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Title Page

Title: A Course-Based Undergraduate Research Experience on the Evolution of Antibiotic Resistance and its Molecular Basis

Running Title: A CURE on Evolution of Antibiotic Resistance

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Figures:

Supplemental Materials: **https://github.com/KatieDickinson/CURE-Evolution-of-Antibiotic-Resistance**

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Conflict of Interest Notification Page

None of the authors declare any conflicts of interest.

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Abstract

Course-based Undergraduate Research Experiences (CUREs) in high-enrollment, introductory classes are a potentially transformative approach to retaining more students in STEM majors. We developed and piloted a CURE in the introductory biology courses at the University of Washington. This CURE focuses on analyzing experimental evolution of antibiotic resistance in *Escherichia coli* and generates data on two topics relevant to clinical practice: compensatory mutations and cross-drug effects. By studying mutations in central cellular machinery that confer drug resistance, students not only gain insight into fundamental cellular phenomena, but also recognize the molecular basis of a medically important form of evolutionary change, connecting genetics, microbiology, and evolution.

Keywords

CURE, undergraduate research, natural selection, experimental evolution, molecular biology, genetics, structure-function relationships, introductory biology, laboratory exercise.

INTRODUCTION

Participating in undergraduate research experiences (UREs) has positive effects on students, providing benefits that increase both affect (1, 2) and retention (3, 4, 5) in STEM disciplines. However, many UREs involve a small number of participants and most are only available to upper-level students. To make scientific research experiences more accessible to all students there has been a recent call for the creation of course-based undergraduate research experiences (CUREs) where research is integrated into the course curriculum. At the introductory level, CUREs can increase the academic performance and retention of students in STEM (e.g. 6).

CUREs are a recent innovation in STEM education, and most published CUREs have been small-scale or focused on discovery-based science, such as mutant screens or barcoding organisms for biodiversity surveys. To expand the scope of CURE options available, we designed and tested a hypothesis-driven, large-scale CURE that focuses on experimental evolution of antibiotic resistance in *Escherichia coli*. In addition to having important real-world applications, antibiotic resistance in bacteria is an ideal system for studying evolution in the classroom. Bacteria reproduce quickly, have large population sizes, and are easy to grow in culture -- all attributes that make them optimal for studying evolution in action.

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We designed the CURE described here to provide a context for discussing important concepts in introductory biology such as mutation, evolution by natural selection, fitness, and the molecular underpinnings of resistant phenotypes. Students identify the initial mutations that confer antibiotic resistance, allow the resistant populations to evolve over time, gather data to explore changes in fitness and resistance level, and evaluate whether collateral effects are occurring. Collateral effects involve pleiotropy across drugs, in which resistance to one antibiotic is associated with resistance or sensitivity to a second drug. Cross-resistance and cross-sensitivity can be important in the design of sequential or drug-cocktail treatment regimens. Because each team in a lab section works with a different, randomly-selected mutant strain, students can also broaden their understanding by exploring an open question in biology about the repeatability of evolution.

This CURE has four broad goals:

1) Scale the benefits of participating in undergraduate research and make those benefits available early in the undergraduate experience;
2) Introduce specific laboratory skills; such as sterile technique, serial dilution, and spread plating, along with professional practices, such as scientific communication and basic statistical analyses;
3) Engage students in applying key biological concepts by connecting genotype (DNA sequence) to phenotype (protein structure/function) to fitness (competitive ability) in the context of a medically relevant topic; and
4) Produce preliminary data that advanced undergraduates or graduate students can use in research projects intended for publication.

Because the student-generated datasets from each term are cumulative, instructors gradually build a large dataset on antibiotic resistance. These data can inform the design of more advanced follow-up studies (described in Goal 4) but can also be used as backup data for the introductory course in the event of experimental failure.

