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Upregulation of Antibiotic Activity of a Streptomyces sp. Via Co-Cultures with Challenge Pathogens

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Abstract

Marine natural product drug discovery has begun to play an important role in the treatment of diseases. Early drug discovery from natural products came primarily from plants, but after the discovery and development of penicillin, scientists started looking at natural products from microorganisms. Numerous natural products have been discovered from members of the order Actinomycetales, particularly in the genus *Streptomyces*, due to their metabolic diversity in the production of biologically active secondary metabolites. Ascidians, also known as tunicates, are marine invertebrates that contain many host-associated microbes. Adult tunicates are sessile, which makes them vulnerable to predators, and thus, they are hypothesized to use host-associated bacteria and their secondary metabolites for chemical defense. Many secondary metabolites cannot be produced under laboratory conditions because growth conditions in a flask culture differ from conditions of the natural environment. One method, mixed fermentation, has been show to increase yields of previously described metabolites, cause production of previously undetected metabolites, and increase antibiotic activity of co-cultured extracts. A *Streptomyces* sp. from a Panamanian tunicate was isolated and subsequently co-cultured with challenge organisms *Bacillus subtilis*, methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* and extracted. The minimum inhibitory concentration of the extracts and the LC-MS profiles show upregulation of the challenge extracts, particularly the *Streptomyce*s sp. co-cultured with MRSA. The competitive interactions of co-cultures may enhance metabolite production and further our understanding of microbial interactions.

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List of Abbreviations

MSSA: Methicillin-sensitive *Staphylococcus aureus*

MRSA: Methicillin-resistant *Staphylococcus aureus*

BS: *Bacillus subtilis*

PA: *Pseudomonas aeruginosa*

MIC: Minimum Inhibitory Concentration

LC-MS: Liquid Chromatography Mass Spectrometry

HPLC: High Performance Liquid Chromatography

ACN: Acetonitrile

R2A: Reasoner's 2A

IO: Instant Ocean

YSP: Yeast Starch Peptone

MeOH: Methanol

DCM: Dichloromethane

OD: Optical Density

DMSO: Dimethyl Sulfoxide

UV: Ultraviolet

AntiSMASH: Antibiotics and Secondary Metabolite Analysis Shell

PCA: Percent Control Activity

EIC: Extracted Ion Chromatogram

Introduction

Natural products produced from microbes and microbial interactions have played a significant role in drug discovery and the treatment of diseases (Newman and Cragg 2012). Almost two-thirds of all small molecule drugs launched from 1981 to 2006 were derived from natural products (Pettit 2009). Early drug discovery from natural products focused primarily on plants, but after the discovery and development of penicillin, scientists started looking at natural products from microorganisms (Cragg and Newman 2013). New antimicrobial drugs need to be discovered and developed due to the increasing resistance in pathogenic bacteria (Malapaka et al. 2007).

Microbial communities in marine invertebrates have been extensively studied and have been shown to contain many bioactive compounds. Ascidians, a type of marine invertebrate, have been found to contain various secondary metabolites with pharmaceutical potential (Donia et al. 2011). Also known as tunicates, ascidians become sessile when they mature and inhabit many different marine environments. Although not closely related to sponges phylogenetically, they adopt a similar sedentary, filter-feeding life style (Schmidt and Donia 2010). A common trait of marine invertebrates is the symbiotic microbial communities they typically house (Erwin et al. 2014). These invertebrates provide nutrients and an environment to inhabit, and the structural and functional diversity of these communities provide evidence that the host provides fertile microbial niches (Erwin et al. 2014). Being sessile, tunicates are vulnerable to predators and thus they are hypothesized to use host-associated bacteria and their secondary metabolites for chemical defense. Approximately 1,000 marine natural products have been isolated from ascidians (Schmidt and Donia 2010). Many of these secondary metabolites have been shown to be important for biotechnology and drug discovery (Erwin et al. 2014).

Bacteria within the order Actinomycetales are very abundant in the marine environment and have found to been a great source of natural products due to their metabolic diversity in the production of biologically active secondary metabolites (Nett et al. 2009). Actinobacteria have been found to be well adapted to living in marine invertebrates, such as sponges, corals, and ascidians (Valliappan et al. 2014). In a study that analyzed 203 16S rRNA gene sequences from GenBank, approximately 34 genera within 16 families of the order Actinomycetales were associated with tunicates (Valliappan et al. 2014). Of all the marine bacteria discovered, actinomycetes comprise approximately 10%, but extensive research on actinobacteria has resulted in 45% of the 22,000 biologically active compounds obtained from microbes (Subramani and Aalbersberg 2013). Actinobacteria continue to be a potential source for undiscovered biologically active compounds.

Actinomycetes are often part of host-associated microbial communities that produce secondary metabolites hypothesized to protect the host from the external environment. They produce a wide variety of natural products including polyketides, isoprenoids, phenazines, peptides, indolocarbazoles, and sterols (Valliappan et al. 2014). Many of these compounds have been shown to have antimicrobial, anticancer, antiviral, antiparasitic, antioxidant, anti-HIV, and many other biological activities (Manivasagan et al. 2014). Actinomycetes have been discovered to dedicate up to 10 percent of their genome to secondary metabolite biosynthesis (Carlson et al. 2015). Much of the research on actinomycetes has been devoted to the genus *Streptomyces*, and thus, of the secondary metabolites produced by actinomycetes, the majority of the compounds isolated have been produced by *Streptomyces* sp. (Fenical and Jensen 2006). Even so, only approximately 3% of the natural product potential of *Streptomyces* sp. has been discovered, thought to be due to the silencing of genes under laboratory conditions (Gontang et al. 2010).

Actinomycetes, particularly in the genus *Streptomyces*, have the genetic capability to produce marine natural products, most of which have not been discovered.

Even with the genetic capacity to produce metabolites, one issue with natural product discovery is that bacteria have the ability to produce many secondary metabolites that cannot be produced under laboratory conditions because growth conditions in a flask culture differ from conditions of the natural environment (Shank and Kolter 2009). Genomic analyses reveal a great abundance of biosynthetic gene clusters indicative of the potential to produce secondary metabolites, especially in *Streptomyces*, but only a small portion are expressed under common laboratory conditions (Hoshino et al. 2015).

Microorganisms interact with each other in the natural environment, and these interactions are thought to be an ecological driving force for the production of bioactive secondary metabolites (Oh et al. 2007). Many marine microorganisms produce these bioactive secondary metabolites in response to environmental conditions, such as limited nutrients, space, or pathogenic fungi or bacteria. Numerous methods have been used to maximize the chemical diversity of bacteria. One method, mixed fermentation, has been show to increase yields of previously described metabolites, causing production of previously undetected metabolites, and increasing antibiotic activity of co-cultured extracts (Pettit 2009). Competition in co-cultures may result in the induction of secondary metabolites that cannot be produced while cultured independently (Oh et al. 2005). The competitive interactions in co-cultures may enhance metabolite production and thus may further our understanding of microbial interactions.

Bacterial-bacterial interactions have been shown to induce production of secondary metabolites. Table 1 summarizes literature reports of co-culture experiments and the secondary metabolites that were produced. In one experiment, 10 mL of *Nocardiopsis* sp. and 10 mL of

Actinokineospora sp. were co-cultured in 1 L of media and incubated for 7 days (Dashti et al. 2014). Three secondary metabolites were induced in the co-culture that were not detected in the monocultures (Dashti et al. 2014). In another study, *Streptomyces* sp. cultivated with mycolic acid containing bacterium resulted in the isolation of three novel cytotoxic butanolides, chojalactones A-C (Hoshino et al. 2015). One mL of each individual culture was added to 100 mL of broth and cultured for 6 days (Hoshino et al. 2015). Another mixed fermentation experiment also used a mycolic acid containing bacterium, *Tsukamurella pulmonis*, to induce secondary metabolite production in a *Streptomyce*s sp. leading to the production of a novel antibiotic, alchivemycin A (Onaka et al. 2011). Mycolic acid is thought to localize in the outer cell layer of the inducer, influencing secondary metabolite production in the *Streptomyces* sp. (Onaka et al. 2011).

In another study testing bacterial-bacterial interactions, 95 actinomycete strains were co-cultured with Proteobacteria, Firmicutes, and Actinobacteria (Carlson et al. 2014). Results showed that 65% of Proteobacteria were able to induce the production of the antibiotic resistomycin, compared to 5.9% of Firmicutes and 9.1% of Actinobacteria (Carlson et al. 2014). This indicates that not all challenge pathogens are able to induce production of secondary metabolites. However, these results show that a certain phylum, Proteobacteria, was able to induce the production of resistomycin more than others at 65% (Carlson et al. 2014).

