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STIMULATION OF AQUATIC BACTERIA FROM MAMMOTH CAVE, KENTUCKY, BY SUBLETHAL CONCENTRATIONS OF ANTIBIOTICS

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Abstract

Many microorganisms secrete secondary metabolites with antibiotic properties; however, there is debate whether the secretions evolved as a means to gain a competitive edge or as a chemical signal to coordinate community growth. The objective of this research was to investigate if select antibiotics acted as a weapon or as a chemical signal by exposing communities of aquatic cave bacteria to increasing concentrations of antibiotics. Water samples were collected from six cave locations where actinobacterial mats appeared to be plentiful. Bacterial growth was measured using colony counts on 10 % tryptic soy agar augmented with increasing concentrations of erythromycin, tetracycline, kanamycin, gentamicin, or quaternary ammonia compounds (QAC). Colony counts generally decreased as the gentamicin, kanamycin and QAC dose increased. In contrast, the colony numbers increased on agar plates supplemented with 0.01 mg L^{-1}, 0.10 mg L^{-1} and 1.00 mg L^{-1} erythromycin or tetracycline. A 10.00 mg L^{-1} dose of each antibiotic treatment reduced bacteria colonies by 98 % or more. Community-level physiological capabilities were evaluated using Ecolog plates inoculated with cave water dosed with either 0.00 mg L^{-1} or 0.10 mg L^{-1} of erythromycin. Incubation with the antibiotic almost doubled the number of food substrates used in the first 24 hours. There was a significant increase in the use of acetyl glucosamine, arginine, and putrescine when bacteria were exposed to 0.10 mg L^{-1} erythromycin triggered by the antibiotic acting as a chemical messenger. Principal component analysis confirmed a shift in substrate preferences when erythromycin was added. A conceptual ecological model is proposed based on the response of aquatic cave bacteria to sublethal antibiotics.

INTRODUCTION

Bioactive secondary metabolites, such as antibiotics, are low molecular-weight compounds, often produced by one microorganism and harmful to other microorganisms at high concentrations. Escalating use of these compounds in medicine and agriculture has resulted in the increased occurrence of antibiotics in sludge, soils, surface water, groundwater, and a concomitant increase in antibiotic-resistant bacteria (Pruden et al., 2012; Rinsky et al., 2013; Amos et al., 2014; Perry and Wright, 2014). Yet, for all the concern surrounding increased antibiotic use and growing resistance, there is almost no information regarding natural background concentrations or the ecological function of secondary metabolites in the environment.

Many antibiotics are small bioactive molecules that are naturally produced as secondary metabolites of microorganisms such as bacteria and fungi (Davies, 2006; Davies and Davies, 2010; Bibb, 2005). The majority of antibiotics were discovered by subjecting isolated soil microorganisms to extremely unnatural laboratory environments, resulting in the elevated production of secondary metabolites. The soil bacteria phylum Actinobacteria, previously referred to as Actinomycetes, accounts for approximately 40–60 % of the known 23,000 bioactive secondary metabolites (Berdy, 2005). Secondary metabolites with antibiotic properties have been the subject of intense research for the past 70 years and form the foundation of modern antimicrobial therapy (Sengupta et al., 2013). Despite their usage for medical purposes, the ecological role of secondary metabolites is not clear. Antibiotic-producing microorganisms such as actinobacteria and fungi are common in cave microbial communities (Lavoie and Northup, 1994; Cheeptham et al., 2013). The antibiotics produced by actinobacteria are categorized into several major structural classes including amino glycosides (e.g., streptomycin and kanamycin), ansamycins (e.g., rifampin), anthracyclines (e.g., doxorubicin), β-lactam (cephalosporins), macrolides (e.g., erythromycin), and tetracyclines (Chaudhary et al., 2013). Traditionally, it has been thought that these secondary metabolites contributed to microbial defense, species fitness, interference, and competitiveness, hence their designation as antibiotics (Oliveira et al., 2015).

Other researchers have proposed that these secondary compounds evolved as microbial communication molecules, also called quorum sensors (Linares et al., 2006; Mlot, 2009; Davies, 2006; Davies and Davies, 2010; Burnier and Surette, 2013), and that quorum sensor molecules coordinate the growth and establishment of microbial biofilm communities. At high concentrations, the molecules exhibit lethal antimicrobial effects on susceptible species. The same molecules at sublethal concentrations stimulate or induce diverse beneficial responses in bacteria. This biphasic

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dose-response to a chemical agent, characterized by low-dose stimulation and high-dose inhibition, is referred to as hormesis (Mattson, 2008).

Scientists have used genetically modified microorganisms and molecular techniques to study the hormesis response to secondary metabolites. The artificial test conditions can be rationalized because soil microbiology and chemistry are complicated and teeming with extraneous, small, organic molecules that make it difficult to correlate a particular physiological response to a single variable like a sublethal dose of antibiotics (Davies and Davies, 2010; Mlot, 2009). For example, Davies (2006) inserted bioluminescence genes into *Salmonella* sp. to verify that sublethal concentrations of particular antibiotics can stimulate gene expression. Low, sublethal concentrations of the test antibiotics produced a strong stimulation of transcription from specific groups of promoters, resulting in bioluminescence. At higher concentrations (near-inhibitory), the transcription and bioluminescence patterns changed again, indicating a dose-dependent response. Other studies have also shown a connection between sublethal antibiotic dosages and transcriptional regulation in *Escherichia coli*, leading to changes in bacterial growth patterns such as a shift to biofilm development or swarming (Dietrich et al., 2008; Nadell et al., 2009). Gude et al. (2020) describe how motility, reproduction rate and antibiotic compounds interact to influence biofilm development. One limitation of these studies is the use of enteric bacteria, as opposed to bacteria from the cave or soil environment where actinobacteria flourish. Another study exposed the soil actinobacteria, *Streptomyces* sp., isolated from nine different geographical areas, to sublethal concentrations of antibiotics and observed a shift in substrate use (Vaz Jauri et al., 2013). The use of laboratory axenic cultures, as opposed to complex microbial communities found in natural environments, limits the conclusions from the Vaz Jauri et al. study. Despite the use of artificial test systems, these previous studies demonstrate that low dosages of antibiotic compounds are inducing changes in gene expression, protein synthesis, and metabolism in bacterial cultures.

