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Screening Environmental Bacteria For Naturally Occurring Antibiosis: Identifying Producers And Their Compounds

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Abstract

The rapidly escalating problem of antibiotic-resistant bacterial pathogens threatens to limit the clinical treatment of infectious disease, as many bacteria have evolved resistance to overprescribed and improperly used antibiotics. Alarmingly, the rate of increasing resistance far exceeds the current rate of development for new treatments. One solution to this problem may, quite literally, lie at our feet. Soil bacteria are recognized as one of the richest sources of naturally produced antibiotic compounds and produce many antibiotics currently in clinical use. In the current study, bacterial strains are cultured from various plant-, rhizosphere- and aquatic- environments throughout Western North Carolina and screened for antibiotic production using a high-throughput antagonism assay against *Staphylococcus aureus* and *Escherichia coli*. Within natural soil, microbes exist in complex, mixed-species communities, and recent research revealed that interspecies communication and competition may drive antibiotic production. Thus, we additionally screened bacterial co-cultures—pairwise combinations of soil bacteria—to activate cryptic biosynthetic gene clusters and enhance chemical diversity for drug discovery. To date, 184 bacterial isolates have been isolated, purified, and screened for antibiotic production with 9% exhibiting antibacterial activity against either a Gram-positive or Gram-negative bacterial agent. Bacteria not capable of antibiotic production in pure culture were tested in pairwise combinations for induced antibiotic production. We tested a total of 3,292 possible co-cultures and found several combinations of interest. However, we were unable to replicate the results in follow-up testing. Additional characterization of isolates of interest included 16S rDNA sequencing for phylogenetic identification, as well as an examination of soil properties that may have influenced our findings.

Keywords: bacteria, co-culture, antibiotic

1. Introduction

Antibiotic resistance poses an ever-increasing threat to global public health.[1,2,3] We have relied heavily on antibiotic medicines for the last half-century to treat disease, protect food supplies, and reduce overall exposure to known pathogens in the name of protecting human health.[2] However, heavy advertising by the pharmaceutical industry led to patient pressuring of doctors for inappropriate prescription and/or overprescription of broad-spectrum antibiotics.[4] Industrialized food production uses antibiotics to diminish loss to disease during processing, with the effect of those medicines eventually reaching the human population.[5] Unnecessary use of antibiotics in consumer household products additionally contributes to a systemic overexposure to these medicines and thus fosters the emergence of antibiotic resistant bacteria.[6,7] As a result, we now see widespread resistance to nearly all clinical antibiotics which formerly provided effective treatment.[1,2,6,8]

To combat the rise of antibiotic resistance, renewed interest in antibiotic drug discovery is needed. However, many pharmaceutical companies have cut funding in this area, as antibiotic treatments tend to be less profitable and require substantial investments of time.[9] A large portion of current research falls to universities.[9]

We may be able to make use of advances in microbial ecology to find a solution to this problem. One recent area of research is microbial co-cultures. In a natural setting, microbes live in mixed cultures where they face ecological competition and the threat of limited resources.[10, 11] The stress of nutrient limitation drives the production of antibiotic compounds as a combative strategy.[10, 12, 13, 14] Thus, expression of genes for antibiotic production may remain silent under monoculture conditions, with production occurring only during mixed-culture growth.[15] Many studies have used soil as a microbial source, since soil contains both high density and diversity of bacteria.[13, 16] Tyc et al. found that interactions had a substantial influence on antibiotic production in soil bacteria, with induced antibiosis in 6% of all tested combinations.[13] Novel compounds produced in such interactions can potentially be applied to the existing problem of antibiotic resistance in human pathogens.

We patterned our study after Tyc *et al*. using a high throughput assay with environmentally-sourced bacteria from Western North Carolina. We sampled soils at a variety of locations ranging from an urban stream bed to pristine wilderness and included two soil samples from outside the region. Concurrent research provided aseptically collected fluid from within wild *Sarracenia* pitcher plants, allowing us to include environmental bacteria from non-soil sources. By co-culturing bacteria from disparate sites, we expected to see an equal or perhaps elevated measure of antibiosis in the face of foreign competition. We measured soil characteristics in order to quantify the degree of difference between environments. We predicted that organic carbon content of soil samples would correlate directly with microbial abundance since that carbon acts as a food source for microbial life. We predicted an inverse correlation between carbon content and antibiotic production, since the reduced carbon would foster greater competition for resources. Given the high level of biodiversity that exists throughout the Appalachian region, we hoped to identify bacterial isolates capable of producing novel antibiotics for the prevention of disease, whether in pure culture or induced through co-culturing.

