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A CURE for antibiotic resistance: Using metabolite supplementation to increase antibiotic efficacy

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Abstract

Drug resistant microbial infections play a role in millions of deaths worldwide each year. As time passes, more pathogenic strains of bacteria acquire antibiotic resistance genes. The development of novel antibiotic drugs cannot keep up. As a result, strategies to maintain or increase the efficacy of existing drugs are one potential way to fight against antibiotic resistant bacterial infections. Previous work has shown that supplementing bacteria with single metabolites can potentiate inhibition or killing by antibiotics that were previously ineffective. For example, several sugars and amino acids have been shown to increase the effectiveness of aminoglycoside antibiotics. To date, no comprehensive survey of combinations of metabolites and antibiotics has been conducted to identify these possible combination therapies. The studies required to establish candidates for this type of therapy only need common and inexpensive microbiological techniques. They are also simple enough for undergraduates to perform and are part of many existing microbiology laboratory curricula. Thus, the undergraduate microbiology laboratory may serve as a potential screening platform for these therapies. This setup allows students to conduct authentic, inquiry-driven research in their teaching lab. Here we describe a multi week laboratory module developed for an undergraduate microbiology lab course. This work outlines the biological background and experimental procedures for this module.

Keywords: CURE, inquiry-driven, microbiology, bacteriology, antibiotics, antibiotic resistance, antibiotic tolerance, metabolism

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INTRODUCTION

Antibiotic resistance is a serious problem that causes millions of illnesses and thousands of the deaths in the United States alone (Centers for Disease Control and Prevention (U.S.) 2019). In the near future, that number is

only expected to grow, making it a global public health threat (World Health Organization 2014; World Health Organization 2015). Current antibiotics are losing their effectiveness as antibiotic resistance genes are rapidly shared between microbes. At the same time, novel antibiotic development has slowed (Cooper and Shlaes 2011). As a result, there is an urgent need to develop new strategies to combat antibiotic resistant bacteria. One of these is to increase the efficacy of existing antibiotic drugs [reviewed in (Schäberle and Hack 2014)]. This strategy is attractive because it involves the use of drugs that are already approved for use in humans. For years, experts in the field have suggested that combination therapies may provide a way to increase the efficacy of existing drugs [reviewed in (Mehta et al. 2014). We also know that the antibiotic tolerance or sensitivity of some microbes can be affected by their metabolic state and the metabolites that are available to them (Stokes et al. 2019). However, to date, there has been no exhaustive study of metabolite and antibiotic combination therapies. Such an undertaking does not require techniques that are very difficult, but it does require time. Therefore, it is a perfect type of project to deploy in the undergraduate teaching lab.

Introducing authentic research into teaching labs through inquiry driven experiments and course-based undergraduate research experiences have repeatedly shown positive impacts on students such as increased motivation and persistence in STEM (Hunter et al. 2007; Lopatto 2007; Auchincloss et al. 2014; Corwin et al. 2015). Here we describe the development of a laboratory module that acts as a pipeline to test the synergistic activity between metabolites and existing antibiotics. By implementing such a module, instructors can motivate students to participate in authentic scientific process that addresses one of the key concepts in microbiology, antibiotics and antibiotic resistance, and a major public health threat, the rise of antibiotic resistant infections worldwide.

Metabolite Addition Improves the Efficacy of Some Antibiotics

Previous work has shown the potential of synergistic combinations of amino acids and aminoglycoside antibiotics (Peng et al. 2015; Ye et al. 2018; Guan et al. 2023; Kuang et al.). Thus, these pairings were prioritized for the initial development of this module. Existing studies have shown combination therapies as effective for gamma-proteobacteria. While the exact molecular mechanisms that underlie this synergy are not clear in every case, the prediction is that the presence of a metabolite in growth media causes changes to the bacterium's physiology or cell structure that makes them more susceptible to antibiotics. For example, in one of these systems, kanamycin sensitivity increases in the presence of glucose and/or alanine. Kanamycin can be transported into the cell using the same transporters as glucose and alanine and these transporters are upregulated when their normal substrates are present (Peng et al. 2015). Kanamycin is a commonly available aminoglycoside antibiotic that is frequently used in lab environments for genetic selection and is sometimes used in the clinic as a treatment for bacterial infections including *E. coli.* The first three metabolites we chose to test in the development of this module were three inexpensive, commonly available, and water-soluble amino acids: alanine, glycine, and the related compound monosodium glutamate (MSG).

A Pipeline for Testing Metabolite-Antibiotic Interactions

Course Context: Experimental Microbiology Laboratory (BIO SCI M118L)

This inquiry-based laboratory module was designed for an upper-level biology laboratory course that is typically taken by 4th year students majoring in Biological Sciences or a related discipline. Many of these students are taking the course to satisfy their major's laboratory requirement and the laboratory requirements of healthcare professional schools. The pre-requisites for this course include introductory biology as well as one year of additional biology training (genetics, biochemistry, and molecular biology). An introductory or upper-level microbiology lecture course is not a pre- or co-requisite for this class.

