Chapter 4

Isolating Multiple Strains of *Escherichia coli* for Coliphage Isolation, Phage-typing, and Mutant Recovery

Richard A. Levin

Department of Biology
Oberlin College
Oberlin, Ohio 44074

Richard is a Professor of Biology at Oberlin, where he has taught for 25 years. In addition to participating in the team-taught General Biology course, he teaches Genetics, Microbiology, and a Colloquium in Sexually Transmitted Diseases. He received his B.A. from Harvard, an M.S. in Genetics and an M.A. in Music Composition from the University of Washington, and his Ph.D. in Microbiology from the University of Iowa. He has won an excellence in teaching prize at Oberlin, and a State of Ohio teaching excellence award. His research interests include the genetics of *Agrobacterium tumefaciens*, genetics of *Saccharomyces cerevisiae*, and ethical concerns with technological advance.

Contents

Introduction

In an age when the miracles of biotechnology simply boggle the imagination, it is sometimes disconcerting to discover that there are investigators who have formidable skills involving "hi tech" procedures with state-of-the-art equipment and yet lack even an elementary appreciation of some of the most basic tools of their trade. There are, for instance, elegantly trained biochemists and molecular biologists for whom the bacteria and viruses used in their research are merely reagents that can be purchased in a multiplicity of genotypes from biological supply houses and can then be flushed down the drain at the end of the experiment.

Recent spectacular advances in biotechnology have had an impact on perceptive students, and it is natural that these young people are impatient to use the elaborate and expensive equipment and to do the sophisticated experiments they have read about in the literature. But being trained to use the equipment is not the same as being able to think through the problems and implications of scientific research. Many important biological questions can be addressed with simple tools. This chapter presents several examples of investigative experiments that are inherently interesting because they involve bacteria from our own bodies in contexts that have a ring of familiarity and an aura of relevance to the student. Yet they can be accomplished with simple equipment and inexpensive reagents. The experiments are instructive at many levels. Furthermore, they are open-ended, in the sense that the successful completion of one experiment can lead onward to another, and another, almost indefinitely. Each result suggests something new to do.

Materials

The materials needed for the experiments I have described are quite minimal and inexpensive. They are generally the kinds of things that would be a part of any reasonably equipped stockroom in a Biology Department that offers a course in Microbiology.

Clearly, one must have an autoclave for the sterilization of media, and at least one incubator that can be set at 37°C. I assume that one may take for granted such pedestrian items as test tubes, plastic petri dishes, disposable syringes, and pasteur pipets.

For the experiments involving the isolation and testing of bacteria and viruses, the media needs are modest. I use commercially available nutrient agar and nutrient broth. The selective and

differential medium referred to in the isolation procedure can be either Eosin-Methylene Blue Agar (EMB Agar), or Endo Agar, both available commercially, and both inexpensive.

For filtering viruses and for filter-sterilizing antibiotics, I use membrane filters that come in a filter holder in a self-contained disposable unit, which allows a Luer-lock kind of connection with a hypodermic syringe. I buy the cheapest kind available.

The antibiotics I use are all readily soluble in water, inexpensive, and reasonably non-hazardous. My list is not cast in stone. Many combinations of antibiotics will do.

The materials listed for the following experiments assume 20 students per laboratory:

Experiment 1

Rectal swab kits (small capped tube containing one Q-tip in 0.5 ml of water), sterile (20)
Petri plates containing 25 ml of Levine's Eosin-Methylene Blue Agar (40)
Petri plates containing 25 ml of nutrient agar (hereafter known as *nutrient agar* plates) (20)
Small screwcapped vials containing 5 ml of nutrient agar that has been tilted as it cools so that when it hardens, a firm slanted surface is present (20)
Capped test tubes packed with wooden applicator sticks (these should be sterilized in the autoclave) (100 or more)
Capped tubes of nutrient broth, sterile (20)

Experiment 2

Liquid cultures of the individual *E. coli* strains isolated in Experiment 1, grown in 5 ml of commercial nutrient broth for 24–48 hours (hereafter these will be called *nutrient broth* cultures) (20)
Capped or plugged tubes of nutrient broth, sterile (20)
Packets of plugged sterile 9-inch pasteur pipets, about 6 pipets per packet   (20)
Rubber bulbs for pasteur pipets (30–40)
Fresh raw sewage (1 beaker of about 50–100 ml will be plenty)
Petri plates (40)
Syringes, 5 ml (40)
Membrane filters (pore size of 0.45 μm) in filter holders with Luer lock connection (these come in a sterilized, disposable unit; I buy the cheapest kind available from most scientific supply companies) (40)
Sterile tubes, capped or plugged (40)

Experiment 3

24- to 48-hour nutrient broth cultures of each student's *E. coli*
Nutrient agar plates (20)
Sterile packets of plugged sterile 9-inch pasteur pipets, 3 or 4 per packet (20)
Rubber bulbs (30–40)

Experiment 4

Nutrient agar plates (450)
Tubes of melted semi-soft nutrient agar (0.8% agar dissolved in nutrient broth), cooled to 48–50°C (450)
Sterile packets of plugged sterile pasteur pipets, about 6 per packet (20)
Rubber bulbs (30–40)

Experiment 5

Nutrient agar plates (20)
Capped or plugged test tubes filled with applicator sticks, all sterile (20)