Competition experiments were also conducted to test istamycin production in *Streptomyces tenjimariensis* with competitive species inoculated 24 prior, 24 after, and co-inoculated (Slattery et al. 2000). Twelve of the 53 species of marine bacteria caused an increase in production of istamycins, with significantly higher levels of production when *S. tenjimariensis* was pre-established (Slattery et al. 2000). Twelve of the 53 species tested induced increased

production of the istamycins, and there was higher istamycin production when the challenge organism was inoculated after *S. tenjimariensis* was already pre-established (Slattery et al. 2000).

Fungal-bacteria interactions can also be employed with either the fungus or bacterium being the challenge organism. A fungus, *Pestaliotia* sp. was cultured in 1 L of media and challenged with 10 mL of a bacterial strain after 24 hours and incubated for 6 additional days (Cueto et al. 2001). This induced the production of a new antibiotic, pestalone, in response to bacterial challenge (Cueto et al. 2001). In another bacterial challenge, 1 mL of a 3-day bacterial culture was added into 1 L of a 3-day fungal culture and incubated for 2 days (Oh 2005). The cytotoxic diterpenoids, libertellenones A-D, were produced by the marine-derived fungus in response to challenge with a proteobacterium (Oh et al. 2005). With the same method, a marine actinomycete challenge organism induced the production of emericellamides A and B in a marine-derived fungus (Oh et al. 2007).

On the other hand, fungi can be used as challenge organisms against bacterial strains. To induce production of secondary metabolites in a mine drainage-derived *Sphingomonas* bacterial strain, 250 μL of a fungus was inoculated into a 2-day culture of the bacterium and incubated for 15 days (Park et al. 2009). The mixed fermentation resulted in isolation of an antibiotic-antitumor metabolite, glionitrin A (Park et al. 2009).

Fungal-fungal interactions have also been found to cause induction of new secondary metabolites. One method employed co-cultures of agar plugs containing mycelial growth in liquid media where a new xanthone derivative was isolated from two mangrove fungi (Li et al. 2011). In another study, a new antibacterial alkaloid, aspergicin, was also produced in mixed fermentation of two marine-derived mangrove epiphytic fungi (Zhu et al. 2011). Using a similar co-culture method in another experiment, a novel alkaloid, marinamide, and its methyl ester were

produced with co-culture of two endophytic fungi from mangroves in the South China Sea (Zhu and Lin 2006). Another method employed agar co-cultures of two fungi for the formation of new lipoaminopeptides, acremostatins A, B, and C (Degenkolb et al. 2001).

Table 1: Co-culture experiments and secondary metabolites in literature.

Bacterial-Bacterial Interactions					
Organism	**Challenge Organism**	**Method of Co-Culture**	**New Compound(s) or Increased Production**	**Activities of Compound**	**Reference**
Actinokineospora sp. (B)[1]	*Nocardiopsis* sp. (B)[1]	10 mL of each culture in 1L added together	3 new compounds[2]	Antibiotic (Phenazine)	Dashti et al. 2014
Streptomyces sp. (B)	Mycolic acid containing bacterium (B)	1 mL of each culture in 100 mL added together	Chojalactones A-C	Cytotoxic	Hoshino et al. 2015
Streptomyces lividans (B)	*Tsukamurella pulmonis* (B)	3 mL of bacteria and 1 mL of challenge organism in 500 mL culture	Alchivemycin A	Antibiotic	Onaka et al. 2011
Streptomyces sp. (B)	Four strains of Proteobacteria (B)	Co-culture for 4-14 days (volume and time dependent on bacteria used)	Resistomycin	Antibiotic	Carlson et al. 2014
Streptomyces tenjimariensis (B)	Marine bacteria (B)	Challenge organism inoculated 24 prior, 24 after, and at the same time with equal cell concentrations	Istamycin A and B	Antibiotic	Slattery et al. 2000

[1] Organism is either organism or challenge organism and cannot be determined.
[2] *N*-(2-hydroxyphenyl)-acetamide, 1,6-dihydroxyphenazine, and 5a,6,11a,12-tetrahydro-5a,11a-dimethyl[1,4]benzoxazino[3,2-b][1,4]benzoxazine.

Table 1 (continued): Co-culture experiments and secondary metabolites in literature.

Bacterial-Fungal Interactions					
Organism	**Challenge Organism**	**Method of Co-Culture**	**New Compound(s) or Increased Production**	**Activities of Compound**	**Reference**
Sphingomonas sp. (B)[1]	*Aspergillus funigatus* (F)[1]	After 2 days, 250 µL of challenge added to 500 mL culture	Glionitrin A	Antibiotic, antitumor	Park et al. 2009
Libertella sp. (F)	Marine proteobacterium (B)	After 3 days, 1 mL of challenge into 1 L culture	Libertellenones A-D	Cytotoxic	Oh et al. 2005
Emericella sp. (F)	*Salinospora arenicola* (B)	After 3 days, 1 mL of bacterial challenge into 1 L culture	Emericellamides A and B	Antibiotic	Oh et al. 2007
Rosenvingea sp. (F)	Unidentified marine bacterium	After 24 hours, 10 mL of bacterial challenge into 1 L culture	Pestalone	Antibiotic, cytotoxic	Cueto et al. 2001
Fungal-Fungal Interactions					
Organism	Challenge Organism	Method of Co-Culture	New Compound(s) or Increased Production	Activities of Compound	Reference
Mangrove fungus (F)	Mangrove fungus (F)	Co-inoculation of agar plugs	Xanthone derivative	Antibiotic	Li et al. 2011
Mangrove endophytic fungus (F)	Mangrove endophytic fungus (F)	Co-inoculation	Marinamide and its methyl ester	Antibiotic	Zhu and Lin 2006
Mangrove endophytic fungus (F)	Mangrove endophytic fungus (F)	Co-inoculation of agar plugs	Aspergicin	Antibiotic	Zhu et al. 2011
Acremonium sp. (F)[1]	*Mycogone rosea* (F)[1]	Surface co-cultures on agar	Acremostatins A, B, and C	Lipoamino-peptides	Degenkolb et al. 2001

[1] Organism is either organism or challenge organism and cannot be determined

We have isolated a Panamanian tunicate-associated *Streptomyces* sp. that has extensive secondary metabolite biosynthesis potential, as discovered via whole genome sequencing. The bacterial extract also shows potent antimicrobial activity against several human pathogens. We hypothesize that challenging this strain with human pathogens, such as *Bacillus subtilis* (BS), methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* (PA), may increase production of antimicrobial compounds and thus increase activity against the pathogens. Co-cultures with small quantities of challenge organisms with cultures of this *Streptomyces* sp. will be performed and extracts will be tested against the challenge pathogens to determine the minimum inhibitory concentration (MIC). We will also analyze the extracts for increased production of compounds or novel compounds.

Experimental

General Experimental

Liquid chromatography mass spectrometry data was collected on an Agilent ESI single quadrupole mass spectrometer coupled to an Agilent high performance liquid chromatography (HPLC) system with a G1311 quaternary pump, G1322 degasser, and a G1315 diode array detector using an Eclipse XDB-C_{18} (4.6 × 150 mm, 5 μm) RP-HPLC column with a solvent gradient of 25-100% acetonitrile (ACN) with 0.1% formic acid over 75 minutes. All dry media and agar for culturing were purchased from Difco and subsequently prepared with water and autoclaved. All reagents and HPLC grade solvents were purchased from Sigma Aldrich. All bacterial products were purchased from ATCC.

Tunicate Collection and Bacterial Isolation

The tunicate, *Styela canopus*, was collected in a mangrove from Bastimentos Park in Bocas de Toro, Panama (9°17.398' N 82°11.106' W). The tunicate was rinsed with ethanol then sterile seawater, homogenized, and plated onto a Reasoner's 2A (R2A) plus instant ocean (IO) agar plate with cycloheximide (80 mgl^{-1}) and nalidixic acid (20 mgl^{-1}) to reduce fungal contamination and fast growing bacteria. The R2A medium contained yeast extract 0.5g, proteose peptone no. 3 0.5g, casamino acids 0.5g, dextrose 0.5g, soluble starch 0.5g, sodium pyruvate 0.3g, dipotassium phosphate 0.3g, and magnesium sulfate 0.05g in 1 liter of Millipore water (Becton, Dickinson and Company, New Jersey, USA). PTY087I2 was isolated onto R2A + IO agar and stored on nutrient agar slants. The bacterium was re-streaked onto yeast starch peptone, YSP +IO agar, a nutrient rich agar. This medium contained soluble starch 5g, trypticase peptone 1g, yeast extract 2g in 1 liter of Millipore water (Becton, Dickinson and Company, New Jersey, USA). The bacterium was stored in liquid nitrogen and -80 C cryogenic vials.

