Bacteriophage as instructional organisms in introductory biology labs

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Designing lab exercises for introductory biology classes requires balancing the need for students to obtain results with a desire to provide unpredictable outcomes to better approximate actual research. Bacteriophage are particularly well suited for this as many species are well-understood but, with their hosts, represent a relatively complex interacting system. I have designed a seven week series of lab exercises that allow students to select bacteriophage resistant mutant hosts, isolate and sequence the corresponding receptor gene to identify the specific bacterial mutation from a large number of potential mutations. I also examined the possibility of collecting useful mutant strains for other studies. After two semesters, the lab series is working well with over 90% of students successfully isolating mutant bacteria and about half identifying the specific mutation. Here I discuss the advantages of using bacteriophage in an introductory class, the specific labs in this series and future plans.

Background

One goal of many introductory science courses is to give students a feeling for what doing science is really like.^{1,2} Counter to this goal, many laboratory exercises in introductory classes tend to be “cookbooked” to ensure that students obtain expected results. It is clearly frustrating for students to complete an exercise and get no results. On the other hand, ensuring results often means that laboratory exercises tend to be student-run demonstrations with all students obtaining the

same or similar results. These exercises can be valuable in demonstrating a particular point but fail to capture the sense of discovery that is an important aspect of scientific research. Thus, one of the challenges in designing laboratory exercises for these biology classes can be generation of novel experimental results in association with a high expectation that each student will also produce pedagogically useful results.

A number of excellent biology curricula have been developed to address this need. These include the Phagehunters/PHIRE/SEA-Phages programs (<http://phagesdb.org/phagehunters/>³) and the Small World Initiative (<http://cst.yale.edu/swi>). These programs tend to span multiple courses and semesters. They therefore may require some curriculum revision to fully integrate into a program. The goal of the work described here was to design a lab for a single introductory biology class that: 1) Can be performed by students with limited laboratory skills; 2) Demonstrates important course concepts; 3) Is robust in reliably producing results; 4) Is unpredictable as to the specific result; 5) Allows students to identify their result with minimal instructor intervention; and 6) Ties in to some research project so as to allow the students a participatory role. This last point was especially useful as the specific project undertaken was done at an undergraduate-only institution for Biology so students doing research have much less time for research than graduate students will have. The laboratory exercises could supply a research student with, essentially, a large number of helpers generating independently isolated mutant strains.

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I began teaching the introductory biology course at Ashland University a year ago. This course is taken by beginning Biology majors but also by pre-med (in many majors), Dietetics, Exercise Science, Psychology and other students since it is a prerequisite for a variety of graduate programs. Previous sections of the course had used a series of labs in which students identified mutant yeast strains and sequenced the key gene but this series had a fairly high failure rate in producing the mutants. Taking the basic idea of 1) generate a mutant, 2) characterize it phenotypically, and then 3) sequence the likely mutated gene, I looked for a bacteriophage system that would be compatible in both my research area of host range evolution and with the lab requirements described above.

Overall, there are a number of advantages to using bacteriophage in an introductory class. First, *E. coli* and lytic coliphage are relatively simple to propagate. Second, both viruses and host grow rapidly and therefore results can be available in time for a weekly lab series. As well, this rapid growth can allow for students (or the instructor) to fix problems before the problems derail progress in the multi-week lab series. Third, the biology of many phage/*E. coli* (and other host) pairs is well understood supporting the necessary predictability for designing effective lab procedures. Fourth, working with microorganisms usually means working with much larger populations than is possible with plants or animals. This allows a reasonable expectation of observing even low probability events. These last two points support the desire for experimental robustness, that is, the achievement of pedagogically useful results with high probability. Finally, and especially importantly for working with beginning students, bacteriophage and the appropriate strains of *E. coli* are not pathogenic so can be used in BSL 1 teaching laboratories.⁴

There are many classic genetic experiments using bacteriophage such as recombination of T4 rII mutants⁵; isolation of clear plaque mutations of temperate phage⁶ and other plaque morphology mutations⁷; transduction⁸; and ploidy effects on resistance to DNA damaging agents.^{9,10} I chose to adapt the Luria-Delbruck fluctuation

assay to generate mutant strains of *E. coli* that are resistant to bacteriophage infection.¹¹ Having often seen resistant bacterial colonies when using too many phage during titring, I knew this has the necessary predictability and robustness needed for this lab series. Because its receptor is known to be a single protein (FhuA), I chose bacteriophage T5 as the bacteriophage.