BIO SCI M118L: Experimental Microbiology Laboratory is taught over a 10-week session. During the academic year we teach 8 sections of 20 students each. Other topics covered in the class include basic laboratory safety, bacterial culturing, identification and antibiotic susceptibility testing. As a result, this module addresses some of the broader objectives of the class such as understanding the requirements and limits for bacterial growth, the function of antibiotics, and an appreciation for the growing problem of antibiotic resistance in bacteria. Our biosafety guidelines allow students to work with approved biosafety level 1 organisms in this course with appropriate personal protective equipment including gloves, lab coats, and protective eyewear.

Since this is a large course, it is not feasible to provide students with total freedom for experimental design, but they are given choice about which metabolite they use for their studies. However, the project includes other components of inquiry-driven science as defined by Spell and colleagues including hypothesis generation, data analysis, critical thinking, and scientific communication (Spell et al. 2014). Experiments have been optimized to fit the structure of the course which is two sessions per week, one 2 hours and 50 minutes and the other 50 minutes. This module replaced another 4-week module in the course and occurs concurrently with a module on brewing beer using yeast.

Pipeline Development

Because the majority of evidence for synergistic metabolite-antibiotic combinations comes from work with gamma-proteobacteria species, we used *E. coli* as the model organism for this study. We used a commercially available strain, SJ358, which is a K-12 derivative and classified as biosafety level 1. The authors recommend using SJ358 specifically for this module because other common model *E. coli* strains (for example, MG1655) contain mutations that disrupt growth under certain metabolic conditions, while SJ358 is wild-type at these loci (Soupene et al. 2003; Deatherage and Barrick 2014; Brown and Jun 2015). Since one goal of the project is to force bacteria to undergo metabolic changes due to media supplementation, we want to ensure that they are performing all normal metabolism. In this new module, bacteria are cultured in M9 minimal media with relatively few nutrient sources prior to and during each experiment. This is essential because our goal is to cause changes in bacterial metabolism based on metabolite addition. In a rich media, bacteria may have no use for the metabolite added due to the availability of a variety of other nutrients. As mentioned above, kanamycin was chosen as the antibiotic for these studies based on previous work and the fact that is a commonly used in many teaching and research microbiology labs. Kanamycin is a bacteriostatic aminoglycoside antibiotic that targets the bacterial ribosome. Over time we plan to test a range of amino acid molecules for synergistic effects with kanamycin. Alanine, glycine, and MSG were chosen for this initial work because alanine was previously shown to increase the efficacy of kanamycin treatment in some bacteria (Peng et al. 2015). All three were also already available from our teaching lab's existing chemical stocks. As explained in the conclusion and instructor notes, the type of bacteria used, types of metabolites tested, and the test antibiotic could all be changed based on the specific interests of the instructor and students with some modifications and optimizations to the pipeline described here.

We chose to use two different types of experiments in this module that are often used when characterizing the effects of antibiotics. Students first perform a test to determine the Minimum Inhibitory Concentration (MIC), the lowest concentration that inhibits the growth of bacteria. There are several different accepted methods for determining MIC (Kowalska-Krochmal and Dudek-Wicher 2021). We used a direct visual inspection of culture tubes for turbidity because it does not require any additional equipment such as spectrophotometers. This method also works well for the teaching lab because observations can be done in one class session, rather than relying on the measurement of colony-forming units (CFUs) which takes at least 1 day for incubation. As a result, students can set up the MIC experiment in one lab session and then check their results when they return for the second lab session of the week.

The second set of experiments in this module provides a different look at growth inhibition. Here, students start with an *E. coli* culture in M9 glucose media and create 4 different incubation conditions: a control culture incubated without kanamycin or metabolite, a culture incubated with 500 μg/ml kanamycin, a culture incubated with 40 mM metabolite, and a culture incubated with both 500 μg/ml kanamycin and 40 mM metabolite. At the end of the class period, they serially dilute the cultures, plate a spot of each culture on an LB agar plate, and calculate the CFUs for each treatment condition in the next lab class period. Because colonies can only arise from the growth of viable cells, this type of study allows students to determine whether the various treatments allow for bacterial growth or result in the death of the bacteria due to the bacteriocidal action of kanamycin. In our class, we refer to this assay as the "killing assay".

A range of kanamycin concentrations were tested for these studies based on literature values for the MIC of other *E.coli* in the range of approximately 0.5-10 μg/ml (Oie et al. 1997; Garza-Cervantes et al. 2020; Pitsiniaga and Sullan 2022). It is important to note that MIC measurements depend on the specific type of bacteria as well as media conditions, so any deviation of student-determined MIC measurements from those reported in

the literature with other strains of *E. coli* and different growth conditions is not necessarily unexpected. Each metabolite (alanine, glycine, or MSG) was provided at a final concentration of 40 mM, which was based on previous studies that have shown that low millimolar levels of metabolites have yielded the synergistic effects with antibiotics. Further optimization of the metabolite concentration is an open area of investigation and could even be incorporated into part of a future iteration of the lab module.