Intended audience and prerequisite knowledge

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This CURE was designed for introductory-level undergraduate students who have completed high-school-level biology and intend to major in the life sciences. All relevant concepts are addressed in the laboratory exercises, so no additional prerequisites are required. We recommend that instructors and students complete biosafety level I training required by their institution.

This laboratory curriculum aligns with core learning objectives included in most introductory biology lecture course(s) on evolutionary and/or molecular and cellular biology (7) see Appendix 7. It functions well as either a standalone laboratory course or as an accompaniment to lecture course(s), and also offers the flexibility of scheduling into one semester or two quarters.

Learning time

This CURE was developed as a 12-part series, with one session scheduled each week for 1.5 to 2 hours. To support experimental evolution, students also drop into the lab for 15-35 minutes to transfer their bacterial cultures. These brief drop-in visits are required on non-lab weekdays during weeks 2, 3, 4, and 5.

Learning objectives

Upon completion of this CURE, students will be able to:

1. Explain the connection between genotype, phenotype, and fitness, using specific examples;
2. Perform basic laboratory techniques safely and effectively;
3. Analyze data and interpret results via graphing and statistical testing;
4. Communicate hypotheses, methods, results, and the implications of a dataset in a poster presentation to both scientific and lay audiences;
5. Propose a follow-up project, which demonstrates agency in research and experimental design, and express confidence in their ability to do research.

Learning objectives for each lab can be found in the student lab manual, Appendix 1; learning objectives' alignment with AAAS "Vision and Change" is provided in Appendix 7.

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PROCEDURES

Materials

Required supplies and equipment include gloves, agar plates, media, pipettes, tubes, freezer, refrigerator, and a 37˚C incubator. In addition, students need to access a thermocycler, gel box for agarose gel electrophoresis, and a gel viewing system. Computers are used in several lab sessions; we recommend one laptop for every two students. The cost per lab for this CURE was comparable to the traditional lab costs. Lab preparation details can be found in Appendix 6.

Student instructions

A student lab manual that includes background information and lab protocols is available in Appendix 1.

Figure 1 provides a visual overview of the CURE activities. Working in teams of four, students meet once per week. The lab series is broken into three, connected phases. Phase I includes experimental evolution and data analysis. Students are first introduced to the experimental design and rationale, along with safety considerations and basic lab skills. Each group of students also establishes the three lines of bacteria that they will study: an antibiotic-sensitive control, and two lines that are each singly-resistant to an antibiotic that targets a different component of the cellular machinery. To do this, teams plate bacteria from a liquid culture onto solid media that contains either no antibiotic, or one of the two antibiotics. After incubation, students select a resistant colony from each antibiotic plate to propagate along with an antibiotic-sensitive control.

Experimental evolution is conducted during Weeks 2 through 4. First, the instructor freezes a sample of each group's ancestor bacteria (progenitors), for later comparison to the same lines after propagation (their descendants). Then, for 10-20 days, students perform daily transfers, to allow evolution to occur. While daily bacteria transfers continue, instructors introduce basic statistical analysis concepts and students practice data-processing in R using RStudio. The curriculum concurrently focuses on biological concepts that are central to the experiment, such as evolution by natural selection, fitness, levels of drug resistance, and collateral effects.

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In Labs 5 and 6, students perform and analyze two types of experiments on the revived progenitors and their descendants: a level-of-resistance assay and a competitive fitness assay. In the latter assay, students count colony forming units (CFUs) and perform calculations to determine relative fitness. In the level-of-resistance assay, students test growth in antibiotic gradient plates to estimate the minimum inhibitory concentration (MIC) -- a standard metric for quantifying resistance -- in each of the drugs. In Lab 7, students analyze their data using the statistical skills they learned during Labs 3 and 4. By the end of Phase I, students have measured the fitness cost of resistance in their strains, estimated the level of resistance in both their progenitors and descendants, and analyzed evidence for collateral effects.