The Lab Exercises

The laboratory section of the introductory biology course meets once per week for two hours. All or parts of seven of these labs during the 15 wk semester are used for the bacteriophage lab series described here. The labs are scheduled weekly except between labs five and six when a two week break allows time to ensure sequencing is completed. The lab procedures vary from approximately 30 min to the full two hours. Shorter procedures are paired with a second, unrelated lab for that session. The first bacteriophage lab is begun during week 5 of the semester. In this first lab, students infect *E. coli* with bacteriophage T5 at a multiplicity of infection of 10 or more to ensure that every cell is infected. The cell/phage mixture is plated immediately after infection and cells with a mutation in the receptor protein, FhuA, are the most likely to survive. The next day students collect a colony which they use to inoculate 5 mL of broth. While plates could be refrigerated for the next week's lab, I typically take the few minutes needed from the next lecture for students to complete this step to move the lab sequence slightly faster. The culture is incubated overnight and then used in the second lab to confirm the phenotype and species of the bacteria. Students mix an aliquot of the broth culture with about 10³ bacteriophage T5 in molten top agar and plate the mixture. The absence of plaques after incubation confirms the resistance phenotype. To confirm that their bacteria is *E. coli*, students streak their bacteria on eosin-methylene blue (EMB) agar plates. EMB agar is somewhat selective for gram-negative bacteria and *E. coli* colonies on EMB agar show a metallic green sheen due to absorption of the eosin and methylene blue dyes. I generally do not

have students also streak control bacteria to minimize the chance of contaminating their cultures. These controls (*E. coli* and some other species of bacteria) could easily be used during the demonstration of the technique at the start of the lab if one desired to show these controls after that plate was incubated. Colony appearance is sufficient to screen out the most common contaminating bacteria which are typically gram-positive cocci. These cocci also do not support T5 growth so this step allows discussion of proper controls as well as phage host specificity.

The third lab has students extracting DNA from their bacteria using a commercial extraction kit. At the end of the lab, students add an aliquot of the DNA to a reaction tube containing a PCR master mix. The master mix contains primers for the middle third of the FhuA gene which includes the amino acid segments responsible for T5 binding.¹²⁻¹⁴ The expected product size is about 900 bp to facilitate complete sequencing from both ends of the product. PCR reactions are run outside of class and reactions stored for the next lab. In this fourth lab, students analyze 10 μ L of the 50 μ L reaction by gel electrophoresis using a 0.7% agarose gel. The gel is finished during lab and students are able to see if their PCR reaction worked. Large insertions or deletions could also be detected at this stage although this has not been seen. The remainder of the PCR reaction is stored for the fifth lab in which students purify their PCR product using a PCR cleanup kit. Purified PCR products are then sent to a commercial lab for sequencing using both PCR primers. We use Functional Biosciences (Madison, WI) which typically posts sequences with three days of receiving samples. The charge is \$7 per reaction for purified DNA and each sample is sequenced twice.

During the sixth lab students follow a template to guide them through the analysis of both sequences. The reverse sequence is converted to a sense orientation, translated to protein sequence and both are aligned with the wild-type sequence using software available online (Molecular Toolkit, <http://arbl.cvmbs.colostate.edu/molkit/index.html> and Clustal Omega, <http://www.ebi.ac.uk/Tools/msa/clustalo/>). Finally, in the last lab, students add

Figure 1. Location of mutations identified in T5 resistant *E. coli*. Table 1 has detailed descriptions of each mutation. Black boxes indicate positions of protein segments involved in bacteriophage T5 binding.^{13,15} An annotated FhuA gene sequence showing the identified mutations can be found with the supplemental materials online at <http://personal.ashland.edu/phyman/Bacteriophage%20supplement.htm>.

T5 phage to an aliquot of their mutant *E. coli* strain at a high MOI and plate the mixture to see if any they can select for mutant phage that have regained the ability to infect the mutant *E. coli*.

After two semesters with a total of 47 students (who typically work in pairs) 23 resistant strains of *E. coli* were isolated. Twenty-one were successfully sequenced and mutations for half of these were identified in class. The mutations for the others were located outside of the target 900 bp region and were identified by other students outside of the class as part of their research projects. No mutant bacteriophage have been identified.