Some Students Observe Enhanced Kanamycin Inhibition After Metabolite Addition

As a control, each group calculated the MIC of kanamycin without any metabolite present. The three metabolites available to students were alanine, glycine, and monosodium glutamate (MSG). As part of the lab activity, students saved their results as part of a class wide Google spreadsheet. Most groups (n=41) determined an MIC of 50 μg/ml for kanamycin when no metabolite was added (Figure 1). This is higher than the values found in literature for the *E. coli* kanamycin MIC (approximately 0.5 – 10 μg) [see (Oie et al. 1997; Garza-Cervantes et al. 2020; Pitsiniaga and Sullan 2022) for examples]; however this literature value is only valid for a specific strain and specific set of growth conditions. A review of literature did not reveal any published MIC value for kanamycin treatment of *E. coli* grown in M9 minimal media specifically. Only one student group determined a different MIC (0.5 μg/ml), while another group reported no growth in any of their cultures. In contrast, the MIC varied when 40 mM of metabolite was added to the *E. coli* cultures with kanamycin. Interestingly, a reduction in MIC was only observed for groups that chose MSG as their metabolite. Approximately half of the MSG groups determined the MIC of kanamycin to be 5 μg/ml while three groups determined an MIC of 0.5 μg/ml. 100% of the groups that added glycine or alanine determined the same MIC for kanamycin with or without metabolite addition. The MIC experiment is relatively quick, which leaves enough time in the lab session for other experiments. In our course, students were still able to finish another project on antibiotic susceptibility (week 6) and set up a brewing experiment (week 7) in the same class session.

This module was tested in Fall 2022. Unfortunately, due to a teaching assistant union strike starting in week 8 in the University of California system, we were unable to finish this module with our academic year students. Previous testing of some of the lab protocols with Summer 2022 students shows that they can complete the killing assay as part of this module. This portion of the module involves a 2-hour incubation. During this time, students continued their brewing experiment or worked on a scientific writing project. The experimental results from these Summer 2022 student groups were not collected and, therefore, are not reported here. We hope to report these findings after future, uninterrupted iterations of the course.

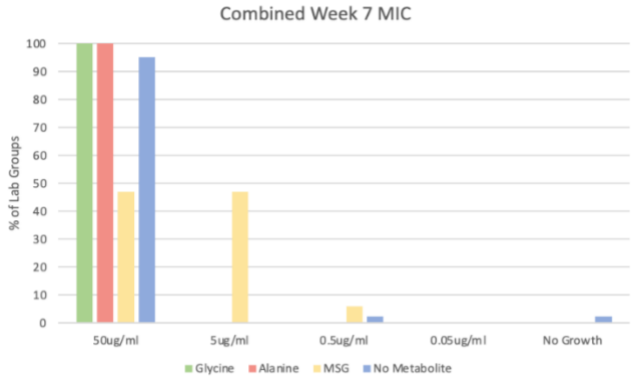


Figure 1. Student determination of kanamycin Minimum Inhibitory Concentration (MIC). Student groups tested the addition of one of three metabolites, glycine, alanine, or monosodium glutamate (MSG), to an *E. coli* culture treated with kanamycin. All metabolites were tested at 40 mM. Results are presented as the percentage of student groups that identified each concentration of kanamycin as the MIC (n = 41 groups).

Effects of the Module on Student Experiences

To assess the impact of this new module on our microbiology lab students, we utilized a CURE survey

instrument (Denofrio et al. 2007). Prior to the start of this module, students took the pre-survey, then they completed two replicates of the MIC and killing assays before taking the post-survey. Because of the aforementioned teaching assistant strike in Fall 2022, we have reported the Summer 2022 student survey data. Selected results of the scientific identity survey are shown in Figure 2 (n=40 students). This work was considered exempt, self-determined by the UC Irvine Institutional Review Board (protocol #1354).

Notably, students reported experience gains with skills associated with the scientific practices and the process of science (Figure 2A-B). A larger group of students reported much or extensive experience with collecting data after participating in the new module than in the pre-survey. We also observed shifts toward more experience with presenting data in written papers or results. At the conclusion of this project, students completed an individual worksheet about the project that involved free-response answers and asked them to produce an abstract on the project. While they did not write a full manuscript on this project, they were still revising and completing a journal article style-manuscript on the project completed in the first half of the quarter. Therefore, this shift may be related either to the worksheet (Appendix A) completed as part of this new module, the manuscript on the project from the first half of the quarter, or a combination of both assignments. Together, we take this data to indicate that the new module allows students to build experience with the practice of science including data collection and communicating results.

We were interested in whether group work and collaboration skills related to the practice of science showed experience gains as well (Figure 2C-D). Students also responded that they gained experience in reviewing and critiquing the work of peers. Survey items related to collaboration skills such as working in small groups and critiquing the work of others also showed a shift towards higher degrees of experience. In contrast to these other characteristics of authentic, inquiry-driven science, students reported minimal or no changes in their experience level with skills associated with science coursework in general (Figure 2E-F). For example, most students reported having much or extensive experience with discussing reading materials in class and listening to lectures. These are skills that we did not design our hands on, in-lab module to target or emphasize. The proportions of students in these categories showed only minor changes in the post-survey relative to the pre-survey. We interpret this to mean that students were not simply reporting experience gains with all skills in general, even those not addressed as part of this module. We will continue to collect assessment data from students to evaluate the impact this new module has on students.

