An evaluation of co-culture parameters effecting antibiotic production in soil microbes

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An Evaluation of Co-Culture Parameters Effecting Antibiotic Production in Soil Microbes

by

Rebecca Lindow

Thesis
Submitted to the Department of Biology
Eastern Michigan University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
Biology
Concentration in Cellular and Molecular Biology

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Abstract

The rise of infections caused by antibiotic resistant bacteria, compounded by a reduction in antibiotic discovery and development, jeopardizes human health. Historically, antibiotics derive from secondary metabolites produced by soil microbes in pure culture, but recent genetic evidence suggests that microbes can produce more secondary metabolites than are currently observed. The modified crowded plate technique directly identifies antibiotic-producing soil microbes that were co-plated with a target pathogen. Here, this technique was refined by testing the effect of a D-alanine auxotrophic target pathogen rather than a prototrophic pathogen as well as investigating conditions most conducive to antibiotic production. Antibiotic producing conditions are most favorable with the use of a D-alanine auxotrophic pathogen that was pre-incubated for one week. Antibiotic-producing microbes isolated using these new parameters were cultured in single and mixed fermentations to compare secondary metabolite production. Furthermore, mixed fermentations with multiple antibiotic producers is an effective means to stimulate antibiotic production.
Table of Contents

Acknowledgments........................................................................................................................ ii

Abstract ........................................................................................................................................ iii

List of Tables.................................................................................................................................. v

List of Figures.............................................................................................................................. vi

INTRODUCTION ......................................................................................................................... 1

MATERIALS AND METHODS................................................................................................ 13

RESULTS ...................................................................................................................................... 21

DISCUSSION ............................................................................................................................... 34

CONCLUSIONS ......................................................................................................................... 40

Literature Cited ........................................................................................................................... 41
List of Tables

Table 1. Different media types and their ingredients ............................................................ 14

Table 2. Bacterial strains used in this study ............................................................................ 14

Table 3. Primer Sequences of S. aureus Alr1, Alr2, and Pkor1 ............................................. 18

Table 4. mCPT derived isolates active against a panel of pathogens ................................. 23

Table 5. Pure cultures and their associated sequencing data ............................................... 31

Table 6. Mixed fermentations of non-producing pure culture microbes ............................ 32
List of Figures

Figure 1. Antibiotic production from 1980 to 2019 ............................................................... 2
Figure 2. MRSA infection incidences .................................................................................... 5
Figure 3. Purified antibiotic producers tested for activity against S. aureus ................. 6
Figure 4. Bacterial D-alanine metabolic pathway ................................................................. 8
Figure 5. Growth of a D-alanine auxotroph ....................................................................... 9
Figure 6. Initial mCPT soil screen using B. subtilis as a target organism ......................... 21
Figure 7. ESKAPE pathogen safe strain screening of soil microbes ............................... 22
Figure 8. Juxtaposition of prototrophic versus auxotrophic production ....................... 25
Figure 9. Comparison of incubation period and media type on antibiotic production ... 26
Figure 10. Isolated microbes from initial mCPT soil screens were tested against B. subtilis and S. aureus .............................................................. 27
Figure 11. Co-occurrence of B. subtilis and S. aureus ....................................................... 28
Figure 12. Construction of homologous recombination plasmids for the deletion of alr1 and alr2 in S. aureus ........................................................................ 29
Figure 13. Antibiotic testing of chemical extracts in single- and mixed-culture fermentations ........................................................................................................ 30
Figure 14. Chemical extracts from mixed-culture fermentations with or without supplemental Mn ......................................................................................... 33
INTRODUCTION

Background: The threat of antibiotic resistance

Infections caused by antibiotic-resistant bacteria have become a global health threat due to the misuse of antibiotics (Spellberg et al., 2008). The World Health Organization (WHO) recently noted that antibiotic resistance seriously jeopardizes progress in modern medicine because the loss of antibiotic effectiveness will make common medical procedures, such as surgeries, remarkably risky (Ventola, 2015). Furthermore, those with weakened immune systems are more vulnerable to such infections, but less able to fight them. In addition to making diseases more arduous to treat, antibiotic resistance is also associated with inflated medical costs as well as increased morbidity and mortality (Li and Webster, 2018). Conservatively, it is estimated that health care costs attributed to antibiotic resistance near tens of billions of dollars spent on treatment annually; some of these expenses are attributed to extended stays in hospital facilities (Li and Webster, 2018). In the United States alone, antibiotic resistant pathogens are responsible for over 30,000 deaths per year, and this number is expected to continue to increase (CDC, 2019). ESKAPE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, cause many antibiotic-resistant nosocomial infections, and thus are listed as top-priority pathogens for antibiotic development (Boucher et al., 2008). Alarmingly, as the rate of infections caused by antibiotic resistant microbes continued to climb, the development of new available antibiotics has practically ceased; only two antibiotics were approved by the FDA between 2005 and 2009 (Figure 1) (Fischbach and Walsh, 2009). In 2012, the GAIN Act was passed to try to incentivize antibiotic research and development. While more
antibiotics have been approved since its passage, few of them are new classes offering novel ways to attack bacteria (Darrow and Kesselheim, 2019).

![Figure 1: Antibiotic production from 1980 to 2019.](image)

The lack of incentive for pharmaceutical companies to invest in antibiotic research derives from high investment costs with few financial rewards (Boucher et al., 2009). Antibiotic development averages billions of dollars, but the medications only cover a short period of time and the reimbursement rate for treating antibiotic infections with new, expensive antibiotics is meager (Sukkar, 2013). Consequently, a new antibiotic can be on the market for a decade and yield little to no return on investment. Achaogen Inc., for example, went out of business due to this financial structure; the risk of bankruptcy heightened caution in the antibiotic industry (Mullard, 2019). For the same upfront costs, pharmaceutical companies can develop more lucrative drugs that
treat chronic illnesses, such as heart conditions and autoimmune diseases, where the profit margin is substantially larger (Sukkar, 2013). Although the root cause of antibiotic resistance must be remedied, overcoming the scientific challenges of identifying new antibiotics is critical to confront the burgeoning threat posed by antibiotic resistance.

Currently, a variety of methods have been deployed in an effort to minimize the spread of antibiotic resistance, including stricter control of antibiotics in humans and livestock, and the use of vaccines. One strategy in humans requires that a proper prescription, one that prescribes appropriate drugs and is signed by a practicing doctor, must be present in order to administer the antibiotics (Davies and Davies, 2010). Over the counter antibiotics are readily available in developing countries, contributing to the remarkable resistance threat because people are able to take antibiotics for any illness (Ayukekbong et al., 2017). Vaccines are another approach to combat resistance, but some types cannot be administered to immunocompromised individuals as they may fall ill. Vaccines may also be met with debate from the anti-vaccination movement (Kata, 2010). On the other hand, the food industry uses antibiotics as a feed-additive to increase livestock yields. Although there are regulatory practices in place to prevent sub-therapeutic use of antibiotics in agriculture, many antibiotics can be purchased without a veterinarian’s prescription to feed to the animal (Davies and Davies, 2010; Sneeringer et al., 2015). The misuse of antibiotics in livestock can support resistant bacteria that may affect humans through either contaminated meat after slaughter or through environmental infections (CDC, 2019). With these concerns, the identification of new antibiotics with therapeutic potential plays a strategic role in the welfare of society.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is of particular interest since it is now classified as a high-priority, multi-drug resistant pathogen known to cause a
variety of skin and soft tissue infections (SSTI) as well as many life-threatening diseases such as pneumonia and septic shock (Lowy, 1998). SSTI’s range from mild (impetigo) to severe (necrotizing fasciitis) (Styjewski and Chambers, 2008). Over 120,000 cases of MRSA are reported in the United States per year, with nearly 20,000 of these diagnoses culminating in death; the fatalities attributed to MRSA now outnumber those from HIV/AIDS (Boucher and Corey, 2008; CDC, 2019). Fortunately, the number of nosocomial MRSA infections has declined because of hospital infection control; however, community-acquired infections have remained nearly constant for the past 15 years, eliciting the threat of community-acquired MRSA infections (Figure 2) (Hassoun et al., 2017; Mehta et al., 2014). Due to its heightened virulence, most of the community and hospital-acquired outbreaks of MRSA derive from a single clonal lineage of MRSA; USA300 (Tenover and Goering, 2009). The extensive antibiotic resistance of MRSA USA300 derives from its fairly plastic genome; it has acquired an assortment of resistance genes that aid in its survival (Kuroda et al., 2001). Thus, there is a considerable demand to discover antibiotics effective against this pathogen in an effort to control this pathogen.
Antibiotic-producing bacteria may reside in soil