2. Methodology

2.1. Sample Collection And Isolation Of Bacteria

Using aseptic collection techniques, soil samples were gathered from a variety of regional sites, including an urban stream bed (RALPH), a compost pile (RGC), underneath *Sphagnum* moss (MOSS), near a waterfall (FALLS) and on a forested hillside (HILL). For each site, 0.5 g of soil was suspended in sterile, deionized water by vortexing for 3 min. A series of tenfold dilutions (100%, 10%, 1%, 0.1% and 0.01%) were made for each sample and spread onto 1/10 tryptic soy agar media (1/10 TSA; 3 g Difco Tryptic Soy Broth per liter solidified with 15 g Difco Agar) to simulate the low nutrient environment in soil. Plates were incubated at 25°C and individual colonies, representing all unique colony morphologies, were harvested from the plates as they appeared. Colonies were further isolated by quadrant streaking on 1/10 TSA to ensure purity.

Concurrent research provided three samples of fluid harvested from within wild pitcher plants (*Sarracenia spp.*) found in Western North Carolina. Two dried soil samples from outside the Appalachian region were also tested, however sterile collection techniques were not guaranteed. An identical dilution sequence and plating process was used to isolate bacteria from these additional sources.

2.2. Assessing Antibiotic Production By Pure Cultures

Because the ultimate goal was to identify antibiotic production induced by interaction, each isolate was first tested in pure culture for the production of antibiotics against both a Gram-positive target, *Staphylococcus aureus*, and a Gram-negative target, *Escherichia coli*. We used one of two methods for testing for antibiotic production by pure cultures: spread plates or soft agar overlays.

Antibiotic production was identified by the appearance of a zone of inhibition surrounding a spot of bacterial culture placed onto a field of the target organism (Figure 1). Isolates showing solo production were eliminated from further testing, as were isolates that showed excessive motility that could impair interpretation of results.

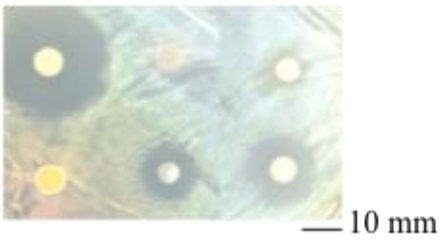

Figure 1. Various environmental isolates cultured on 1/10 TSA against a field of *S. aureus*. Several isolates are capable of antibiotic production in pure culture, apparent by the zone of inhibition surrounding the colony. The isolate at bottom left does not produce an antibiotic that affects the target.

2.2.1. *spread plate technique*

We transferred a single colony of the target bacteria into a test tube with 3-4 ml of sterile 1/10 Tryptic Soy Broth (1/10 TSB) and placed the test tube under agitation in a room-temperature water bath, allowing the culture to grow overnight. Environmental isolates for testing were cultured in an identical manner. An aliquot of 60-100 µl of the target organism was transferred to a 1/10 TSA plate, either 100 mm or 150 mm in size, and spread evenly with a sterile spreader bar to provide a bacterial lawn for antagonism. The spread plate was allowed to dry before spotting with 2 µl of the environmental isolate, making three replicates of each.

2.2.2 *soft agar overlay technique*

As above, both target organisms and environmental isolates were cultured overnight in 1/10 TSB. Prepared 1/10 TSA plates were warmed to 37°C. Individual polyethylene tubes were prepared with 4 ml of soft agar (solidified with 0.6% agar) in each and held at 50°C before adding 100 µl of the target culture, vortexing for 1 second and pouring over the warmed 1/10 TSA plates, swirling to ensure even distribution. Once the overlay agar had set and cooled, plates were spotted as above with 2 µl of a cultured environmental isolate to test for antibiotic production against the embedded target.

2.3. Testing For Co-Culture Production Of Antibiotics

A 96-well plate was used to create a master plate containing all of the environmental isolates by suspending a single colony of each environmental isolate in 150 µl of sterile 1/10 TSB. A known antibiotic producer was included in the array as a positive control, while other wells were left empty to serve as a negative control. This master plate was incubated overnight at 25°C. The master plate was replicated by transferring 20 µl from the wells of the master into a fresh 96-well plate and supplementing with an additional 180 µl of sterile 1/10 TSB. The replicated master plates were subsequently incubated overnight at 25°C. At this point, each well contains only a single isolate, each different.