Conclusion

We have created a new multiweek module for the microbiology lab that allows students to conduct an inquiry-driven study of antibiotic efficacy. It represents a pipeline for testing combinations of metabolites and antibiotics with the potential to review synergistic pairings that may represent a new tool in the fight against antibiotic resistant bacteria in healthcare. Students can complete this project in four weeks, meeting twice a week. The timing of these experiments also allows for the implementation of this module at the same time as another project, with the MIC assays being very quick to set up and interpret and the killing assay having a 2-hour incubation time. Thus, this project is amenable to a course schedule in which students work on more than one project at a time. It is important to note that this combination therapy testing pipeline should be adaptable to other combinations of metabolites and antibiotics. Past work has shown that bacterial persister cells regain antibiotic tolerance when treated with certain carbohydrates and sugar alcohols (Allison et al. 2011), so this may be another area for study. Also, these experiments can be done with other types of bacteria if an appropriate minimal media is selected for that organism. Essentially every component: metabolite, antibiotic, and organism can be substituted depending on the materials available at the institution, biosafety requirements, or student and instructor interest. Because of this flexibility, this pipeline and the overall line of scientific inquiry would allow for a large variety of options for students and a long longevity for this project in the teaching lab.

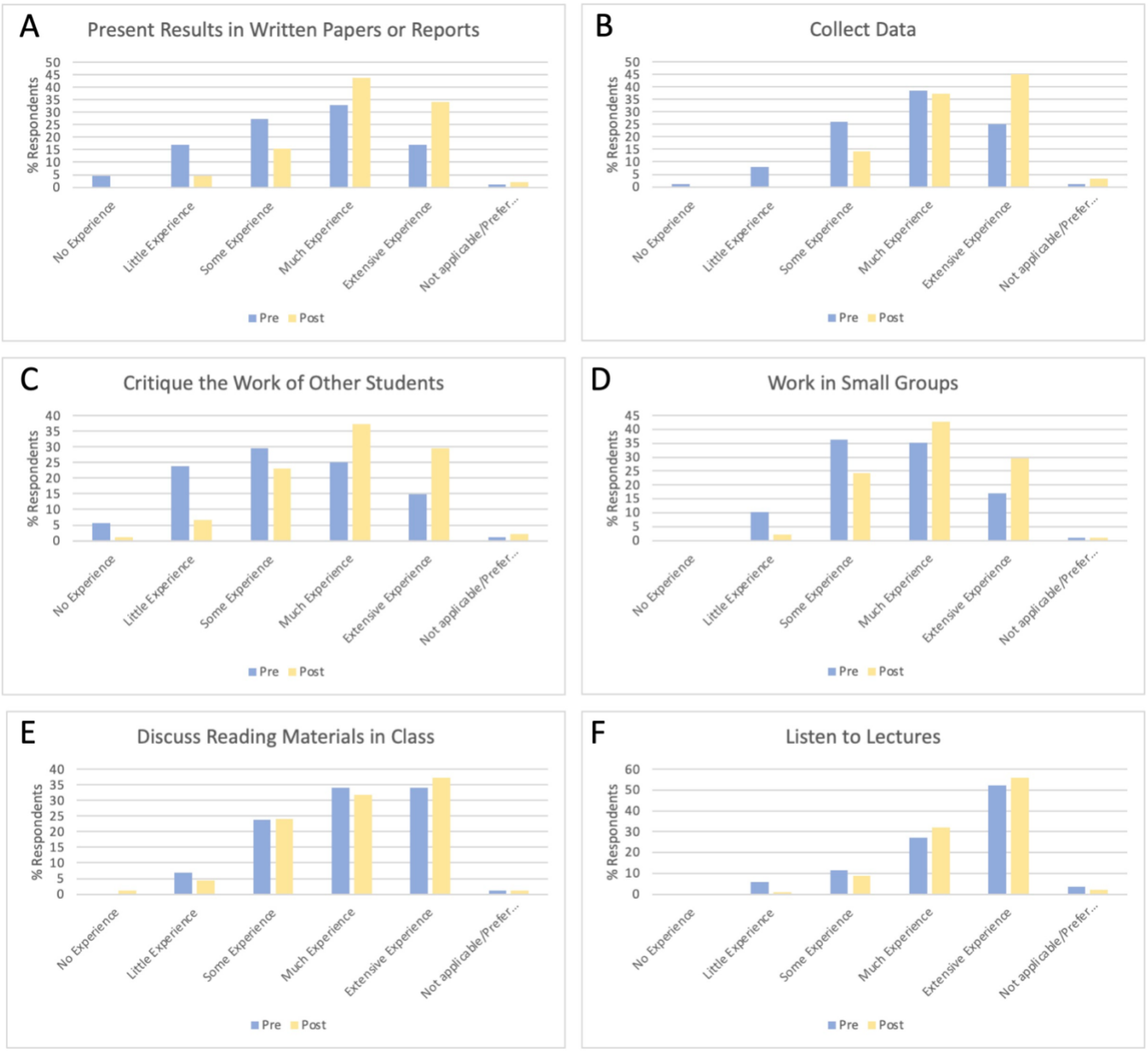


Figure 2. Student skills before and after module completion. Students were surveyed before (blue) and after (yellow) completing the new module. Responses to selected prompts are shown in terms of percentage of students that reported that level of experience with each item. Selected survey items such as those related to skills involved in the process of science (A, B), collaborative work (C, D), and traditional lecture course skills (E, F).

