Functional or Just Plain Pretty? A Reevaluation of Bacterial Pigment Activity

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Abstract

Serratia marcescens and *Chromobacterium violaceum* are both gram-negative opportunistic pathogenetic bacteria that produce the pigments prodigiosin and violacein, respectively. These secondary metabolites have been demonstrated to act as antibiotic compounds effective against pathogenic bacteria, which could be of future therapeutic importance. In this study, the pigments were purified environmental samples of *S. marcescens* and *C. violaceum* previously identified as antibiotic producers. These pigments were tested for antibiotic activity under three conditions: individually, together, and in combination with the antibiotic neomycin. No antibiotic activity or enhancement of neomycin was seen in either pigment. Efforts to reactivate the antibiotic production included altering the growth conditions for *S. marcescens* and *C. violaceum* and challenging these bacteria with other competing bacterial species. While pigment production was robust, antibiotic activity was not restored. These results demonstrate that the pigment alone does not induce bacteriostatic or bactericidal properties, indicating other cofactors may play a role in antibiotic production within the bacteria.

Keywords: Prodigiosin, violacein, antibiotics, secondary metabolite

Introduction

Antibiotics are molecular compounds that inhibit the growth and spread of microorganisms. In recent years, antibiotic resistance has become a pressing issue in the medical field, and pathogenic bacteria are becoming resistant to all available antibiotics. Each year, there are approximately 2.8 million infections, with 35,000 deaths, from resistant bacteria in the United States (Center of Disease Control, 2019). The pace of production of novel antibiotics is not fast enough to replace existing, ineffective antibiotics. Currently, there are 43 novel antibiotics in clinical trials. However, are all derivatives of existing antibiotics and are likely to not substantially impact the global crisis. Ultimately only one or two may ever make it to market (World Health Organization, 2021).

In an apparent contradiction, most of the existing antibiotics in use have been isolated from bacteria. These microorganisms naturally produce secondary metabolites, such as antibiotics and pigment compounds, that are not required for the organism to survive, but can enhance the organism's selective advantage (Demain & Fang, 2000). Therefore, antibiotics produced in bacteria do not harm the producer but may kill off other bacteria competing for resources in the environment. One new approach to combat antibiotic resistant bacteria involves the discovery of novel antimicrobial agents through the culturing of environmental bacteria found in soil. Another approach focuses on the development of known secondary metabolites, such as pigments, to enhance or replace our existing arsenal of antibiotics.

The Tiny Earth Network was established in 2012 and focuses on student led research in identifying novel antibiotics from bacteria found in soil samples. To identify potential antibiotics, soil bacteria are tested against non-pathogenic bacterial laboratory strains (safe relatives) of pathogenic bacteria of grave concern. These pathogenic bacteria are known by the

acronym ESKAPE to represent the taxa of these organisms (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter*). Through the Tiny Earth Network, over 13,000 isolates have been identified as being effective against pathogenic bacteria (Tiny Earth Network, 2022). Recently, it has been noted that when bacteria are removed from their native environment for an extended period of time, the production of antibiotics decreases. This may be due to reduced competition, increased nutrient resources, or a combination of these and other factors (B. Gasper, personal communication). Current investigations are underway testing various techniques to turn antibiotic production back on in these bacteria. Approaches include growing bacteria in conditions designed to mimic their original environment, challenging them with competing bacteria, and introducing mycolic acid into the growth medium (B. Gasper, personal communication).

Pigment production in bacteria has been extensively studied and correlated with different characteristics, including pathogenicity and antibiotic production (Choi et al., 2021; Demain & Fang, 2000; Durán & Menck, 2001). Testing for antibiotic activity can be done in vivo, by challenging the pigment producers alongside competing bacteria (such as ESKAPE safe relatives) and in vitro, by extracting the pigment from the cells and testing against ESKAPE safe relatives. Two such pigments are prodigiosin, produced by *Serratia marcescens,* and violacein, produced by *Chromobacterium violaceum*. These pigments are of special significance as they have been extensively researched and have properties of interest to the medical community (Aruldass et al., 2018; Choi et a., 2021; Ji et al., 2015; Subramaniam et al., 2014)

Serratia marcescens is a species of gram-negative, rod-shaped bacteria from the family *Enterobacteriaceae*, and is commonly found in multiple different environments such as soil and

water. It is responsible for one to two percent of nosocomial infections, acting as an opportunistic pathogen in clinical settings (Khanna et al. 2013). Prodigiosin is the red pigmentation found in *S. marcescens*. It is characterized by its tripyrrole structure, which contains three 5 carbon organic molecules. The ideal conditions for prodigiosin production have been well studied (Giri et al., 2004; Elkenawy et al., 2017). The biosynthesis of the red pigment is regulated by a gene cluster known as *pig,* with the master regulator of the operon being PigP (Sakai-Kawada et al., 2019). Pigment production increases the hydrophobicity of *S. marcescens*, determining how strongly the bacteria can adhere to surfaces and be transported through the air (Burger & Bennett, 1985).

Prodigiosin has been found to have antimalarial, antifungal, immunosuppressant, and antibiotic activity, and has been found to be cytotoxic against cancer cells (Sakai-Kawada et al., 2019). As an antibiotic, prodigiosin directly disrupts the bacterial cell's plasma membrane, penetrating the membrane and causing the cell to lyse (Soenens & Imperial, 2019). Prodigiosin is effective against a variety of bacteria, including gram negative *E. coli,* and multiple gram-positive species including *S. aureus, B. subtilis* (Sakai-Kawada et al., 2019). The effectiveness of prodigiosin in inhibiting the growth of bacteria can be observed by a zone of inhibition, which measures the amount of bacteria killed on an agar plate after an antibiotic compound has been placed on the plate. With increasing concentrations of prodigiosin, a greater zone of inhibition will be produced against the tested bacteria (Othman et al., 2019). Prodigiosin is non-toxic to eukaryotic cells in culture and does not affect *C. elegans* (Seah et al., 2016). Therefore, prodigiosin is of interest as an antimicrobial for use in humans.

Chromobacterium violaceum is a species of gram-negative, rod-shaped bacteria from the family Neisseriaceae, and is a rare opportunistic pathogen that infects both humans and animals.

C. violaceum produces three different antibiotic compounds: violacein, aerocyanidin, and aerocavin (Durán & Menck, 2001). Violacein is a secondary metabolite composed of 2 modified L-tryptophan molecules, and is the violet pigment produced in *C. violaceum* (Duran et al., 2016). The biosynthesis of the pigment is done through the gene cluster vioABCDE. The violacein producing pathway is considered to be unstable, as regulation can be modified spontaneously in violacein producers. This pathway is also responsible for quorum sensing and biofilm production within *C. violaceum*. Violacein has been shown to kill or slow the growth of bacteria, fungi, protozoa, viruses, and tumor cells (Jiang et al., 2010).

As an antibiotic, violacein inhibits the growth of gram-positive and some gram-negative bacteria (Durán & Menck, 2001; Subramaniam et al., 2014). Violacein kills bacteria by accumulating on the membrane, entering the cell, and causing the cell to lyse, which leads to cell death (Aruldass et al., 2018). Violacein has been shown to have additive or synergistic relationships with antibiotics, and when used in combination the efficacy of commercial antibiotics is increased (Subramaniam et al., 2014; Choi et al., 2015). While violacein does show promise against bacterial cells, it may be harmful to eukaryotes, as it shows toxicity to *C. elegans*, causing developmental and behavioral changes within the worms (Choi et al., 2015). Violacein was also seen to be toxic at high levels to human embryonic kidney and fetal lung fibroblast cells (Aruldass et al., 2018; Nakazato et al., 2019). Therefore, its potential use in humans will need to be carefully investigated.

