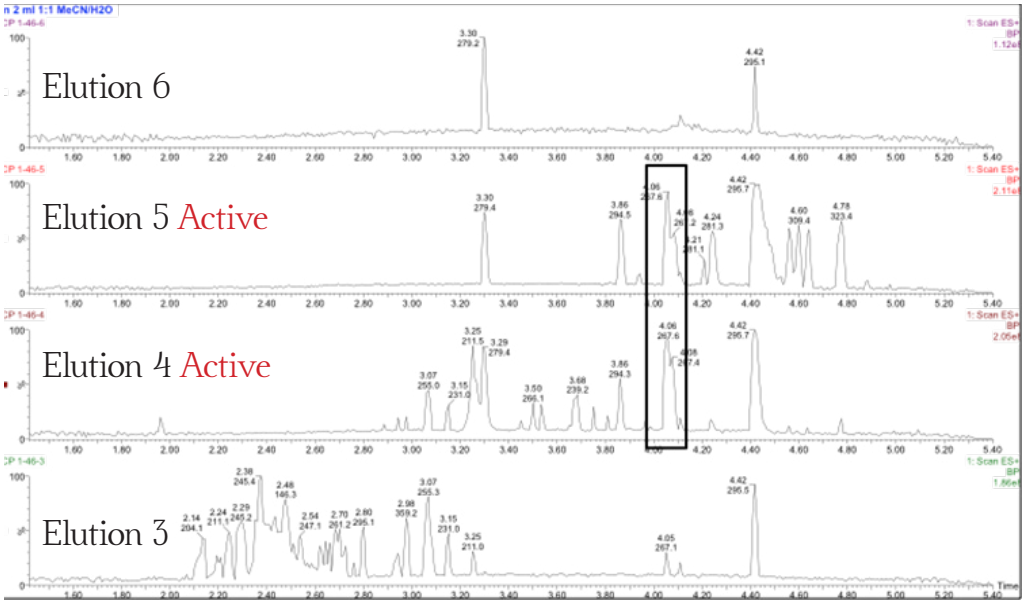

Figure 5. UPLC-LRMS chromatogram results from 60%, 80%, 100% MeOH fractions, and the 100% acetone extraction (elutions 3, 4, 5, and 6, from top to bottom). The boxed area is a selected peak of interest with a mass of 267.6 m/z in the 80% and 100% MeOH fractions.