Co-Culture in Liquid Media and Extraction

A confluent culture of PTY087I2 was prepared prior to the start of the experiment. In addition, the challenge pathogens were streaked out onto agar plates. On day one, 1 mL of culture was added to 25 mL of YSP+IO broth in a 125 mL baffled flask to the monoculture and each co-culture (Derewacz et al. 2015). One colony of each pathogen was inoculated into 5 mL of YSP+IO broth. On day two, 200 µL of each challenge pathogen was added to the respective co-culture and challenge pathogen monoculture. The cultures were incubated at 30 ºC at 200 RPM for 6 days after challenging. After confluent growth in the large culture, HP20 Diaion beads were prepared by washing them in a frit filter with methanol and water. The beads were then added to the culture and placed on a shaker for 24 hours. The purpose of the beads was to

temporarily attach to secondary metabolites produced by the bacteria. The beads were first washed with 60 mL of deionized water. The metabolites were then released from the beads using organic solvents. The beads were washed with 60 mL of methanol (MeOH), 60 mL of dichloromethane (DCM), and 60 mL of acetone. All organic solvents were combined and dried in vacuo.

96 Well Bioassays

All bioassays used 96 well plates flat bottom plates from Costar™. Extracts were prepared at stock concentrations of 50 mg/mL. The 96 well bioassays were modified from Zgoda and Porter 2011. Pathogens were inoculated into 5 mL of broth at an optical density (OD) of 0.08-0.10 equivalent to 0.5 McFarland Standard (approximately 1 x 10^8 CFU/mL). To prepare the master mix, 1.6 mL of the inoculum were added, along with 7.84 mL of sterile water, and 6.4 mL of broth into a 50 mL falcon tube. With a multi-channel pipette, 198 µL of master mix was aliquoted into the designated wells except the sterility and media wells. 98 µL of sterile water and 100 µL of broth was added into the sterility control wells. 2 µL of dimethyl sulfoxide (DMSO) was added to the sterility and negative controls, 2 µL of antibiotic (X, Y, or Z) was added to positive controls, and 2 µL of extract was added to sample wells. All of the controls and samples were tested in triplicate for a minimum of two times. The outer wells were filled with sterile broth. The plates were read at 0 and 24 hours at 600 nm in a plate reader using a Synergy H1 Hybrid Reader from Biotek. All wells were compared with the DMSO negative control.

Whole Genome Sequencing

PTY087I2 was inoculated into YSP+IO media and incubated at 30 °C for a confluent culture. DNA was extracted from 1 mL of confluent culture using the Promega Wizard Genomic DNA Purification Kit according to manufacturer's protocol (Promega Corporation, Wisconsin, USA).

Subsequently, 200 ng of DNA was used with Truseq. The phylogenetic tree was constructed using ORFcor pipeline (Klassen and Currie 2013), fasttree and iTol.

Antibiotics and Secondary Metabolite Analysis Shell (AntiSMASH) Genome

The genome was analyzed using anti-SMASH 3.0.5 for identification of secondary metabolite clusters and percent similarity (Weber et al. 2015).

Results and Discussion

The Panamanian actinomycete (PTY087I2) was originally isolated from an R2A+IO agar plate. Upon reisolation onto a nutrient rich YSP+IO agar plate, we noticed that the bacterium was powdery white and turned the agar blue (Figure 1). PTY087I2 was cultured in liquid YSP+IO media and extracted (Figure 1). The extract showed strong inhibition against MRSA and *Bacillus subtilis* (Table 4), and thus was chosen as the bacterium of interest for co-culture challenge experiments.

The bacterium resembled an actinomycete based on the white powdery spores. The DNA of the bacterium was extracted and sequenced to determine the genus and species. After the whole genome was sequenced, the bacterium was found to be a *Streptomyces* sp., which was 94.67% identical to a known species, *Streptomyces griseus* accession number ADFC00000000. In addition, antiSMASH analysis shows several gene clusters in PTY087I2 that were similar to known gene clusters (Table 2). The genome was shown to contain 2 bacteriocin, 8 NRPS, 2 NRPS-T1PKS, 1 otherKS-NRPS, 4 terpene, 3 lantipeptide, 1 melanin, 2 T3PKS, 2 ectoine, 2 siderophone, 1 T2PKS, 1 butyrolactone, 1 otherKS-T1PKS, 1 thiopeptide-lantipeptide, 2 lassopeptide, 1 lantipeptide-melanin, 1 T1PKS-NRPS, 1 ladderane-arylpolyene, and 1 other cluster. Of those clusters, 30 of them were similar to known clusters with 7 clusters having 100% similarity for SGR PTMs, melanin, ectoine, griseobactin, SRO15-2005 (lassopeptide), SRO15-

2005 (NRPS), and amfS biosynthesis (Table 2). The antiSMASH analysis shows that the *Streptomyces* sp. has great potential to produce a variety of antibiotic secondary metabolites.

After co-cultures and extraction, extracts were analyzed via LC-MS. The UV chromatograms of each challenge culture extract were overlayed with the UV chromatogram of the monoculture (Figure 2A-D). The peaks of interest were determined to elute at 5, 10, 11, 13, 16, 34, and 68 minutes and labeled A-G (Figure 3). The peaks heights for all challenge extract peaks appear higher than the peaks of PTY monoculture. The areas under the peaks were measured via integration (Table 3). All the areas of the peaks were significantly higher in the challenge extracts as compared with monoculture extracts for peaks B, C, D, and E. For peaks F and G, PTY+BS, PTY+MSSA, and PTY+MRSA had significantly higher areas. For the highest peak, A, at 5 minutes PTY+BS and PTY+MSSA had higher area under the peak than PTY. Interestingly, most of the areas under the peaks for the challenge extracts were higher than the mono-culture extract with the exception of peak A of PTY+PA.

Figure 1: PTY087I2 on YSP+IO agar plate (left) and in YSP+IO liquid media (right).

Table 2: antiSMASH genome of PTY087I2 with most similar known gene clusters and percent similarities.

Most Similar Known Cluster	Percent Similarity	Molecular Weights (g/mol)
SGR PTMs	100	
Melanin	100	318.283
Ectoine	100	142.156
Griseobactin	100	
SRO15-2005 (Lassopeptide)	100	
SRO15-2005 (Nrps)	100	
AmfS	100	
Granaticin	83	444.388
Desferrioxamine B	80	560.684
Coelibactin	72	
Coelichelin	72	565.58
Hopene	69	410.718
Nataxazole	59	
Labyrinthopeptin A1, A2, A3	40	
Skyllamycin (Ladderane-Arylpolyene)	26	
Skyllamycin (other)	26	
Pristinamycin	23	IA 866.96 IB 852.93 IIA 525.59 IIB 527.62
Kinamycin	22	484.499
Steffimycin	19	574.532 B 588.557
Calcium-dependent antibiotic	15	
Enduracidin	10	A 2355.61 B 2369.329
Skyllamycin (Nrps)	10	
Herboxidiene (Nrps)	9	438.597
Herboxidiene (T3pks)	8	438.597
Guadinomine	7	A 518.281 B 502.565 D 544.297
Oxazolomycin	6	655.778
Herboxidiene (T3pks)	6	438.597
Daptomycin	4	1620.67
Tetronasin	3	602.798
Phosphonoglycans	3	