Most antibiotics in use today were originally derived from soil microbes. Penicillin, produced by the soil fungus, *Penicillium*, serves as the archetype of such antibiotic exploration because it was the first true antibiotic to be discovered (Clardy et al., 2009). Early discovery efforts generally followed the “Waksman Platform” method, in which soil microorganisms were plated alongside pathogenic bacteria and zones of inhibition (ZOI) were detected (Figure 3). These ZOI imply the ability of a microbe to produce an antibiotic that prevents the growth of the pathogen. The resulting strains, and antibiotic compounds they produced, could then be characterized (Valiquette and Laupland, 2012). However, by the 1960s, many scientists assumed that most of the cultivatable bacteria that could produce antibiotics had been identified (Nichols et al, 2010). Fortunately, soil is a remarkably diverse environment, as elicited by metagenomic studies; this implies that there are a variety of bacteria living in this
habitat that may possess novel qualities (Daniel, 2005). Because there are so many different bacteria in the soil, there are various survival strategies prevalent, namely, competition via antibiotic production (D’Costa et al., 2006). The crowded plate technique (CPT), an antibiotic screening method developed by Waksman, exploits these routine functions of bacteria. To do so, soil microbes are plated and grown for several days, at which point the plates are observed for ZOIs. Although simplistic, this method is limited by its lack of specificity. Furthermore, the isolated antibiotic producers from this practice were seldom active against pathogens following purification and testing in isolation. Soil microbes are a largely untapped source for producing novel antibiotics; but refined methods to discover these antibiotic-producing microbes are needed.

Recently, the Price lab developed a variant of the CPT by simultaneously inoculating a target pathogen with a diluted soil sample on the media to directly screen for antibiotic producers that are active against a pathogen, as indicated by a ZOI. These adjustments were termed the modified crowded plate technique (mCPT). These
fundamental changes improved the effectiveness of the method to identify antibiotic-producing bacteria in a complex stimulatory environment. These isolated antibiotic-producing bacteria can then be further tested against other microbes, such as the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species*). However, isolating the antibiotic producing microbe is often complicated by the presence of the target organism; this makes it difficult to test the producer against other pathogens. Further refinement to the mCPT could aid in establishing a more efficient antibiotic discovery assay.

The mCPT has been introduced into two labs (BIO112 and BIO328) via the Tiny Earth curriculum at Eastern Michigan University (EMU), allowing students to engage in inquiry-based learning while also supplying many soil samples (Davis et al., 2017). This educational program is partially based on the original methods, such as the “Waksman Platform,” employed by scientists to discover antibiotics from soil microbes and it proposes to use a crowdsourcing strategy to further explore the soil microbiome in hopes of discovering new antibiotics (Valderrama et al., 2018; Peek et al., 2018). At EMU, the mCPT has been intermingled into this curriculum to better exploit the routine ecological duties of antibiotics. The Price lab advances this classroom research to determine the novelty of the antibiotic producing microbes and their secondary metabolites. A contingent of the Price lab is also developing new tools and methodologies that can then be taken back into the classroom, further promoting safe learning and discovery for students with introductory laboratory skills and experience.

**D-alanine auxotrophs may function as a safe screening tool**

One such approach, developed by undergraduate researchers in the Price lab at Eastern Michigan University (EMU), involves the use of D-alanine auxotrophic bacteria
as target organisms during the initial mCPT screening process for antibiotic producers. Auxotrophic bacteria are strains that require an extra nutrient for basic cellular function that other bacteria do not. D-alanine is an enantiomer of L-alanine and is found in the cell wall of almost all bacteria, contributing to the architecture of bacterial peptidoglycan and providing resistance to most known proteases (Cava et al., 2011). When using D-alanine auxotrophs, the strains have been mutated such that they are unable to convert environmental L-alanine to D-alanine for use in peptidoglycan synthesis; this function is primarily carried out by the alanine racemases, Alr1 and Alr2, for use in peptidoglycan synthesis (Figure 4) (Strych et al., 2000; Moscoso et al., 2018). Such mutations are lethal because without D-alanine, a bacterial cell will lyse since the cell wall is compromised.

Purification of antibiotic producers off of mCPT plates transpires with ease because the D-alanine auxotrophic bacteria are unable to survive on media lacking D-alanine; most of the environmental alanine exists in the L-enantiomer conformation.
Because D-alanine auxotrophs are easily eliminated during purification steps by not supplementing the media with D-alanine, slow-growing organisms are able to be recovered more frequently. In addition, D-alanine auxotrophs add a level of safety to pathogenic strains for undergraduate research. Attesting to the safety of such a mutant, current D-alanine MRSA auxotrophs are being investigated for potential vaccine use (Moscoso et al., 2018). Therefore, D-alanine auxotrophic mutants can be used to screen for antibiotic producers on media supplemented with D-alanine, thus acting as a constructive platform on which to identify and purify antibiotic producing microbes (Strych et al., 2000; Moscoso et al., 2018). However, the Price lab does not currently have any D-alanine auxotrophic strains of *S. aureus* available for screening antibiotic producers and the aforementioned exploratory companies do not take requests for their auxotrophic strain. Following the early success of using a D-alanine auxotroph to identify antibiotic-producing bacteria compared to the prototrophic *B. subtilis*, this research has begun construction of a D-alanine auxotroph MRSA mutant.

![Figure 5: Growth of a D-alanine auxotroph](image)

*Figure 5: Growth of a D-alanine auxotroph.* When plated on minimal media (MM), the auxotrophic strain is unable to grow. However, when media is supplemented with D-alanine, mutant colonies are observed.
Culturing techniques for antibiotic production

The looming threat of antibiotic resistance requires the construction of novel antibiotic screening tools, as well as the efficient identification of bioactive substances. Many bioactive substances are generated as secondary metabolites, compounds that are not directly involved in growth and development of the cell, but they promote survival functions within the environment, such as corporative or competitive cell-to-cell communication within the environment, often through the use of antibiotics (Netzker et al., 2015). Secondary metabolites are encoded by cryptic biosynthetic gene clusters (BGCs), which in some bacteria, such as Actinomycetes, constitute roughly 5% of the genome (Traxler et al., 2013). Many BGCs, however, are not expressed under normal axenic laboratory conditions, including many encoding proteins that produce antibiotics as they can be self-harming. Research focused on inducing expression of cryptic BGCs indicates that certain ecological or chemical cues within a microbe’s environment may be necessary to stimulate their expression (Cornforth and Foster, 2015; Van der Meij et al., 2017). Thus, in order to encourage the production of secondary metabolites and antibiotics, culture methods need to closely mimic the complexity experienced in a microbe’s natural environment.

Co-culturing seeks to stimulate bioactive substances by encouraging interspecies interactions. In contrast to axenic cultures, which have been the basis for most antibiotic discovery methods, co-cultures involve growth of different microbes together, thereby encouraging interspecies interactions and potentially stimulating the expression of cryptic BGCs (Van der Meij et al., 2017). Assuming that antibiotic production is the product of a competitive environment, and many antibiotics are self-harming, then the use of axenic cultures likely restricts antibiotic expression as interspecies interactions that encourage antibiotic production are absent (Abruden et al., 2015; Seyedsayamdost
et al., 2012; Nai and Meyer, 2017). For example, some microbes, such as *Streptomyces* when grown with mycolic acid-containing bacteria, require cell-to-cell contact to produce antibiotics that are otherwise not produced in axenic cultures (Onaka et al., 2011). Under co-culturing conditions, the expression of BGCs and the concentration of secondary metabolites increase, suggesting that their induction and production may be triggered by interspecies interactions, such as commensalism and competition (Ueda and Beppu, 2016). As a testament to the impact of co-culture, antibiotic activity was only observed in bacteria from the leaf microbiome of a plant with no known antibiotic activity when they were grown in co-cultures (Helfrich, 2018). This implies that changes in cultivation conditions can change the metabolic profile of a microbe (Bode et al., 2002). Co-culturing, then, may allow for increased production of novel secondary metabolites that can be used to combat antibiotic resistance (Onaka et al., 2011).