STUDENT OUTLINE

Objectives

To determine whether a small molecule modulates the Minimum Inhibitory Concentration (MIC) of an antibiotic.

To determine whether a small molecule modulates the ability of an antibiotic to decrease the viability of bacteria during short-term treatment.

Introduction: Can metabolites affect the antibiotic sensitivity of bacteria?

As you have already seen first-hand through your Kirby Bauer tests, different strains of bacteria may be more or less susceptible to antibiotics. This is a big problem when it comes to treating bacterial infections and other situations in which antibiotics may be used. Rising rates of antibiotic resistance among pathogenic bacteria are a looming public health concern. In 2019, the Centers for Disease Control and Prevention estimated that *antibiotic-resistant bacteria caused a minimum of ~2.8 million illnesses in the United States*. 10-15% of these illnesses *directly* resulted in death (Centers for Disease Control and Prevention (U.S.) 2019).

The number of antibiotic resistant bacterial strains has been on the rise since the implementation of antibiotics for routine medical and agricultural use in the 1940s [reviewed in (Schäberle and Hack 2014)]. Many commonly used antibiotics halt metabolic pathways or growth processes to eliminate bacteria. For example, quinolones, one of the most prescribed antibiotic drugs in the United States, target DNA replication activity (Aldred et al. 2014). However, not all bacteria in a population are replicating and dividing, leaving the dormant cells within a population unaffected (Coates and Hu 2008). These dormant cells may begin proliferating later, causing disease relapse, and leaving a population of living bacteria that may acquire or develop antibiotic resistance (Grant and Hung 2013).

So what can we do about this big problem? Since metabolic changes are one way that bacterial cells may evade the effects of antibiotics, it is possible that we can force metabolic changes that can instead make bacteria *more* susceptible to those same antibiotics. In this module, you will help test combinations of metabolites and antibiotics to evaluate whether any of these combination therapies are effective modulators of antibiotic susceptibility a non-pathogenic strain of *E. coli*.

Methods and Data Collection

Experiment A – Calculating Minimum Inhibitory Concentration (MIC) of Antibiotic With and Without Small Molecule Treatment

Purpose: To determine whether a small molecule modulates the MIC of an antibiotic.

Calculating Minimum Inhibitory Concentration (MIC)

One way to quantify the effectiveness of an antibiotic is to calculate the minimum inhibitory concentration (MIC). MIC is defined as the minimum concentration of a substance capable of inhibiting microbial growth. Calculating an MIC is often one of the first steps in the pre-clinical workup of a new drug candidate. One common way to perform an MIC calculation is to conduct a broth dilution assay. In this type of assay, a microbe is inoculated into growth media with the potential antibiotic substance. Multiple concentrations of the potential antibiotic substance are used, some of which may be too low to inhibit microbial growth and some of which may be high enough to prevent any visible growth. Visible inspection for the cloudiness or turbidity associated with microbes in broth culture is sufficient to determine which concentrations of antibiotics can inhibit growth.

For your project, you are comparing the MIC of an antibiotic alone versus an antibiotic combined with a metabolite. You will be working with kanamycin, a commonly used antibiotic that inhibits bacterial protein synthesis. Many types of bacteria are naturally sensitive to kanamycin, including *E. coli*. In your first experiment, you will determine whether the addition of a metabolite alters the MIC of kanamycin in *E. coli*.

Procedure (day 1):

Pay close attention to what media you are adding in each step as there are several different types available today. You will be working in groups of 4 people to complete this experiment. Two members of the group should perform the serial dilutions today. Whichever group members did not perform the serial dilution last week should perform it this week.

Part 1: MIC With Antibiotic Alone (No Metabolite) Setup

1. Set up 5 tubes for your antibiotic only experiment. For each tube, take a piece of labeling tape and write "ABX only" and your tube number (between 1 and 5, #1-5).
2. To the "ABX only #1" tube, add 5.5 ml of sterile minimal media with kanamycin (50 μg/ml).
3. To 3 of the other 4 "ABX only" tubes (#2-4), add 4.5 ml of sterile minimal media (no additives).
4. To create the appropriate dilutions for tubes 2-4, you will perform serial 1:10 dilutions from tube 1. To start, use your P1000 pipet to remove 500 μl of minimal media with kanamycin from "ABX only #1". Add this liquid to "ABX only #2".
5. Swirl "ABX only #2" to mix.
6. Use your P1000 pipet to remove 500 μl of minimal media with kanamycin from "ABX only #2". Add this liquid to "ABX only #3".
7. Swirl "ABX only #3" to mix.
8. Use your P1000 pipet to remove 500 μl of minimal media with kanamycin from "ABX only #3". Add this liquid to "ABX only #4".
9. Swirl "ABX only #4" to mix. Do not remove any liquid from #4 when you are done.
10. To "ABX only - 5", add 5 ml of sterile minimal media (no additives).
11. Put your 5 "ABX only" tubes to the side for now.