Antibiotic-producing bacteria are typically found in complex soil ecosystems, and chemical signals are hypothesized to play a substantial role in bacterial community structure and function (Keller and Surette, 2006). Not surprisingly, the vast majority of studies that examined soil microbial response to antibiotics have focused on human health-related issues, such as the increasing occurrence of antibiotic resistance (Chee-Sanford et al., 2009; Bhullar et al., 2012; Rinsky et al., 2013), and not on the changes in the microbial community function. Investigating the ecological role of these secondary metabolites in their natural environment is hindered by the complexity of soil ecosystems. Only two studies have been found in published literature that consider a soil microbial ecosystem response to antibiotics. Thiele-Bruhn and Beck (2005) used concentrations of sulfonamides and tetracycline that approximated the dosage lethal to 50 percent of the microbial population. They reported a reduction in respiration rates after 24 hours of exposure and also found that sulfonamides and tetracycline added to soil manure caused a shift in microbial community from bacteria to fungi. A study by Underwood et al. (2011) found that trace concentrations of the synthetic antibiotic sulfamethoxazole, commonly found in wastewater, significantly reduced microbial denitrificiation in a sandy aquifer. The findings from Thiele-Bruhn and Beck (2005) and Underwood et al. (2011) substantiated that sublethal concentrations of anthropogenic antimicrobial compounds can adversely affect microbial communities and disrupt biogeochemical processes in the environment. While important, these studies do not address the role of natural secondary metabolites as weapons or signals in the soil ecosystem.

If the primary ecological role of secondary metabolites is to serve as a weapon for defense or competition, then natural antibiotics should be present in subsurface environments, especially ecosystems rich in actinobacteria. For the secondary metabolites to serve as competitive weapons, the metabolites must be present in toxic concentrations for sufficient periods of time. Anything less than acute concentrations would result in natural selection for antibiotic resistance. This premise is exemplified in clinical therapy where patients are told that failure to complete their prescription as directed may result in increased antibiotic resistance of the pathogen (Washington, 1979). A rigorous review of the literature did not yield a single study reporting the detection of natural antibiotics in pristine soils or waters (Haack, 2009; Alvarez et al., 2014; Barber et al., 2013). Perhaps this is because of a lack of examining natural sites under the right conditions and the complexity of soil ecosystems. Alternatively, the secondary metabolites could have been present below standard detection thresholds while serving a function other than as a weapon. Perhaps natural analogs of the pharmaceutical antibiotics were released into the surrounding water but went undetected because most analytical methods are very specific for anthropogenic antibiotics, and even minor structural changes in the molecule would preclude them from being identified.

The present study examines how cave aquatic microbial communities respond to sublethal concentrations of secondary metabolites, some of which humans exploit as pharmaceutical antibiotics (Linares et al., 2006; Davies, 2009). This study avoided many of the complications associated with soils by using water from cave passages in Mammoth Cave National Park rich in actinobacterial mats (Northup and Lavoie, 2001; Rusterholtz and Mallory, 1994; Engel, 2010). Microbial assemblages adapted to wet cave passages maintain abundant diverse microbial communities that function within a normal range and respond to changes in nutrition, moisture, or geochemistry (Engel, 2011; Byl et

al., 2014). However, little research has been conducted on the response of aquatic microbial communities in caves to sublethal concentrations of secondary metabolites. From 2012 to 2014, the U.S. Geological Survey, in cooperation with Mammoth Cave National Park and Tennessee State University, conducted a study with two purposes: (1) to determine if natural antibiotics could be detected in cave water directly in contact with actinobacteria and (2) to determine if sublethal levels of select antibiotics stimulated or inhibited growth and metabolite functions in aquatic cave bacteria. Aquatic microbial communities at six locations in Mammoth Cave rich in actinobacterial mats were subjected to varying doses of antibiotics, and colony growth and community-level physiological capabilities were quantified. This study attempts to address whether secondary metabolic compounds (i.e., natural antibiotics) may have evolved as weapons or communication signals in cave microbial ecosystems. Improving our understanding of cave aquatic microbial ecology and the role of natural antibiotics will provide a greater awareness of the consequences of releasing sublethal concentrations of antibiotics into karst environments.

METHODS AND MATERIALS

Description of Study Sites

Water samples were collected from 6 locations in a small watershed contained within the boundaries of Mammoth Cave National Park, Kentucky, in mid-level passages of the cave. All the sites are located in the Historic Section but have not been part of show-cave tours for more than 40 years. Each sampling site had standing, seeping, or flowing water. A selection criterion for sample sites was the presence of visible colonies of actinobacterial mats (Fig. 1) growing on the cave surface adjacent to each site (Rusterholtz and Mallory, 1994; Barr, 1976). A large, healthy actinobacterial community might release one or more of the secondary metabolites into the surrounding water, possibly exposing some of the local microbial community to trace amounts of natural antibiotics.

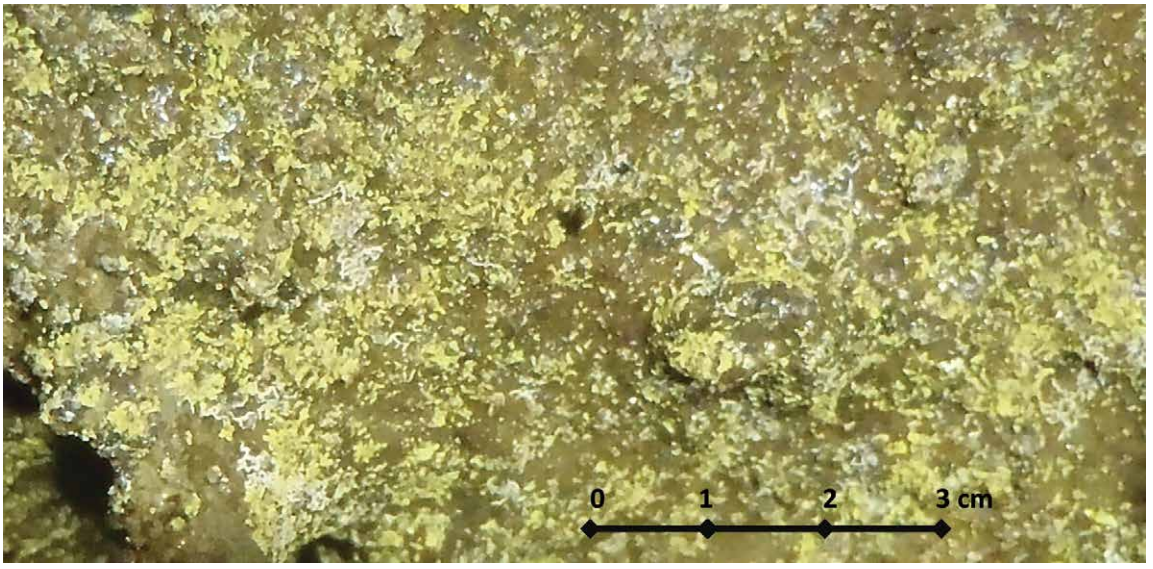

Figure 1. Photograph of white and gold actinobacteria colonies as described by Barr (1976) that were visible in the vicinity of the sampling sites. *White colony* and *Gold colony* labels are below the colonies. The location shown here is a cave wall in the Shaler's Brook area of Mammoth Cave.