**Study objectives**

As a result of the formidable demand to discover new antibiotics effective against multi-drug resistant pathogens, this research aims to advance the robustness of the mCPT screening method to identify more antibiotic-producing bacteria. To do so, the effectiveness of the auxotrophic mutants was compared to the prototrophic pathogens, and culture conditions were optimized to improve the sensitivity of the mCPT screening method. The differences in antibiotic production between axenic and co-culturing methods of antibiotic production were also evaluated. Using the improved screening methods, the antibiotic properties of the secondary metabolites, released by antibiotic-producing bacteria grown under single and mixed fermentations, were isolated and characterized. Construction of the D-alanine MRSA auxotroph has begun with cloning the plasmids. This auxotroph will broaden the scope of the mCPT
screening method as a way to safely and effectively screen for and isolate antibiotic-producing bacteria.
MATERIALS AND METHODS

Soil collection

Soil samples were collected at an air temperature of 15 °C in May 2019 from five locations on the Eastern Michigan University Campus in Ypsilanti, Michigan (Halle Library, Student Center, Rec/IM, Pray Harrold, and the Science Complex). The locations were sampled at a depth of one inch into the soil.

Soil screening

One gram of each soil sample was serially diluted 1:10,000 with ddH_2O, and 100 μL was plated on TYME (Table 1) pre-inoculated with B. subtilis (TE-Bs) or S. aureus (PP667) (Table 2). Natamycin (20 μg/mL; NataMax SF, DuPont-Danisco USA Inc.: Kansas, USA) was added to these plates to prevent fungal growth. For experiments involving B. subtilis D-alanine auxotrophs (PP655), TYME media was supplemented with 100 μg/mL D-alanine (Matrix Scientific, Colombia, SC, USA) (Table 2). For experiments examining the potential of gellan gum (VWR: Radnor, VA, USA) to generate solid media, 7 g/L gellan gum was added in place of agar and solidified by adding CaCl_2 to a final concentration of 5 mM. These plates were grown in closed containers at 30 °C for three months, with antibiotic production checked at both two weeks and three months of incubation.
Students in the Fall 2019 sections of Tiny Earth incorporated labs (BIO112 and BIO328) followed the same protocol, but the BIO112 students used prototrophic *B. subtilis* (TE-Bs) and the BIO328 students used auxotrophic *B. subtilis* (PP655) (Table 2). Both of these courses are introductory biology labs; BIO112 is geared towards biology majors while BIO328 is designed for nursing majors.

### Table 1: Different media types and their ingredients

All chemicals were sourced from VWR: Radnor, VA, USA.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>TYME</th>
<th>EPSM</th>
<th>TY</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients</strong> (per 1L ddH₂O)</td>
<td>0.5g Glucose 0.5g Yeast extract 0.5g Tryptone 1mL KH₂PO₄ 1mL CaCl₂ 1mL MgSO₄ 1mL Minor salts 12g Agar (for solid media)</td>
<td>0.5g Potato starch 0.5g Yeast extract 0.5g Tryptone 1mL KH₂PO₄ 1mL CaCl₂ 1mL MgSO₄ 1mL Minor salts 12g Agar (for solid media)</td>
<td>6g Tryptone 3g Yeast extract 0.5g CaCl₂·2H₂O 12g Agar (for solid media)</td>
<td>10g Tryptone 5g Yeast extract 10g NaCl</td>
</tr>
</tbody>
</table>

### Table 2: Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP655</td>
<td><em>Bacillus subtilis</em> 168 dal-1 sigB:erm [from AG232 (46)]</td>
<td>Alan Grossman (Massachusetts Institute of Technology)</td>
</tr>
<tr>
<td>PP665</td>
<td><em>Klebsiella pneumoniae</em> ATCC 13883</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>PP666</td>
<td><em>Pseudomonas aeruginosa</em> ATCC 27853</td>
<td>ATCC</td>
</tr>
<tr>
<td>PP667</td>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>ATCC</td>
</tr>
<tr>
<td>PP673</td>
<td><em>Mycobacterium smegmatis</em> MC2155</td>
<td>Miriam Braunstein (University of North Carolina at Chapel Hill)</td>
</tr>
<tr>
<td>TE-Ps</td>
<td><em>Pseudomonas putida</em></td>
<td>Tiny Earth</td>
</tr>
<tr>
<td>TE-Ec</td>
<td><em>Escherichia coli</em> ATCC 1775</td>
<td>Tiny Earth</td>
</tr>
<tr>
<td>TE-Bs</td>
<td><em>Bacillus subtilis</em></td>
<td>Tiny Earth</td>
</tr>
<tr>
<td>RN4220</td>
<td><em>Staphylococcus aureus</em> RN4220</td>
<td>Eric Skaar (Vanderbilt University)</td>
</tr>
</tbody>
</table>
Screen against pathogen panel via spread patch plate technique:

Antibiotic-producing isolates identified following the mCPT screen, both at two weeks and three months post-incubation, were partially purified and tested, via a spread-patch assay, for secondary screening against a panel of pathogens that include: *S. aureus* (PP667), *B. subtilis* (TE-Bs), *M. smegmatis* (PP673), *P. putida* (TE-Ps) and *E. coli* (TE-Ec) (Table 2). Purified antibiotic-producing bacteria were patched on top of a target pathogen. These plates were grown at 30 °C for two weeks and bacteria were examined for antibiotic production at days one, three, and seven post-inoculation.

**Isolation of antibiotic producers**

For antibiotic-producing isolates that presented a clear zone of inhibition (ZOI) on any of the pathogens in the aforementioned panel, the size of the ZOI was measured in millimeters using a ruler. The antibiotic producers were then isolated to pure culture via three-phase streaks and frozen down in 20% glycerol and stored at -80 °C.

**Bacterial identification**

Isolated colonies were identified using 16S rRNA gene sequencing colony PCR with 1X GoTaq Green (Promega: Madison, WI, USA), 0.125 μM forward primer (27F 5’ AGRGTTTGATYMTGGCTCAG 3’), 0.125 μM reverse primer (1492R 5’ GGYTACCTTGTACGACTT 3’), brought to a final volume of 10 μL with ddH2O, and a colony was mixed in. The thermocycler parameters were set as follows: initial denaturation at 95 °C for 2 minutes, 35 cycles of 30 seconds at 95 °C for denaturation, 30 seconds at 50 °C for annealing, and 1 minute at 72 °C for extension, followed by a final extension period of 2 minutes at 72 °C. The PCR products were confirmed on a 1% agarose gel containing 1X GelRed (VWR: Radnor, VA, USA). For isolates that did not yield sufficient PCR product for Sanger sequencing, InstaGene matric (BioRad,
Hercules, CA, USA) was used to prepare partially purified genomic DNA for PCR according to the manufacturer’s instructions. An ExoSAP-IT clean-up procedure was used to prepare all PCR products for Sanger sequencing by adding 1X ExoSAP-IT (Thermo Fisher Scientific: Waltham, MA, USA) to the PCR tube, then placing in the thermocycler for 15 minutes at 37 °C then 15 minutes at 80 °C. Sanger sequencing was performed by Eton Biosciences (Union, New Jersey, USA). Sequencing results were run through a nucleotide BLAST analysis using the 16S rRNA sequences database to identify the bacteria (Altschul et al., 1990).

**Pure and co-cultures**

A culture consisting of an antibiotic producing colony from the mCPT screen was placed in 5 mL TYME and incubated 3-5 days at 30 °C at 300 rpm. Subsequently, 1 mL of the overnight culture was then added to 100 mL of fresh ESPM, a TYME media substituting 5 g/L potato starch for glucose (VWR: Radnor, VA, USA), in a 250 mL Erlenmeyer flask covered with surgical paper (VWR: Radnor, VA, USA) (Table 1). After three days of incubation at room temperature at 200 rpm, a sterile amberlite bag was added to the culture.

Co-cultures followed the same steps; however, 1 mL of three randomly selected antibiotic-producing bacteria from the mCPT screen were added to 100 mL liquid EPSM in a single 250 mL Erlenmeyer flask, rather than one. For experiments comparing EPSM with and without supplemental Manganese in the form of MnCl$_2$ (1 mM final concentration), MnCl$_2$ was added to the culture media prior to autoclaving.

**Amberlite™ bag assembly**

Amberlite XAD16N resin (Sigma Aldrich, St. Louis, MO, USA) was enclosed in small, 1 x 1 inch bags using heat sealed strips of Unitherm 1.5, a polyester/polyethylene cloth-like material (Midwest Filtration Company, West Chester Township, OH, USA).
15 g/L (w/v) Amberlite™ was measured out for each culture. The Amberlite™ was then washed with acetone three times and once with ddH₂O. The new mass of the Amberlite™ was then equally distributed amongst the bags and autoclaved prior to addition to liquid cultures.