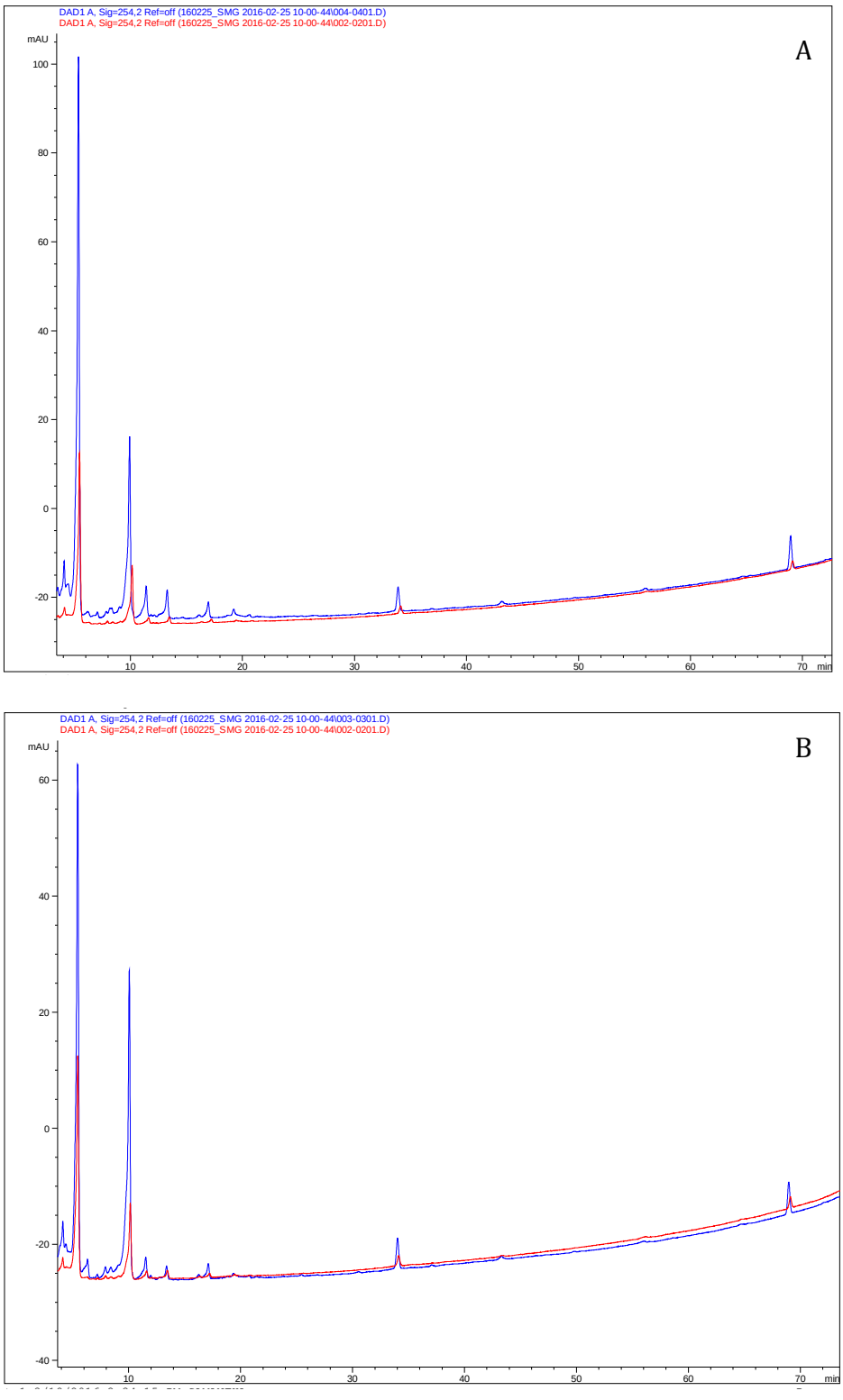
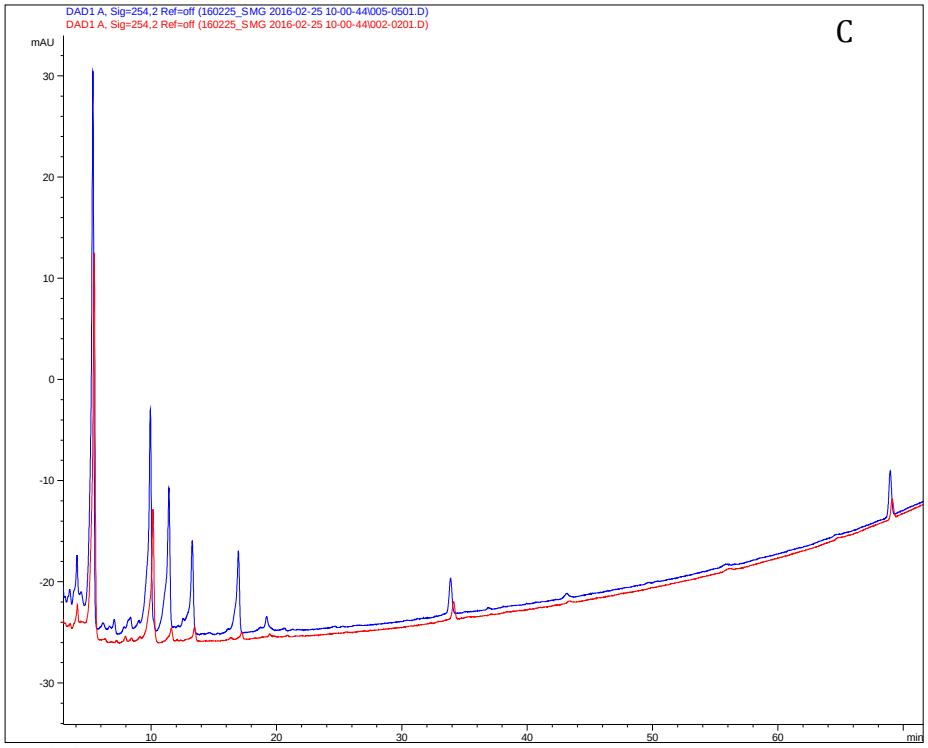


Figure 2: LC-MS UV chromatogram overlay of challenge culture against monoculture. A: PTY (red) and PTY+BS (blue). B: PTY (red) and PTY+MSSA (blue). C: PTY (red) and PTY+MRSA (blue). D: PTY (red) and PTY+PA (blue).



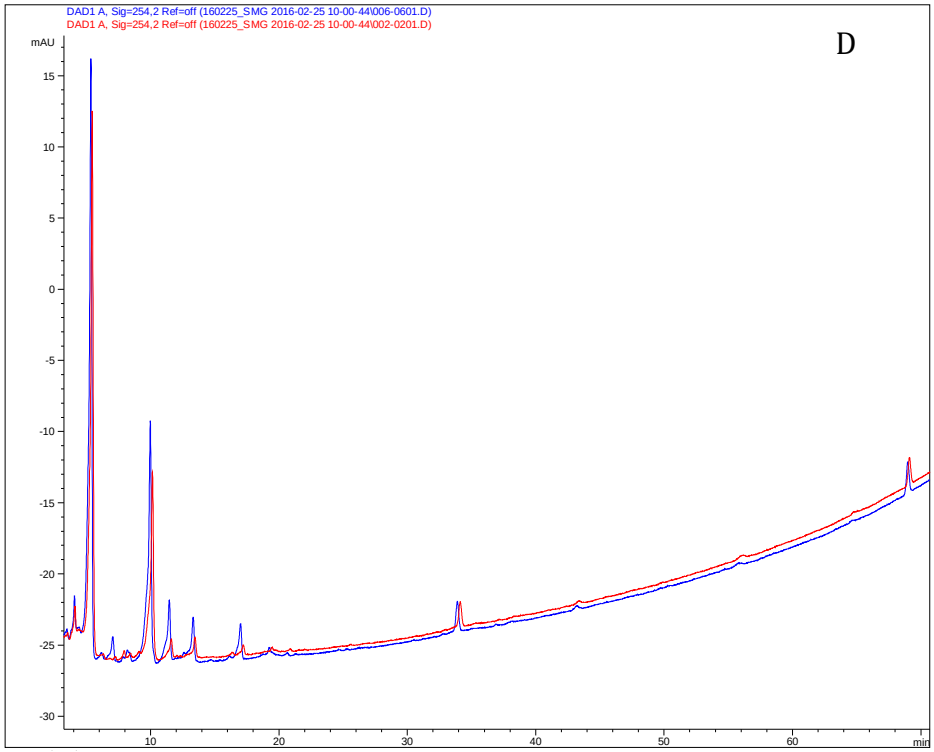


Figure 2 continued: LC-MS UV chromatogram overlay of challenge culture against monoculture. A: PTY (red) and PTY+BS (blue). B: PTY (red) and PTY+MSSA (blue). C: PTY (red) and PTY+MRSA (blue). D: PTY (red) and PTY+PA (blue).