Comparing data across student teams can inspire rich discussions. For example, after transferring their resistant bacteria in antibiotic-free media, some teams have observed no change in level of resistance in descendants compared to progenitors, but a lowered fitness cost of resistance. This is evidence that compensatory mutations, which lower the fitness cost of resistance, have occurred in some of the experimental strains but not in others.

In Phase II, the molecular analysis phase (Labs 8-10), students proceed with just one drug-resistant strain and delve into the molecular changes associated with resistance to the focal drug. Although other drugs could be used, sensitivity or resistance to rifampicin is the focus of the protocols published here. Rifampicin, which inhibits RNA polymerase, is a frontline drug used to treat patients with tuberculosis and other infectious diseases.

To explore the molecular basis of resistance, in Lab 8, students PCR-amplify a region of a candidate gene in which resistance-conferring mutations to rifampicin are likely to occur, and the instructor sends the PCR-products to an external lab for Sanger sequencing. Students focus on the *rpoB* gene: this gene encodes the β subunit of RNA polymerase, which contains the rifampicin binding site. In Lab 9, to identify mutations that may be associated with resistance, students use the freely-available version of Benchling software to compare sequence data from their rifampicin-resistant strain versus that of a rifampicin-sensitive ancestor.

By documenting the location and nature of mutations in their strains, including nucleotide change(s) and the corresponding amino acid replacement(s), students can predict consequences for the structure and function of the

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RNA polymerase. In Lab 10, students use educational-use-only PyMOL software to visualize the three-dimensional structure of the RNA polymerase β subunit and model amino acid differences between their sensitive and resistant strains, paying particular attention to amino acid replacements that alter hydrophobicity, stereochemistry or electrostatic interactions. Because changes in RNA polymerase structure are likely to change the enzyme's transcription rate, students can make predictions about the fitness effects of the observed mutation(s). Furthermore, it is possible that a given mutation can simultaneously impact rifampicin binding (promoting drug resistance) and compromise transcription efficiency (leading to a cost of resistance). Such pleiotropic effects provide opportunities for students to connect core topics in evolutionary and molecular biology.

In Phase III of the CURE, students synthesize their results into a scientific poster, which they present in a live public forum. Therefore, Lab 11 focuses on using data that the students have generated to link genotype to phenotype to fitness. By conducting assays on both sensitive and resistant isolates, the students gain an understanding of the genetic and phenotypic causes of drug resistance. Further, because students also generated data on fitness and level of resistance to a second antibiotic – here, streptomycin -- they can analyze whether collateral effects are occurring. Some student teams have found, for example, that their rifampicin-resistant strains are more resistant to streptomycin than their rifampicin-sensitive strains are. Each team is also challenged to propose future experimental directions. Finally, students practice and then present their experimental results in a poster presentation session held during Week 12. As they visit each other's posters, the students see data from a wide array of resistance mutations, including many that affect fitness and resistance in ways that contrast with the strain that their own team analyzed. Seeing the wide variety of outcomes selected via an identical drug treatment reinforces students' awareness that mutations occur randomly, and that, despite mutations' occurring in the same gene, the specific molecular underpinnings of resistance often differ.

Faculty instructions

Guidance for instructors, including scientific background information and other resources, is available in Appendix 2 and Appendix 6. As a general note, it is important for the instructor or lab coordinator to monitor cultures between scheduled lab sections to make sure there is bacterial growth to use in the labs. Although students do not use a flame in lab, all lab preparations should be done under sterile conditions to lessen the chance of contamination. If graduate

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teaching assistants will be teaching in the labs, a TA training meeting in which the TAs practice the student protocols accomplishes two tasks: 1) giving the TAs required background information and skills, and 2) creating "backup" strains or data to use in case of a large-scale experimental error.

Although we advise instructors to keep the basic framework of the experiment intact, faculty are encouraged to change the protocol in biologically- and medically-interesting ways; see "Potential modifications and extensions" for suggestions. Over time, the goal of the CURE is to build a database of mutations that influence resistance to a wide array of antibiotics.