**Chemical extractions of Amberlite™ bags:**

On day 8 of incubation, Amberlite™ bags were removed from their respective cultures, rinsed in cold water, and patted dry. The bags were then placed in pre-weighed vials, to which 20 mL of 50/50 ethyl acetate/methanol was added. This shook for 30 minutes at room temperature at 300 rpm, after which the Amberlite™ bags were removed and the samples were dried in a Thermo Fisher Scientific SpeedVac at 45 °C for 8 hours. After reweighing the vials, the mass of the dried product was calculated and the samples were resuspended in DMSO at 15 mg/mL to obtain the final natural product extract.

**Antibiotic activity**

To test for antibiotic activity, 10 μL spots of chemical extract were placed on TYME agar plates and allowed to dry. *S. aureus* (PP667) or *E. coli* (TE-Ec) were then inoculated over top the samples and the plates grew for 7 days at 37 °C (Table 2). The plates were checked at days one and seven of incubation for ZOI and signs of developing antibiotic resistance.

**Comparison of pre-incubation time and media type for mCPT screening**

Auxotrophic *B. subtilis* streaks were grown on LB, TY, TYME, and EPSM (Table 1) for either 1 or 7 days at 30 °C. mCPT screening plates were prepared as described above with natamycin and D-alanine. These plates incubated for two weeks at 30 °C. Size and number of ZOI were then recorded.
Construction of a D-alanine auxotrophic *S. aureus* mutant:

**Primer design:** The alanine racemase genes, *alr1* and *alr2*, of MRSA USA300 (accession number: CP000255) were located on the *S. aureus* genome. Primers for clean deletion mutations for Alr1 and Alr2 using the pKOR1 plasmid were then designed with melting temperatures ($T_m$) around 60 °C (Table 3).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>$T_m$ °C</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>oPP494</td>
<td>GATCGGTACCCTTAGAAGGTAATGGCTCACATACGATAGC</td>
<td>60</td>
<td><em>S. aureus</em> Alr2 upstream KpnI</td>
</tr>
<tr>
<td>oPP495</td>
<td>TGCACCAACACCCCATGTGTGCTGCAATGTATTACACCTC</td>
<td>58.6</td>
<td><em>S. aureus</em> Alr2 upstream</td>
</tr>
<tr>
<td>oPP496</td>
<td>AGCAACATGGGTGTTGCGCAAAATTCTGAACAAATTAAGT</td>
<td>57.6</td>
<td><em>S. aureus</em> Alr2 downstream</td>
</tr>
<tr>
<td>oPP497</td>
<td>GATCGAATTCGCCTCAATAAAGTGAGTTCTGATATG</td>
<td>60</td>
<td><em>S. aureus</em> Alr2 downstream EcoRI</td>
</tr>
<tr>
<td>oPP498</td>
<td>GATCGGTACCAGAAGCCATCAATGATTAACTAAATCAGCGC</td>
<td>58.7</td>
<td><em>S. aureus</em> Alr1 upstream EcoRI</td>
</tr>
<tr>
<td>oPP499</td>
<td>AGATTCGCGGACATATTACTTCTTTCCAATGATTTGAGGAG</td>
<td>58.6</td>
<td><em>S. aureus</em> Alr1 upstream</td>
</tr>
<tr>
<td>oPP500</td>
<td>AGTAATATGTCCGCGCGAATCTATGATGCTGTGATCAAC</td>
<td>59.9</td>
<td><em>S. aureus</em> Alr1 downstream</td>
</tr>
<tr>
<td>oPP501</td>
<td>GATCGAATTCGATGAAATCGGAGATGCTATGCTTGAGGCAAG</td>
<td>58.4</td>
<td><em>S. aureus</em> Alr1 downstream EcoRI</td>
</tr>
<tr>
<td>oPP502</td>
<td>GTGAGCGGATAACAATTCACACAGGAAC</td>
<td>61</td>
<td>Pkor1 Plasmid Forward</td>
</tr>
<tr>
<td>oPP503</td>
<td>CTGAACCGACTTCTCTTTTTCGCCCTTC</td>
<td>61</td>
<td>Pkor1 Plasmid Reverse</td>
</tr>
<tr>
<td>oPP504</td>
<td>CATCGCGTCAATTACATCAACATCTTG</td>
<td>60.4</td>
<td><em>S. aureus</em> Alr2 check</td>
</tr>
<tr>
<td>oPP505</td>
<td>GCGCATCACATTATAGGATTTGACACTTG</td>
<td>60.3</td>
<td><em>S. aureus</em> Alr1 check</td>
</tr>
</tbody>
</table>
**Plasmid preparation:** S. aureus genomic DNA was isolated using the Purelink Microbiome DNA Purification kit for Gram-positive organisms (Thermo Fisher Scientific: Waltham, MA, USA) using 2 mL of a S. aureus (PP667) overnight culture grown in LB (Table 2). The target regions were amplified via PCR at a volume of 20 μL consisting of 1.25 ng/μL isolated gDNA, 10 μM dNTPs (VWR: Radnor, VA, USA), 25 units per liter Q5 polymerase (NEB: Ipswich, MA, USA), 1X Q5 Buffer (NEB: Ipswich, MA, USA), 0.5 μM of each primer and brought to the final volume with ddH2O. The thermocycler conditions were as follows: initial denaturation at 95 °C for 2 minutes, 35 cycles of 30 seconds at 95 °C for denaturation, 30 seconds at 55 °C for annealing, and 1 minute at 72 °C for extension, followed by a final extension period of 2 minutes at 72 °C. Splicing by overlap extension (SOE) PCR using the same PCR conditions was used to generate a single PCR product for cloning into the pKOR1 vector. Primers oPP494, oPP495, oPP496, and oPP497 (Table 3) were used to construct the clean deletion plasmid for alr2 and oPP498, oPP499, oPP500, and oPP501 (Table 3) for alr1. Following agarose gel electrophoresis, PCR products were purified with Zymoclean Gel DNA Recovery Kit using manufacturer’s instructions (Zymo Research, Irvine, CA, USA).

**Plasmid Construction:** pKOR1 and the corresponding SOE PCR products were enzymatically digested with EcoRI and KpnI (NEB: Ipswich, MA, USA). Digested PCR product was purified with Zymoclean Gel DNA Recovery Kit using manufacturer’s instructions (Zymo Research, Irvine, CA, USA). This product was then ligated using T4 DNA ligase (NEB: Ipswich, MA, USA) and transformed into chemically competent NEB-5α E. coli (NEB: Ipswich, MA, USA) cells following the manufacturer’s protocols.

**Sanger Sequencing of Potential Clones:** Colony PCR was used to amplify clean deletion insertions into pKOR1 using oPP502 and oPP503 (Table 3). Sanger sequencing,
as described above, was used to verify the correct insertions. Final constructs were
designated pPAP003 for the alr2 deletion construct and pPAP004 for the alr1 deletion
construct.

Transformation in RN4220 competent S. aureus cells: To obtain the correct
methylation pattern, pPAP003 and pPAP004 were transformed into electrocompetent
RN4220 S. aureus cells (Bae and Schneewind, 2006) (Table 2). To construct competent
RN4220 S. aureus cells, 30 mL TSB (BD GmbH: Tullastrasse, Germany) was inoculated
with 300 μL of a RN4220 overnight culture in TSB and incubated at 37 °C for 3 hours.
The sample was centrifuged at 8000 rpm for 10 minutes at 4°C. The supernatant was
discarded, and 30 mL ice-cold 10% glycerol diluted with ddH2O was added to
resuspend the pellet; this step was repeated three times, suspending the final cells in 3
mL 10% glycerol. Competent cells were then aliquoted and stored at -80 °C. For the
transformation, 5 μL of purified plasmid DNA was electroporated into 100 μL
competent RN4220 cells at 2,900 V, 25 μF, and 100 ohms. After electroporation, the cells
were recovered in 400 μL of TSB and plated on TSA (BD GmbH: Tullastrasse, Germany)
supplemented with chloramphenicol (10 μg/mL) (VWR: Radnor, VA, USA). Plasmid
preparations from these cells were then used to transform S. aureus USA300 using the
same procedure.