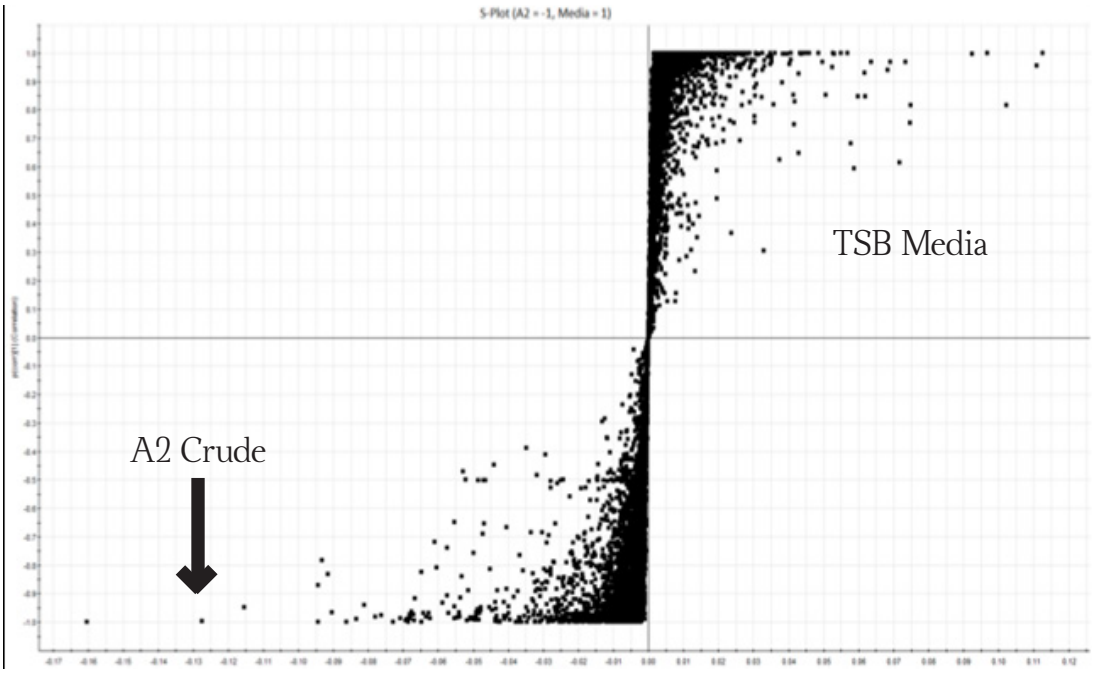

Figure 6. S-plot from small-molecule analysis of triplicate cell-free extracts of A2 culture supernatants (bottom left quadrant) and the TSB media control for comparison (top right quadrant). Arrow indicates compound at 265.23 m/z, which was identified as monolauryl maleate.

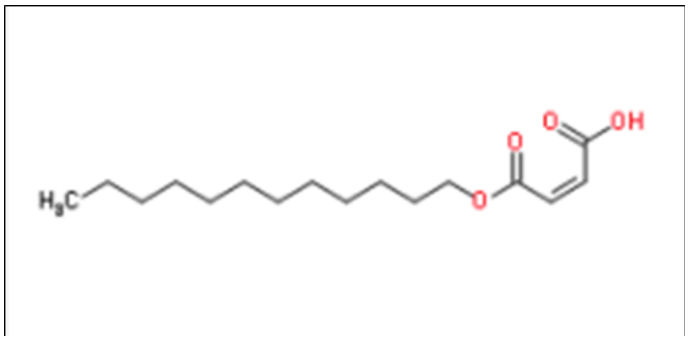

Figure 7. Chemical structure of monolauryl maleate from Progenesis QI software, a compound identified in bioactive C-18 column fractions from A2 cell-free, ethyl acetate extracts.

fied by small-molecule analysis. A cleavage product of this compound was also revealed with a 44 m/z ratio difference, which likely represents a potential carboxylic acid group in the complete compound (data not shown). This finding was consistent with QI Progenesis prediction (97% probability) of the identity of the unknown 266 m/z compound as monolauryl maleate, which has a molecular formula $C_{16}H_{28}O_4$ with a probable carboxylic acid group attached to a 12-carbon chain (Fig. 7).

DISCUSSION

In response to the growing global problem of antibiotic-resistant ESKAPE pathogens in healthcare institutions, soil samples were collected in coastal North Carolina and investigated for the presence of novel antibiotic-producing bacteria. One promising isolate (A2) found in anthill soil inhibited a variety of Gram-positive and Gram-negative bacteria in cross-streak tests (Fig. 3). Broad-spectrum antimicrobial activity is likely beneficial to ants that become colonized by this microbe from sediment manipulation for protection against various pathogenic bacteria, as suggested previously (1). Detection of this antibiotic-producing species on the heads or bodies of imported fire ants could reveal a new mutualistic relationship, similar to ones observed in other ant species. The fact that A2 also inhibited five of six ESKAPE pathogens or their surrogates suggests that anthills may warrant further investigation for bioactive natural products.