Cave sites are traditionally identified by names rather than latitude-longitude coordinates because of overlapping cave passages and the time it takes to accurately survey three-dimensional coordinates into a cave. Following this tradition, the six sampling sites used in this study are Devil's Cooling Tub (DCT), Stagnant Pool (SP; so named by the authors; also known as Lake Purity), Charlotte's Dome (CD), and a cave stream called Shaler's Brook which flows through 3 sampling sites, from Annette's Dome (AD) to Man Cave (MC; so named by the authors) and then Lee's Cistern (LC). Each site along Shaler's Brook is composed of a modest room-sized chamber, separated by 9–15 m of bedrock with Shaler's Brook flowing through a 10–15 cm high bedding-plane opening along the cave floor. The cave surfaces had water seeping down the sides or dripping from the ceiling. During storms, the water volume in Shaler's Brook increased due to storm runoff and rapid infiltration.

The other sites (DCT, SP, and CD) are in separate areas of the cave, away from Shaler's Brook, and they represent independent sampling sites with their own unique hydrology. DCT was formed by water flowing from an opening in the cave ceiling (Fig. 2A), dropping 1.3 m, and eroding a pool in the floor. The ceiling hole was large enough to insert a camera and capture images of the small conduit (Fig. 2B). SP is a small pool of water (4.5 m long $\times$ 2 m wide $\times$ 15 cm deep) in Gratz Avenue with a mean water residence time of 3.5 mo. (Solomon, 2015). The stable water level in the pool is maintained by water slowly seeping in from a nearby breakdown pile. White colonies of actinobacteria (1–2 mm) were floating on the surface at an approximate density of 1 colony ft^{-2} (Barr, 1976). CD is in Brigg's Avenue, is a large chamber with 30 m high ceilings and water persistently cascading down one corner and saturating adjacent walls.

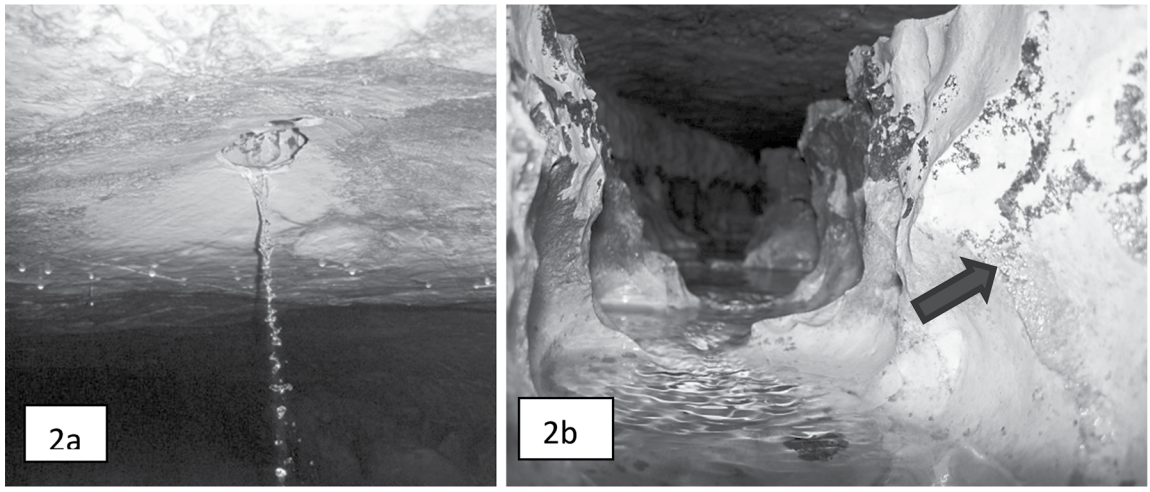

Figure 2. (a) Water was collected as it flowed from the cave ceiling at the Devil's Cooling Tub. The area in the photo is approximately 1 m wide. (b) A hand-held camera inserted into the opening captured this image of water flowing in a small conduit. Note the colonies thought to be actinobacteria on the walls (black arrow). The conduit is approximately 13 cm wide.

Water Sampling and Laboratory Analysis

Water samples for geochemical and microbiological analysis were collected two to three times during the summers of 2012, 2013, and 2014. Grab samples were collected using 250 mL, sterile, clean bottles. The samples were stored at 4–8 °C no longer than 1–2 days until analysis of microbial growth (Byl et al., 2013). In July 2014, seven water samples were collected for antibiotic analysis at DCT, SP, AD, MC (duplicates), LC, and CD, and in August, 2014, three water samples were collected at DCT, SP, and MC. Sampling for antibiotics included filtering 125 mL of water through a 0.7 µm glass fiber filter into pristine 125 mL amber glass bottles with Teflon-lined caps as described by Shelton (1994). Samples were shipped overnight on ice to the U.S. Geological Survey Organic Geochemistry Research Laboratory in Lawrence, Kansas. The laboratory used solid-phase extraction with liquid chromatography-electrospray ionization mass spectrometry capable of accurately identifying and measuring 31 antibiotic compounds down to the nanogram-per-liter level (Meyer et al., 2007). The analytes included chloramphenicol, lincomycin, ormetoprim, trimethoprim, five macrolides, six sulfonamides, six quinolines, four tetracycline antibiotics, six antibiotic degradation products, and two pharmaceuticals, carbamezpine and ibuprofen.

Bacteria Dose-Response Tests

Microbial analysis included bacterial plate counts using 2 % agar (2 g in 100 mL water) augmented with dilute tryptic soy nutrients (10 % tryptic soy agar (TSA)). Previous work by Byl et al. (2014) found karst groundwater bacteria grew better on 10% strength media than on full-strength TSA. Appropriate quantities of sterile-filtered stock solutions of quaternary ammonia compounds (QAC mix composed of 60 % dodecyl dimethyl ammonium chloride and 40 % n-alkyl dimethyl benzyl ammonium chloride), tetracycline, gentamicin, kanomycin, and erythromycin, (Sigma-Aldrich, Inc.) were mixed into the 10 % TSA just prior to pouring the agar into 9 × 50 mm petri plates. Supplementing the 10 % TSA with antibiotics resulted in final nominal concentrations of 0.00 mg L^{-1}, 0.01 mg L^{-1}, 0.10 mg L^{-1}, 1.0 mg L^{-1}, and 10 mg L^{-1} of agar media, with the exception of QAC having nominal concentrations of 0.00 g L^{-1}, 0.07 g L^{-1}, 0.13 g L^{-1}, 0.67 g L^{-1}, and 1.33 g L^{-1} of agar media. There were 3 replicate plates per treatment. Prior to inoculating the plates with 10 µL of raw water from each site, the water samples were shaken for a minute to resuspend the bacteria. The bacteria were evenly spread over the agar using a sterile, bent-glass rod. The plates were labeled, inverted, and placed in an incubator at 25 °C. The bacteria colonies were counted at 1 day, 2 days, and 3 days. The results are reported as colony-forming units per 10 µL. A Student *t*-test was used to determine significant differences between the different antibiotic treatments and the control ($p < 0.05$).