Statistical methods:

Statistical analyses were calculated at a significance level of P < 0.05. Due to
sample sizes, chi-squared tests were used for frequency analyses. To compare sizes of
ZOI, either a Mann Whitney U test or a Kruskal Wallace test was used, depending on
the number of samples. Lin’s correspondence correlation compared the similarity of
antibiotic production.
RESULTS:

**Initial soil screening using the mCPT screening method**

Soil continues to be a viable source for identifying microbes that produce antimicrobial compounds (Daniel, 2005). Consequently, five soil samples from the Eastern Michigan University campus were diluted and screened for antibiotic-producing bacteria using the mCPT screening method. The initial soil microbe screening using *B. subtilis* and *S. aureus* as target organisms yielded 35 antibiotic producers at two weeks post-inoculation, 26 (74.2%) of which were active against *B. subtilis* following purification using the spread-patch assay (Figure 6). At three months post-inoculation, an additional 33 antibiotic producers were identified, 28 (84.8%) of which were active against *B. subtilis* following purification. Of the 68 total antibiotic producers screened, 54 (79.4%) continued to actively inhibit *B. subtilis* during the spread-patch secondary screening assay. In addition to *B. subtilis*, the 68 isolated producers from this initial screen were further isolated against a panel of ESKAPE pathogen relatives.

![Figure 6: Initial mCPT soil screen using *B. subtilis* as a target organism. Arrows indicate ZOI at two weeks post-inoculation. The ZOIs imply antibiotic-producing microbes.](image)
Secondary screen against a safe relative ESKAPE panel

Antibiotic-producing bacteria can exhibit a broad or narrow spectrum of activity against a variety of pathogens. Therefore, isolated antibiotic-producing bacteria were screened for activity against additional safe relatives of Gram-negative and Gram-positive ESKAPE pathogens. Of the 35 microbes from the initial two-week checkpoint, 22 (62.8%) inhibited at least one member of the ESKAPE pathogen safe relatives (Figure 7 and Table 4). Antibiotic production in this experiment was slightly lower than that seen during the first and secondary screen using only *B. subtilis* as a target organism.
Table 4: mCPT derived isolates active against a panel of pathogens

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identity</th>
<th>% Identity</th>
<th>Size of Halo (mm)</th>
<th>mCPT Target</th>
<th>Activity Against Panel Pathogen</th>
<th>Size of Halo(s) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Pseudomonas soli</em></td>
<td>80%</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>S. aureus</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>100%</td>
<td>1</td>
<td>B. subtilis</td>
<td>S. aureus</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td><em>P. soli</em></td>
<td>99.80%</td>
<td>1</td>
<td>B. subtilis</td>
<td><em>B. subtilis,</em> and <em>S. aureus</em></td>
<td>1.5, 2.5</td>
</tr>
<tr>
<td>4</td>
<td><em>Bacillus subtilis</em></td>
<td>100%</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>S. aureus</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>N/A</td>
<td>0.25</td>
<td>B. subtilis</td>
<td><em>B. subtilis,</em> and <em>S. aureus</em></td>
<td>1, 1</td>
</tr>
<tr>
<td>6</td>
<td><em>P. soli</em></td>
<td>99.80%</td>
<td>0.5</td>
<td>B. subtilis</td>
<td>S. aureus</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>N/A</td>
<td>N/A</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>N/A</td>
<td>N/A</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>N/A</td>
<td>N/A</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
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<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>11</td>
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<td>N/A</td>
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<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td><em>Pseudomonas alcaligenes</em></td>
<td>100%</td>
<td>3</td>
<td>B. subtilis</td>
<td><em>B. subtilis</em> and <em>S. aureus</em></td>
<td>2, 3</td>
</tr>
<tr>
<td>13</td>
<td><em>P. soli</em></td>
<td>99.80%</td>
<td>0.5</td>
<td>B. subtilis</td>
<td><em>B. subtilis</em> and <em>S. aureus</em></td>
<td>1.5, 2</td>
</tr>
<tr>
<td>14</td>
<td><em>B. subtilis</em></td>
<td>100%</td>
<td>0.5</td>
<td>B. subtilis</td>
<td><em>S. aureus</em> and <em>P. putida</em></td>
<td>0.5, 1</td>
</tr>
<tr>
<td>15</td>
<td><em>P. soli</em></td>
<td>99.74%</td>
<td>0.5</td>
<td>B. subtilis</td>
<td><em>B. subtilis</em> and <em>S. aureus</em></td>
<td>1, 0.5</td>
</tr>
<tr>
<td>16</td>
<td><em>P. soli</em></td>
<td>99.75%</td>
<td>0.25</td>
<td>B. subtilis</td>
<td><em>B. subtilis</em> and <em>S. aureus</em></td>
<td>1, 1, 1</td>
</tr>
<tr>
<td>17</td>
<td><em>Chitinophaga caseinilytica</em></td>
<td>97.26%</td>
<td>1</td>
<td><em>S. aureus</em></td>
<td>B. subtilis</td>
<td>0.5</td>
</tr>
<tr>
<td>18</td>
<td><em>B. subtilis</em></td>
<td>99.69%</td>
<td>1</td>
<td>B. subtilis</td>
<td><em>B. subtilis</em> and <em>S. aureus</em></td>
<td>1.2</td>
</tr>
<tr>
<td>19</td>
<td><em>Brevibacillus brevis</em></td>
<td>99.57%</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>P. putida</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td><em>B. subtilis</em></td>
<td>100%</td>
<td>0.5</td>
<td>B. subtilis</td>
<td><em>S. aureus</em> and <em>P. putida</em></td>
<td>0.5, 1</td>
</tr>
<tr>
<td>21</td>
<td><em>Bacillus tequilensis</em></td>
<td>100%</td>
<td>0.5</td>
<td><em>S. aureus</em></td>
<td><em>S. aureus</em></td>
<td>0.5</td>
</tr>
<tr>
<td>22</td>
<td><em>Brevibacillus reuszeri</em></td>
<td>99.44%</td>
<td>0.5</td>
<td><em>S. aureus</em></td>
<td>P. putida</td>
<td>0.5</td>
</tr>
<tr>
<td>23</td>
<td><em>S. aureus</em></td>
<td>N/A</td>
<td>1.5</td>
<td><em>S. aureus</em></td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>24</td>
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<td><em>S. aureus</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>25</td>
<td><em>B. subtilis</em></td>
<td>N/A</td>
<td>0.25</td>
<td><em>B. subtilis</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>26</td>
<td><em>B. subtilis</em></td>
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<td>B. subtilis</td>
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<td>N/A</td>
</tr>
<tr>
<td>27</td>
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<td><em>B. subtilis</em></td>
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<td>N/A</td>
</tr>
<tr>
<td>28</td>
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<td>N/A</td>
<td>0.25</td>
<td>B. subtilis</td>
<td>N/A</td>
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</tr>
<tr>
<td>29</td>
<td><em>S. aureus</em></td>
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<td><em>B. subtilis</em></td>
<td>N/A</td>
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<tr>
<td>30</td>
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<td><em>B. subtilis</em></td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>31</td>
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<td><em>B. subtilis</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>32</td>
<td><em>Pseudomonas monteillii</em></td>
<td>100%</td>
<td>0.25</td>
<td>B. subtilis</td>
<td><em>B. subtilis</em></td>
<td>1</td>
</tr>
<tr>
<td>33</td>
<td><em>P. soli</em></td>
<td>N/A</td>
<td>0.5</td>
<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>34</td>
<td><em>P. soli</em></td>
<td>N/A</td>
<td>0.5</td>
<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>35</td>
<td><em>P. soli</em></td>
<td>N/A</td>
<td>0.5</td>
<td>B. subtilis</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>36</td>
<td><em>P. soli</em></td>
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<td>0.5</td>
<td>B. subtilis</td>
<td>N/A</td>
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</tr>
<tr>
<td>37</td>
<td><em>P. soli</em></td>
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<td>0.5</td>
<td><em>S. aureus</em></td>
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<td>N/A</td>
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<tr>
<td>38</td>
<td><em>P. soli</em></td>
<td>N/A</td>
<td>0.5</td>
<td><em>S. aureus</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>39</td>
<td><em>P. soli</em></td>
<td>N/A</td>
<td>0.5</td>
<td><em>S. aureus</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>40</td>
<td><em>P. soli</em></td>
<td>N/A</td>
<td>0.5</td>
<td><em>S. aureus</em></td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Optimization of the mCPT technique

Comparison of gellan gum to agar: Previous literature suggested that when gellan gum is used as a medium solidifying agent, it is more effective for isolating actinomycetes and stimulating antibiotic production than agar (Suzuki, 2001). Gellan gum-based and agar-based media were compared for their effectiveness in the mCPT screening method. While media solidified using agar yielded 35 antibiotic producers after two weeks of incubation, gellan gum only yielded five antibiotic producers from the same diluted soil sample. This indicates a significant difference in antibiotic production between the two media types (Corrected $X^2 = 56.077, p < 0.05$ (1, N = 2436)). In addition, because gellan gum has a much narrower pH range at which it remains solid, the media becomes less solid over time, making the mCPT method more difficult over time (Picona and Cunha, 2011).