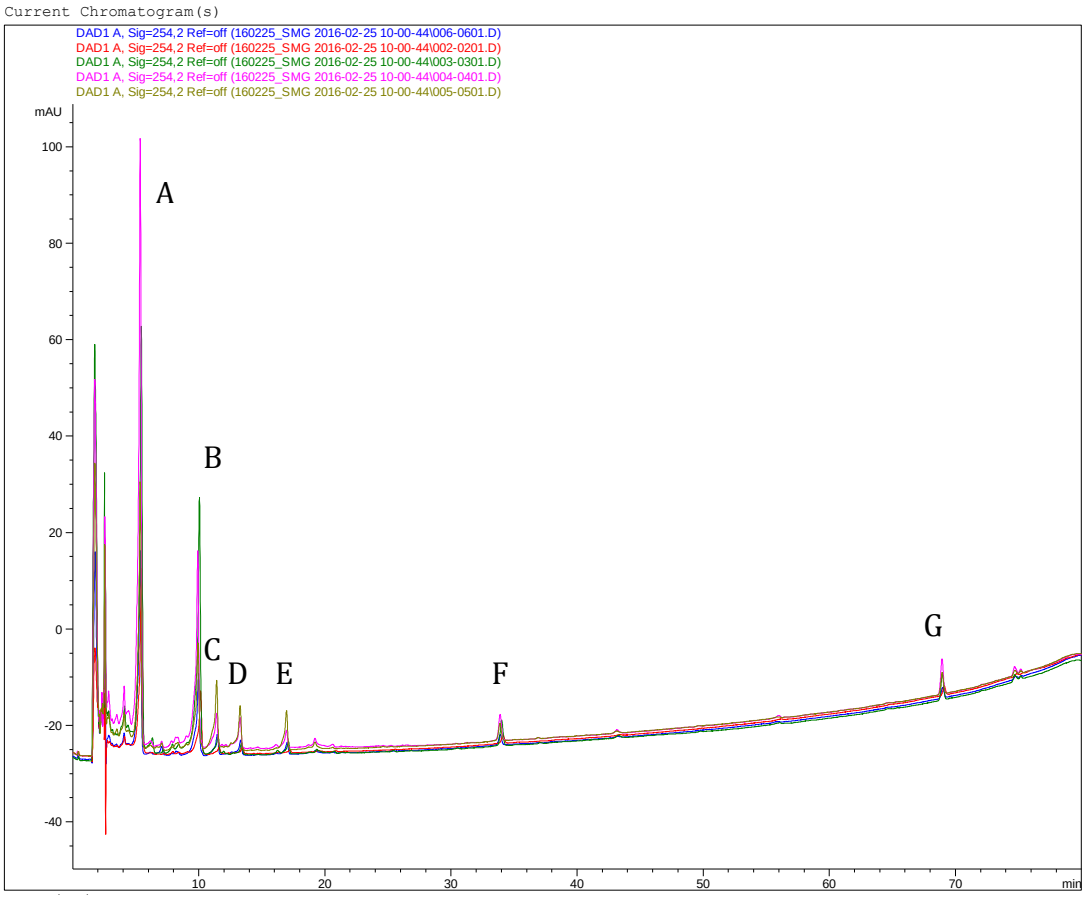


Figure 3: LC-MS UV chromatogram overlay of all extracts showing peaks of interest A-G. PTY (blue), PTY+BS (green), PTY+MSSA (red), PTY+MRSA (pink), PTY+PA (brown).

Table 3: Area of peaks of interest in UV chromatogram of co-culture and monoculture extracts.

Area of Integrated Peaks of Interest on LCMS Chromatogram							
Peak / Extract	A (5 mins)	B (10 mins)	C (11 mins)	D (13 mins)	E (16 mins)	F (34 mins)	G (68 mins)
PTY	930.4	231.3	15.1	13.2	5.4	27.3	29
PTY+BS	2117.8	826.0	208.4	149.7	58.7	86.6	110.7
PTY+MSSA	1480.5	1107.4	83.6	25.9	38.2	84.2	81.9
PTY+MRSA	962.2	463.2	349.3	206.2	139.6	56.4	67.1
PTY+PA	739.0	335.1	87.4	49.7	39.3	33.3	33.8

The fold increase for the peak areas of the co-cultures compared to the monoculture was also calculated (Figure 4). The figure shows the most substantial fold increases in peaks C-E, particularly in the PTY+MRSA extract. The peaks C, D, and E in the PTY+MRSA extract showed a 23.1, 15.6, and 25.9-fold increase in area, respectively. The peaks for PTY+BS, PTY+MSSA, and PTY+PA also showed moderate fold increases, as compared to the monoculture. The challenge pathogens were able to induce an increase production of compounds that are potentially antibacterial at the peaks of interest A-G.

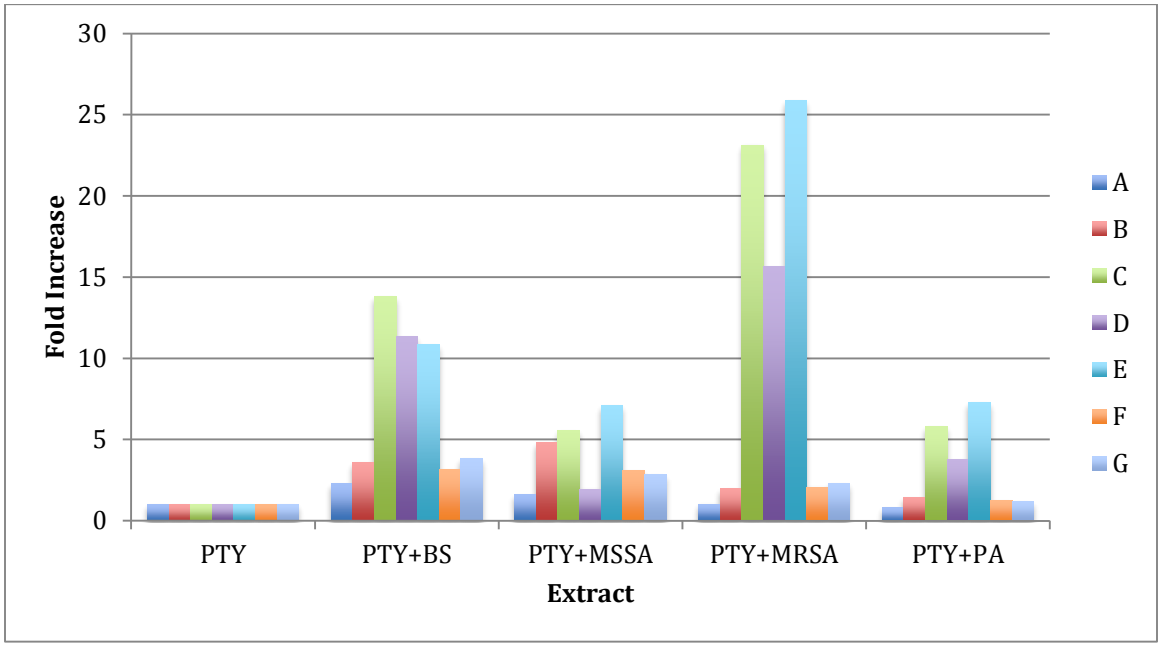
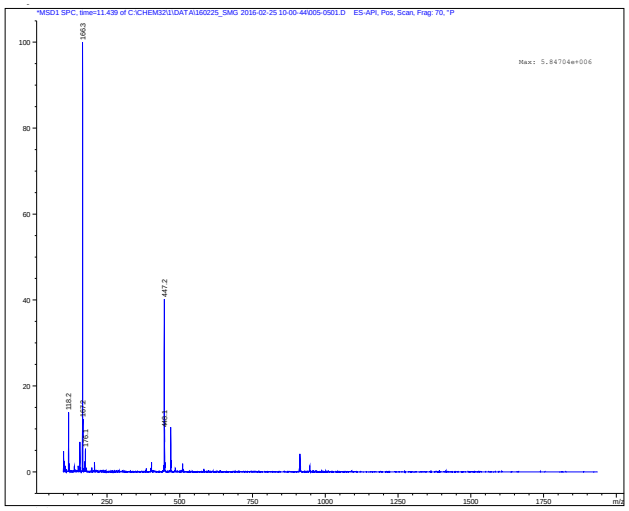


Figure 4: Fold increase of area under integrated peaks of interest, A-G, from Table 3 above of the co-culture extracts against the monoculture extract.

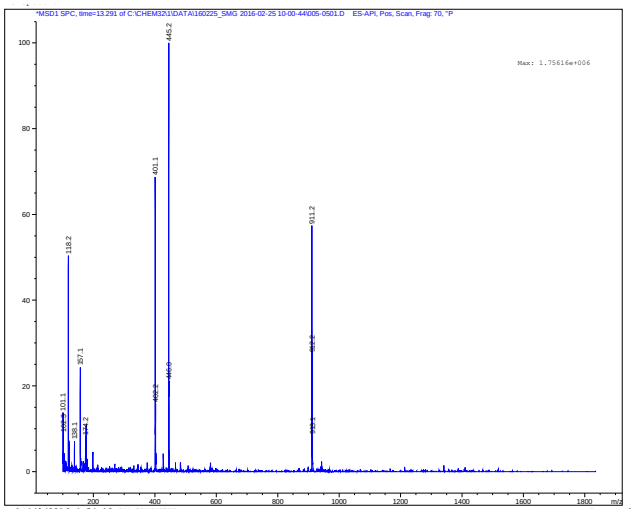
Positive ion mass spectrometry data was obtained for peaks C-E to determine the molecular weights of compounds C, D, and E (retention times 11, 13, and 16 minutes, respectively). Peak C has a $[M+H]^+$ of 447.2, peak D has a M+H of 445.2, and peak E has a M+H of 447.1. Based on the molecular weights of the known compounds found in the *Streptomyces* sp. genome, the mass spectrometry data for peak D matched with granaticin (Table 2). The gene cluster has an 83 percent similarity to a known cluster, and the molecular weight of

granaticin is 444.388, matching the mass spectrometry of peak D (Figure 5). The extracted ion chromatogram (EIC) for the molecular weight of granaticin shows the peaks that match with that particular molecular weight (Figure 6). The extracted ion in positive mode shows that peaks A, B, and D match with the molecular weight with peak D having the highest abundance (Figure 6). The extracted ion in negative mode shows that only peak D contains a compound with that molecular weight (Figure 6). The EIC results confirm the mass spectrometry data of the potential presence of granaticin in peak D. Granaticin is an antibiotic that was originally isolated from *Streptomyces olivaceus* and produced by many other actinomycetes (Snipes et al. 1979). It has shown to have antibiotic activity against Gram-positive bacteria but have little or no activity against Gram-negative bacteria.