Community-Level Physiology Profiles (Ecolog Plates)

The metabolic capabilities of the microbial communities were characterized using Ecolog plates (Biolog, Inc.) to determine community-level physiological profiles. Each plate had three sets of 31 different substrates and a reference well, and three replicates of each treatment (Stefanowicz, 2006) for a total of 96 microtiter wells per plate. Metabolism of a particular food substrate resulted in a color change of tetrazolium dye that was measured as absorbance readings ($A_{595\ nm}$) after 24 h, 48 h, 72 h, 96 h, and 120 h. incubation at 25 °C. The bacteria inoculum densities were normalized by diluting the individual site water with sterile distilled water to a standard turbidity of one nephelometric turbidity unit. (The normal range of unfiltered cave water was 1–5 units.) Standardizing the inoculum concentration by turbidity, as described in Haack et al. (1995), helped to ensure that observed differences in community-level physiological profiles were primarily because of distinctions in cellular respiration, not differences in initial inoculum concentration (Byl et al., 2013).

Color change data (representative of metabolic capabilities) were interpreted using richness, Gini coefficients (Harch et al., 1997), average well-color development (AWCD) (Stefanowicz, 2006), and principal component analysis (PCA) (Preston-Mafham et al., 2002). Richness is a measure of how many substrates the bacterial communities use after a specified incubation time. The Gini coefficient index is a measure of substrate use that takes into account the number of substrates consumed and how evenly the substrates are used. On a scale of 0 to 1, a Gini coefficient of 0 indicates equal use of all 31 substrates, and a value near 1 implies uneven consumption of a low number of substrates. Displaying the Gini coefficients through time provides a measure of substrate equitability as the microbial community matures. AWCD is a simple way to assess the kinetics of substrate utilization by the microbial community (Stefanowicz, 2006) and takes the average of the 93 substrate wells after compensating with the color in the three reference (blank) wells. The objective of PCA is to reduce the 31 substrates (variables) for each microbial community to identify meaningful variables that distinguish the community or treatment. It does this by identifying substrate-use patterns that distinguish each site, by noting the presence or absence of erythromycin treatment, and by looking for maximum differences in substrate use between the sites with and without erythromycin treatment.

To formally test whether the addition of antibiotics led to shifts in the consumption of particular substrates, multivariate regression analysis was used to isolate the effect of the antibiotic in the 2013 samples. The 2013 Ecolog results were used for the multivariate regression analyses because the timing between storms and sampling events was more uniformly distributed than in the 2012 or 2014 sampling seasons, resulting in more stable results between sampling events. The ordinary least-squares method of regression was used on the well-color development data from the 3 replicates of the 31 substrates both with 0.10 mg L^{-1} of erythromycin and without any erythromycin. For the ordinary least-squares analysis, data from five sampling sites in the cave (AD, CD, DCT, LC, and SP) were used for a total of 930 observations. The statistical method modeled changes in consumption within each substrate (as opposed to between substrates) and modeled the difference between the control samples and the samples treated with 0.10 mg L^{-1} erythromycin. Indicator variables for each sampling site were also included to control for ways in which consumption may vary across areas in the cave. Statistical analysis was performed using Stata statistical software package version 13. Heteroscedasticity-robust standard errors were used and coefficient plots were created using the Coefplot plugin (Jann, 2014). Data used to support the findings in this paper are available from Byl and Byl (2020).

RESULTS

Water Sampling and Laboratory Analysis

Although Mammoth Cave in Kentucky has a large population of actinobacteria growing on the cave walls, very few antibiotic compounds were detected in water samples from each of the six sites. Trace amounts of azithromycin, which is an analog of erythromycin, were detected twice at DCT (July and August 2014) and once at CD (July 2014). The concentration detected in these waters ranged from 0.006 µg L^{-1} to 0.014 µg L^{-1} and was several orders of magnitude lower than concentrations used in this study. Trace amounts of the quinolone antibiotics, ciprofloxacin, enrofloxacin, and ofloxacin, were also detected in August 2014 at DCT (concentration range 0.006 µg L^{-1} to 0.016 µg L^{-1}). No ibuprofen or other analyzed compounds were detected in water samples from any of the six sites. Laboratory and trip blanks had no detected antibiotics. The azithromycin and quinolones detected are each considered synthetic, and additional studies are needed to determine the source of those antibiotics in the cave water.

Bacteria Dose-Response Tests

The number of bacteria colonies growing on the control (0.00 mg L^{-1}) agar plates varied over the three-year sampling period for each site, possibly because of varying meteorological influences during each sampling seasons. For example, the three-year average colony count of SP water without any antibiotic augmentation was 291 colony-forming units per 10 µL, with a standard deviation of 281. All of the sites experienced large standard deviations in bacteria counts over the three-year study period. The large standard deviations in the three-year averages masked statistically significant differences between treatments. However, there were significant differences between many treatments and the controls within a single sampling event. Thus, dose-response figures (Fig. 3A–E) are shown as individual sampling events, and significant differences are noted. To save space and avoid redundancy, we limit the figures to one dose-response graph for each antibiotic. The figures are representative of the general pattern observed in the other dose-response assays for the same antibiotic.

Increasing concentrations of QAC (Fig. 3A), gentamicin (Fig. 3B) and kanamycin (Fig. 3C) mixed into the agar media generally elicited a decrease in the number of colony-forming units as compared to the control treatment with no antibiotic added. This response to QAC, gentamicin, and kanamycin was typical for all of the sites with the exception of kanamycin occasionally stimulating colonies for samples collected in 2013. The microbial response to media augmented with tetracycline and erythromycin was typically an increase in colony-forming units as concentrations increased to 0.1 mg L^{-1} or 1.0 mg L^{-1} and then a significant drop at 10 mg L^{-1} (Fig. 3D–E). The exception to this pattern was observed

