Elicitation of Antibiotically Active Secondary Metabolites from Co-cultured Marine Bacteria

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ELICITATION OF ANTIBIOTICALLY ACTIVE SECONDARY METABOLITES FROM CO-CULTURED MARINE BACTERIA

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ABSTRACT

In the past, research has frequently demonstrated the production of antimicrobial agents from single cultures of marine organisms. This has traditionally been shown to occur in stationary phase growth, i.e. when nutrients are scarce and cell populations reach a steady state. In the present study, we chose to screen for antibiotic activity in cultures containing two simultaneously growing marine organisms. In this way, we mimic the competition for space and nutrients in bacterial communities that reside in biofilms on the surfaces of marine seaweed and algae. Of particular interest are those marine bacteria that show no activity when cultured singly and induced antibiotic activity when cultured in the presence of a competitor. To search for such pairings, a novel co-cultured matrix screening technique was employed with sixteen unidentified bacterial species isolated from algae samples collected from the shores of O'ahu. Using this technique, three pairings of interest have been identified. Two follow-up experiments were conducted with a subset of these pairings—one to identify the inducer and the producer of antibiotic activity and the other to chart the time course of elicitation and production. In the future, the matrix screening design presented here could be expanded to screen a far greater number of marine bacterial pairings in the search for novel antibiotics.

INTRODUCTION

A recent study in the *New England Journal of Medicine* reported that U.S. physicians write close to 80 million prescriptions for approximately 12,500 tons of antibiotics annually. Give the widespread misuse and over-prescription of antibiotics by the medical community, antibiotics available today are rapidly becoming less and less

effective in the face of emerging multi-drug resistant pathogens of clinical concern. New antibiotics are urgently needed to combat these pathogens.

This urgency is compounded by the fact that few cellular targets of antibiotic action have been found in the last seventy years since the discovery of penicillin. Only four key aspects of cellular machinery are targeted by today's antibiotics arsenal: inhibition of cell wall biosynthesis, disruption of protein synthesis at the ribosome, inhibition of DNA replication, and disruption of metabolic pathways leading to folic acid production. Thus, not only are new antibiotic structures sought, but also novel targets of attack.

Indeed, if the objective is to the find novel antibiotic structures with new targets, new ways of screening for these compounds must be developed and implemented. In the present study, we consider the community of marine bacteria on surfaces as a potential source for novel antibiotics. Marine bacteria have been screened extensively in the past, but the traditional methods for antibiotic discovery have essentially remained the same: once a single, isolated bacterial culture is grown up to stationary phase, it is screened for antibiotic activity. Rarely is the surrounding community environment from which the bacteria originated taken into account. This study joins the few other studies that have considered the surface community chemical signaling of marine bacteria as a source for novel antibiotics.

Marine bacteria that live on the surfaces of seaweed and algae are in constant competition for limited space and nutrients. We chose to artificially create this community environment by growing two pure bacterial species together in a cell suspension co-culture. Our hypothesis is that this will trigger a secondary metabolic response in one or both species as they compete for limited space and nutrients. This hypothesis is well-supported by references in the literature which draw on chemical signaling and secondary metabolic activity in other marine bacteria communities. Lemos et. al. studied the interaction between antibiotic-producing and non-producing marine bacteria in co-cultures. Mearns-Spragg et. al. exposed various species of antibiotic-producing marine bacteria to live and heat-killed cells of *S. aureus*, *P. aeruginosa*, and *E. coli*. Burgess et. al. screened over 400 strains of surface-associated marine bacteria for antibiotic activity; they reported that some of the non-producing bacteria could be

induced to produce antibiotics by exposing them to live cells, supernatants from other bacterial cultures, or other chemicals.

The key innovation presented here is the introduction of a matrix co-culturing technique that exhausts all possible co-culture combinations given an initial set of bacteria. Once the co-cultures in the matrix are allowed sufficient time for incubation, the supernatant of each well can be isolated and screened for antibiotic activity against *Staphylococus aureus*. Usage of this matrix technique could open up a whole new avenue for antibiotics discovery that may potentially lead to novel structures and targets.