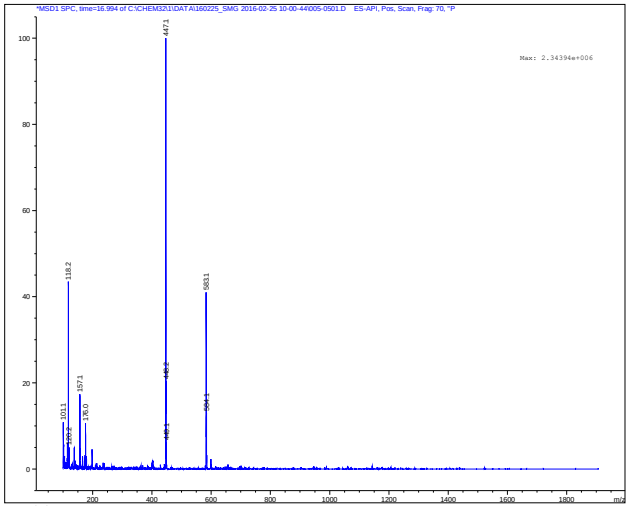
Furthermore, the mass spectrometry of peaks C and E showed similar molecular weights to granaticin, which could indicate a possible derivative of granaticin. A derivative of granaticin that could potential have a $[M+H]^+$ of 447 g/mol is granatomycin D with a molecular weight of 446.4041 g/mol. The EIC for the molecular weight of granatomycin D with a range of ±0.1 g/mol shows that there are peaks that match with the molecular weight (Figure 7). The extracted ion in positive mode shows that peaks C and E match with the molecular weight with both peaks having high abundance (Figure 7). The extracted ion in negative mode shows that only peak C contains a compound with the molecular weight of granatomycin D (Figure 7). The EIC results confirm the mass spectrometry data of the potential presence of granatomycin D in peak C (both positive and negative ion mode) and peak E (positive ion mode only). Granatomycin has similar physicochemical properties of granaticin and has shown to have antibiotic activity against Gram-positive and Gram-negative bacteria (Fleck et al. 1980).



Peak C



Peak D



Peak E

Figure 5: Positive ion mass spectrometry of peaks C-E in PTY+MRSA chromatogram.

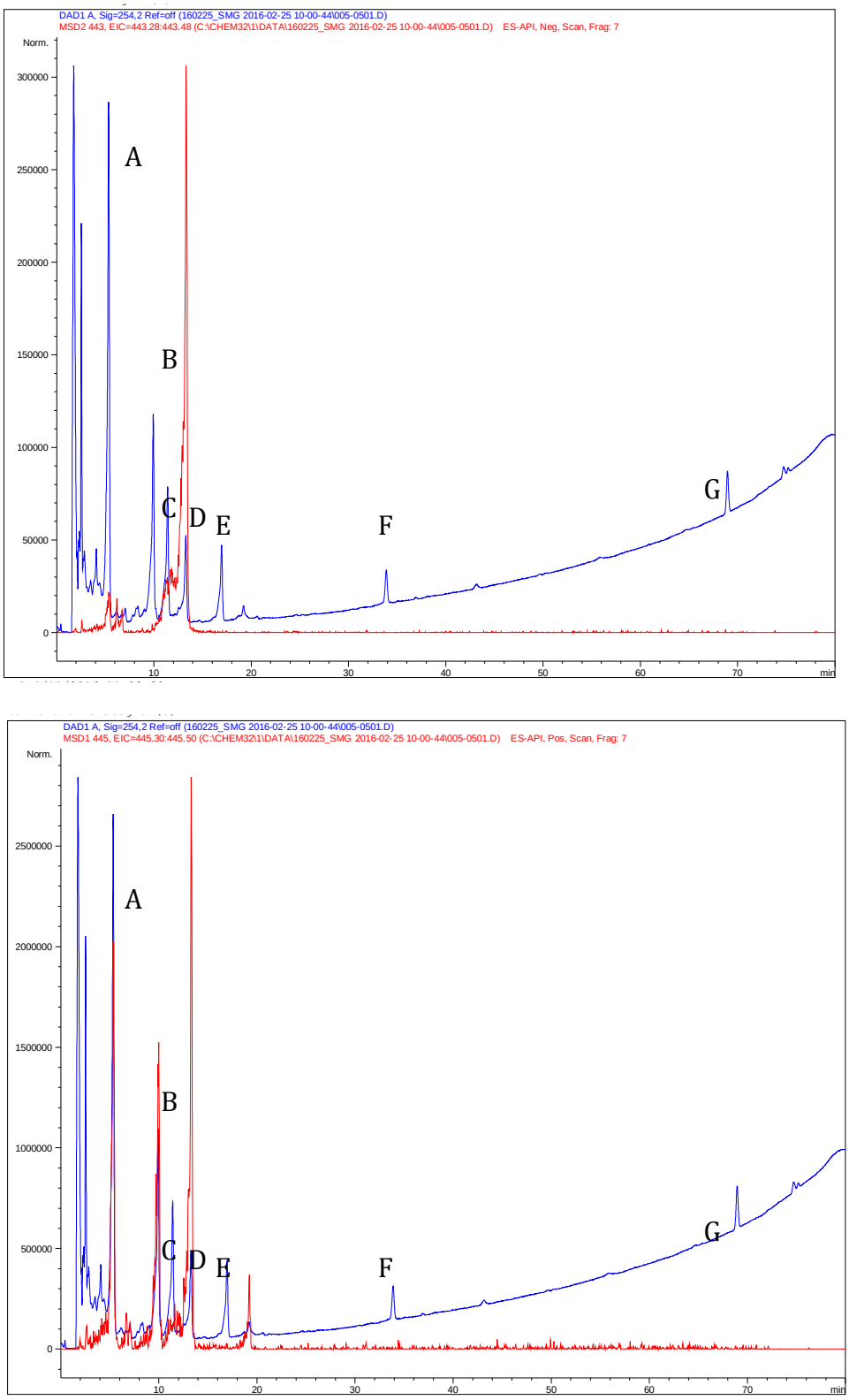


Figure 6: Extracted ion chromatogram (EIC) of granaticin molecular weight (444.3882 g/mol) in negative ion (top) and positive ion (bottom) mode for PTY+MRSA sample.

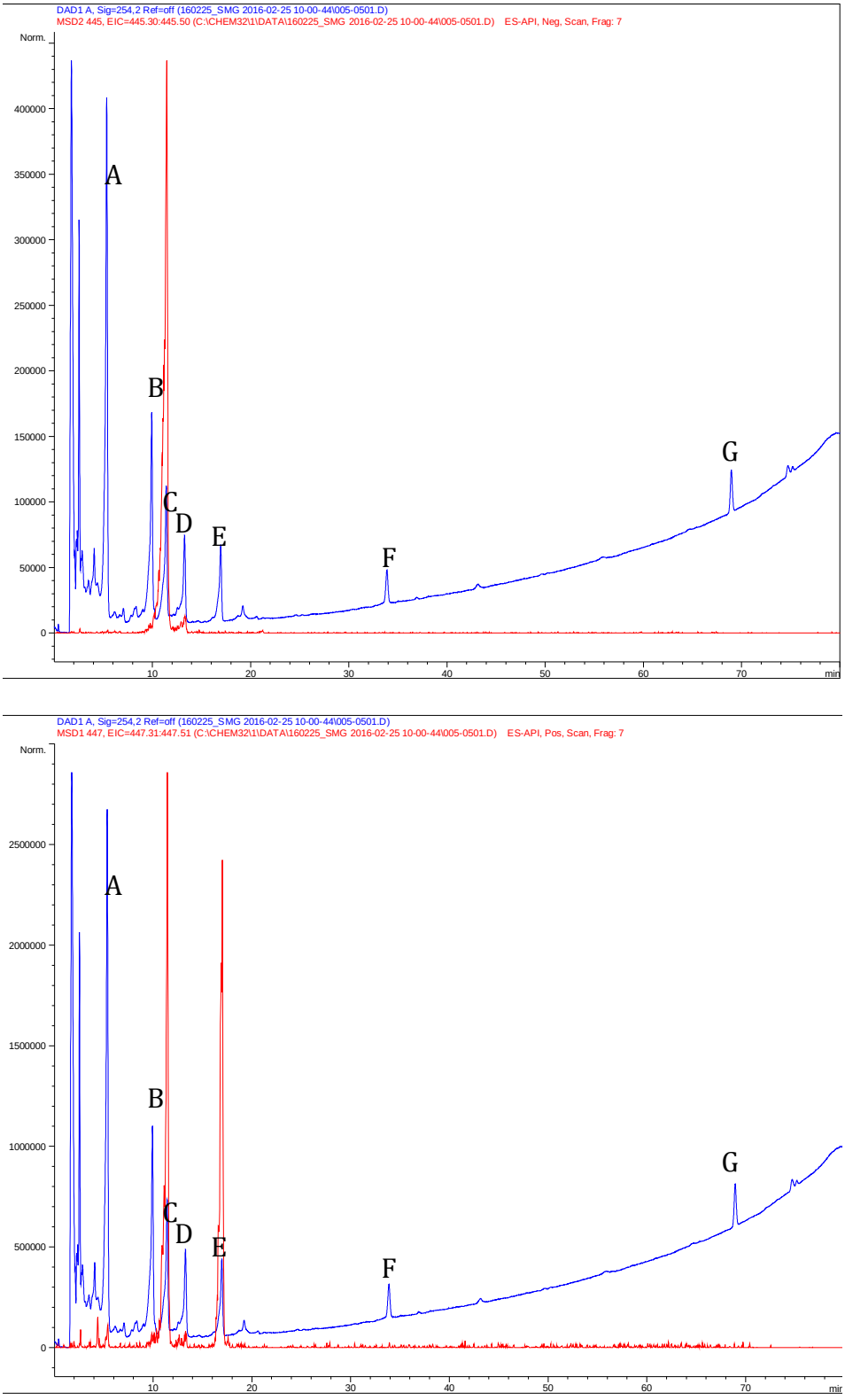


Figure 7: Extracted ion chromatogram (EIC) of granatomycin D molecular weight (446.4041 g/mol) in negative ion (top) and positive ion (bottom) mode for PTY+MRSA sample.