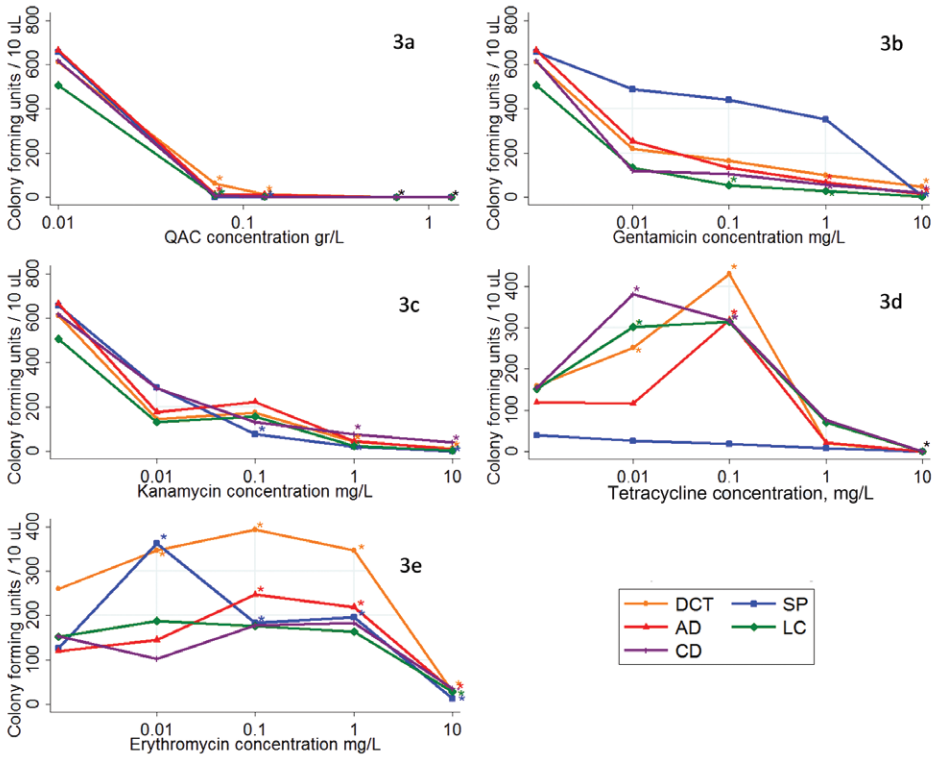

Figure 3. Dose response of bacteria from 5 locations in Mammoth Cave, each plotting the number of colony-forming units per 10 µL against increasing doses of (a) QAC, (b) gentamicin, (c) kanamycin, (d) tetracycline, (e) erythromycin. Different lines on each graph represent different cave locations. The cave locations are the Devil's Cooling Tub (DCT), Stagnant Pool (SP), Annette's Dome (AD), Lee's Cistern (LC), and Charlotte's Dome (CD). *Asterisks* indicate significant differences from the control ($p < .05$, Student *t*-test).

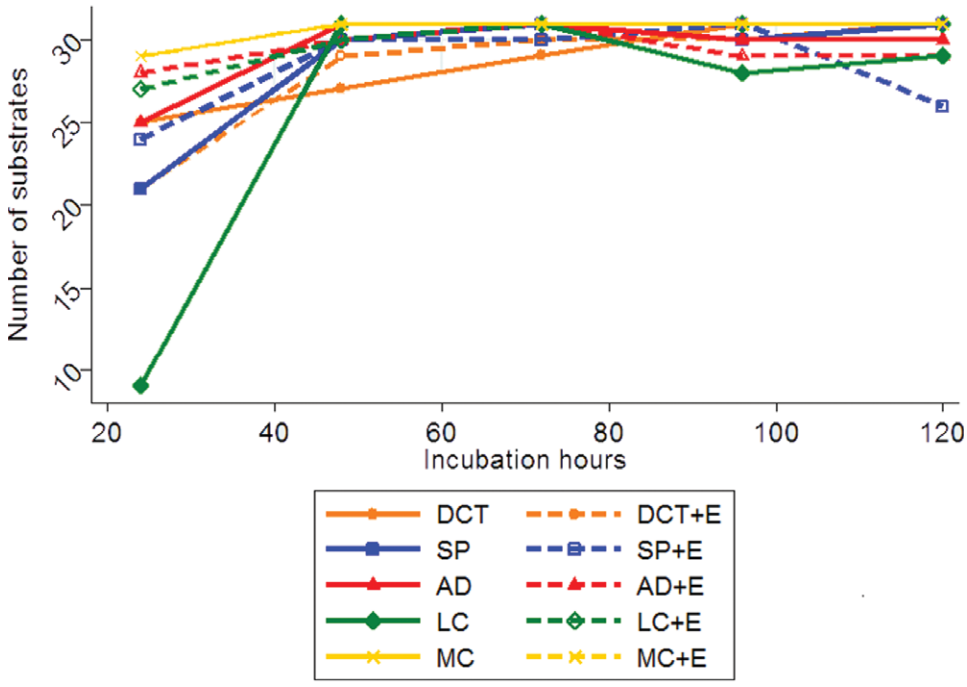

Figure 4. Richness of substrate use by the microbial communities from Mammoth Cave with and without added erythromycin (dashed and solid lines, respectively) from five locations in Mammoth Cave. The number of substrates used is plotted against time of incubation. The cave locations are the Devil's Cooling Tub (DCT), Stagnant Pool (SP), Annette's Dome (AD), Man Cave (MC), and Lee's Cistern (LC). +E indicates 0.1 mg L^{-1} erythromycin dose.

for samples from the SP site. The numbers of bacteria colonies from SP were relatively unaffected by 0.01 mg L^{-1} to 1.0 mg L^{-1} doses of tetracycline and erythromycin with the rare exception when 0.01 mg L^{-1} of erythromycin increased colony-forming units in August 2014 (Fig. 3E).

Community-Level Physiology Profiles (Ecolog Plates)

Based on the dose-response studies, it was noted that erythromycin, a macrolide secondary metabolite produced by actinobacteria, gave the most consistent hormesis response of the antibiotics tested. It was therefore selected as the antibiotic treatment for the Ecolog community-level physiology profile studies. Bacteria exposed to 0.10 mg L^{-1} concentrations of erythromycin often had a slight increase in richness (mean number of substrates consumed) over the untreated samples in the first 24 hours (Fig. 4). The ability to use a larger number of substrates in the first 24 hours would provide a slight competitive advantage at the beginning of the growth phase. However, any erythromycin-induced increase in richness was no longer noticeable after 24 hours when all the microbial communities and treatments generally used 27 or more of the 31 substrates (87 %).

Exposing the bacteria to 0.10 mg L^{-1} erythromycin resulted in lower Gini coefficients than the coefficients for the untreated samples from AD, LC, and DCT (Fig. 5). Erythromycin raised the Gini coefficient for samples from SP and CD. The lower Gini coefficient values at AD, LC and DCT implied a greater evenness of substrate use for microbial communities upon exposure to 0.10 mg L^{-1} erythromycin. Higher Gini coefficients for the erythromycin-treated samples from SP and CD indicated that sublethal erythromycin induces uneven consumption of a lower number of substrates. The