Part 2: MIC With Antibiotic + Metabolite Setup

1. Set up 5 tubes for your antibiotic + metabolite M experiment. For each tube, take a piece of labeling tape and write "ABX + M" and your tube number (between 1 and 5, #1-5).
2. To the "ABX + M #1" tube, add 5.5 ml of sterile minimal media with kanamycin (50 μg/ml) + M (40 mM alanine, glycine, or MSG).
3. To 3 of the other 4 "ABX + M" tubes (#2-4), add 4.5 ml of sterile minimal media + M.
4. To create the appropriate dilutions for tubes 2-4, you will perform serial 1:10 dilutions from tube 1. To start, use your P1000 pipet to remove 500 μl of minimal media with kanamycin from "ABX + M #1". Add this liquid to "ABX + M #2".
5. Swirl "ABX + M #2" to mix.
6. Use your P1000 pipet to remove 500 μl of minimal media with kanamycin from "ABX + M #2". Add this liquid to "ABX + M #3".
7. Swirl "ABX + M #3" to mix.
8. Use your P1000 pipet to remove 500 ml of minimal media with kanamycin from "ABX + M #3". Add this liquid to "ABX + M #4".
9. Swirl "ABX + M #4" to mix. Do not remove any liquid from #4 when you are done.
10. To "ABX + M #5" add 5 ml of sterile minimal media + M.

Part 3: Adding Bacteria to Start the Experiment

11. Collect all 10 of your tubes on one test tube rack. Now, it is time to inoculate your tubes with *E. coli*.
12. Swirl the *E. coli* culture provided to mix it well. Use your P20 pipet to remove 5 μl of *E. coli* culture and add it to each tube for the MIC experiment. Make sure to use aseptic technique and change tips between tubes.
13. At the end of the class, place each tube in your section's rack for incubation at 37 °C.

Procedure (day 2):

Retrieve the 10 tubes you used to set up your MIC experiment in the previous lab section and place them in a test tube rack.

Separate them in the test tube rack based on whether or not metabolite X was added and order them based on the amount of antibiotic they contain.

Visually inspect each tube for cloudiness/turbidity. If the tube is cloudy, then bacterial growth occurred. The MIC is the lowest concentration of antibiotic that inhibits growth. Record it below. Be sure to include units.

If no growth is observed in any tubes, continue to incubate at 37 °C with shaking for another class period and then reobserve.

MIC for kanamycin: _____ MIC for kanamycin + M: _____

Experiment B – Quantifying Decreases in Bacterial Viability With and Without Small Molecule Treatment (Killing Assay)

Purpose: To determine whether a small molecule modulates the ability of an antibiotic to decrease the viability of bacteria during short-term treatment.

Quantifying Antibiotic Inhibition

Kanamycin works as an antibiotic by inhibiting normal protein synthesis. Over time, bacteria treated with kanamycin cannot make the normal protein they need to live and replicate. Another way that antibiotics are characterized is to determine how they affect the number of viable cells in a bacteria culture. There are many ways to quantify microbial cells, but not all of them are conducive to determining which cells are viable and which cells are nonviable or dead. In this experiment, you will determine whether the presence of a metabolite makes kanamycin more or less effective at reducing the number of viable bacteria in a culture.

Procedure (day 1):

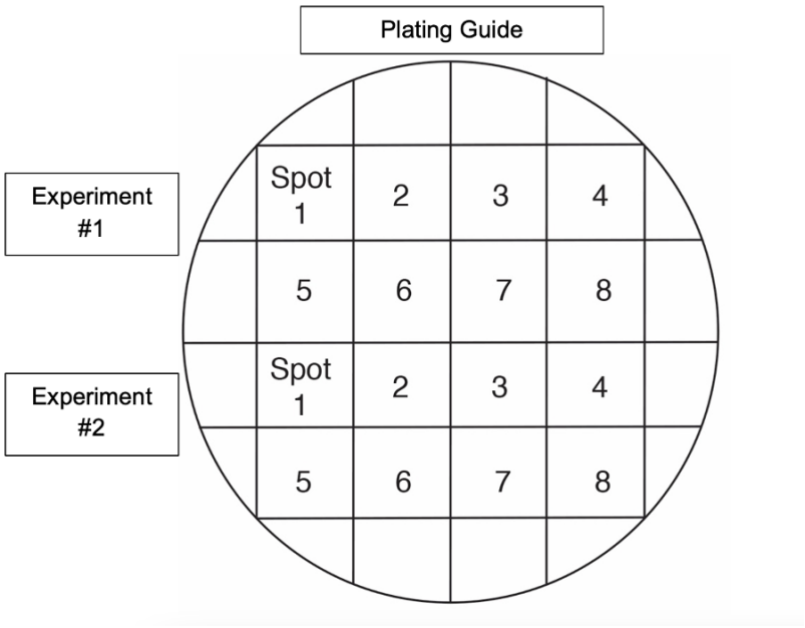
Make sure to use the same metabolite that you used in your previous experiments.