MATERIALS AND METHODS

Growth and Isolation of Marine Bacteria

Four different sites on shores of O'ahu were visited to build a collection of algae: Malaekahana Beach Park, Kaimana Beach Park, Kahala Beach Park, and the research pier at Oceanic Research Institute. The algae were randomly collected 0-10 m from the shoreline and at depths ranging from 0-2 m.

After washing the samples with autoclaved sea water (as described in Burgess *et. al.*) to remove any loosely-bound organisms, surface scrapings were taken by means of a sterile applicator and streaked on 100% DIFCO Marine Agar plates which were allowed to incubate for 3 d at 25 °C. After successive re-streaking, seventeen different isolates of marine bacteria based on differing morphological characteristics such as color, shape, etc. were isolated. Representative single colonies were used to inoculate 3 mL 100% DIFCO Marine Broth cultures which were then incubated overnight at 25 °C. A frozen culture library (in 20% glycerol in marine broth) of all bacterial species was maintained at -70°C.

Co-culture Matrix Experiment

The bacteria were cultured in a 16x16 marine broth matrix (using 24-well Coring Costar plates) consisting of 16 axenic well cultures on the main diagonal and 240 off-diagonal co-cultured wells of all possible pairings in duplicate. Each well had a total volume of 1.8 mL, with 50 μL used for each inoculum and 1.7 mL Marine Broth. To ensure approximately equal cell density, the innocula were taken from cultures which were adjusted by dilution to roughly equal optical densities at 590 nm.

The plates were allowed to incubate for four days at room temperature on a low-speed plate shaker. Before analysis, the well cultures were transferred to 2.2 mL

Eppendorf tubes and centrifuged at 3000 RPM for 5 min to pellet whole cells. The supernatants were decanted and screened for antibiotic activity. In some cases, the supernatant was filtered through a 0.22 μm filter disk prior to screening.

Time Course Experiment

In this experiment, both members of a bacterial pairing were grown up separately in 50 mL Marine Broth shaker flasks (1.5 mL overnight culture used as inoculum, shaker speed: 200 RPM). After 3 days of growth, the shaker flask culture supernatants were isolated by vacuum filtration through 0.22μm syringe filters. Every twelve hours, a 900 μL aliquot of the supernatant was added to progressively older well cultures (0.8 mL Marine Broth, 100 μL inoculum) of the complementary bacterium within each pairing. After five time points, the supernatant from each well was isolated and screened for antibiotic activity.

Antibiotic Screening Assays

The test strain used in these assays—methicillin-susceptible *Staphylococus aureus*—was provided as a generous gift by Clay Wakano of the University of Hawai'i Department of Microbiology. The bacterium was cultured in tryptic soy broth, kept on a shaker, and maintained at 25 °C. Every 2 d new cultures were inoculated, but only vigorously dividing overnight cultures were used for the screening assays.

A standard well-diffusion assay based on the method described by Ivanona et. al. was used to screen for antibiotic activity. 30 μL aliquots of an overnight *S. aureus* culture were used to inoculate tryptic soy soft agar enriched with yeast extract (30 g/L tryptic soy, 0.8% Agar, 0.7% yeast extract) just before solidifying. Circular wells (diameter 10 mm) were cut into the agar plates and 0.1 mL of each supernatant was added into each well. After incubation at 37 °C for 24 h, the plates were inspected for circular zones of clearing which indicated inhibition of *S. aureus* growth.

RESULTS AND DISCUSSION

The motivating hypothesis behind this project was that growing two bacterial species together in a co-culture would trigger certain secondary metabolic pathways leading to the production of antibiotics in one or both bacteria. These antibiotics would be ones that would not otherwise be produced by either bacterium in separate single

cultures. This idea was motivated by observing the stable marine bacteria community environments that exist in biofilms on the surfaces of marine seaweed and algae.

Seventeen unidentified, morphologically distinct isolates of marine bacteria were taken from the surfaces of algae samples randomly collected from four sites on the shores of O'ahu. One of the seventeen was discarded due to cultivation and screening difficulties. The bacterial species (arbitrarily labeled A-P) were cultured in a 16x16 matrix of culture vessels containing marine broth. The matrix consisted of 16 axenic well cultures on the main diagonal and 240 off-diagonal co-culture wells of all possible pairings in duplicate. After four days of growth, the supernatant of each well was isolated and screened for antibiotic activity against methicillin-susceptible *Staphylococus aureus* using a standard well-diffusion assay that gives zones of clearing to indicate antibiotically active samples.