The extracts were all tested against the challenge pathogens in 96 well bioassays. Table 4 and Figures 8-11 summarize MIC values of the challenge extracts against BS, MSSA, MRSA, and PA. None of the extracts showed activity against *Pseudomonas aeruginosa* (MIC > 400 μg/mL). All of the extracts showed strong activity against *Bacillus subtilis* (MIC = 3.125 μg/mL for PTY+MRSA and MIC = 6.25 μg/mL for the other four extracts). PTY+MSSA and PTY showed the same activity against MSSA and MRSA, MIC = 50 μg/mL. PTY+BS, PTY+PA, and PTY+MRSA showed increased activity against MRSA and MSSA with lower MIC values (Table 4). PTY challenged with MRSA had the highest activity against both MSSA and MRSA. Figure 6 displays the different concentrations of challenge extracts tested against MRSA. The percent control activity (PCA) is set against the negative control (1% DMSO). The MIC was determined from the lowest concentration at which the PCA value was less than 0, which means that there was inhibition (no growth) of MRSA in the wells. PTY+MRSA had the lowest MIC value of 6.25 μg/mL versus the PTY monoculture of 50 μg/mL. PTY+MRSA also had a lower MIC value of 3.125 μg/mL compared to the other co-cultures and monocultures. This suggests that MRSA was able to challenge the *Streptomyces* sp. and increase production of the antibacterial compound.

Table 4: MIC values from 96 well bioassays for PTY087I2 challenge extracts against challenge pathogens.

Minimum Inhibitory Concentration of Challenge Extracts Against Pathogens (μg/mL)				
Pathogen / Extract	*Bacillus subtilis*	MSSA	MRSA	*Pseudomonas aeruginosa*
PTY	6.25	50	50	>400
PTY+BS	6.25	25	25	>400
PTY+MSSA	6.25	50	50	>400
PTY+MRSA	3.125	6.25	6.25/12.5	>400
PTY+PA	6.25	12.5	12.5	>400

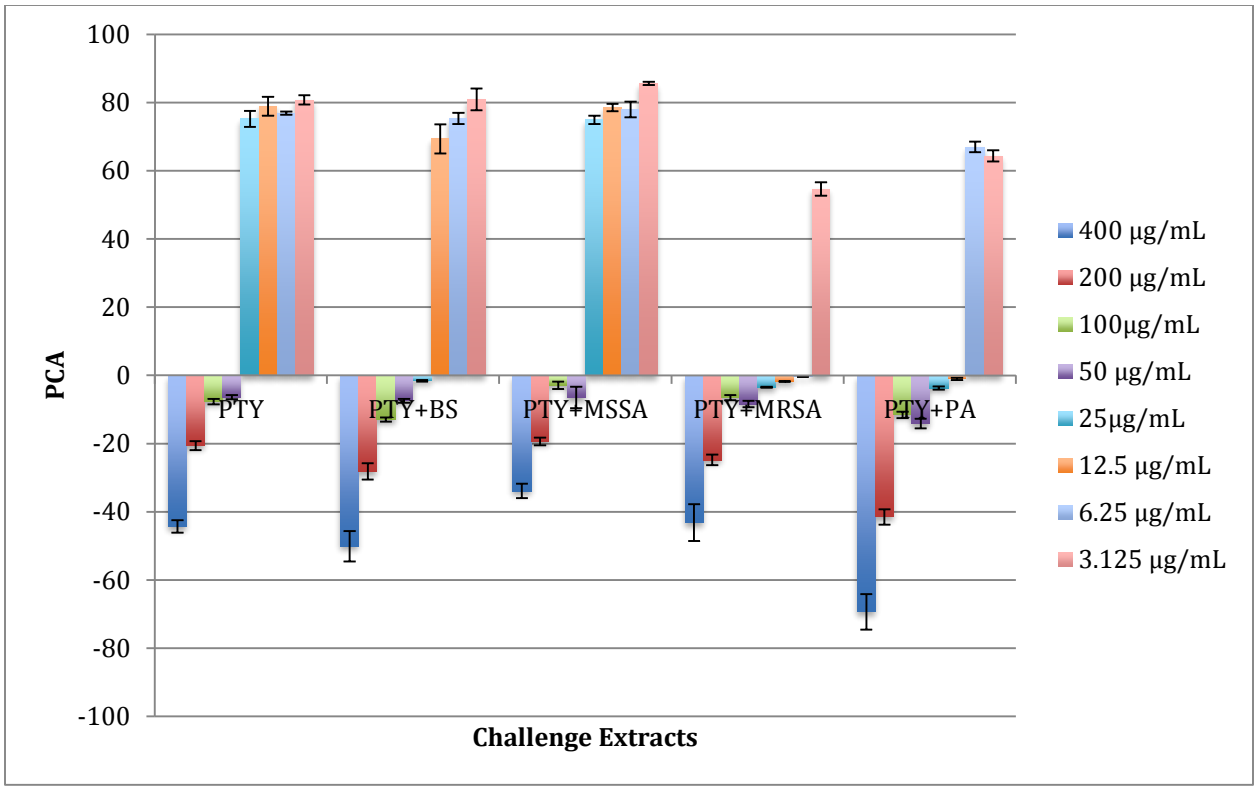


Figure 8: 96 well bioassay of PTY087I2 challenge extracts against MRSA at different concentrations (µg/mL).

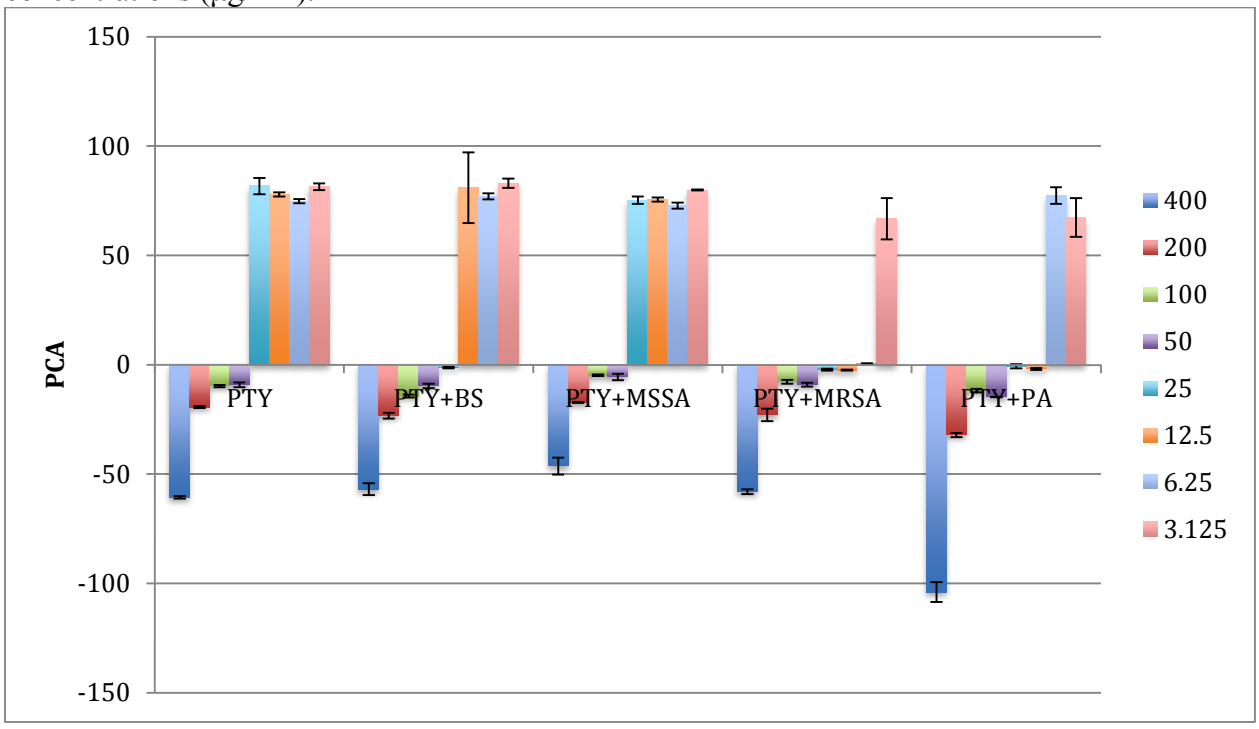


Figure 9: 96 well bioassay of PTY087I2 challenge extracts against MSSA at different concentrations (µg/mL).