Additional 96-well plates were used to combine each environmental isolate with all the others from the master array (1:1). To do this, all wells were loaded with 45 µl of a single environmental isolate to be tested. From the master plate, 45 µl was extracted using a multipipette tool from each well and transferred to the corresponding well containing the environmental isolate. At this point, the plate contains two isolates, with one being the environmental isolate to be tested, identical across the plate, and the other being one from the master array, each different (Figure 2).

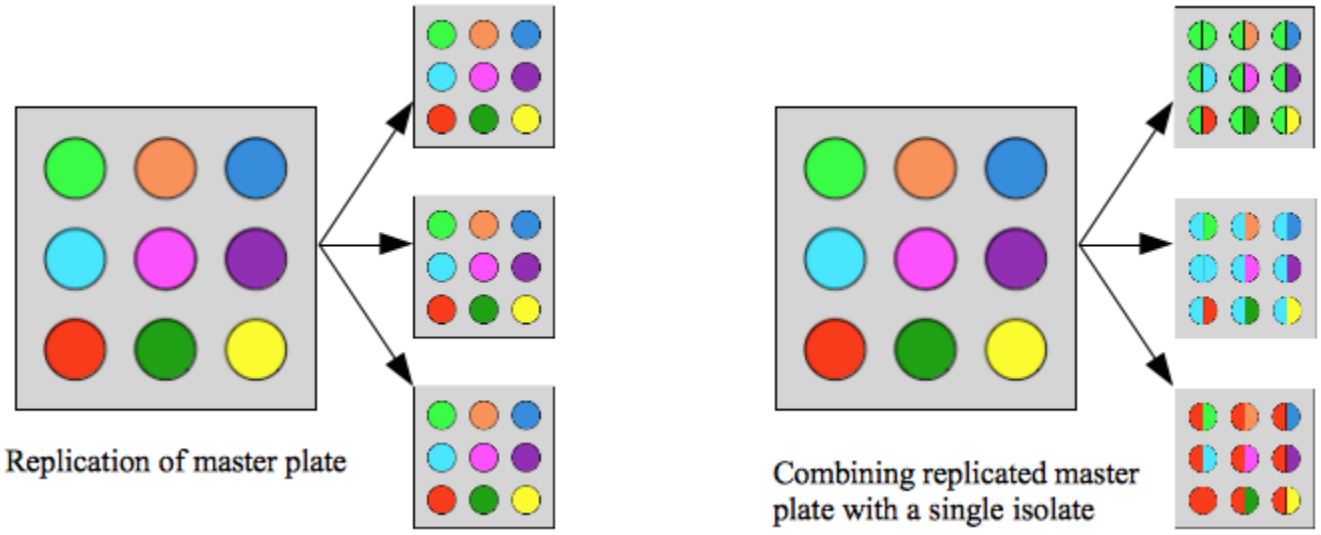

Figure 2. A 96-well master plate (simplified here to 9 wells) containing each isolate was replicated multiple times before combining the array contained in the master plate with a 96-well plate containing a single organism. This process allowed us to ensure that each isolate was screened against all others.

From the combined 96-well plate, the array was transferred, using a pin replicator, onto a prepared spread plate of a target organism, either *S. aureus* or *E. coli*. Plates were incubated at room temperature and examined daily for up to three weeks using backlighting and magnification to look for zones of inhibition.

Bacterial combinations noted to produce zones of inhibition were retested using the soft agar overlay technique to verify results. In retests, 2 µl of each pure culture was spotted alongside 2 µl of a combined culture, with three or more replicates of each.

2.4. Phylogenetic Identification Of Bacteria Of Interest

Isolates of interest were identified to the genus level via PCR amplification and DNA sequencing of the 16S rDNA region. DNA was extracted using the 5Prime ArchivePure DNA extraction kit, following the protocol for Gram-positive bacteria. The genomic DNA was then used as the template in a PCR using universal bacterial 16S primers 27F and 1429R.[17] Reactions consisted of OneTaq Hot Start Master Mix (New England Biolabs), 1 µM each of forward and reverse primers, and 100 ng of template DNA. Thermocycler settings were 5 min at 95 ºC, followed by 30 cycles of 30 sec at 95 ºC, 30 sec at 50 ºC, and 90 sec at 72 ºC. Gel electrophoresis was used to ensure the presence of ~1450 base pair product before sending the product out for DNA sequencing through GeneWiz. PCR products were sequenced in both directions, sequences were merged and subsequently compared to known sequences using the online National Center for Biotechnology Information's Basic Local Alignment Search Tool for 16S ribosomal DNA sequences.[18] Isolates were identified to the genus level wherever possible.