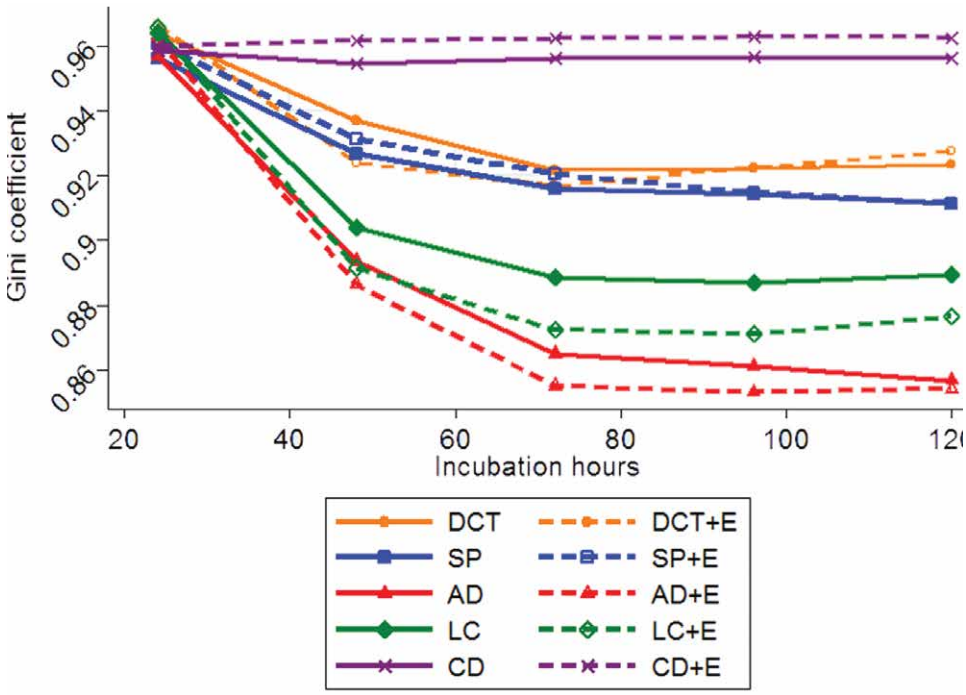

Figure 5. Gini coefficient through time for microbial communities from five locations in Mammoth Cave with and without erythromycin (dashed and solid lines, respectively). Gini coefficient is plotted against time of incubation. The cave locations are the Devil's Cooling Tub (DCT), Stagnant Pool (SP), Annette's Dome (AD), Lee's Cistern (LC), and Charlotte's Dome (CD). +E indicates 0.10 mg L^{-1} erythromycin dose.

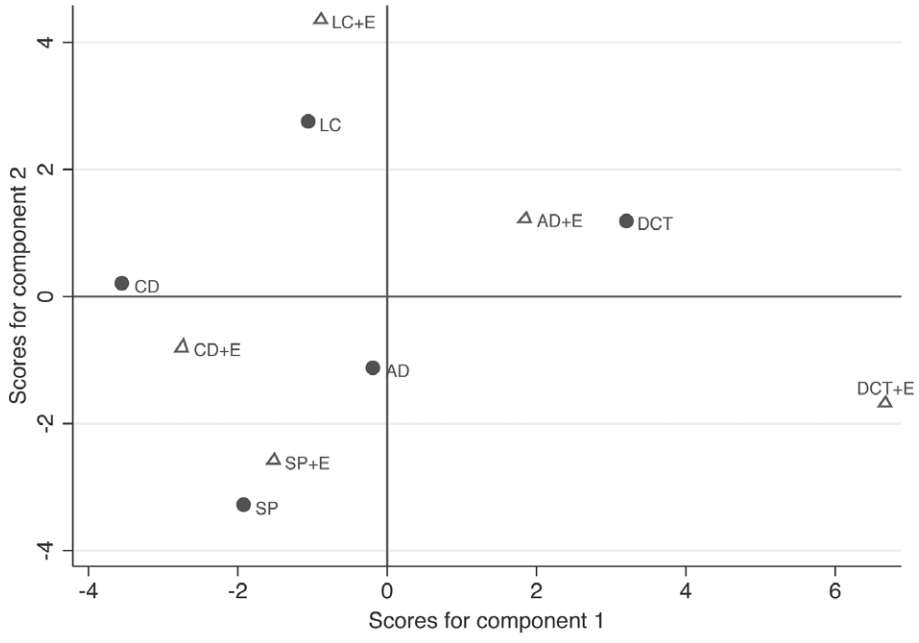

Figure 6. Principal component analysis of bacteria from five locations in Mammoth Cave. The Ecolog assay was run with and without 0.10 mg L^{-1} erythromycin (solid circles and open triangles, respectively). Scores for component 2 are plotted against scores for component 1. The cave locations are the Devil's Cooling Tub (DCT), Stagnant Pool (SP), Annette's Dome (AD), Lee's Cistern (LC), and Charlotte's Dome (CD). +E indicates 0.10 mg L^{-1} erythromycin dose.

tendency for Gini coefficients to drop over the first three days in the samples treated with 0.10 mg L^{-1} erythromycin was consistent within all the data with a few exceptions. Approximately a third of the time, Gini coefficients would level off after 96 hours (Byl et al., 2013). Leveling off of the Gini coefficients implied the microbial communities reached a steady state for substrate consumption. Here the term steady state means the microbial community settled upon which of the 31 substrates they used and which they did not use.

The presence of 0.10 mg L^{-1} erythromycin in the inoculum slightly increased or had no effect on the average well-color development (AWCD) compared to the untreated samples (Fig. 6). In most cases, the rate of substrate use as indicated by color development through time was best illustrated with a sigmoidal curve that showed the lag phase (initial portion of curve), growth phase (steep slope of curve) and stationary phase (asymptote). However, the difference in AWCD curves between samples with and without erythromycin treatments for each cave site was minimal, making it difficult to assign metabolic benefits from sublethal erythromycin. The apparent similarity may be due to the oversimplification of the AWCD plot, which averages all 31 substrates.

The richness, Gini coefficients, and AWCD results indicate that there is a change in substrate use as a result of augmenting the cave waters with 0.10 mg L^{-1} erythromycin. Principal component analysis (PCA) was used to reduce the multivariate data set, which included multiple sites, 2 erythromycin treatments, 31 substrates, and 5 absorbance readings through time, into a small number of principal components that in turn accounted for the variation in substrate use between sites and by erythromycin treatment. The 24-hour absorbance data were used for PCA (Fig. 6) because the antibiotic-induced colony stimulation occurred within 24–48 hours. Any metabolic activity that contributed to the erythromycin-induced growth should manifest itself in the first 24 hours. The

substrates were organized into categories (carbohydrates, carboxylic acids, polymers, and miscellaneous) for ease of interpretation (Preston-Mafham et al., 2002). Organizing the substrates into categories provided a simple way to scan the data for metabolic patterns associated with different food groups.

The *x* axis in Figure 6 represents the 1st principal components (PC1), accounting for 39 % of the variance in substrate use. For PC1, there were positive loadings on xylose, glucosaminic acid, erithritol, and threonine, and negative loadings on asparagine, glycerol phosphate, pyruvic acid, and malic acid. The *y* axis in Figure 6 represents the 2nd principal components (PC2), accounting for 33 % of the variance in substrate use. For PC2, there were positive loadings on hydroxybutric acid, pyruvic acid, asparagine, and serine. There were high negative loadings on phenylethyl-amine, acetyl glucosamine, methyl glucoside, and polysorbate 80 (Tween 80). Closer proximity of the points on the *x* and *y* axes indicates a tendency for comparable substrate use patterns. As indicated by their relative positions on the *x* and *y* axes in Figure 6, there is a noticeable difference in substrate use between the sites and the presence or absence of erythromycin treatment. For three of the five microbial communities incubated with 0.10 mg L^{-1} erythromycin, there was an increase in PC1 (*x* axis) relative to the untreated communities for each site. The erythromycin-induced shift on the *x* axis was small for LC. The largest shift in response to erythromycin was for the DCT samples with a shift to the right on the *x* axis, indicating a greater difference in substrate preferences. The unique metabolic response by each microbial community with or without erythromycin noted in the PCA results is probably a reflection of unique microbial populations at each sampling site. Other studies using a single species (as opposed to the community level used in this study) found sublethal concentrations of antibiotics influenced gene expression or stimulated certain metabolic functions (Dietrich et al., 2008; Dandekar et al., 2012; Oslizlo et al., 2014; Vaz Jauri et al., 2013). Likewise, erythromycin stimulated or inhibited biochemical processes within the different microbial populations from each cave sampling site community. Although PCA was useful in showing there were differences in substrate use, additional statistics were needed to assess substrate-use similarities in response to exposing the microbial communities to 0.10 mg L^{-1} erythromycin.