Suggestions for scaffolding and measuring student learning

Examples of prelab quizzes, worksheets, and discussion questions, all of which are designed to deepen student conceptual understanding of the experiment, are provided in the appendices along with a poster presentation template, sample clicker and exam questions. Rubrics developed to assess students' understanding of the central dogma of molecular biology and its connection to evolution by natural selection are reported elsewhere (Sievers et al., in prep).

Sample Data

Each team of students collects data on their sensitive and drug resistance strains, both from the beginning and end of the evolution experiment. The MIC assay provides data on the level of resistance to two drugs and the competitive fitness assay allows students to calculate relative fitness. Students also compare fitness in resistant and sensitive progenitors to determine if there is an initial fitness cost of resistance in the mutant they are analyzing.

For simplicity, students do targeted sequencing on their rifampicin-resistant strains and compare the sequencing results to the reference strain, which is the antibiotic-sensitive ancestor. Students also visualize RNA polymerase.

The sample data shown in Figure 2 illustrate the richness of a typical student-generated dataset:

- Not surprisingly, the level of resistance did not significantly change as the sensitive strain evolved in an environment lacking antibiotic.

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- The resistant strains had lower fitness than the sensitive strain, indicating a cost of resistance.
- Surprisingly, the level of resistance did not change as the resistant strain evolved in an environment lacking antibiotic (despite the potential for a reversion to sensitivity to erase the cost of resistance). Instead of declining, a high level of resistance was maintained.
- Both sensitive and resistant strains displayed comparable increases in relative fitness over the course of experimental evolution.
    - In isolation, the observation of continued high resistance with an increase in fitness would suggest that compensatory mutations occurred in the resistant strains. However, this interpretation is clouded by the observation that the same pattern occurred in the sensitive strain.
    - This pattern inspires students to consider that beneficial mutations may exist (irrespective of the strains' resistance status); alternatively, the nature/identity of beneficial mutations may differ in the sensitive and resistant backgrounds.
    - These data highlight the importance of replicating experimental results, to increase one's confidence that the data are representative and reliable.
- The combination of DNA sequence data and inferred protein structure suggest that an aspartic acid to glycine change engendered resistance. This replacement mutation appears to have occurred near the rifampicin binding site in the beta-subunit of RNA polymerase, suggesting that the drug can now bind less tightly.

Safety

In this CURE, students work with a BSL-1 organism -- specifically the MG1655 strain, although another *E. coli* strain could be used. Risk of glassware breakage and spilling of cultures is reduced by having students grow the bacteria in microtiter plates. Students wore closed-toed shoes and latex gloves as standard laboratory protection, at all times. As a safety precaution, students do not work with Bunsen burners during sterile procedures, instead, contamination is minimized by working carefully and quickly, keeping cultures covered, and using sterile disposable pipette tips.

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Contaminated waste was autoclaved and disposed of according to the Institution's policy and regulations. At the beginning and end of the experiments, students were instructed on the disposal procedures of all biohazardous material. When students made observations about their MIC plates, they were required to wear goggles, and instructed to keep the plates level and never remove the lids.

DISCUSSION

Field testing

Multiple integrations of the CURE lab series have been tested in the first two courses of the three-part introductory biology series; total CURE enrollees per offering ranged from 24 students to almost 600 students. Bio1 and Bio2 lectures address Ecology and Evolutionary Biology, and Cell and Molecular Biology, respectively. Faculty taught the lectures while graduate teaching assistants led the lab sections, with each lab section containing a maximum of 24 students. CURE students' comments, and comparison of their conceptual mastery compared to that of peers who completed a traditional lab series, are described below.