2.5. Soil Testing

Soil properties were examined to gain a better understanding of the microbial environment in situ. At the time of original collection in each location, a separate and larger sample was acquired without concern for sterility. Each was air-dried and sieved to 2 mm prior to testing for texture, pH and organic carbon content as factors that might influence microbial growth.

2.5.1. *soil texture*

To determine soil texture, approximately 50 g of soil was mixed with 100 ml of 5% sodium hexametaphosphate to separate soil particles; this was mechanically stirred at low speed for 5 min to mix thoroughly. The fluid was

transferred into a 1000 ml graduated cylinder which was then filled with deionized water. A control solution was created using 100 ml of 5% sodium hexametaphosphate, 900 ml of deionized water, and no soil. Parafilm was applied to the top of each graduated cylinder before inverting to suspend the soil particles.

A hydrometer was used to find the relative density at 30 and 60 seconds after suspension, when only the sand particles would have settled out. Another hydrometer reading was taken after 90 min, once smaller particles also had a chance to settle, and again after 24 hours. Temperatures were recorded along with the relative density. Readings were adjusted to account for the reading from the control solution (subtract the control reading), and for a difference in temperature between the two reading times (± 0.36 per ºC difference from 20ºC).

The percent of soil separates was determined using the following formulas. Calculations were completed for each reading, with the final value for each soil fraction being determined by an average of the 30 sec and 60 sec results for sand, and an average for the 90 min and 24 hr results for clay.

$$\%\ \text{sand} = 100 - \frac{[\text{30 sec or 60 sec hydrometer reading (corrected)} \times 100]}{\text{sample weight}}$$

$$\%\ \text{clay content} = \frac{\text{90 min or 24 hr hydrometer reading (corrected)} \times 100}{\text{sample weight}}$$

$$\%\ \text{silt content} = 100 - (\text{averaged \% sand} + \text{averaged \% clay}).$$

2.5.2. *soil pH*

To determine soil pH, 5 g of soil was mixed with 5 ml of distilled water, stirred and allowed to equilibrate for 30 min. The pH of each sample was then measured with a digital pH meter using a standard electrode.

2.5.3. *soil organic carbon*

Soil organic carbon was measured using the loss-on-ignition method.[19] Briefly, 15-ml crucibles were weighed empty and filled with 5 g of soil. Samples were heated to 105ºC for one hour, cooled in a desiccator and weighed. Samples were combusted at 360ºC in a muffle furnace for two hours, followed by an additional hour at 105ºC, cooled in a desiccator and re-weighed. Loss-on-ignition was calculated as follows:

$$\text{oven dry weight} = 105ºC\ \text{weight} - \text{crucible weight}$$

$$\text{weight after combustion} = 360ºC\ \text{weight} - \text{crucible weight}$$

$$\%\ \text{soil organic carbon} = \frac{\text{oven-dry weight} - \text{weight after combustion}}{\text{oven-dry weight}} \times 100$$

2.6. Testing For Resistance To Other Targets

Additional follow-up work included re-testing select single producers against an expanded array of targets, including *Pseudomonas fluorescens* and *Micrococcus luteus*, using the soft agar overlay technique.

3. Data and results

3.1 Bacterial Testing

September sampling included three fresh soil samples, two air dried soil samples, and three liquid extracts from *Sarracenia* plants. Fresh samples included soil taken from an urban Asheville stream bed (RALPH), alongside the University garden compost pile (RGC), and underneath a bed of sphagnum moss in the Shining Rock Wilderness (MOSS). Dried samples were donated and described as coming from an orchid garden (POG) and a wetland (PW)

from outside the Appalachian region. *Sarracenia* phytotelmata extracts, identified as 023, 053 and 083, were obtained from natural populations of pitcher plants in an undisclosed location in Western North Carolina. From these samples, a total of 107 bacteria were isolated.

Of these 107 isolates, 10% (11 isolates) were identified as capable of antibiotic production in pure culture, and thus eliminated from co-culture screening. Other isolates were eliminated because they proved excessively motile under assay conditions (23 isolates), or were eliminated for other reasons (4 isolates). The remaining 69 bacterial isolates were advanced to co-culture screening, during which they were mixed in all possible pairwise combinations and screened for antibiotic production against both *S. aureus* and *E. coli*. Twelve interactions were noted to produce zones of inhibition and were retested, however, the results were not replicated.