The Ecolog data from each site were combined to compare the use of 31 substrates between the control and 0.10 mg L^{-1} erythromycin treatment using the ordinary least-squares regression model. The absorbance readings ($A_{595\ nm}$) of the untreated controls are normalized to zero and the increase or decrease in substrate consumption induced by 0.10 mg L^{-1} erythromycin (as measured by absorbance, $A_{595\ nm}$) is shown relative to the controls (Fig. 7). Results indicate that the erythromycin stimulated a statistically significant increase in consumption of 3 substrates: acetyl glucosamine, arginine, and putresceine. The absorbance increased by 0.064 absorbance units (standard error 0.037) in the acetyl glucosamine replicates compared to the controls without erythromycin (control average $A_{595\,nm}$ = 0.047, stan-

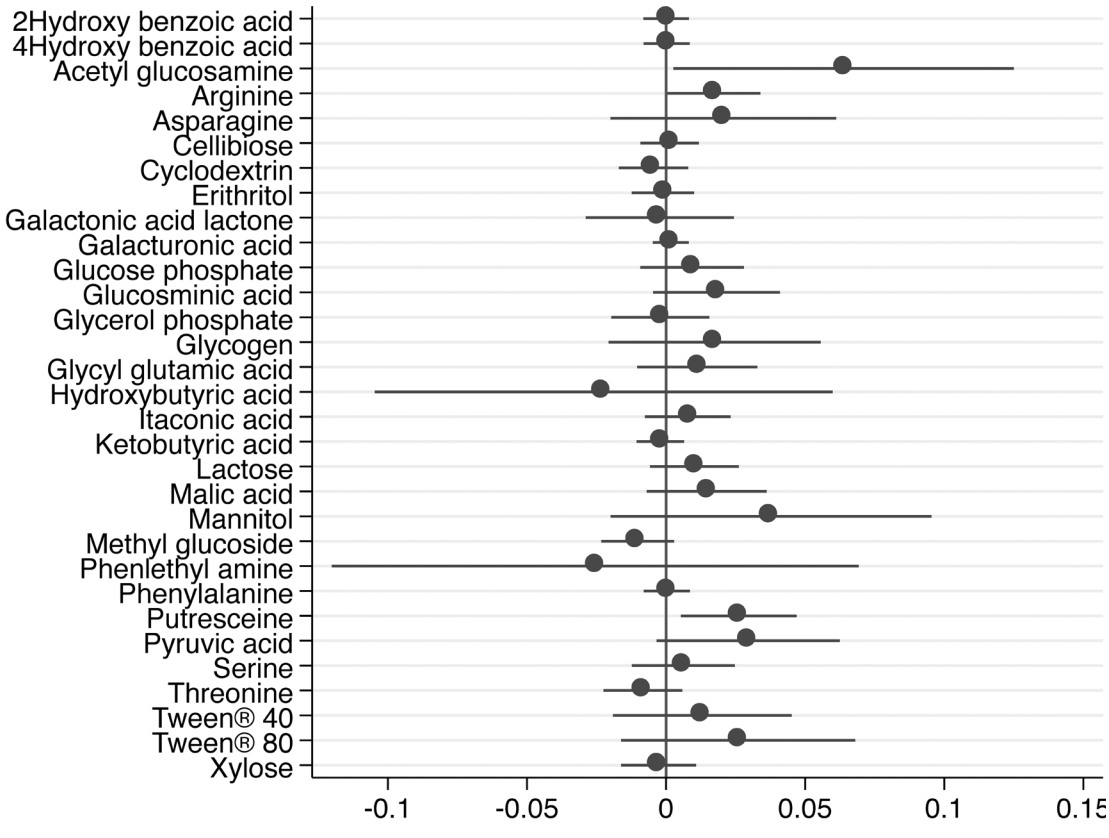

Figure 7. Regression coefficient plot showing the change in substrate consumption at 24 hours due to erythromycin after controlling for the different sampling sites (AD, CD, DCT, SP, and LC) and fixed effects for each substrate. The coefficients (dots with 95 % horizontal confidence interval bars) show the estimated change in each substrate use from adding 0.10 mg L^{-1} erythromycin compared with the control of no added erythromycin (center line at 0); n = 930; R^2 = 0.33.

dard deviation 0.104). Absorbance values of the arginine wells increased by 0.017 ($A_{595\,nm}$ = 0.056, standard error 0.010) when exposed to erythromycin relative to a control mean of 0.039 $A_{595\,nm}$ (standard deviation 0.038). Absorbance values for putrescine increased by 0.026 absorbance units (standard error 0.013) when exposed to erythromycin relative to a control mean of 0.044 $A_{595\,nm}$ (standard deviation 0.042). Based on the absorbance values, erythromycin is estimated to have had positive and negative effects on consumption of other substrates (Fig. 7), but these other effects were not statistically significant ($p > .05$). Sampling sites tended to have statistically significant differences from each other with consumption lower at CD ($p < .01$) and SP ($p < .01$) than at AD, and consumption was higher at DCT ($p = .04$) than at AD. There was no statistical difference in consumption between LC and AD.

CONCEPTUAL MODEL AND CONCLUSIONS

The goals of this investigation were to determine if natural antibiotics could be detected in cave water and to evaluate the effect of sublethal concentrations of antibiotics on growth and metabolism of cave bacteria. Cave sampling sites were selected based on the visual presence of water and microbial communities including actinobacterial mats. Analysis of the cave water found trace amounts of antibiotics that appear to be from an unknown anthropogenic source. Antibiotic dose-response assays show that bacteria collected from certain sites exposed to increasing concentrations of erythromycin elicited a classic hormesis growth response. Metabolic studies using Ecolog plates found cave bacteria incubated in water containing 0.10 mg L^{-1} erythromycin increased the number of substrates (richness) metabolized by the indigenous microbial communities in the first 24 hours. This increase in substrate richness agrees with the findings of Vaz Jauri et al. (2013), where they noted use of more substrates by soil *Streptomyces* after exposure to sublethal concentrations of antibiotics. The community-level physiological profile varied from site to site indicating variations in the microbial communities at different sampling locations in the cave, which agrees with the diversity found by Rusterholtz and Mallory (1994) in their Mammoth Cave study. Antibiotic stimulation of gene expression observed in previous molecular studies (Anderson and Hughes, 2014; Davies, 2006; Linares et al., 2006) may include some of the enhanced catabolic pathways we observed in the first 24 hours.