Prodigiosin also acts in combination with commercial antibiotics, as seen in a greater inhibition of the growth of *S. aureus* (Gohil et al., 2020). This is hypothesized to occur through different mechanisms, such as moderation of the pH or inhibition of cell growth during various stages of the cell cycle. Prodigiosin combined with ampicillin and with penicillin both showed

synergistic effects at suppressing the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), which may be due to the inhibition of cell wall growth (Ji et al., 2015). No combinations of prodigiosin with commercial antibiotics created an antagonistic effect on the efficacy of the antibiotic, unlike what was seen with violacein (Gohil et al., 2020).

The synergistic effects of violacein can be observed through the larger zones of inhibition and the decrease in the minimum inhibitory concentration during in vitro assays (Subramaniam et al., 2014). Crude violacein has the ability to inhibit the growth of *S. aureus* (Choi et al., 2015; Gohil et al., 2020) and a combination of violacein and vancomycin was shown to act synergistically to inhibit the growth of *S. aureus* in comparison to the antibiotic or pigment alone (Durán & Menck, 2016; Subramaniam et al., 2014). However, when combined with some antibiotics, there can be a decrease in the efficacy of the antibiotic (antagonism), which occurs in rare cases. Violacein also shows synergistic or additive effects against *Staphylococcus epidermidis,* reducing the required minimal concentrations of the antibiotics by 16-fold (Dodou et al., 2017).

Florida Southern College (Lakeland, FL) has participated in the Tiny Earth Network curriculum since 2014 to identify novel antibiotics. In a screen for antibiotic production in soil bacteria, two bacterial strains, AC1 and AG1, were isolated in the fall of 2018 and 2019, respectively. AC1 was isolated from a single red colony grown on R2A medium and was shown to have antimicrobial activity against *Bacillus subtilis and Pseudomonas putida*. AG1 was isolated from a colony producing a purple pigment grown on AIA medium, and was shown to have antimicrobial activity against *Enterococcus faecalis* and *Acinetobacter baylyi*. Both isolates underwent PCR and subsequent sequencing of the 16s rRNA gene. Based on a BLAST search,

AC1 was identified as *S. marcescens* and AG1 was identified as *C. violaceum* (unpublished data).

This study investigated the ability the environmental strains of *S. marcescens* (AC1) and *C. violaceum* (AG1), and the pigments purified from them, to inhibit the growth of ESKAPE safe relatives. The results call into question the accepted paradigm that these pigments, on their own, affect bacterial growth.

Methods

Bacterial Growth

Media

Commercially available media (LB, TSA, R2A, AIA and Muller-Hinton agar) were prepared in agar or liquid form as directed by the manufacturer or altered as noted. Soil Agar was made by collecting soil from near the shore of Lake Hollingsworth in Lakeland, FL. The 10% TSA soil agar (modified from a HiMedia Laboratories recipe) was made with 250 mL of DI water, 4.44 g soil, 0.75 g TSB and 4.5 g agar.

Bacterial samples

Glycerol stocks, stored at -80˚C, of environmental bacteria isolated from a screen for antibiotic production were used in these studies (unpublished data). AC1 (*S. marcescens)* and AG1 (*C. violaceum)* were grown on TSA at 30°C unless otherwise noted.

ESKAPE safe relatives (*Escherichia coli, Bacillus subtilis, Staphylococcus epidermidis,* and *Pseudomonas putida*) and *Serratia marcescens* were obtained from laboratory stocks at Florida

Southern College and grown by FSC students. *Mycobacterium smegmatis* was a generous gift from E. Warrick (State College of Florida).

Growth Curves

For growth curve analysis, isolated colonies of the environmental bacteria were inoculated into 100 mL of LB broth or TSB. The flasks were kept in a shaking incubator at 30°C. After two hours, samples were removed every 20 minutes for OD readings. AC1 was measured at 535 and 600 nm, and AG1 was measured at 585 and 600 nm.

Extraction of Violacein and Prodigiosin

Agar extraction: AC1 was heavy streaked on four TSA plates and incubated at 30°C overnight for optimal growth of a bacterial lawn and for maximal pigment production. The agar was cut up and transferred to a 100 mL flask and placed in a -20°C freezer overnight. 30 mL of room temperature 95% ethanol was then added to the flasks and incubated at room temperature on a shaker. The solvent phase was extracted and stored at 4°C while the agar was discarded.

Liquid extraction: 250 mL of TSB was inoculated with 1 mL of an overnight bacterial culture of AG1and grown overnight at 30°C. The broth was separated into five 50 mL vials and centrifuged for 15 minutes at 4,000 rpm. The supernatant was discarded. The cells were then washed with 10 mL of DI water and separated into 1.5 mL vials. The samples were then centrifuged for 10 minutes at 8,000 rpm. The supernatant was discarded, and the pellets were resuspended in 1 mL of ethanol. Each sample was allowed to incubated for a minimum of 30 minutes at room temperature before being centrifuged for 10 minutes at 10,000 rpm. The supernatant (solvent phase) containing the pigment was collected and pooled, and the pellets were washed with ethanol and centrifuged again until no pigment was shown in the solvent phase. All supernatants

from the washed samples were pooled and added to the original supernatant. Prodigiosin isolated in a similar manner from laboratory strains of *S. marcescens* was a kind gift of T. Peristerditis and J. Tuckerman.

Once the pigment was collected, the samples were placed in a chemical fume hood to allow for the ethanol to evaporate for two weeks at room temperature. The dried pigment was resuspended with 10-15 mL of 95% ethanol and stored at 4^0 C.

Antibiotic Testing against ESKAPE Safe Relatives

Overnight cultures of *S. epidermidis, E. coli, B. subtilis,* and *P. putida* were plated onto Muller-Hinton agar to perform disk tests for the effectiveness of 9 antibiotics (Streptomycin 10 µg, Chloramphenicol 30 µg, Neomycin 30 µg, Ciprofloxacin 5 µg, Tetracycline 30 µg, Erythromycin 15 µg, Novobiocin 30 µg, Kanamycin 30 µg, and Penicillin 10 µg, all obtained from Carolina Biological Supply Co.). The diameter of each zone of inhibition was measured and antibiotic effectiveness was assessed using an Antimicrobial Disk Diffusion Zone Interpretation Guide. Each bacteria was designated as being resistant, intermediate, or susceptible to the antibiotic based on these industry accepted standards.

Antibiotic Trials with purified pigments

Neomycin and blank disks were dipped into ethanol, prodigiosin, violacein, or a mix of prodigiosin and violacein. Disks were placed into a sterile petri dish to air dry. *E. coli* was heavy streaked on Muller Hinton agar plates and disks were placed on top of the *E. coli*. Plates were incubator at 30°C for approximately 24 hours. Zones of inhibition for each sample were measured and recorded. Each trial was repeated 20 times.

Enhancement of antibiotic production

Attempts to increase antibiotic production included plating AC1 and AG1 on soil media and low nutrient media (R2A, AIA, and 10% TSA) at room temperature. Alternatively, each environmental bacteria was plated in close proximity to ESKAPE safe relatives on low nutrient agar plates or directly on top of a growing lawn of *Mycobacterium smegmatis* on TSA. All plates were incubated at 30°C for 2 days.