Comparison of prototrophic and auxotrophic B. subtilis strains: The initial antibiotic screens used a prototrophic strain of B. subtilis as the target organism in the mCPT screen, but the Price lab had also started using a D-alanine auxotrophic mutant of B. subtilis as the target strain to aid in the downstream purification process. This study’s initial mCPT screen resulted in fewer antibiotic producers than those isolated with the D-alanine auxotrophic strains of B. subtilis. Therefore, a course wide experiment was designed to determine the potential differences between using prototrophic and D-alanine auxotrophic strains of B. subtilis as a target organism in the mCPT screen. Students in the BIO328 sections of the Tiny Earth lab were provided with the D-alanine auxotrophic strain whereas the students in the BIO112 sections of the Tiny Earth lab were provided with the prototrophic B. subtilis strain. Overall, compared to prototrophic strains of B. subtilis, the D-alanine auxotrophic mutants produced more
and larger ZOIs. There were 346 ZOI averaging 3.96 mm in size on the mCPT plates using prototrophic *B. subtilis*. There were significantly more, and larger, halos with the auxotrophic *B. subtilis* overlay with 677 halos averaging 7.20 mm in size (Corrected $\chi^2 = 128.9103$, $p < 0.05$ (1, $N = 41752$); Mann-Whitney U test, $Z = -8.571$. $n_1 = 346$, $n_2 = 677$, $p < 0.05$, one-tailed) (Figure 8). This implied that there may be an interaction between the microbes and supplemental D-alanine that might be weakening the cell wall of the pathogen. This may make the target pathogen more susceptible to antibiotics.

![Figure 8: Juxtaposition of prototrophic versus auxotrophic antibiotic production.](image)

(A) Antibiotic production against the prototrophic *B. subtilis* reveals halos averaging 3.96mm. (B) Antibiotic production against auxotrophic *B. subtilis* unveiled halos averaging 7.20mm.

**Comparison of incubation time and media type:** The Price lab also observed variation in the performance of the mCPT screening method over time. Based on these observations, it was hypothesized that more antibiotic producers would be identified with increasing culture age. The effects of the incubation time (one or seven days) of the D-alanine auxotrophic mutant culture and the media type (LB, TY, TYME, or EPSM) it was cultivated on prior to inoculating the screen were tested to determine whether pre-incubation conditions influenced antibiotic production during the mCPT screen. Overall, a seven-day pre-growth period yielded the most antibiotic producers,
regardless of media type, although there was a trend toward TY and TYME generating more antibiotic producers (Figure 9). These data suggest that there is an interaction between media and pre-incubation time (G-test = 316008.908, \( p < 0.05 \) (7, \( N = 8176 \))) and the number of antibiotic producers observed during the mCPT screening method. Overall, the largest number of antibiotic producers was identified on TYME media when auxotrophic \( B. \text{subtilis} \) cultures were pre-grown for seven days.

![Figure 9: Comparison of incubation period and media type on antibiotic production. Testing for antibiotic production against auxotrophic \( B. \text{subtilis} \) that has been incubated for one day yields low numbers of ZOI, with a minor trend in media type. When grown for seven days, antibiotic production was strongest on TYME media.](image)

Comparison of antibiotic producers ssing either \( B. \text{subtilis} \) or \( S. \text{aureus} \) as the target organism

Unpublished data from the Price lab suggest a correlation between the ability of antibiotic-producing microbes to inhibit the growth of both \( B. \text{subtilis} \) and \( S. \text{aureus} \) upon secondary screening when the D-alanine auxotrophic mutant of \( B. \text{subtilis} \) is used initially as a screening tool. However, when this relationship was tested using prototrophic \( B. \text{subtilis} \) or \( S. \text{aureus} \) as target organisms, there was a poor correlation between the antibiotic producers that inhibited both \( B. \text{subtilis} \) and \( S. \text{aureus} \) (Lin’s
correspondence; N = 35, r = -0.5525) (McBride, 2005) (Figures 10 and 11). Although not all antibiotic producers kill both organisms all of the time, *B. subtilis* can still be used as an initial tool to screen for antibiotic producers with the understanding that the isolated antibiotic producers from such experiments may not inhibit true pathogens like *S. aureus*. Consequently, the construction of a D-alanine auxotrophic mutant of *S. aureus* would likely provide the added antibiotic sensitivity observed with *B. subtilis* while potentially increasing the likelihood of recovering antibiotic-producing bacteria that kill MRSA. Furthermore, an auxotrophic mutant would provide a safe way to screen for antibiotic producers that specifically inhibit *S. aureus*.

Figure 10: Isolated microbes from initial soil screens were tested against *B. subtilis* and *S. aureus*. This showed whether the same antibiotic producer was able to kill both pathogens.
Generation of a S. aureus D-alanine auxotrophic mutant

Although there are two alanine racemase genes in B. subtilis, the mutant in the Price lab effectively functions as an auxotroph with only the alr1 gene mutated. However, for the purposes of the mCPT antibiotic screening tool in a classroom setting, both alanine racemase genes, alr1 and alr2, in the S. aureus strain will be deleted using homologous recombination with the plasmid pKOR1 (Bae and Schneewind, 2006). Briefly, 1KB upstream and downstream of each alr gene was amplified using PCR to increase the likelihood of a recombination event (Figure 12A). The individual upstream and downstream PCR products were combined used splicing by overlap extension (SOE) PCR prior to being cloned into pKOR1 and transformed into NEB-5α E. coli cells (3.951 × 10³ transformants per μg of DNA) (Figure 12B). Sanger sequencing was used to confirm each plasmid construct. To generate the right methylation patterns, each plasmid was then transformed into chemically competent S. aureus RN4220 cells (transformation efficiency was 3.453 × 10⁴ transformants per μg of DNA for alr1).
Unfortunately, subsequent steps in the mutation process were unable to be completed due to technical reasons with the chemically competent MRSA strains. Thus, cells were transformed, but no mutants were recovered.

Effect of mixed-culture fermentation on antibiotic production

Many of the antibiotic-producing strains discovered in the Price lab using the mCPT technique require co-culturing to observe antibiotic production. Co-culturing in liquid media offers a convenient way to test the impacts of interspecies interactions on antibiotic production in a more industrial setting. First, single-culture fermentations for 18 unique antibiotic producing bacteria were tested to determine the extent of antibiotic production of each antibiotic-producing microbe in isolation. Chemical extractions from each fermentation were used to measure antibiotic production using a plate-based method similar to the Kirby-Bauer assay, which measures the ZOI of each extract (Bauer et al., 1966). Of the 18 antibiotic-producing microbes grown up in monoculture, only three isolates (80, 83, and 92) produced antibiotics that also inhibited *S. aureus* and five isolates (64, 65, 80, 83, and 99) produced antibiotic that also inhibited *E. coli* (Figure 13A and Table 5). To help determine the potency of the antibiotic and the potential for rapid
antibiotic resistance, the Price lab incubated these assays for seven days. After seven days, chemical extracts from isolates 80, 83, and 92 had *S. aureus* colonies growing within the initial ZOI. Chemical extracts from 64, 65, and 99 had *E. coli* colonies growing within the initial ZOI.

Strains that failed to produce a ZOI using the single-culture fermentation reactions were then selected for mixed-culture fermentation experiments. Groups of three antibiotic producers were used to test the effect of mixed-culture fermentation on secondary metabolite production. With these ten combinations, antibiotic production that inhibited growth of *S. aureus* or *E. coli* was observed for an additional four (40%) chemical extracts (combinations G, I, K, and O) (Figure 13B and Table 6).