Antibiotic Treatment

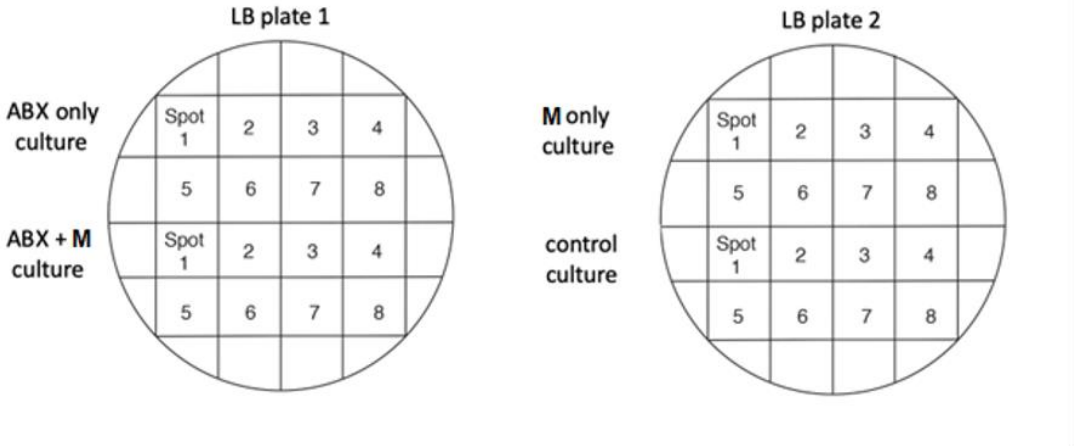
1. Set up 4 tubes for your antibiotic treatment experiment. On a piece of tape give them the following labels:
    a. “ABX only”
    b. “ABX + M”
    c. “M only”
    d. “Control”
2. To the “ABX only” tube, add 4 ml sterile minimal media with kanamycin.
3. To the “ABX + M” tube, add 4 ml of sterile minimal media with kanamycin + M.
4. To the “M only” tube, add 4 ml sterile minimal media with M.
5. To the “control” tube, add 4 ml sterile minimal media (no additives).
6. To each tube, add 1 ml of the *E. coli* culture.
7. Place each tube in your section’s rack for incubation at 37 °C.
8. Incubate your tubes with shaking for 2 hours.

Quantifying Bacterial Viability

Use this plating guide to help you with the following steps.



1. Each group will collect 2 LB plates. Label them “#1” and “#2”.
2. You will plate two cultures on each LB plate, allowing you to plate all four of your experiments.
3. You will plate each experiment one at a time. Mix the tube thoroughly before removing any liquid. You should plate one strain in its entirety before moving on to the next.
4. In an empty Petri dish, you will make eight spots with 90 µl of LB each for each experimental condition. Do not let the drops merge. Eventually you will have 24 spots, which may require you to use both the lid and bottom of the empty Petri dish.
5. Take 10 µl of one of your cultures (start with “ABX only”) and add it into the first drop of LB (spot 1). Pipette up and down and stir the drop with the pipette tip to mix the dilution.
6. Using the same tip, pipette 10 µl of the first drop (from spot 1) onto the LB plate “#1”. You will use the grid below to keep track of your spots.
7. Take a new pipette tip and remove 10 µl of the first drop (spot 1) and pipette into the second drop (spot 2). Mix and pipette 10 µl onto LB plate “#1”.
8. Repeat for drops three through eight.
9. Repeat steps 2-6 for your second culture (“ABX + M”) on LB plate “#1”.
10. Repeat steps 2-6 for your third culture (“M only”) on LB plate “#2”.
11. Repeat steps 2-6 for your fourth culture (“Control”) on LB plate “#2”.
12. Let the plate dry face up. Then flip upside down and incubate at room temperature. If it does not dry in time, leave face up on the room temperature shelf.



Procedure (day 2):

Observe plates 1 and 2. Take a clear photograph of them for your records.

To determine the CFU/ml for viable cells, you will look at your dilution spots. The more concentrated spots are likely completely covered with growth and it is no longer possible to count individual colonies. Instead, select the spot with individual colonies.

Colonies from LB plate 1 (ABX only) : _____ on Spot # _____

Colonies from LB plate 1 (ABX + M) : _____on Spot # _____

Colonies from LB plate 2 (M only) : _____ on Spot # _____

Colonies from LB plate 2 (control) : _____on Spot # _____

Data Analysis

Experiment B – Quantifying Decreases in Bacterial Viability With and Without Small Molecule Treatment (Killing Assay)

Now you need to determine the CFU/ml. Imagine you had 2 colonies on spot 5. The very first spot was created by adding 10 µl of cells to 90 µl LB and plating 10 µl (1/10th) of this dilution. Since you plated 1/10th of that original 10 µl (from the culture), you only added 1 µl of the original culture. This means the growth in the first spot signifies the CFU/0.001ml or CFU/10-3 ml. Spot number two is a 1:10 dilution of the first, so growth in this spot signifies the CFU/10-4 ml.

This means that the 2 colonies on spot 5 signify a value of 2 CFU/10____ ml or _____CFU/ml.

Now, based on this little tutorial, calculate your CFU/ml for all viable cells using the values you noted from your LB plates.