Patch Plating to Test for Antibiotic Production

E. coli or *B. subtilis* were spread on TSA plates to induce a lawn either by heavy streaking or spreading of an overnight liquid culture. Isolated colonies of AC1 and AG1grown on TSA, soil medium, or on TSA in the presence of *M. smegmatis* were individually spread in approximately 2 X 2 cm squares on top of the freshly spread ESKAPE safe relatives. Additional patch plates were made by mixing AG1, AC1, and *Mycobacterium smegmatis* in all possible combinations directly on top of the ESAKAPE safe relative seeded plates. Plates were incubated overnight at 30°C and analyzed for zones of inhibition around the environmental bacteria.

Results

Pigment Purification

An environment strain of *Serratia marcescens* (AC1) was isolated on R2A medium from a soil sample collected on the campus of Florida Southern College (Lakeland, FL) in 2018 and was shown to act as an antibiotic producer against *B. subtilis* and *P. putida* (data not shown). AC1 pigment production was optimal when grown at less than 30°C on LB and TSA. Pigment production was eliminated when cells were grown on nutrient deficient media (R2A, AIA, and 10% TSA) or incubated at or above 30°C (Figure 1).

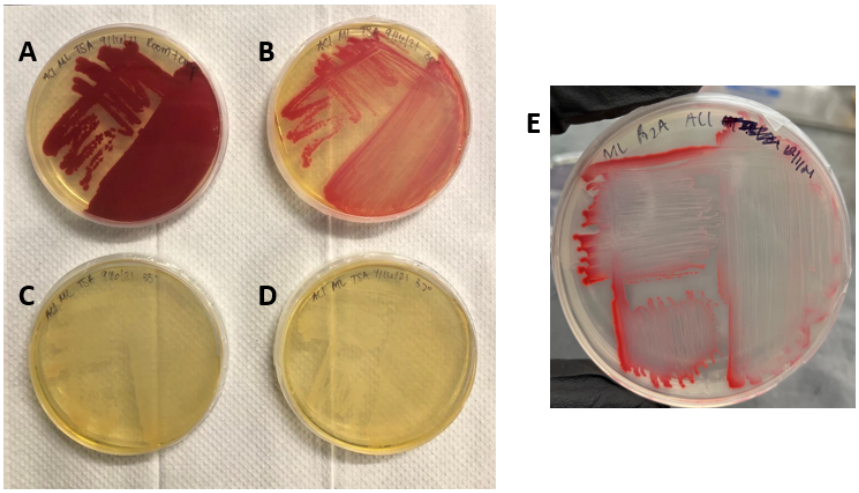

Figure 1: Growth and pigment production in the S. marcescens isolate, AC1. AC1 was grown on different media and at different temperatures. A) TSA at room temperature, ~25°C B) TSA at 30°C C) TSA at 35°C D) TSA at 37°C and E) R2A at 30°C. While the bacteria grew under all conditions, pigment production varied depending on the growth conditions.

Much like with AC1, an environmental strain of *Chromobacterium violaceum* (AG1) was isolated on AIA medium from a soil sample collected on the campus of Florida Southern College in 2019, and produced antibiotic activity against *E. faecalis* and *A. baylyi* (N. Morvillo, personal communication). AG1's growth conditions were not temperature specific; however, media was a limiting factor in growth and pigment production of violacein. AG1 grew well on TSA, but growth and pigment concentration decreased when grown on nutrient deficient media (R2A, AIA, and 10% TSA) (Figure 2).

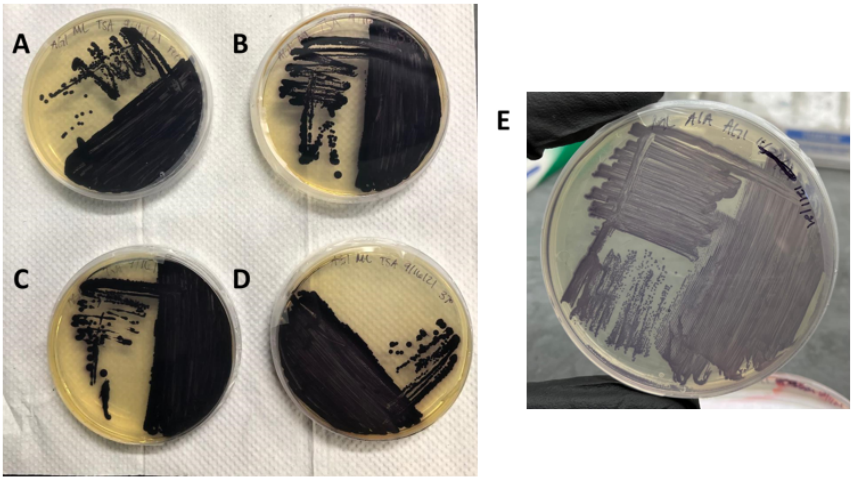

Figure 2: Growth and pigment production in the C. violaceum isolate, AG1. AG1 was grown on different media and at different temperatures. A) TSA at room temperature ~25°C B) TSA at 30°C C) TSA at 35°C D) TSA at 37°C and E) AIA at 30°C. Pigment production was affected by nutrient level in the media, but was not impacted by growth temperature.

Growth curves of both AC1 and AG1 in LB showed that pigment production increased as cell density increased (Figure 3). Samples of AC1 grown over night in LB and kept at 4°C showed an increase in pigment production of the course of two weeks, indicated by a vibrant pink color in comparison to the original broth (Figure 3c). This indicates that when bacterial replication is slowed or halted due to low temperatures pigment production can still occur.

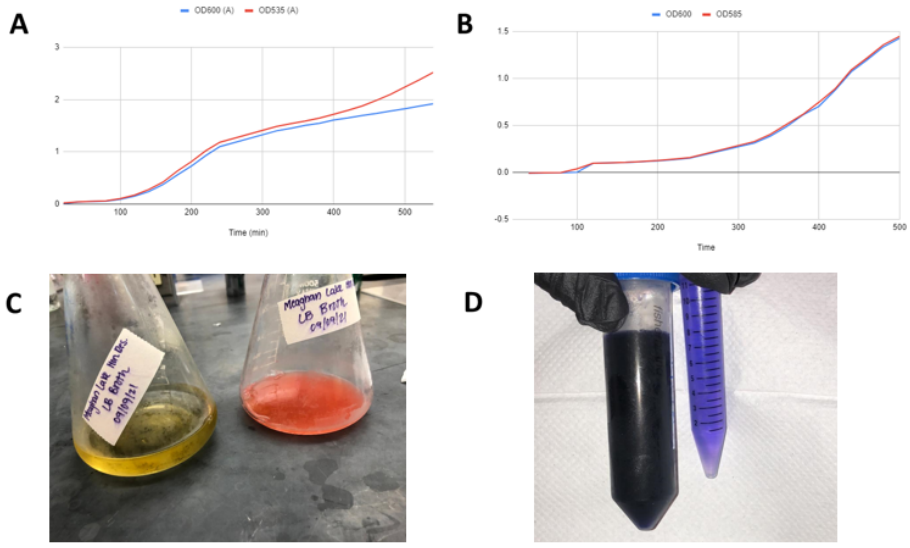

Figure 3: Growth and pigment production in liquid culture. Cells were growth in liquid culture and spectrophotometer readings were taken at regular intervals to monitor growth (Absorbance readings at 600 nm) and pigment production. A) AC1 grown in LB broth at 30°C; prodigiosin measured at 535 nm; B) AG1 grown in TSB at 25°C; violacein measured at 585 nm. C) Pigment

production is seen to increase in AC1 over the course of two weeks. The pigment in the flask on the right shows the remainder of the AC1 growth curve sample after storage at 4^{0}C for two weeks (flask on the left is LB) D) Pigment extraction from AG1 taken after a 3-day culture (left) and an overnight culture (right) All results show the increase of pigment production throughout the growth curve and even when bacteria were stored and presumably quiescent at 4 ^{0}C for two weeks.