Our study suggests that natural antibiotics may play multiple roles in cave microbial ecosystems as a response to evolutionary pressure. For example, erythromycin might function as both a growth signal and a weapon for cave microorganisms. In laboratory studies, production of erythromycin and other secondary metabolites by actinobacteria is triggered once the colonies have matured and start to form spores (Bibb, 2005). The production of toxic secondary metabolites during spore development may have evolved as a weapon to prevent predation of the spores by other microorganisms, to inhibit competitive growth of the spores in close proximity to the parent colony, to prevent other microorganisms from utilizing the same resources, or as a way to kill susceptible bacteria in close proximity for food. These antibiotics-as-weapons theories explain why high concentrations (10.00 mg L^{-1}) of erythromycin inhibited growth but do not explain the observed stimulation effect at low concentrations.

We propose a conceptual model that combines three microbial ecology models, the cue model, the anticipatory regulation model, and the intermediate antibiotic production model (Fig. 8). The cue model, proposed by Oliveira et al. (2015), describes how antibiotics stimulate biofilm development. In the cue model, antibiotics are not secreted to signal coordinated growth, rather, they are secreted to attack competition but inadvertently serve as a cue to other microorganisms at low concentrations. The anticipatory regulation model proposed by Mitchell et al. (2009) states that microorganisms can associate an environmental stimulus with an appropriate response to prepare for the future environment. In this particular case, sublethal erythromycin would be the

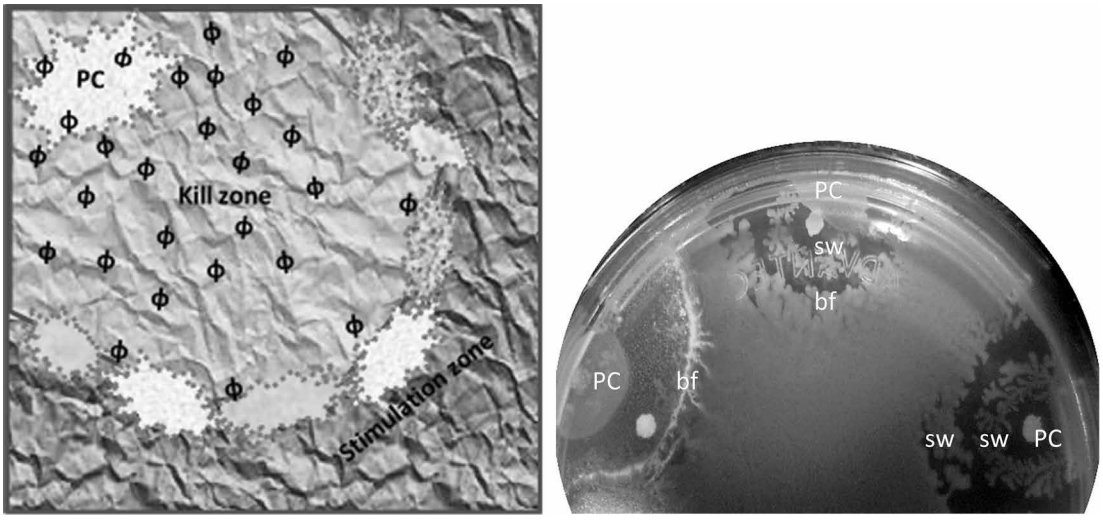

Figure 8. Conceptual model (left) and photo depicting model growth on agar plate (right). Secondary metabolites (natural antibiotics, *Φ*) diffuse from the parent colony (PC) and the concentration transitions from a high, biostatic/lethal concentration (Kill zone) to a low, sublethal concentration (*Stimulation zone*). Stimulated growth includes microbial biofilm (bf) and swarming (sw).

cue or stimulus, and the anticipated response would be stimulation of cell division and certain metabolic pathways in the preparation of substrates released from dying microorganisms. In the anticipatory regulation model, the release of substrates by dying susceptible microorganisms in the antibiotic kill zone is anticipated by bacteria in the sublethal zone before the substrates reach them, thus stimulating growth in the sublethal zone. Evolutionary selection for this genetic trait would provide an advantage because bacteria that were primed by the cue would be ready to metabolize the substrates released by dying bacteria and avoid the lag time characteristic of waiting to encounter the substrates before activating the metabolic genes (Cooper, 2009). The intermediate antibiotic production model developed by Gerardin et al. (2016) shows that optimum antibiotic production is achieved by selection for moderate antibiotic production. They demonstrate how bacteria cells capable of releasing intermediate concentrations of antibiotic compounds may maximize available nutrients for producers while reducing unintended benefits for antibiotic-resistant bacteria.

The proposed anticipation-cue conceptual model depicts sublethal concentrations of erythromycin as a cue, stimulating specific metabolic pathways in anticipation of substrates released by dying bacteria and providing an ecological advantage by reducing the response time. In our studies, multivariate regression analysis showed that exposure of cave bacteria to sublethal erythromycin stimulated a significant increase in consumption of acetyl glucosamine, arginine, and putrescine in the first 24 hours. Acetyl glucosamine is a major component of microbial cell walls, and its release from dying microorganisms in the antibiotic kill zone would be typical. Arginine has been shown to revitalize non-culturable bacteria by stimulating cell growth and division (Tonon and Lonvaud-Funel, 2000). The ability of non-culturable (dormant) bacteria to activate the arginine metabolic pathways in anticipation of arginine release would provide a time advantage over bacteria waiting until the nutrients appeared to initiate gene expression. Likewise, putrescine is a polyamine essential to cell growth and biofilm development (Wortham et al., 2010). Classic microbial responses to antibiotics are biofilm development and swarming (Oliveira et al., 2015; Andersson and Hughes, 2014). If exposure to sublethal erythromycin simply caused stress and higher metabolic rates, then an increase in use of other substrates in the Ecolog test, like glucose-6-phosphate, pyruvate, and malate, which feed directly into the energy-producing tricarboxylic acid cycle pathway, would be expected. However, that did not occur.

The significant increase in use of acetyl glucosamine, arginine, and putrescine 24 hours after exposure supports the hypothesis that sublethal erythromycin served as a cue for the anticipated release of these substrates. Cave bacteria that evolved a genetic link between the probable availability of these 3 substrates with sublethal antibiotics would have an ecological advantage over bacteria that waited for the arrival of the released substrates to turn on their metabolic pathways. Unfortunately, it was beyond the scope of this study to establish whether sublethal erythromycin elicited the anticipatory response at the molecular level. Promotion of acetyl glucosamine-, arginine-, and putrescine-related genes by sublethal erythromycin, especially in the absence of these three compounds, would provide strong molecular support of the anticipatory model. Continued efforts to identify and characterize the distribution and concentration of secondary metabolites (antibiotics) in natural habitats are also essential for determining the functions of antibiotics in microbial ecology. In conclusion, the results provided in this paper suggest that the microbial community of Mammoth Cave may have evolved concentration-dependent ecological roles associated with certain antibiotics.

DISCLAIMER

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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