![Figure 13: Antibiotic testing of chemical extracts of single- and mixed-culture fermentations.](image)

(A) Secondary metabolites extracted from single-culture fermentation tested using *S. aureus*. (B) Secondary metabolites extracted from mixed-culture fermentation tested using *S. aureus*. 
Table 5: Pure cultures and their associated sequencing data.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Closest Relative (16S rRNA)</th>
<th>% Identity</th>
<th>Antibiotic Activity</th>
<th>ZOI Size (mm)</th>
<th>Activity Against</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td><em>Brevibacillus reuszeri</em></td>
<td>99.59%</td>
<td>Yes*</td>
<td>2</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>65</td>
<td><em>Brevibacillus brevis</em></td>
<td>99.25%</td>
<td>Yes*</td>
<td>3.5</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>75</td>
<td><em>Flavobacterium amniphilum</em></td>
<td>98.88%</td>
<td>No</td>
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<td>N/A</td>
</tr>
<tr>
<td>76</td>
<td><em>Streptomycetes zaomyceticus</em></td>
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</tr>
<tr>
<td>78</td>
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<tr>
<td>79</td>
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<tr>
<td>80</td>
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<td>6 and 4.5</td>
<td><em>S. aureus and E. coli</em></td>
</tr>
<tr>
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<tr>
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<tr>
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<td>N/A</td>
</tr>
<tr>
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<td><em>S. aureus</em></td>
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<td><em>Agrobacterium larrymoorei</em></td>
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<td>N/A</td>
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<tr>
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<td>N/A</td>
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<tr>
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<td><em>Psuedoxanthomonas mexicana</em></td>
<td>100%</td>
<td>Yes*</td>
<td>3.5</td>
<td><em>E. coli</em></td>
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<tr>
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<td><em>Tahibacter aquaticus</em></td>
<td>97.98%</td>
<td>No</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A – data not applicable
* resistance observed on Day 7
Manganese (Mn) has been shown to stimulate antibiotic production in various microorganisms (Foster and Woodruff, 1945). Although TYME and EPSM media contain Mn, its concentration is at the lower threshold (0.2 µM) for this stimulation effect. Therefore, it was tested whether the addition of supplemental Mn (2.3 µM) would induce antibiotic production in mixed-culture fermentations. The same mixed-culture fermentation combinations were grown in regular EPSM and EPSM+Mn. The

<table>
<thead>
<tr>
<th>Letter</th>
<th>Isolates</th>
<th>Microbes Co-Cultured</th>
<th>Antibiotic Activity</th>
<th>Activity Against</th>
<th>ZOI size (mm)</th>
<th>Mn</th>
<th>Extract Colour</th>
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</thead>
<tbody>
<tr>
<td>A</td>
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<td>N/A</td>
<td>N/A</td>
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<td>N/A</td>
<td>N/A</td>
<td>Yes</td>
<td>Transparent yellow</td>
</tr>
<tr>
<td>C</td>
<td>76, 78, 79</td>
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<td>No</td>
<td>N/A</td>
<td>N/A</td>
<td>Yes</td>
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<td>N/A</td>
<td>No</td>
<td>Purple</td>
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<td>Yes</td>
<td>S. aureus</td>
<td>2.5</td>
<td>No</td>
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</tr>
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<td>S. aureus</td>
<td>2.5</td>
<td>Yes</td>
<td>Transparent grey</td>
</tr>
<tr>
<td>G</td>
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<td>S. aureus</td>
<td>2.5</td>
<td>Yes</td>
<td>Transparent grey</td>
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<td>Yes</td>
<td>S. aureus and E. coli</td>
<td>8</td>
<td>No</td>
<td>Brown</td>
</tr>
<tr>
<td>I</td>
<td>84, 85, 90</td>
<td>Streptomyces venezuelae, Bacillus toyonensis, and Bacillus mobilis</td>
<td>Yes</td>
<td>S. aureus and E. coli</td>
<td>8</td>
<td>Yes</td>
<td>Brown</td>
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<tr>
<td>J</td>
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<td>N/A</td>
<td>N/A</td>
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<td>Transparent yellow / brown</td>
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<td>90, 91, 95</td>
<td>Bacillus mobilis, Lyso bacter soli, and Agrobacterium larrymoorei</td>
<td>Yes</td>
<td>S. aureus</td>
<td>2</td>
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<td>Transparent yellow / grey</td>
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<tr>
<td>M</td>
<td>90, 91, 95</td>
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<td>Yes</td>
<td>S. aureus</td>
<td>2</td>
<td>No</td>
<td>Transparent yellow / grey</td>
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<tr>
<td>N</td>
<td>91, 95, 84</td>
<td>Lyso bacter soli, Agrobacterium larrymoorei, and Streptomyces venezuelae</td>
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<td>N/A</td>
<td>N/A</td>
<td>No</td>
<td>Yellow</td>
</tr>
<tr>
<td>O</td>
<td>91, 95, 84</td>
<td>Lyso bacter soli, Agrobacterium larrymoorei, and Streptomyces venezuelae</td>
<td>No</td>
<td>N/A</td>
<td>N/A</td>
<td>Yes</td>
<td>Brown</td>
</tr>
<tr>
<td>P</td>
<td>95, 84, 85</td>
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<td>No</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
<td>Transparent yellow / grey</td>
</tr>
<tr>
<td>Q</td>
<td>95, 84, 85</td>
<td>Agrobacterium larrymoorei, Streptomyces venezuelae, and Bacillus toyonensis</td>
<td>No</td>
<td>N/A</td>
<td>N/A</td>
<td>Yes</td>
<td>Transparent yellow</td>
</tr>
</tbody>
</table>

N/A – data not applicable
Mn – addition of Manganese

Effect of manganese on antibiotic production

Manganese (Mn) has been shown to stimulate antibiotic production in various microorganisms (Foster and Woodruff, 1945). Although TYME and EPSM media contain Mn, its concentration is at the lower threshold (0.2 µM) for this stimulation effect. Therefore, it was tested whether the addition of supplemental Mn (2.3 µM) would induce antibiotic production in mixed-culture fermentations. The same mixed-culture fermentation combinations were grown in regular EPSM and EPSM+Mn. The
resulting chemical extracts from each combination displayed different colors depending on the additions of Mn, but antibiotic production was either not affected or diminished (Figure 14 and Table 3). While Mn had an effect on the production of secondary metabolites, as indicated by the extract colors, characterizing the extracts via LC/MS/MS and GNPS analysis may offer insight into the impact of Mn on secondary metabolite production in these cultures.

Figure 14: Chemical extracts from mixed-culture fermentations grown with or without supplemental Mn. The chemical extracts display different colors depending on the additions of Mn. Extracts from cultures lacking Mn are on the left while Mn-supplemented cultures are on the right side of each paired group.
DISCUSSION:

This study improves the efficacy of the mCPT screening method and shows that mixed-culture fermentation can be effective at inducing antibiotic activity when single-culture fermentation fails to produce sufficient antibiotic activity. By optimizing growth conditions, specifically, incubation time and media type, prior to and during the mCPT screen, the improved conditions for enhanced antibiotic production were developed. In addition, the results indicate that the benefits of using D-alanine auxotrophic \textit{B. subtilis} extend beyond safety and the efficiency of colony purification; they also suggest an increased sensitivity to antibiotic producers, resulting in more and larger ZOIs. The results also show that it is possible to use the mCPT screening method to find antibiotics active against \textit{S. aureus}; this pattern was most defined when \textit{S. aureus} was used as an initial target. Consequently, a MRSA D-alanine auxotroph is under construction and will help broaden the range of the mCPT screening method to specifically target MRSA. The results also suggested that otherwise cryptic BGCs (biosynthetic gene clusters) can be stimulated under mixed-culture fermentations when single-culture fermentation conditions fail to yield antibiotic activity.

\textbf{Effectiveness of the mCPT modifications}

One of the primary advantages of the mCPT screening method is the ability to accommodate slow growing bacteria by incubating the mCPT plates for several months (Wollheben et al., 2016; Weiner, 2000). These microbes are often outcompeted by faster growing microbes, however, when grown over a long period of time, slow growers are able to compete with the other microbes via the production of antibiotics (Lazzarini et al., 2000; Wollheben et al., 2016). Slow growing bacteria are rarely represented in antibiotic screens because they are often outcompeted or do not grow over the short
incubation periods normally used. Hence, the secondary metabolites of slow-growing bacteria offer a new potential source for novel antibiotics (Wollheben et al., 2016).

Different neighboring microbes likely resulted in different external stimuli and might explain why microbes may not have produced antibiotics against *B. subtilis* during both the primary mCPT and secondary ESKAPE pathogen screens (Netzker et al., 2015). The number of antibiotic producers observed during the mCPT decreased during the secondary screening using the spread-patch assay with safe relatives of the ESKAPE pathogens. Notably, many of the microbes formed ZOI during the initial antibiotic screen using *B. subtilis* did not necessarily produce a ZOI during the secondary ESKAPE pathogen screen. During the ESKAPE relative screen, the antibiotic producing bacteria are not necessarily near the same microbes they were neighboring during the initial mCPT screen.

Previous research has suggested that the use of gellan gum as a solidifying agent helps stimulate secondary metabolite production in actinomycetes (Suzuki, 2001). However, this study found that compared to agar plates, there was less growth and less antibiotic production. In addition, gellan gum made a comparatively soft media that was not suitable for standard plating procedures. Thus, agar yields better results for the mCPT screening method than gellan gum. This is potentially due to the sensitivity of gellan gum to pH (Picona and Cunha, 2011).