Once optimal conditions for both AC1 and AG1 were identified, large overnight cultures were made for pigment purification. AC1 did not produce enough prodigiosin in the liquid broth to be isolated, so an agar extraction protocol was used (Figure 4). Violacein was purified from AG1 using a liquid extraction method (Figure 5). Attempts to quantify the amount of pigment were unsuccessful due to anomalous spectrophotometer readings, however darkly pigmented extracts indicated successful extraction.

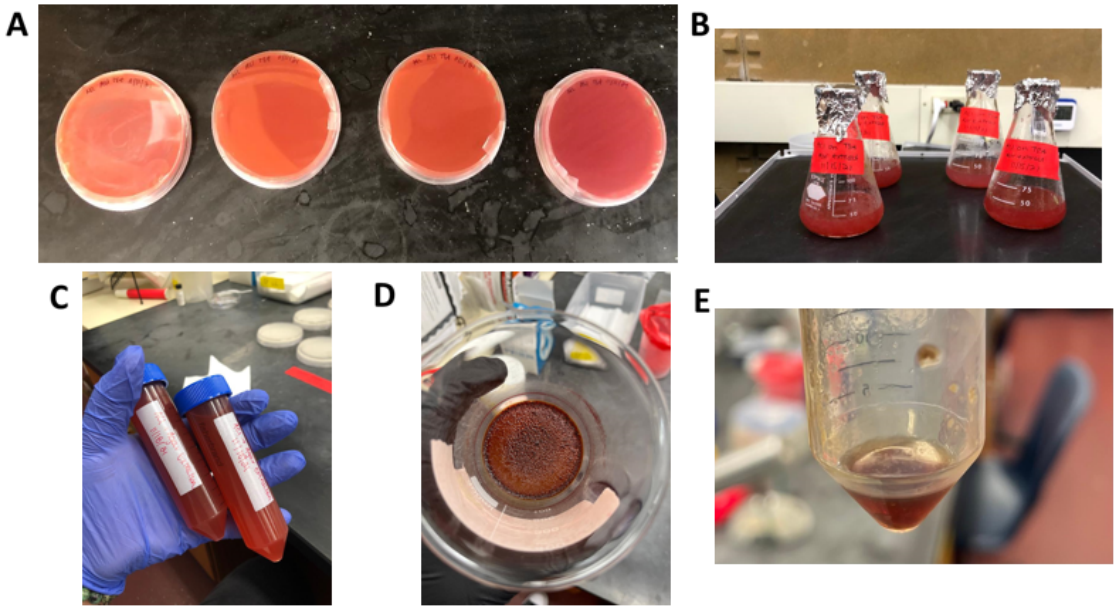

Figure 4: Agar extraction process of prodigiosin from AC1. A) Overnight heavy streaked plates of AC1 grown on TSA. B) Agar plates diced and placed in EtOH on a shaker. C) EtOH and extracted prodigiosin. D) Prodigiosin extract after being evaporated under the fume hood. E) Prodigiosin resuspended in EtOH

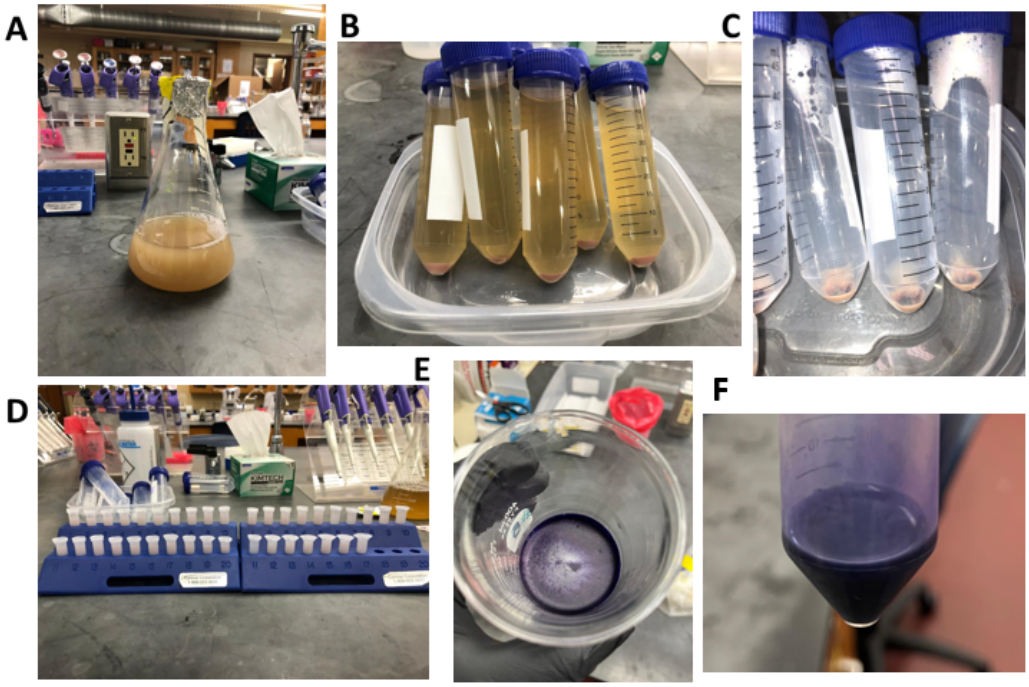

Figure 5: Liquid extraction process of violacein from AG1. A) Overnight culture of AG1 in TSA. B) Samples after centrifugation at 4000 rpm. C) Samples after TSA broth is drained showing the bacterial pellet. D) Samples after being washed with DI water, transferred to smaller tubes and centrifuged at 10,000 rpm. E) Violacein extract after being dried under the fume hood. F) Violacein resuspended in EtOH.

Selection of Antibiotic

To determine the appropriate antibiotics to test the efficacy of the pigments, nine antibiotics were tested against *E. coli, B. subtilis, S. epidermidis,* and *P. putida* in a disk diffusion assay (Figure 6). The diameters of the zones of inhibition were measured, and each ESKAPE safe relative was classified as resistant, intermediate, or susceptible to the antibiotic based on a standard zone size chart (Table 1). *E. coli* and *B. subtilis* were selected for trials as both had the most antibiotics that were intermediate in inhibiting their production. Neomycin was selected as the antibiotic for testing as it demonstrated intermediate effectiveness against *E. coli* and *B subtilis* and based on its common use currently as an antibiotic.