December sampling came from two sites with mixed vegetation within the Coleman Boundary in Barnardsville, NC. One set of soil samples was collected from a dry hillside (HILL), with three samples collected at a distance of approximately 3 m apart. Another set was collected at somewhat higher elevation near a small waterfall (FALLS), with three samples collected at a distance of 30 cm. A total of 77 bacterial types were isolated from the Coleman Boundary samples.

Antibiotic screening eliminated 43% of the isolates for production of antibiotics in pure culture (6), motility (22), or for other reasons (5). The remaining 44 isolates were co-cultured in all possible combinations and tested against both *S. aureus* and *E. coli*. No combinations were seen to produce zones of inhibition.

In all, a total of 184 bacteria were isolated for testing and 3,292 co-cultures have been tested against both Gram-positive and Gram-negative targets. September and December isolates have not been tested against one another as of this writing, but have been tested in all possible combinations within their respective groupings.

Genetic testing was used to identify organisms of interest as *Enterobacter* (1), *Enterobacteraceae* (1), *Pseudomonas* (1), and *Bacillus* (2) species. Two of these were chosen for more extensive testing, with results reported in Table 1.

Table 1. Two isolates originally identified as producing antibiotics against both Gram-positive and Gram-negative targets were further characterized against an expanded panel of targets, with mixed results. G+ targets were *S. aureus (Sa)* and *Micrococcus luteus (Ml);* G- targets were *E. coli* (*Ec)* and *Pseudomonas fluorescens (Pf)*. Y indicates a zone of inhibition was noted against that target. N indicates no apparent inhibition.

			original screen		expanded screen			
isolate	source	identified as	*Sa*	*Ec*	*Sa*	*Ml*	*Ec*	*Pf*
SS 443	*Sarracenia*	*Enterobacteraceae*	Y	Y	Y	N	Y	Y
SS 492	RALPH	*Pseudomonas*	Y	Y	Y	Y	N	N

3.2. Soil Testing

Results of soil testing showed a range of physical and chemical properties which are summarized in Table 2, along with the results of bacterial testing. RALPH soil showed the lowest level of organic carbon (0.112%) and the highest percentage of antibiotic producers; MOSS soil contained the highest percentage of organic carbon (50.8%) and the lowest percentage of antibiotic producers. However, linear regression analysis revealed only weak correlation between carbon content and the percentage of solo antibiotic producers ($f(x) = -0.424x + 23.391$; $r^2 = 0.144$, $p = 0.401$). However, eliminating dried samples from the data set increased correlation to a moderate value ($f(x) = -0.611 + 34.229$, $r^2 = 0.445$, $p = 0.219$). A strong correlation existed between the number of isolates extracted from each sample and the sand content of soil ($f(x) = 0.185x - 1.296$; $r^2 = 0.943$, $p = 0.001$).

Table 2. Physical and chemical properties of soil are combined with the results from bacterial screening for antibiotic production. The sample size from MOSS was insufficient for testing soil texture. Pitcher plant fluid pH readings were not taken at the time of collection.

source	% sand	% silt	% clay	pH	organic carbon content (%)	# isolates	# solo antibiotic producers	% solo antibiotic producers
RALPH	94	3.5	2.5	6.14	0.112	17	9	52
RGC	57	29	14	5.76	9.24	7	1	14
MOSS	-	-	-	3.45	50.8	12	1	8
FALLS	66.5	28	5.5	4.19	17.1	11	3	27
HILL	81.5	14	4.5	5.48	14.7	14	2	14
PW	22.5	59.5	18	5.09	6.27	4	0	0
POG	39.5	47.5	13	5.93	16.8	6	0	0
PP 023	n/a	n/a	n/a	-	n/a	17	2	11
PP 053	n/a	n/a	n/a	-	n/a	25	7	28
PP 083	n/a	n/a	n/a	-	n/a	19	0	0

4. Conclusion

Here, we report isolation of 184 novel environmental bacteria and subsequent screening of these bacteria for antibiotic production in pure and co-culture. It is important to note that the methods used limited our isolates to culturable aerobic and/or aerotolerant bacteria, thus representing only a fraction of the microbial diversity present in the sampled environments. With that in mind, we might have anticipated that sandy substrates would yield a greater number of isolates for our experiment since the larger particle size would allow for faster drainage and/or increased air flow and thus a higher density of aerobic or aerotolerant bacteria. No correlation between microbial abundance and carbon content was detected, contrary to predictions, even when we excluded the dried soil samples. Although a previous study used dried soil samples, fresh soil samples provided significantly more isolates for our work than did the dried samples.[16]