Research Tie-In

As part of their research, especially in learning techniques, two students have been looking at ways to improve the lab exercises. This was done in two ways. First, as noted above, some of the mutations fell outside of the region sequenced in class. Additional sequencing was needed to identify those mutations. Table 1 lists the types of mutations identified (see also Fig. 1). Second, they tested different media to see if the mutation spectrum could be shifted away from mutations that inactivated FhuA to instead missense mutations. Presumably a point mutation in the phage receptor binding protein (selected for in the final lab) would be more likely to bind to an altered FhuA protein than to change to an entirely new receptor when FhuA was not expressed. Studying host range changes due to these mutations is the goal of their research. The media testing also produced new resistant

Table 1. Descriptions of mutations identified in resistant *E. coli*

Designation in Figure 1	Mutation description	Number of isolates ^a
Nonsense mutations		
Am	Amber mutation at nt 1081 (aa 361 Q(CAG → TAG))	3
Op1	Opal mutation at nt 926 (aa 309 S(TCA to TAA))	1
Op2	Opal mutation at nt 951 (aa 317 Y(TAC to TAA))	9
Op3	Opal mutation at nt 2050 (aa 684 K (AAA to TAA))	1
Frameshift mutations		
FS1	11 nucleotide deletion at nt 179 (CTATTGCGGCG) (aa 60)	1
FS2	2 nucleotide insertion after nt 264 (GC) (aa 88)	1
FS3	11 nucleotide deletion at nt 471 (TATGCTGGAAC) (aa 157)	1
FS4	1 nucleotide deletion at nt 499 (G) (aa 167)	1
FS5	Insert of G at nt 552 (aa 185)	2
FS6	4 nt duplication TTCC at nt789 (aa 263)	1
FS7	insertion of C at nt 868 (aa 290)	1
FS8	Single nucleotide deletion of A at nt 1015 (aa 339)	3
FS9	5 nucleotide deletion (GGTTT) begins at nt 1269 (aa 425)	1
FS10	deletion of G at nt 1752 (aa 584)	1
FS11	deletion of G at nt 1995 (aa 665)	3
Other mutations		
IS1	IS1 insertion at nt 148 (aa50)	1
IS1	IS1 insertion at nt 43 (aa 15)	5
	large deletion ^b	2 or 2 different deletions

All mutations were confirmed by DNA sequencing except as noted. ^aSince students share bacterial cultures initially, the same mutation may be isolated by multiple groups. ^bPresumed deletions based on the failure of four different PCR primer pairs. Three primer pairs amplify different segments within FhuA and one set of flanking primers amplifying 2.5 kb of the genome including the FhuA gene.

strains whose mutation needed identification. This includes strains identified using media containing bipyridyl which binds iron and on minimal media that was not supplemented with iron since the normal function of FhuA is transporting iron into the bacterial cell.

None of these mutations is specific for the known sites that interact with the T5 receptor binding protein.¹²⁻¹⁴ Instead, all are various types of knockout mutations. Because of this, the research students are now beginning to look at site-directed mutagenesis to create useful *E. coli* strains.

Their initial work in support of the teaching project did give them the opportunity to learn many of the techniques needed to work with bacteriophage, isolate mutants, sequence genes, etc.

Procedural Challenges

As a teaching exercise, the lab series has been reasonably successful. There are certain procedural steps that some students struggle with. While these errors would be surprising if done by an experienced researcher, these labs are being performed by students with minimal or no experience. For some students, this may be one of only two laboratory science classes required for their program. One technical challenge is the use of pipetters. Even with an earlier lab on pipette use, some students struggle both with proper volume setting and pipette use. Most commonly, they forget the need to use the soft stop as the filling volume and instead fill using the hard stop. Still, the majority of problems come from students not reading or understanding the instructions as they read them, even with demonstrations. For example, bacteriophage are occasionally added to bacterial stocks rather than to melted top agar. This error is less a problem when the virus is added to a phage resistant bacterial stock but is a problem when students then attempt

to plate the bacteria/phage mixture sans top agar. Similarly the DNA extraction kits and PCR cleanup kits are challenging for some students, but walking the entire class through each step as a group minimizes potential errors. Even with these challenges, about 80% of the students complete the lab series without having to redo any labs and 91% (21/23) did have a sequence to be analyzed for the sixth lab. I have also either modified or added more detailed instruction to those steps that students have demonstrated are particularly difficult.

Conclusions and Future Directions

While the lab procedures are working well, there is an additional level of instruction that has not yet been addressed. The protocols and background of the labs address a number of topics that are taught in the course the lab is part of. These include virus-host interactions, protein binding specificity, genes, mutation, and biotechnology (evolution is covered in the other semester of this two course sequence). Ideally, laboratory exercises should complement and enhance instruction. Assessing whether this is true for this lab series and course will need testing at the beginning and end of the semester as well as recruiting other instructors of this course who do not use this lab series to

act as controls. As there are typically five sections of the course with at least three instructors each year, this should be readily achievable.

Copies of the seven lab protocols are available at <http://personal.ashland.edu/phyman/Bacteriophage%20supplement.htm>. Anyone interested in using some or all of them should feel free to adapt them as desired. While there is always room for refinement, in their current version the labs are providing students with a chance to be the first person ever to know a specific fact (in this case the mutation sequence). This is, for me at least, an important part of what makes scientific research enjoyable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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