A

	ESKAPE Safe Relative			
Antibiotic	*E. coli*	*B. subtilis*	*S. epidermidis*	*P. putida*
Streptomycin	14 mm	11 mm	16 mm	18 mm
Chorampehnicol	26 mm	19 mm	28 mm	23mm
Neomycin	14 mm	13 mm	15 mm	19mm
Ciprofloxacin	24 mm	20 mm	30 mm	33 mm
Tetracycline	23 mm	18 mm	22 mm	26 mm
Erythromycin	12 mm	0 mm	10 mm	0 mm
Novobiocin	9 mm	13 mm	10 mm	15 mm
Kanamycin	20 mm	14 mm	21 mm	26 mm
Penicillin	0 mm	0 mm	0 mm	0 mm

B

	Antibiotic Effectivness *E.coli*			Antibiotic Effectivness *B. subtilis*			Antibiotic Effectivness *S. epidermidis*			Antibiotic Effectivness *P. putida*		
Antibiotic	Resitant	Intermediate	Susceptible	Resitant	Intermediate	Susceptible	Resitant	Intermediate	Susceptible	Resitant	Intermediate	Susceptible
Streptomycin		X		X	X				X			X
Chorampehnicol			X			X			X			X
Neomycin		X			X			X				X
Ciprofloxacin												
Tetracycline			X		X				X			X
Erythromycin	X			X			X			X		
Novobiocin	X			X			X			X		
Kanamycin			X		X				X			X
Penicillin	X			X			X			X		

Table 1: Effectiveness of antibiotics on four ESKAPE safe relatives. A) Zones of inhibition from standard antibiotic disks for the ESKAPE safe relatives. B) The effectiveness of each of the antibiotics was assessed as resistant, intermediate, or susceptible based on the standard zones for each antibiotic.

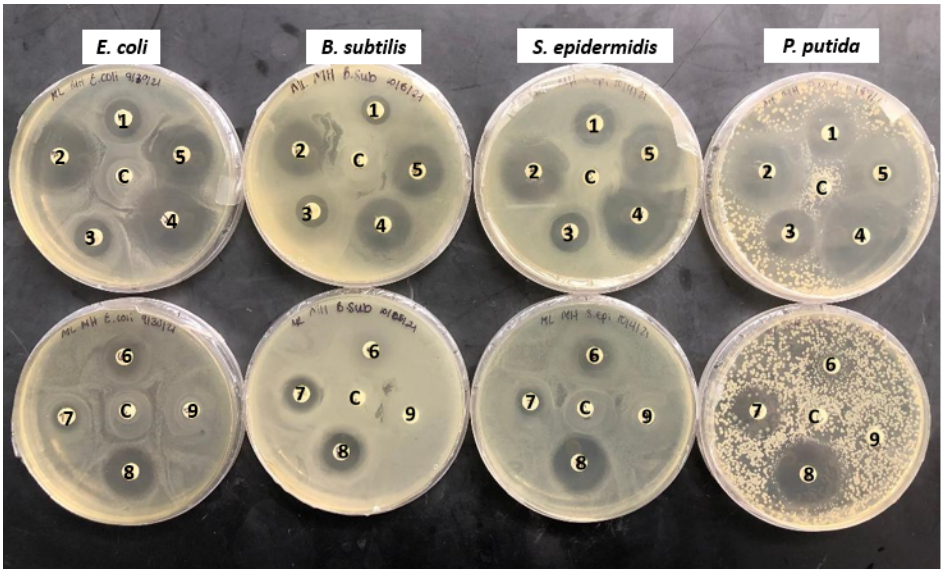

Figure 4: Disk-diffusion assay. Each ESKAPE safe relative (E. coli, B subtilis, S epidermidis and P. putida) was spread on LB agar and antibiotic disks were placed on top of the bacteria. Plates were incubated at 30°Cfor 24 hours. Zones of inhibition around each disk were measured and recorded (see Table 1). C- control, 1- Streptomycin, 2- Chloramphenicol, 3- Neomycin, 4- Ciprofloxacin, 5- Tetracycline, 6- Erythromycin, 7- Novobiocin, 8- Kanamycin, 9- Penicillin.

Pigment Testing

Prodigiosin and violacein were used in a disk-diffusion assay on *E. coli* and *B. subtilis* (data not shown) in combination with neomycin (Table 2). Antibiotic activity or the enhancement of neomycin activity was determined by the diameter of the zones of inhibition

surrounding the disks (Figure 7). Pigments alone did not demonstrate antibiotic activity and did not provide any additive or synergistic impacts with neomycin.

Trial	Pigment	ESKAPE safe-relative	Commercial Antibiotic
1	Prodigiosin	B. Subtilis	N/A
2	Violacein	B. Subtilis	N/A
3	Prodigiosin+violacein	B. Subtilis	N/A
4	Prodigiosin	B. Subtilis	Neomycin
5	Violacein	B. Subtilis	Neomycin
6	Prodigiosin+Violacein	B. Subtilis	Neomycin
7	Prodigiosin	E. coli	N/A
8	Violacein	E. coli	N/A
9	Prodigiosin+Violacein	E. coli	N/A
10	Prodigiosin	E. coli	Neomycin
11	Violacein	E. coli	Neomycin
12	Prodigiosin+Violacein	E. coli	Neomycin

Table 2: Trials to test the ability of the pigments to inhibit pathogens or act synergistically with the antibiotic neomycin. Trials without a commercial antibiotic were listed as N/A. Purified pigments were absorbed onto a blank disk (trials 1-3 and 7-9) or on a disk containing neomycin (trials 4-6 and 10-12). Trials 1-6 were unable to be completed due to lack of time.

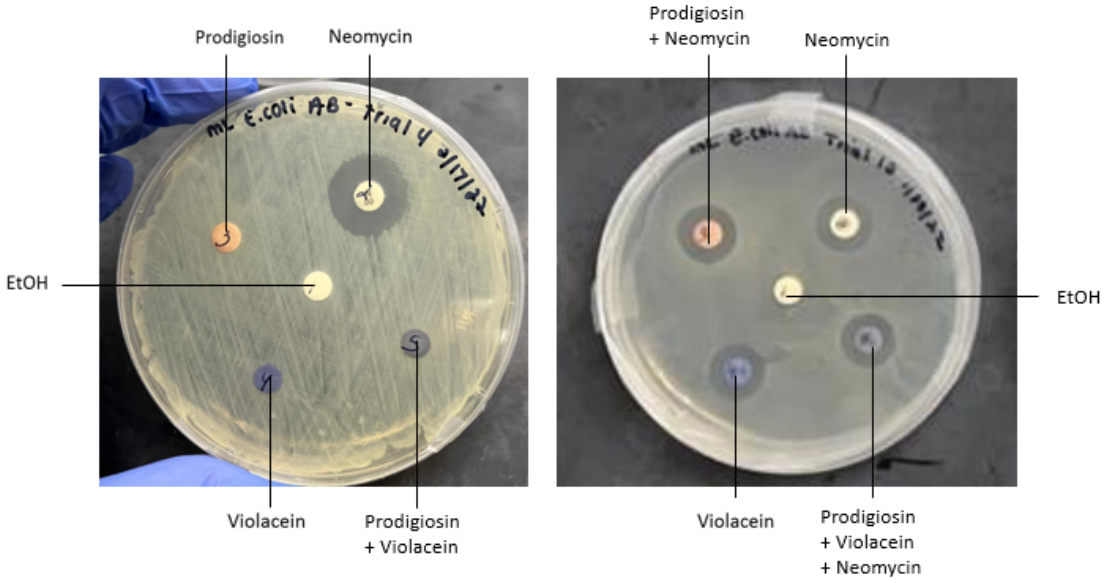

Figure 5: Disk diffusion assays with purified pigments tested against E. coli grown on TSA. Trials were set up as indicated in Table 2. Plate on the left is representative of trials with blank disks. Plate on the right is representative of antibiotic (neomycin) trials. The order of the disks - beginning at the top and going clockwise: Neomycin disks; disks dipped in prodigiosin and violacein; disks dipped in violacein; neomycin disks dipped in prodigiosin. Middle disk: blank disk dipped in ethanol as a control. Remaining trials as indicated in Table 2 are not shown. Each trial was repeated 20 times.