The matrix experiment is designed as an elementary technique to help discover potentially novel antibiotic candidates that are only produced in co-cultured environments. It does this by exhausting all pair-wise co-cultured combinations of the bacteria species inputted. After a standard antibiotic screening assay of all well-cultures in the matrix, one can identify those pairings of bacteria that exhibit induced antibiotic activity as a result of co-cultured growth. In such pairings, the two axenic cultures that lie on the main diagonal of the matrix show no activity, whereas the two corresponding cultures in the off-diagonal co-cultured wells display activity.

To illustrate how these pairings of interest are identified, consider the following hypothetical simplified matrix experiment in which three distinct bacterial species α, β, and γ are cultured and co-cultured in all possible combinations. The nine different co-cultures are summarized in Figure 1. Each square represents a different culture or co-culture inoculated at the beginning of the experiment. After four days, supernatant from each culture is isolated and screened for antibiotic activity. Hypothetical results are shown in Figure 2.

In the 3x3 matrices shown in the two figures, the main diagonal in both runs from the upper left-hand corner to the lower right-hand corner. Along the main diagonal lie the three axenic cultures: α, β, γ. The co-cultures are shown in the off-diagonal squares

of the matrix. Note that the co-cultures shown in the three squares above the main diagonal are all reproduced in the three squares below the main diagonal.

	α	β	γ
α	(α,α)	(α,β)	(α,γ)
β	(β,α)	(β,β)	(β,γ)
γ	(γ,α)	(γ,β)	(γ,γ)

Figure 1: Hypothetical mini-matrix showing all possible co-cultures of bacteria α,β, and γ.

	α	β	γ
α	-	+	+
β	+	-	+
γ	+	+	+

Figure 2: Hypothetical results of antibiotic screening. "+" is active, "-" is inactive

The results in Figure 2 show that α and β are inactive in single cultures (the two -'s along the main diagonal) but display induced activity in the co-cultured off-diagonal wells (the + in (α,β) and the + in (β,α)). Thus, α/β would be a bacterial pairing of interest. Now consider the γ bacterium. The four off-diagonal wells containing γ all show activity (the +'s in (α,γ), (γ,α), (β,γ), (γ,β)). In addition, the axenic culture of γ is also active and thus no induced activity is observed in any of the pairings involving γ.

Note that this hypothetical matrix is internally consistent—the off-diagonal results are mirrored across the main diagonal. This feature of the matrix serves as a reproducibility check and helps safeguard against false negatives.

Let us now consider the results from the actual 16x16 matrix that was the centerpiece of this project. In this experiment, the antibiotic screening of supernatants presented a number of difficulties. Initially, the culture supernatants were isolated by centrifugation of the matrix culture suspensions in order to pellet the whole cells. Following centrifugation, the supernatants were then simply decanted off. For the well-diffusion screening assay, the supernatants are then added into wells that have been drilled into tryptic soy agar plates containing the indicator organism. Any whole bacterial cells that were inadvertently transferred with the supernatant in the decanting step would then have a chance to grow in the tryptic soy agar plates, thereby interfering with an accurate reading of the screening results. In the first round of screening, the results from 81 well-cultures were rendered unreadable by possibly obscured zones of clearing owing to bacterial growth originating from non-sterilized supernatants. Furthermore, 12 instances of inconsistency in antibiotic activity between identical co-

cultured off-diagonal wells were observed. To solve this problem, a second round of screening was conducted with these two error sets. In these experiments the supernatants were sterile filtered through a 0.22μm disk prior to re-screening for antibiotic activity. All antibiotic activity data from the two rounds of re-screening are summarized in Figure 3. These are the complete results from the 16x16 matrix.

The results of interest that support the original hypothesis are those pairings that show activity in the off-diagonal co-cultures and inactivity in the main-diagonal cultures. Three such bacterial pairings (highlighted in Figure 3) are seen in the matrix results: L/A, L/B, L/D. See Figure 4. In these three cases, axenic cultures of L, A, B, and D show no activity but the L/A, L/B, and L/D co-cultures are all antibiotically active.