*B. subtilis* and *S. aureus* are both Gram-positive bacteria in the Firmicutes phylum. Consequently, they share much of their metabolic profile and are susceptible to many of the same antibiotics (Brown et al., 2010; Bosi et al., 2016). They have similar cell wall structures consisting of peptidoglycan, polyribitol phosphate, and wall teichoic acids (WTA) in both constitutes roughly 60% of the cell wall in both species. However, the WTA is produced via different metabolic pathways (Brown et al., 2010).
Unpublished data from the Price lab suggests that many (about 75%) of the antibiotic-producing microbes that inhibited *B. subtilis* also inhibited the growth of *S. aureus*. However, a much lower proportion (28.6%) was observed in this study. Overall, these data suggest that the mCPT can be easily adapted to find antibiotics to target particular species.

Interestingly, when D-alanine prototrophic and auxotrophic *B. subtilis* strains were directly compared using the mCPT screen, the auxotrophic mutants yielded more antibiotic producers with larger ZOI. D-alanine is used to construct both the crosslinking amino acids in peptidoglycan and in wall teichoic acids (WTA). WTAs function in cell support, as well as contributing to the charge and hydrophobicity of the cell wall (Brown et al., 2010). This, in turn, impacts the susceptibility of the microbe to the antibiotics by influencing the binding and flow of secondary metabolites through the cell wall. D-alanine esters are attached to WTAs in a process known as D-alanylation (Brown et al., 2013). In lower concentrations of D-alanine, there is a decline in D-alanylation; this alters the hydrophobicity and charge of the cell wall, thus lending itself to increase the susceptibility of the bacteria to antibiotics (Brown et al., 2013).

When D-alanine is provided in the media, such as the case during the initial antibiotic producer screen, both auxotrophic and prototrophic microbes will preferentially take up the D-alanine from their environment (Khonsari and Kollmann, 2015). This results in local D-alanine depletion on these screening plates because both the soil microbes and the auxotrophic bacteria using the supplemental D-alanine from the media. Following the local depletion of D-alanine from the media, prototrophic bacteria transition to produce D-alanine from L-alanine using D-alanine racemases, continuing to divide and grow on the plate (Moscoso et al., 2018). The auxotrophs, however, rely solely on the supplied D-alanine in the media; when this is depleted, the auxotrophs are no longer
able to grow, likely have weaker cell walls, and may be lysing at a higher rate than prototrophic bacteria. Therefore, as the concentration of D-alanine in the media decreases, there is likely a similar decline in the robustness of the auxotrophic cell wall because D-alanine can be incorporated into the peptidoglycan and the WTAs. Consequently, the auxotroph may be more susceptible to antibiotics following the primary growth of the bacteria. This may result in more and larger ZOIs because the soil microbes are able to inhibit a weakened pathogen with less antibiotic, adding more sensitivity to the mCPT screen. It may be beneficial to construct more auxotrophic pathogens for use as an antibiotic screening tool because they lead to more obvious ZOIs. In doing so, rarer, slow-growing antibiotic producers in the complex stimulatory environment resulting from the mCPT screening method may be identified (Mehl and Cotty, 2013). It is anticipated that the increased sensitivity of D-alanine auxotrophs and the abundant microbe-microbe interactions will increase the ability to identify antibiotic production from otherwise cryptic BGCs. To this end, the process of constructing a D-alanine auxotrophic S. aureus mutant has begun; considerable progress has been made, but mutagenesis has not yet been completed.

**Liquid culturing conditions**

Growth of pure cultures were considered the gold-standard for the production and extraction of secondary metabolites because they provide more controlled growth environments; there is also no question as to which microbe produced the secondary metabolite (Nei and Meyer, 2017). Thus, most antibiotics to date were discovered and produced using this type of single-culture fermentation. Unfortunately, these are relatively barren growth conditions as there are no interspecies interactions or competition to encourage antibiotic production, especially when most antibiotics are also self-harming (Seyedsayamdost et al., 2012). Co-cultures, or mixed-culture
fermentations, provide interspecies interactions that can induce different and potentially novel secondary metabolic pathways. To evaluate the effects of interspecies interactions on antibiotic production, Ueda and Beppu (2016) selected strains that showed very little antibiotic production in pure cultures and used mixed-culture fermentations significantly increase secondary metabolite production. To help control for the production of a desired secondary antimicrobial metabolite, only bacteria that did not produce antibiotics in pure culture were used in the mixed-culture fermentation. These mixed-culture fermentations with non-antibiotic producing bacteria increased antibiotic production, supporting the notion that interspecies interactions increase the expression of various metabolic pathways. However, this process has largely been limited to the increasing production of known compounds; only the Onaka lab has used this process as a mechanism for discovering new antibiotics (Onaka et al., 2011). The dichotomy observed between antibiotic production using the mCPT screening method and the overall lack of antibiotic production in single-culture fermentation results presented in this study suggest that the parameters for mixed-culture fermentation still need to be optimized. While the compounds produced during mixed-culture fermentation are currently unknown, it is encouraging to see increased antibiotic activity when multiple antibiotic producers are combined in mixed-culture fermentation. This suggests that there are still likely new antibiotics to be found and that combining multiple antibiotic-producing bacteria together during mixed-fermentation might provide the best strategy for increasing antibiotic production from these producers (Seyedsayamdost et al., 2012).

While this study focused on optimizing the use of multiple cultures, media modifications are widely used to modify antibiotic production. Manganese (Mn) has been reported to stimulate secondary metabolism, increasing antibiotic production
(Foster and Woodruff, 1945). Although chemical extracts from Mn and non-Mn supplemented culture were different colors, a potential sign of differential expression of secondary metabolites, there was little change in the size of the ZOIs. However, Mn exists in different colors at different oxidation states, so it is possible, too, that the color change is courtesy of a different redox environment (Willard and Greathouse, 1917).

These results suggest that there is an interaction between secondary metabolite pathways and Mn availability. However, without characterizing the chemical extracts, it is hard to say what effect Mn has on secondary metabolite production.

**D-alanine auxotroph construction**

Plasmids for the MRSA D-alanine auxotroph have been constructed, but they have not yet been transformed into MRSA. Thus far, transformations reactions have yielded any colonies. Glycine was added to competent cells in an attempt to optimize culture conditions and a new electroporator was purchased, but no colonies have been observed (Cruz-Rodz and Gilmore, 1990).

One potential explanation for the lack of transformed MRSA colonies is the importance of methylation patterns. Due to the number of restriction modification systems in MRSA, methylation patterns of the plasmid are of utmost importance in order to transform the plasmid into the cell (Jones et al., 2015). Despite using *S. aureus* RN4220, which is supposed to help bypass some of these restriction systems in the MRSA cell that recognize foreign DNA and restrict transformation, there was still an issue with transformation (Jones et al., 2015). For future transformation attempts, it may be worthwhile to investigate plasmid alterations that can circumvent restriction modification in MRSA. One study accomplished this by using plasmids from *E. coli* DC10B; this strain is able to bypass some restriction modifications in *S. aureus* as it does not methylate cytosines (Jones et al., 2015).
CONCLUSIONS

These findings suggest the need to continue to optimize the process of identifying and producing antibiotics from cryptic BGCs in soil microbes. There was a notable increase in efficiency when using a D-alanine auxotrophic mutant of *B. subtilis* during the initial mCPT screening to identify antibiotic producers. This success could thus be furthered by constructing a series of D-alanine auxotrophs in addition to the MRSA auxotroph as this will allow for specific identification of antibiotic producers that are able to kill MRSA and other specific ESKAPE pathogens. Until these auxotrophs are built, however, the *B. subtilis* and *E. coli* auxotrophs can continue to be used in introductory labs as the best means of efficiently identifying antibiotic producers for further study. Furthermore, the use of *B. subtilis* auxotroph in the classroom will allow students to more easily visualize their results, potentially creating a stronger sense of discovery and engagement with concepts taught in the lab.

Given the early success with mixed-culture fermentation using multiple antibiotic producers, this technique may be further optimized; perhaps there are specific interactions that can be harnessed to trigger secondary metabolite production and specifically secondary metabolites that have antimicrobial properties. To get a better understanding of the trends seen in the mixed-culture fermentation cultures, full chemical profiles of the single- and mixed-culture fermentation chemical extracts can be characterized via LC/MS/MS. Modern computational chemistry approaches such as Global Natural Products Social (GNPS) molecular networking can be used to delineate the novelty of these secondary metabolites and potentially useful compounds that specifically induce antibiotic production (Wang et al., 2016).
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