	EtOH	AB	AB+PRO	AB+VIO	AB+VIO+PRO
Mean (mm)	3.800 ± 0.975	13.700 ± 0.147	14.450 ± 0.185	14.050 ± 0.198	13.475 ± 0.168

Table 3: Zones of inhibition from the antibiotic trials shown by Mean +/- 1SEM. Diameters of zones for each condition were measured. The measurements from the 20 trials in E. coli were averaged for each condition (EtOH- ethanol control; AB:

Neomycin disks; AB+PRO; Neomycin disk dipped in prodigiosin; AB+VIO: Neomycin disk dipped in violacein; AB+VIO+PRO: Neomycin disk dipped in both pigments. The mean and SEM for EtOH (control) may be skewed due to the ethanol not completely evaporating before being placed on the plate.

Since antibiotic production was not seen through the in vitro testing of pigments produced by environmental isolates, a lab strain of *S. marcescens* was used to perform in vivo testing of its antibiotic activity via patch plating and in vitro testing after extraction of prodigiosin (Figure 8). Despite the coloration being a very pale pink and not the bright red, *S. marcescens* inhibited the growth of *E. coli* when introduced on a patch plate. However, when this strain was grown for prodigiosin production, and the pigment was purified, the pigment did not display antibiotic activity during the disk-diffusion assay (Figure 8).

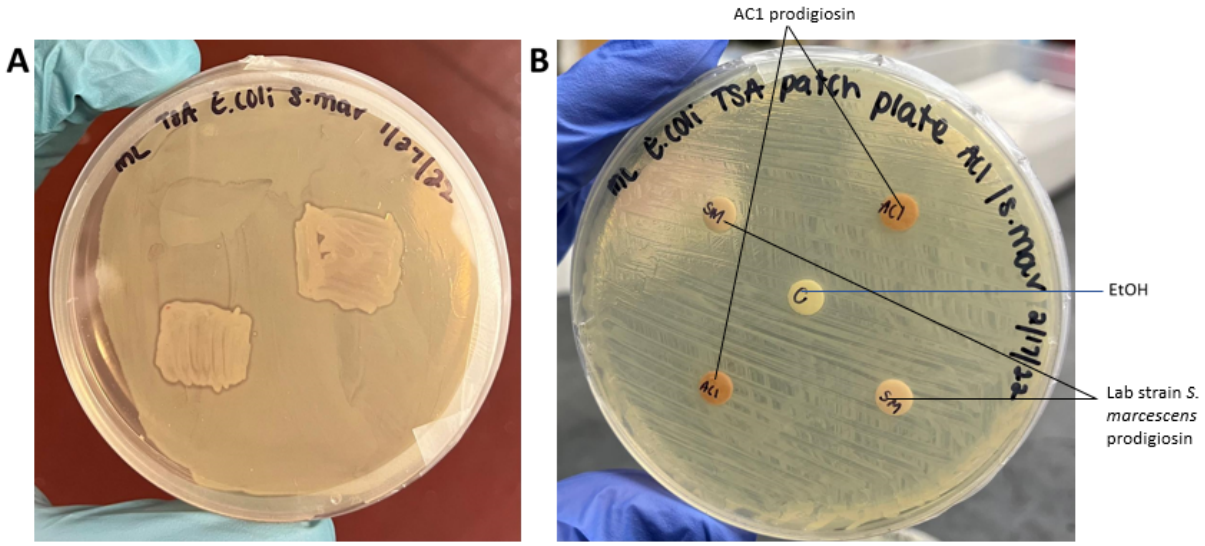

Figure 8: Testing for antibiotic activity in a laboratory strain of S. marcescens grown on TSA: A) A lab strain of S. marcescens was patch plated on E. coli. Zones of inhibition surrounding the patches indicate antibiotic activity. B) Prodigiosin was extracted from the lab strain of S. marcescens and was tested using a disk-diffusion assay against E. coli. No zones of inhibition were seen, indicating no antibiotic activity associate with the extract.

Reactivation of Antibiotic Production

The lack of correlation between the production of pigments and antibiotic activity leads to the hypothesis that the pigments themselves are not solely responsible for the antibiotic activity in *S. marcescens* and C. *violaceum*, and that the antibiotic production in AC1 and AG1

were diminished after laboratory isolation. One reason why this may occur is due to the medium used to grow the bacteria. To test this hypothesis, the original AC1 and AG1 samples that were stored in glycerol stocks at -80°C were regrown on nutrient rich media. These cultures were then patch plated with four ESKAPE safe relatives. No antibiotic activity was noted; however, pigment production was obvious (Figure 9). In attempts to reactivate the antibiotic production, the bacteria were grown on different types of nutrient deficient media as well as media supplemented with soil. After growth on these different media, AC1 and AG1 were patch plated on four EKSAPE safe relatives. Pigment production was expressed in all trials on the different media, but no antibiotic activity was noted (Figure 9). Additional patch plates were done with diluted ESKAPE safe relatives: pigment production was detected in AC1 and AG1, but antibiotic activity was still not detected (data not shown).

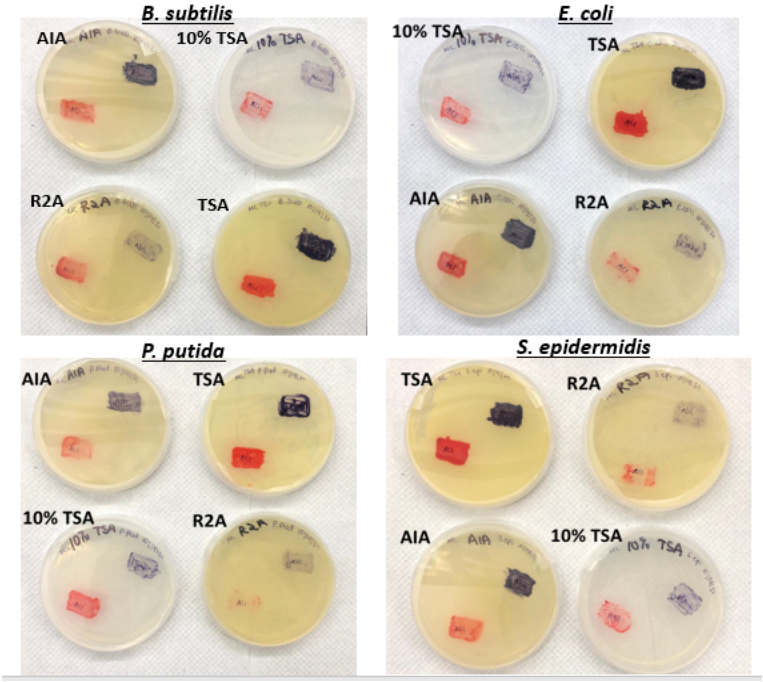

Figure 9: Growth on different media did not reactivate antibiotic production in AC1 or AG1. AC1 and AG1 were grown on four different media (nutrient rich TSA and nutrient deficient R2A, AIA and 10% TSA) and then patch plated on ESKAPE safe relatives (E. coli, B. subtilis, S. epidermidis, and P. putida) grown on the same medium. Pigment production varies based on the medium used, but all conditions show expression of prodigiosin and violacien. However, no zones of inhibition were detected.

Another reason for the reduction in antibiotic activity may be due to lack of competition with other bacteria that may exist in the native habitat of AC1 and AG1, reducing the need for these bacteria to produce the antibiotic. In addition to the traditional patch plating method (Figure 9), AC1 and AG1 were streaked side-by-side next to *E. coli, B. subtilis, S. epidermidis,* and *P. putida* (data not shown), on 10% TSA and R2A. Pigment production was observed, but there was no evidence of inhibition of ESKAPE safe relatives (Figure 10).