Given the reoccurrence of L in the pairings, it is plausible that L is the antibiotic producer and A, B, and D are all capable of inducing antibiotic production by L. There is also some concern that L may actually be a constitutive antibiotic producer at very low titer in axenic culture and that antibiotic production is merely increased as a result of co-culture, rather than elicited de novo. Whether or not this is the case could be determined by growing up separate, axenic cultures of A, B, D, and L and then adding the supernatant of one culture to a stationary phase culture of another. All the supernatant + culture combinations could then be assayed for antibiotic activity in the manner described before. If only the cultures of L with supernatants of A, B, or D added show antibiotic activity, then this would strongly suggest that L is the producer of the antibiotic and A, B, and D are L-inducers.

This experiment was attempted but antibiotic activity screening results were inconclusive due to *S. aureus* growth problems.

Let us consider a brief statistical analysis of the results from the matrix experiment. Of the 240 off-diagonal pairings, 16 are members of mismatch pairs. These types of pairings, which constitute 6.7% of the total pairings, show opposite results of antibiotic activity in the duplicated co-cultures. Six of the off-diagonal wells were part of induced activity pairings of interest. This represents 2.5% of total number of pairings. Of the 16 bacteria that were studied in this matrix experiment, 4 were involved in induced activity pairings. This represents 25% of the total number of bacteria entered into the matrix.

A time course experiment was also conducted with a subset of pairings from the matrix. The pairings used were (O,E), (O,H), and (O,J)—all antibiotically active co-

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-
B	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-
C	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-
D	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
E	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
F	+	+	-	-	+	+	+	-	+	+	-	-	+	+	-	+
G	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
H	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
I	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
J	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-
M	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
N	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
O	-	-	-	-	+	-	+	+	+	+	-	+	+	-	+	-
P	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-

Figure 3: Summary of antibiotic activity against methicillin-susceptible *Staphylococus aureus* for all cultures screened in the 16x16 matrix. + indicates an observable zone of clearing, - indicates the absence of a zone of clearing. The green boxes indicate the axenic cultures of the main diagonal, and the yellow boxes indicate induced activity parings.

Cultures	A	B	D	L	A+L	B+L	D+L
Activity	-	-	-	-	+	+	+

Figure 4: Pairings of interest shown in yellow boxes from the 16x16 matrix above.

cultures. These were reasonable choices for this experiment because the 1.8 mL axenic cultures of O, E, and J consistently showed no antibiotic activity. However, upon screening the supernatant of a 50 mL culture of isolate O used in the time course experiment, it was discovered that O was in fact antibiotically active (see the + value for O on the main diagonal). Since one of the pairing members was active in single culture, this strongly suggested that the activity of the 3 O co-culture pairings was likely not due to induction effects. This incident soundly demonstrates the importance of correctly knowing if the axenic cultures on the main diagonal in the matrix are antibiotically active. Whether or not they are should be repeatedly confirmed. Secondly, this result suggests that aeration and vigorous stirring may be necessary to obtain better results with all cultures in the matrix.

The time course experiment should be repeated with the three L pairings of interest shown above. However, a small modification to the experimental design of the time course experiment is needed. The amount of time that each culture of L incubates with supernatant from the paired bacterial cultures should be uniform and fixed. This means that the cultures in the time course experiment cannot all be screened for antibiotic activity at the same time.

The results from this exploratory research project indicate that it is in fact possible to induce one bacterium to produce antibiotics by allowing it to grow in the presence of another. Of the bacterial species used in the matrix experiment, 25% were involved in induced activity pairings. Reproducing the matrix experiment with a larger sampling of marine bacteria is expected to yield a greater number of induced activity pairings. Secondly, adding inert, solid beads to each co-culture suspension might better mimic the surface community environment. This has been shown to be a successful strategy in single bacterial cultures by Ivanova et. al. Finally, as mentioned before, aeration and vigorous shaking may be necessary as well to increase induction effects.

In order to determine whether or not a novel antibiotic has been discovered which may be clinically useful, it must be first determined which bacterium is the inducer and which is the producer in pairings of interest, what the chemical structures of the elicitor and the antibiotic molecules are, and whether or not the antibiotic shows activity against MRSA. In the future, the matrix screening design presented here could be expanded to

screen a far greater number of marine bacterial pairings in this search for novel antibiotics.

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