Our prediction of inverse correlation between carbon content and antibiotic production was validated with respect to the number of bacterial isolates producing antibiotics when grown in pure culture. Scatterplot graphing provided a negative trend line and, after the exclusion of dried samples, a moderate correlation was detected with linear regression ($f(x) = -0.611 + 34.229$, $r^2 = 0.445$, $p = 0.219$). Thus, an ecological filter is presumed to be in effect, allowing for increased colonization of nutrient limited environments by bacteria with a more suitable phenotype, which would include an adversarial edge.[20] Fierer et al. found correlation between available soil carbon and bacterial genotypes, with the newly coined phyla Acidobacteria showing preference for low nutrient environments.[21, 22]

Two isolates exhibited production of an antibiotic capable of inhibiting both Gram-positive and Gram-negative targets. Both isolates were identified through PCR amplification and DNA sequencing of the 16S rRNA gene and were further tested against an expanded panel of targets. Isolate SS 492, from the urban stream bed (RALPH) that originally tested as producing antibiotics against both Gram-positive and Gram-negative targets was identified as a *Pseudomonas* species. Members of the genus *Pseudomonas* are known as an opportunistic pathogen commonly associated with natural settings including soil and water, as well as food spoilage and hospital infections. In subsequent testing, this isolate displayed antibiosis only against Gram-positive targets, including *S. aureus* as before and also

Micrococcus luteus; antibiosis was not shown against Gram-negative targets in follow-up testing. Research has associated combinations of *Pseudomonas* and *Bacillus* with degradation of petroleum products, a definite plus in an urban stream. [24, 25]

Isolate SS 443, originating from one of the pitcher plants, was also seen to defend against both Gram-positive and Gram-negative targets. It was identified as *Enterobacteraceae*, commonly associated with the digestive tracts of animals as well as soil and water. Since pitcher plant fluid is sterile before opening, the bacteria is understood to be introduced by insect vectors and other prey species.[26] In follow-up testing, this isolate showed short-lived antibiosis against Gram-negative targets *E. coli* and *Pseudomonas fluorescens* as well as sustained antibiosis against Gram-positive *S. aureus*.

One contaminant that led to false positive screenings was excised from the plate, isolated, sequenced and identified as *Enterobacter*.

Most importantly, we are unable to validate the findings of Tyc *et al*, which suggested that co-culturing of bacteria induced antibiosis in approximately 6% of environmental isolates. In that study, 33% of the isolates tested were found to exhibit antimicrobial activity in monoculture; in our study, only 19% of the isolates tested were solo producers.[13] The difference here may be attributable to the variation in soil types. Since several of our soils had higher carbon content and provided a nutrient-rich environment, in-situ evolution may have been less selective for antibiotic production based on a diminished need for interspecific competition via antibiosis.

While we initially believed to have identified several pairwise combinations capable of induced antibiotic production, we were unable to replicate the results in repeat testing. Contamination is suspected as the cause of false positives. Follow-up screening using bacteria excised and re-isolated from the test plates showed that at least one single producer, the suspected *Enterobacter* contaminant, was inhibited in combination with another organism, an outcome that was reported as relatively common in Tyc *et al*., where 22% of interactions resulted in suppression of known microbial activity.[13]

One hypothesis for why our results may differ from those reported by Tyc *et al*. is the diversity of environmental samples selected for our original isolation. Since ecological stability evolves over time under a prescribed set of conditions, our thrusting together of bacteria from unrelated environments may have reduced the likelihood that the respective genomes coded for mechanisms to detect and respond to one another through antibiosis. In our second experiments, we attempted to correct for the disparity of environmental conditions by taking multiple samples from each of two locations, but again we did not find induced antibiotic production. However, failure to replicate results does not necessarily invalidate findings of the original study.[27] While interaction has been seen to provoke expression of antibiotic producing cryptic gene clusters, the precise circumstances of such expression are not entirely understood.[14] Additionally, what appears to be a lack of antibiotic production may actually be antibiotic simultaneously degraded by an enzymatic defense.[14] Lastly, signaling is believed to be more abundant *in situ* than under laboratory conditions.[14]

Recent development of an isolation chip (iChip) allows for in situ culturing of soil bacteria and should allow for more accurate observation of natural processes.[28, 29] Future work may be able to make use of the iChip to increase accuracy of results, as well as the number of culturable organisms.

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