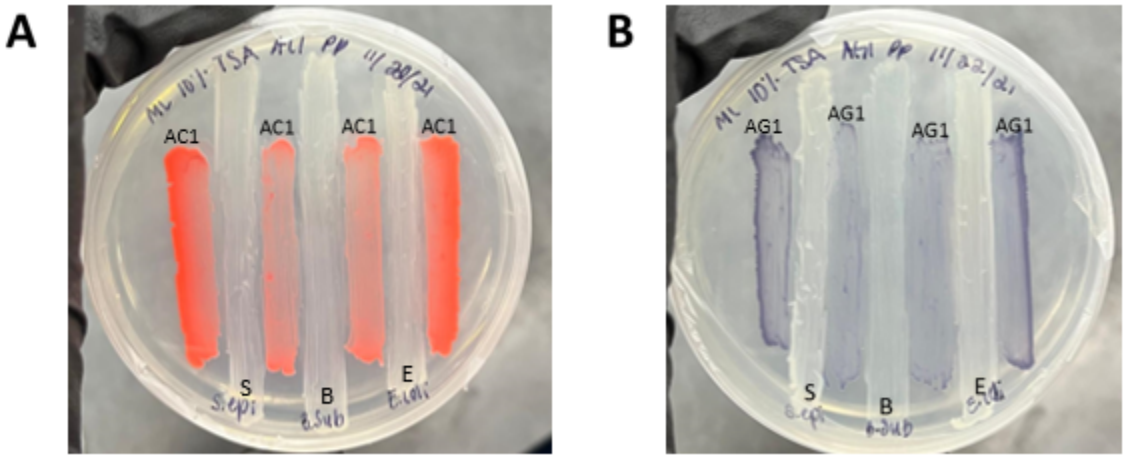

Figure 10: Attempts to reactivate antibiotic production in AC1 and AG1. ESKAPE safe relatives B. subtilis, E. coli., P. putida (not shown), and S. epidermidis were streaked side-by-side next to A) AC1 and B) AG1 on 10% TSA plates. The pigment production of prodigiosin was strong in AC1 but weaker in AG1. No clearing of ESKAPE safe relatives was detected.

Another avenue to reactivate antibiotic production has been done by culturing bacteria in the presence of *M. smegmatis* (B. Gasper, personal communication). To test this, *M. smegmatis* was heavily streaked on TSA and freezer stocks of AC1 and AG1 were patch plated on the lawn. AC1 produced a clear zone of inhibition. The results with AG1 are inconclusive, but the edges of the patch plate were unusual, indicating possible antibiotic production (Figure 11).

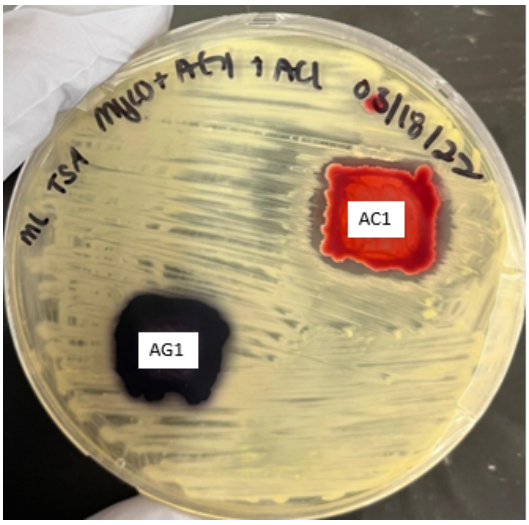

Figure 11: Patch plate of AC1 and AG1 against Mycobacterium smegmatis. Mycobacterium smegmatis was heavy streaked on a TSA plate. AC1 and AG1 were plated on top of the lawn. Antibiotic activity was seen against M. smegmatis in AC1, but the results for AG1 are inconclusive.

To further test the reactivation in AC1, colonies grown on TSA, 10% TSA soil plates, and the patch plate against *M. smegmatis* (Figure 11) were patch plated against *E. coli* and *B. subtilis* on TSA. Despite the previous antibiotic activity seen in AC1 against *M. smegmatis*, the patches did not produce a zone of inhibition against the ESKAPE safe relatives (Figure 12).

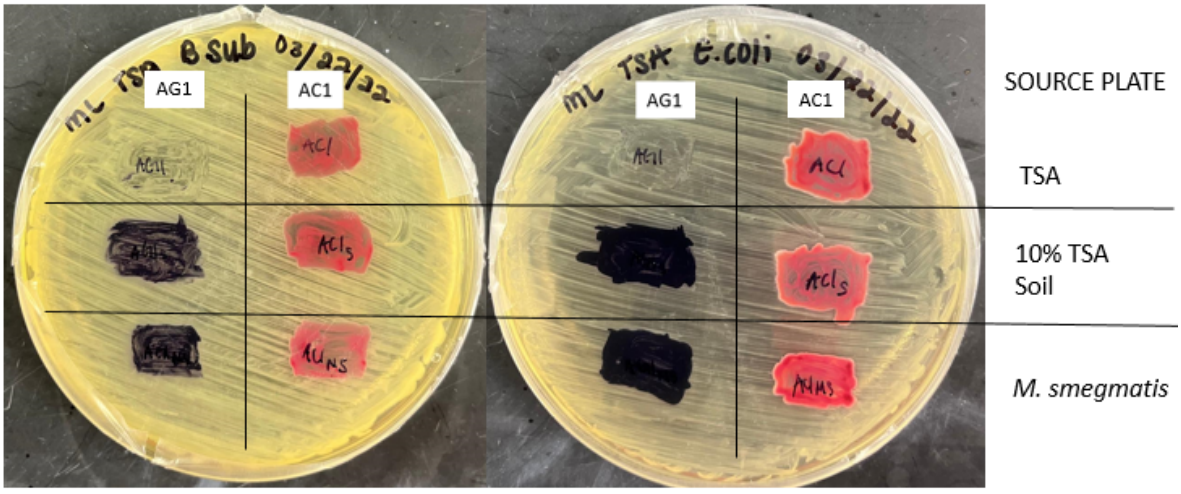

Figure 12: AC1 and AG1 from different sources patch plated against ESKAPE safe relatives. AC1 and AG1 from lab samples, AC1 and AG1 taken from the soil agar, and AC1 and AG1 that had inhibited M. smegmatis were patch plated on top of heavy streaked plates of B. subtilis (left) and E. coli (right) on TSA.

Samples of AC1 and AG1 were taken from the patch plate against *M. smegmatis* that showed inhibition (Figure 11) and mixed in combinations with *M. smegmatis* on top of a heavy

streaked lawn of *E. coli* and *B. subtilis* (Figure 13). None of the trials produced any antibiotic activity against the ESKAPES.