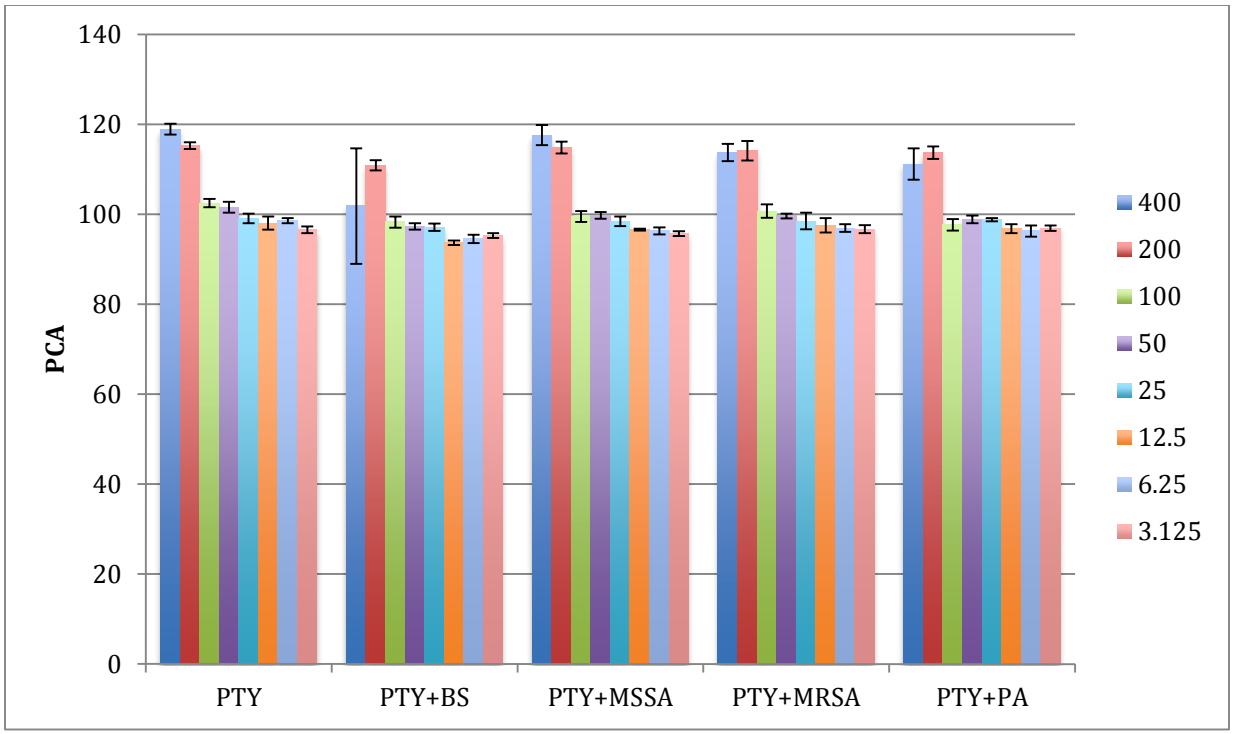


Figure 10: 96 well bioassay of PTY087I2 challenge extracts against *Pseudomonas aeruginosa* at different concentrations (µg/mL).

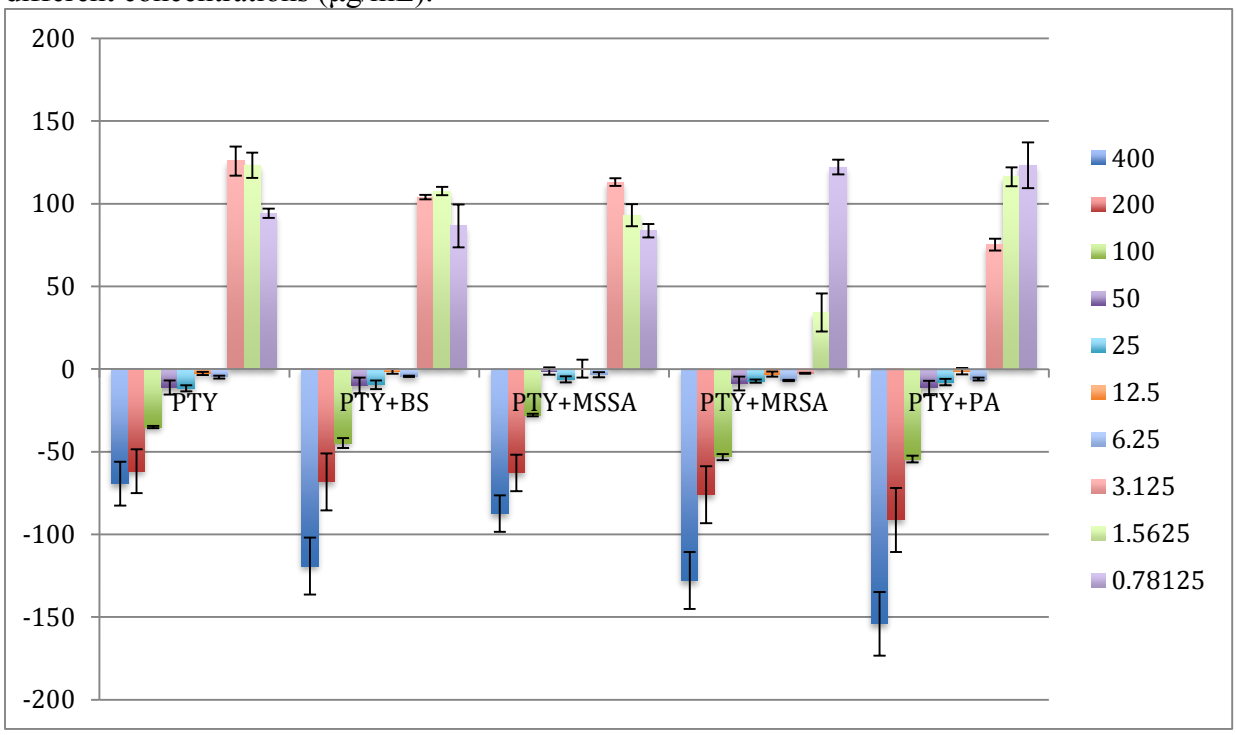


Figure 11: 96 well bioassay of PTY087I2 challenge extracts against *Bacillus subtilis* at different concentrations (µg/mL).

The challenge extracts run through LC-MS exhibited an upregulation of compounds at several peaks of the chromatogram (Figure 3). In antibacterial assays, these same challenge extracts also showed more activity against MRSA, MSSA and *Bacillus subtilis*, particularly the PTY+MRSA co-culture challenge extract (Table 4). PTY+MRSA showed the greatest activity as well as the greatest fold increase on LC-MS. Upon further analysis of the mass spectrometry EIC, this *Streptomyces* sp. likely produces the antibiotic granaticin in peak D that has antibacterial activity against Gram-positive bacteria but not Gram-negative bacteria (Figure 6). In our results, the extracts show activity against the Gram-positive bacteria, MRSA, MSSA, and *Bacillus subtilis* and no activity against the Gram-negative bacterium, *Pseudomonas aeruginosa* (Table 4). Peaks C and E also have likely contain a derivative of granaticin, granatomycin D. The peaks that potentially contain the antibiotics were upregulated in the co-cultures and the upregulation of the production of the compounds were confirmed via antibacterial assays.

Conclusions

The MIC values and LC-MS peaks were indicative of an increase in activity and an increase of production of antibacterial compounds. The antiSMASH analysis and mass spectrometry data showed the possibility of the production of granaticin and granatomycin in peaks C-E. Our results suggest that MRSA induced increased production of antibacterial compounds active against MRSA, MSSA and *Bacillus subtilis* as compared with the *Streptomyces* sp. monoculture. These results indicate that co-culturing with human pathogens can be used as a method to increase yield of antibiotic compounds under laboratory conditions. Co-cultures with small amounts of challenge pathogens may provide an environmental stress that promotes increased biosynthesis of the active antibacterial compounds.

Further co-culture experiments should also be conducted to determine the effect of adding varying amounts of the challenge pathogen. Other future research experiments include optimum timing of challenge pathogen inoculation and optimal growth and culturing conditions. Future direction will also include fractionation and isolation of active compounds against MRSA, MSSA, and *Bacillus subtilis.*

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