Evidence of student learning

We performed two experiments to measure student learning. First, in a "side-by-side" design, students in the same course participated in either CURE labs or traditional labs. During the 2017-18 academic year, the CURE curriculum was taught in four sections in Bio1, while a traditional lab sequence taught in the other 19 lab sections. Due to schedule conflicts and routine attrition only 16 of the 96 students who completed Bio1 CURE labs were enrolled in the CURE sections of Bio2 offered during the subsequent term.

Second, during the 2018-19 academic year, we conducted a longitudinal study, in which different instances of the two introductory courses taught *either* the traditional labs *or* the CURE labs. Specifically, all students in the fall Bio1 and winter Bio2 sequence did traditional labs, while all students in the winter Bio1 and spring Bio2 classes did the CURE labs.

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In both experiments, we used pre-post testing to measure changes in student affect and conceptual understanding. Detailed information on the experimental designs, data analysis, and results on student learning are reported elsewhere (Mukerji et al., in prep; Sievers et al., in prep).

To summarize, compared to students in the traditional labs, students in the CURE:

- showed a more expert-like understanding of both:
    - evolution by natural selection and
    - the culture of scientific research
- rated CURE labs as having more relevance to their career goals than traditional labs

In contrast, there was no statistically-significant difference between treatments in:

- conceptual understanding of experimental design, nor
- self-efficacy or sense of belonging in STEM, nor
- intent to pursue subsequent undergraduate research

In the side-by-side experiment, despite CURE students' loss of time covering traditional lab material tested on exams, there was almost no discernible impact on student exam scores.

Although not formally assessed, the student poster sessions seemed critical for students to solidify their excitement for and understanding of their research. For example, students said:

- "There is still so much to explore and find out and this is just the beginning"
- "Because our results go against our prediction, I hope that we can show that being wrong in science is just as valuable as being right"
- "I am excited to share research that makes my group different from other people. Most science fairs that I myself have attended have people presenting the same results so there is little to no originality…I think my group has a lot to say and our conclusion and next steps were very thought out."

Unexpected Outcomes

Much is known about antibiotic resistance, for example, previous research has identified mutational hotspots for resistance to rifampicin (8), and compensatory mutations also tend to occur in these regions. Therefore, we decided

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to sequence a portion of the *rpoB* gene in the CURE protocol. However, it is not possible to predict which specific mutations will be present in strains that students isolate, nor which mutations will arise during experimental evolution. Thus, for some teams' strains, the molecular basis of resistance may be encoded outside of the region of *rpoB* sequenced in this protocol. In the event that targeted sequencing is not sufficient to explain resistance or compensation of teams' strains, these strains warrant further investigation, such as whole-genome sequencing.

Potential modifications and extensions

This CURE illustrates evolutionary concepts and the molecular basis of adaptation via hands-on discovery. Therefore, scaffolding the CURE sequence with content on evolution, nucleic acids, proteins, and the central dogma enhances its effectiveness. For a multi-part course, these curricular elements can be delivered in lecture, or, for a standalone course, they can be incorporated into an expanded version of the laboratory curriculum presented here.

Alternatively, depending on institutional context and departmental needs, this CURE can be shortened or simplified. For example, the reagent preparation and conceptual complexity could be decreased by studying one drug instead of two. (Here, we included two drugs, to allow analysis of collateral effects; this version adds biological interest and medical relevance, but can be conceptually difficult for students.)

During the pilots and the larger-scale experiments, obtaining consistent results from our competition assay was a challenge. Much of the inconsistency seems attributable to students' errors when performing serial dilutions; therefore, we recommend requiring that students practice these steps using a known concentration of bacteria, before doing the competition assay. Depending on institutional context, instructors could consider alternative methods for estimating relative fitness, such as marking the resistant and sensitive strains appropriately and using a flow cytometer or spectrophotometer (9) to collect data from the competitive fitness experiments.

Although we removed most glassware from the protocols for safety reasons, daily transfers of populations could be done in test tubes that hold larger volumes and thus support more generations of evolution. Similarly, some instructors may be able to restore the use of open flames in the lab so that students learn proper sterile technique and reduce the risk of cultures becoming contaminated.