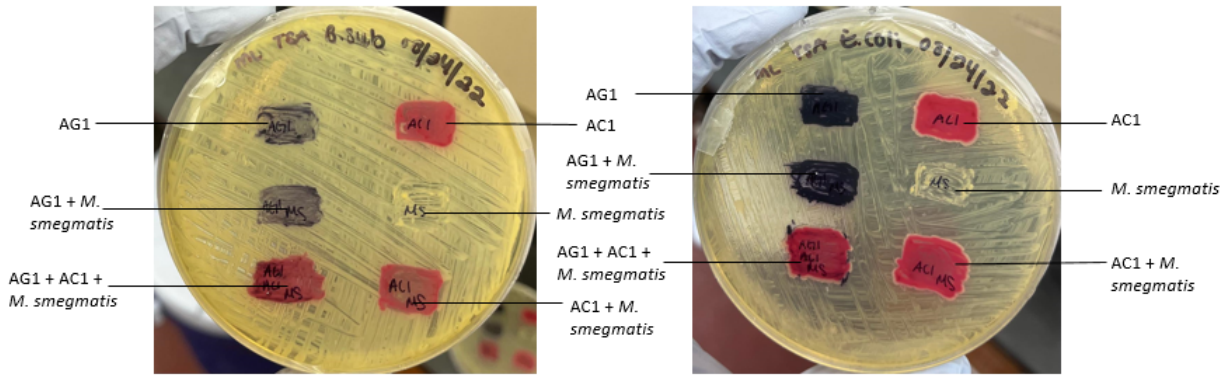

Figure 13: Mixtures of bacteria tested against ESKAPE safe relatives. Samples of AC1 and AG1(grown in the presence of M. smegmatis), AC1 mixed with M. smegmatis, AG1 mixed with M. smegmatis, M. smegmatis alone, and AC1, AG1, and M. smegmatis mixed were patch plated on heavy streaked lawns of B. subtilis (left) and E. coli (right) on TSA to test for antibiotic activity.

Discussion

Antibiotic properties of the pigments prodigiosin and violacein were used in both in vivo and in vitro assays to test for potential synergistic effects of the combination of the pigments. Our results contradict previous research that indicates the pigments produced by *S. marcescens* and *C. violaceum* demonstrate antibiotic activity. When the pigments from AC1 and AG1 were isolated, there was no evidence of antibiotic activity against *E. coli*. This was also noted in lab strains of *S. marcescens* that had previously shown antibiotic activity against *E. coli*: while the bacteria showed a zone of inhibition on a patch plate, no inhibition was seen during the disk-diffusion assay with prodigiosin isolated from this lab strain. This does not appear to be an issue with concentration, as both pigments were produced in abundance in the environmental strains, and the lab strain of *S. marcescens* showed antibiotic activity in vivo with very light pigmentation production. Also, the difference in the prodigiosin extract concentration between

AC1 and the lab strain of *S. marcescens* indicated that prodigiosin was not inhibitory at various concentrations.

One possibility that could lead to the lack of antibiotic activity is a mutation in the biosynthetic pathways of both environmental isolates that allowed for the pigment to still be produced, but ultimately shut off the antibiotic activity. In AC1 (*S. marcescens*), the biosynthetic pathway that regulates the production of prodigiosin, which is made up of 14-15 different genes, is the combination of pathways MAP and MBC (Pan et al., 2020). The pathway itself is complex, and ultimately a disruption in the pathway would end with the pigment no longer being produced. Similarly, in AG1 (*C. violaceum*) the pathway is composed of five different genes, and has been considered unstable due to spontaneous modifications in its regulation, which can impact the production of violacein (Jiang et al., 2010). While these modifications could impact the metabolite, it seems as if a disruption in the pathway would inhibit the pigment entirely, rather than just its antibiotic activity.

In the case that an enzyme in the metabolic producing the pigment did undergo a mutation causing a change in its activity, it could be possible that the antibiotic activity was lost. However, the likelihood of this occurring in two different bacterial strains to have the exact same impact on the antibiotic activity is highly unlikely. This hypothesis can also be contradicted by the observation that antibiotic production was induced in AC1 when streaked with *M. smegmatis*. If the pathway was completely blocked, the antibiotic activity would not have reappeared in the presence of mycolic acid. This makes mycolic acid of special importance in the future of antimicrobial reactivation, especially as the antibiotic activity turned back off when streaked against the ESKAPE safe relatives after being reactivated.

Another possibility is the requirement for cofactors to work simultaneously with the pigments in order for activity to be expressed. In *S. marcescens*, serratomolide, a compound produced by the SwrW gene and also known as serrawettin W1, has been shown to have antimicrobial properties (Kadouri & Shanks, 2013). Serratomolide could potentially work in combination with other microbial components, such as prodigiosin, to activate the antibiotic within the bacteria. However, the crude extracts prepared in this study were done using standard protocols from other published articles on prodigiosin and violacein (Dodou et al., 2017; Giri et al., 2004; Gohil et al., 2020; Mathlom et al., 2018; Park et al., 2012; Subramanian et al., 2014), where antibiotic activity was seen in the isolated pigments. Because these were not purified pigments, any potential necessary cofactors would likely still be present in the extract. Further research is necessary to determine the exact role prodigiosin and violacein play in these bacteria, and what component(s) allow for the bacteria that produce them to demonstrate antibiotic activity.

Anecdotal evidence from the Tiny Earth Network indicates that the antibiotic production in isolated lab or environmental bacterial strains are increasingly becoming difficult to maintain (B. Gasper, personal communication). While the exact reason behind this diminished activity is not well researched, it could be attributed to multiple factors. Generally, most of the environmental isolates express antibiotic activity relatively close to when they were originally isolated; however, as these strains are stored for an extended period of time, they may become too far removed from their original isolation conditions. AC1 and AG1were isolated in fall of 2018 and 2019 respectively, and stored as glycerol stocks at -80°C until fall of 2021. Upon restreaking the isolates and testing them for activity, neither strain produced antibiotics as seen through the results from traditional patch plates or side-by-side patches. This loss of antibiotic

production could be due to the environmental strains no longer having an intrinsic motivation to produce antibiotics due to the lack of competition (B. Gasper, personal communication). Despite being introduced to competing bacteria, the testing conditions and previous storage of the strains had provided enough nutrients to make it plausible that antibiotics became nonessential and the pathways were shut down. With environmental isolates originally producing antibiotics and losing this ability over time, further research needs to be done to determine how long they can be stored before the pathway breaks down and how it can be reactivated.

Another major consideration that is impactful on this study is the presence of publication bias. There has been no published data about the pigments used in this study lacking antibiotic activity, nor has there been published reports about antibiotic producers losing this ability. This reflects a critical problem with publication bias. Publishing negative or inconclusive data within the scientific community is not a priority, and indeed is widely avoided. Publication bias within academic journals leads to selective reporting, the situation where it is unlikely that null or negative results are published (Bernard et al., 2020). Selective reporting may lead to the misconception that specific scientific methods are always successful, and to the practice where researchers are more likely to omit negative results from their reports, only including what worked or was deemed significant. This is a growing problem within the community: as research expands and other scientists run into complications, selective reporting eliminates a valuable resource that could be provided by the reporting of “failed” attempts. If more problems were reported, along with the strategies that were used to resolve them, many resources could be saved as researchers would not have to waste time and funding attempting to repeat work that had already been done but not reported in the literature. It is unknown how serious this bias may be in stifling the progress of science. It also has impacts on the reputations of researchers and labs.

Although attempts may fail, this does not negate the creativity, hard work, and innovation that are required to make these attempts. The impact of these negative results not being published can, unfortunately, be measured in lack of future funding and in tenure and promotion decisions in academia. It may also lead to rushed results, or, at worst, fabricated and falsified data, due to the pressures of "publish or perish" seen in the scientific community. The questions raised in this study are impossible to answer with the current published data available, and what is published is of no help in providing ideas or additional avenues of research that could be attempted. This highlights the need to publish more than just "significant" results. Negative results are just as powerful and important as positive results, and the ability to overcome publication bias would provide a huge advantage to researchers and ultimately benefit the scientific community as a whole.

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