BLAST analysis of 16S rRNA gene sequence of A2 showed a match with *P. koreensis*, a newly defined species first discovered in Korean farming soil (8). Genome sequence analysis of another *P. koreensis* isolate from a Korean rice paddy revealed a putative gene cluster for the synthesis of cyclic lipopeptide (CLP), which is a biosurfactant shown to have antagonistic properties (9). *Pseudomonas* species are known to produce a variety of CLPs with antimicrobial activity that likely inhibit colonization of ants by pathogens in the environment (12). One of three *P. koreensis* isolates from a water basin in Mexico demonstrated biosurfactant activity, but inhibition of bacterial growth was limited to two Gram-positive species, *Bacillus subtilis* and *Exiguobacterium aurantiacum* (13). In fact, no broad-spectrum antibacterial activity has been described previously for this species. In this study, not only did the A2 isolate inhibit several different bacterial species, including ESKAPE pathogens, crude and fractionated cell-free extracts from A2 inhibited *S. aureus* (Fig. 3 & 4). Genomic comparison of the two Korean *P. koreensis* strains demonstrated genetic diversity among *Pseudomonas* strains, with 631 putative genes unique to one isolate (9), so it is expected that the North Carolina anthill isolate possesses genetic elements and metabolic capabilities not present in the Korean or Mexican isolates. One of the potentially active compounds, monolauryl maleate, produced by isolate A2 is structurally distinct from CLPs and represents a novel finding in *P. koreensis*. Whether this new compound has similar biosurfactant activity to CLPs might be determined in the future with an in vitro biofilm inhibition assay, or potentially through the development of an ant colonization model. Genomic sequencing of A2 would also reveal differences and help identify a gene cluster involved in antibiotic synthesis.

It was curious that the A2 cell-free extract did not inhibit *E. coli* in a disk diffusion assay, since the A2 isolate did inhibit E. coli in a cell-based, cross-streak assay rather well. This finding indicates that the broad-spectrum activity demonstrated in cross-streaks may represent two or more distinct antibacterial compounds produced by A2 – one absent from cell-free extract that is active against *E. coli* and other Gram-negatives, and another present in cell-free extract that is active against *S. aureus* and other Gram-positives. The compound(s) that inhibit Gram-negative bacteria may not have be soluble in ethyl acetate or may have remained with the cell pellet during collection of the culture supernatant. In hindsight, *K. pneumoniae*, which displayed the greatest inhibition in cross streaks, should have been first Gram-negative tested. Unfortunately, the extraction yielded only enough material to complete a preliminary round of disc-diffusion assays against one Gram-positive and one Gram-negative species. It will be important to revisit the extraction technique to

explore other options for isolating bioactive metabolites and increasing yield, including modifying culture conditions and performing extractions on lysed cells in the pellet. Furthermore, these extracts should also be tested for antibacterial activity against other Gram-negative ESKAPE pathogens, *K. pneumoniae* and *A. calcoaceticus*, which were also inhibited in the cross-streak assay.

A potential novel antimicrobial compound, monolauryl maleate, was identified by small-molecule analysis and low-resolution mass spectrometry of a cell-free extract of isolate A2. Mass spectrometry, UV light absorbance, and small-molecule profiling of isolate A2 provided evidence of a potential antimicrobial compound in the S-plot with a 266 m/z ratio that was characterized through QI Progenesis software. There was a 97% match with the compound, monolauryl maleate (Fig 7). This compound consists of a carbon chain connected to a carboxylic acid group, as confirmed by a 44 m/z fractionation with low-resolution mass spectrometry. No findings had been previously reported regarding the properties of monolauryl maleate as an antibiotic agent. Monolauryl maleate is not available commercially for testing purposes at this time. Synthesis of monolauryl maleate may be possible and could be valuable for future comparative studies. This is one of thirteen potential antimicrobial compounds residing within the active elutions 4 and 5 (Fig. 5). HPLC purification and NMR analysis (3) on the compounds of interest in these two fractions, including peak 294.5 m/z, will be required to ultimately confirm the identity of the active compound(s) and to characterize antimicrobial activity against the ESKAPE pathogens that were inhibited in the cell-based assays. Once identified, the potential of any active A2 compound as a therapeutic agent can be explored.

In 2005, there were 8,987 cases of methicillin-resistant *S. aureus*, or MRSA, infections in hospital and communal locations across the United States, and there were a total of 988 deaths from complications of these infections (6). The constant battle against antibiotic-resistant infections in the healthcare community must be addressed, in part, through the discovery of novel antibiotics. One method includes studying environmental bacteria and their natural products from unique habitats around the world (4, 11). In this study, antimicrobial activity against *S. aureus*, and other ESKAPE pathogens, was discovered in a *Pseudomonas* species isolated from an anthill. The preliminary identification of monolauryl maleate may represent the initial step in the discovery of the next novel antimicrobial agent to address the increasing prevalence of antibiotic-resistant infections.

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