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We used the drugs rifampicin and streptomycin in the protocols reported here and, for Labs 8-10, focused specifically on rifampicin-resistant mutants. However, the protocol inherently lends itself to exploration. An array of extensions and modifications are possible so that over time, students can explore different aspects of the biological and medical questions that form the experiment's core. For example, the protocols and lab prep could be modified so that students:

- Complete the daily transfers with drug present;
- Explore collateral effects further by selecting mutants that are resistant to antibiotics other than rifampicin and streptomycin;
- Target a different part of the genome for sequencing; and/or select mutants of interest for full genome sequencing.

When completing the protocols published here, or pursuing modifications like those suggested above, students should be aware that they are collecting preliminary data that will need to be replicated and augmented with additional assays to be publishable.

Finally, we urge instructors to add curriculum to support positive changes in student affect during the CURE. During our side-by-side and longitudinal experiments, we deliberately avoided any messaging about belonging, identity, self-efficacy, or using the CURE as a springboard to more intensive UREs. Our goal in doing so was to keep the CURE and traditional treatments as identical as possible with respect to the constructs we were measuring, in order to avoid reporting results that would appear self-fulfilling and thus biased or trivial. However, outside of the context of an education research study, instructors are free of this constraint and can speak candidly about the types of benefits that students can gain from the CURE.

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FIGURES

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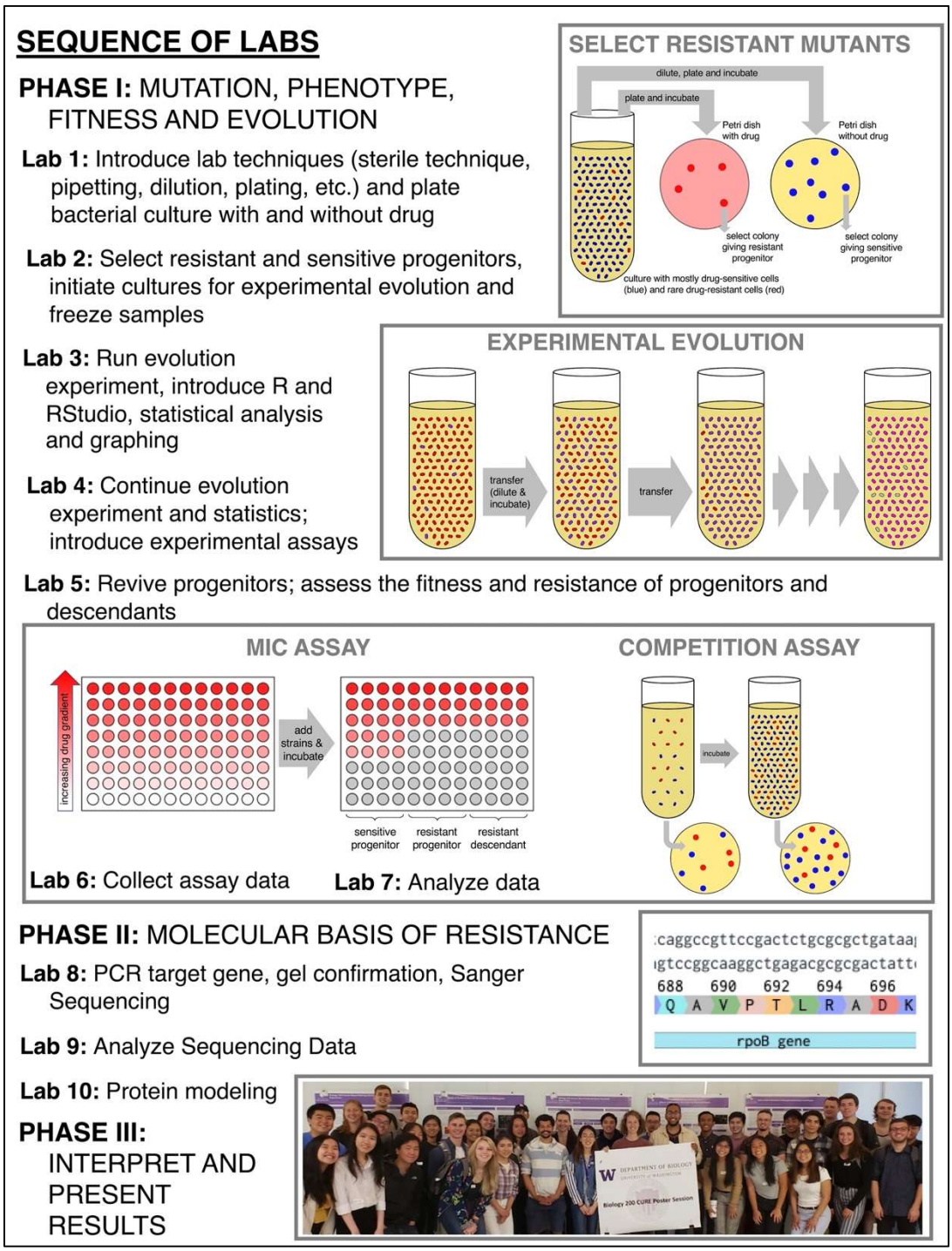