ABX only CFU/ml: ABX + M CFU/ml:

M only CFU/ml: Control CFU/ml:

Discussion

Students will repeat these exercises an additional time. They will collect their data on MIC and changes in viability and post them to a class wide spreadsheet online. This will allow them to compare their group's results between the first trial and the second trial as well as compare their group's results to the groups of others in their section and across sections. Finally, students will use this data to complete the project report worksheet (Appendix A).

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MATERIALS

Materials required (per class):

Item	Quantity	Notes
E. coli SJ358	1 freezer stock, cultured in M9 Glucose media for experiments	Available from Addgene (item number: 67755, $85 USD)
Stationary incubator or environmental room (37°C)	1	Experiments can be conducted at room temperature if no incubator is available, but growth will occur more slowly. Longer incubation times may be required.
Shaking incubator	1	

Materials required for each 20-person section (4 lab groups):

Item	Quantity
Culture tubes (5 ml capacity)	20 per group
Micropipettes (P10 or P20, P100 or P200, P1000)	1 set per group
Pipettor and serological pipettes (10 ml)	1 pipettor and >2 pipettes per group
Empty Petri dishes	2 per group
Liquid bacteria growth media (LB, M9 glucose)	500 ml per type per section, autoclaved
Solid bacterial growth media (LB agar)	2 plates per group, autoclaved
Metabolites (alanine, glycine, MSG)	100 ml of 1 M solution (sterile-filtered)
Kanamycin	500 μg/ml in water (sterile-filtered)
LB broth	5 ml per group
M9 glucose	50 ml per group

NOTES FOR THE INSTRUCTOR

In this set of experiments, students are assessing whether the addition of a metabolite changes the antibiotic sensitivity of a bacterium. In the protocols described, students work with antibiotic sensitive *E. coli.* The recommended strain (SJ358) is a K-12 derivative, non-pathogenic, and is typically used under biosafety level 1 conditions.

Students test for kanamycin sensitivity in the presence and absence of metabolites (in this case, amino acids). In this multi week exercise, students work in a group to determine the minimum inhibitory concentration of kanamycin with and without these antibiotics. Then they determine whether the metabolite has any effect on the kanamycin's ability to kill bacteria by assessing the number of viable cells by counting colony forming units after antibiotic treatment. They will repeat each of these experiments to see if their results are reproducible. This exercise is flexible and may be adapted to other types of bacteria, antibiotics, and metabolites. At the end of the unit, students were assigned a worksheet (Appendix A), but a longer lab report could be assigned.

Kanamycin sulfate stock solution should be aliquoted and stored at -20°C. Media containing kanamycin should be stored (for no longer than 2 weeks) at 4°C until use. Kanamycin begins to lose effectiveness within a few days at room temperature. M9 glucose media is used for both the MIC and killing assays based on the standard recipe from Cold Spring Harbor (https://cshprotocols.cshlp.org/content/2010/8/pdb.rec12295.short). When starting a fresh *E. coli* culture, a rich media like LB is recommended so that robust growth can be achieved, however, the *E.* coli should be subcultured into M9 glucose to create fresh cultures for each experiment.

This assignment requires common equipment found in most labs equipped for working with microorganisms. The actual experiments are relatively straightforward and can be completed by a range of students at both lower and upper levels after introductory training with micropipettes. The content works best with students that have already had some exposure to normal microbial growth and the idea of antibiotic resistance. The developers of this module

used it with upper-level microbiology students. The protocols used here were designed for a course structure in which the students met for 2 hours and 50 minutes for one lab class session in a week and then returned for a 50-minute lab class session two days later (for example, Tuesday and Thursday or Wednesday and Friday). These students also had a 1 hour and 50-minute lecture session on Monday each week. Each lab section had 20 students and these students worked in groups of four (due to limited shaking incubator space for culture tubes). If storage and shaking incubator space is not an issue, students could conduct these experiments in pairs or individually. Links to additional resources and updated protocols (when available) will be posted to https://faculty.sites.uci.edu/rachaelbarry/education-resources/.

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Rachael Barry started as an Assistant Professor of Teaching at UC Irvine in Fall 2019. She teaches microbiology and biochemistry courses and co-directs the Microbiology & Immunology major. In addition to bringing authentic science into the classroom, she also studies faculty-student interactions.

APPENDIX A

Can metabolites affect the antibiotic sensitivity of bacteria?

Project Report Worksheet

1. What metabolite did you choose? What role does this metabolite normally play in cells?

2. In 1 sentence, what is your hypothesis or statement of purpose for this project?

3. Based on your data, do you think that the metabolite makes kanamycin more or less effective? Clearly state your conclusion and link it back to specific data.

4. Suppose you are working on this project as an independent study student. Based on the data you have collected thus far, what is the next experiment that you would like to try? Why?

Note: This should be a logical next step that could be done in the teaching lab. This means, no clinical trials or animal testing. If you are proposing another experiment similar to the ones you have already done, explain how it would teach you something new and important about the system we are studying.

Your answer will be evaluated based on three criteria:

1) Is the experiment feasible/doable?
2) Is the experiment novel (compared to what you have done already)?
3) Is the experiment one that could yield interesting or significant results?

5. The final part of this worksheet is to write an abstract describing your work on this project. Your abstract should be between 200-300 words.

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