Figure 1. CURE experiment workflow over the 12 weeks. There are 3 phases to this CURE. First, experimental evolution and analysis (Labs 1-7). Second, analyzing the molecular basis of resistance (Labs 8-10). Lastly, integrating connections amongst genotype, phenotype, and fitness and communicating results to the public (Lab 11-12). For simplicity, we show the workflow with one antibiotic; however, students also perform single-drug selection with another antibiotic during Lab 1, so that they can explore cross-drug effects of resistance.

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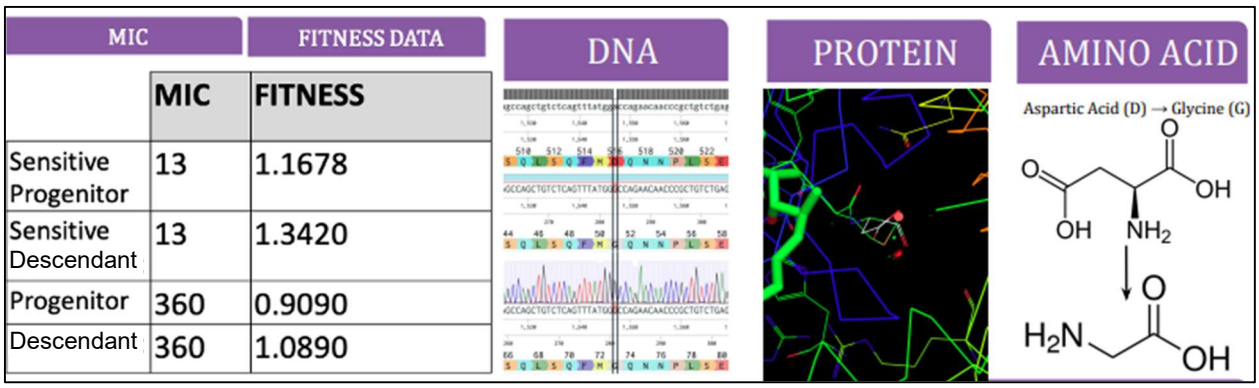

	MIC	FITNESS
Sensitive Progenitor	13	1.1678
Sensitive Descendant	13	1.3420
Progenitor	360	0.9090
Descendant	360	1.0890

Figure 2. Example of students' collected data. "MIC" is the level of drug resistance to rifampicin (in ug/mL). "Fitness" represents the relative fitness of the resistant and the reference strain, which is the antibiotic-sensitive ancestor.