Experiments 6 and 7

Bulk samples of several inexpensive antibiotics. Examples include ampicillin, streptomycin, nalidixic acid, tetracycline, and bacitracin. A bottle of 5–20 g may cost under $20 US. The available form of each antibiotic that is most soluble in water should be selected; for example, streptomycin sulfate is considerably more convenient than streptomycin.
Spatulas
Weighing paper
Top-loading balance capable of weighing in the milligram range
As many 5 ml syringes and membrane filter set-ups as there are antibiotics to be tested
Capped or plugged test tubes, sterile (about 24)
Sterile flasks of nutrient agar, 50 ml of agar per 125 ml or 250 ml flask,
     melted and cooled to 48–50°C (60)
24- to 48-hour nutrient broth cultures of each *E. coli* isolate
Bent glass rods, called "hockey sticks" (20)
Beakers, 250 ml (10)
Absolute ethanol, 95% (500 ml)
Sterile petri plates (120)

<div align="center">

Experiment 1:
Isolating *Escherichia coli*

</div>

For the first in this series of experiments, each student isolates his or her own strain of *Escherichia coli* as quickly as possible. So, during the first lab period, after a discussion about where *E. coli* is found "in nature," each student takes a little "kit," composed of a moistened Q-tip in a sterile tube, to the restroom and obtains a rectal swab. After returning to the lab, the student learns the streak plating technique by transferring the fecal sample on the Q-tip to a petri plate containing a solid growth medium that will permit *E. coli* to grow as colonies while inhibiting many other kinds of bacteria, and that will also impart to *E. coli* colonies some visible attribute that allows them to be identified. In other words, this medium must be both selective and differential. Several commercially-available media, for example, Endo Agar, or Eosin-Methylene Blue (or EMB Agar), meet these specifications. They select against those bacterial species known as Gram positive, which are unusually sensitive to certain dyes in the medium, and allow Gram negative species, of which *E. coli* is one, to grow. Furthermore, the dyes react with certain metabolic byproducts of *E. coli*, imparting a metallic green sheen appearance to the dark *E. coli* colonies. This kind of reaction does not occur with other species. If the streaked plate is incubated at 37°C, the colonies will begin to appear within about 24 hours, and *E. coli* can be identified within the next 12 hours or so.

Streaking plates is a simple but indispensable technique used by microbiologists to separate different organisms from a mixture. The underlying principle is that individual cells, when placed on a nutritionally adequate medium that contains nothing harmful, will divide repeatedly until either some key nutrient is depleted or some toxic end product accumulates. When such a population that has been derived from an individual cell becomes visible as a mass of cells, it is known as a colony.

Sometimes mixed colonies result from two or more cells that have were deposited so close to one another that they grew together in a common mass. These impure colonies are not helpful for our purposes. We are looking for clones, which are colonies derived from single cells. By definition, clones are pure cultures. In our experiment, the rectal swab, which is the source of *E. coli*, may contain millions or even billions of cells from dozens of bacterial species. The streak plate technique aims at distributing microorganisms on the surface of the agar in a way that will maximize the probability that colonies will be pure cultures. Plate streaking and the Gram stain procedure are both routine bacterial procedures, described in any bacteriology laboratory manual. Although students need not carry out the staining, it will be instructive to point out that whether a cell is Gram positive or negative depends on the composition of the cell wall and also has implications for such features as sensitivity
or resistance to drugs and a variety of dyes.

Once the colonies appear, they can be restreaked on the EMB agar just described as a first step in the purification of the isolate. When this subculture has grown, a sample from a single colony can be streaked on a commercially available non-selective nutrient agar, and after 24 hours, a purified isolate, now assumed to be a clone, can be recovered. This will be the bacterial strain that the student will work with for the rest of the semester. Once the bacterial strains are isolated, there are many different kinds of experiments that can be done. Some interesting ones that are easily performed are described below.

Experiment 2:
Isolating a Virus Specific for *E. coli*

Bacterial viruses are known as "bacteriophages," which means "bacterial eater," or simply as "phages." Those phages which specifically destroy *E. coli* are known as "coliphages." Viruses can be recovered where their cellular hosts are plentiful, and since *E. coli* from every person in town is flushed into the sewage system, any raw sewage sample will be teeming with coliphages. Since waste disposal is an urgent concern of our society, it is surely a timely topic for any biology student. Thus a tour of a local sewage treatment facility is a highly interesting and relevant experience, and at the conclusion of the tour, a small sample of raw sewage, not more than a few milliliters per student, can be collected for use as a source of coliphages.

Each student inoculates a tube of nutrient broth with his or her own strain of *E. coli*, adds a few milliliters of sewage, and incubates the mixture at 37°C for 24–48 hours. The point of adding *E. coli* is to provide the specific bacterial strain that serves as host for the particular coliphage being cultivated. Since bacteriophages multiply rapidly once they infect bacterial cells, the coliphages that can attack each student's particular strain of *E. coli* will multiply abundantly. In other words, this procedure is an enrichment technique for a particular coliphage.

After this mixture incubates for 24–48 hours, it is carefully poured into a petri plate, taken up in a disposable syringe, and a few milliliters of the liquid is filtered into a sterile tube through a membrane filter with pores no larger than 0.45 µm. These pores are large enough to let viruses pass through, but bacteria, which are much larger, will be held back. The crystal clear filtrate is labeled "crude phage prep." To it is added more nutrient broth, and more *E. coli*, and the mixture is incubated for another 24–48 hours in order to increase even further the concentration of the particular coliphage being isolated. Once again, the mixture is filtered through a membrane filter into a sterile tube, and the tube is labeled "enriched phage prep." This enriched phage prep may be stored in a refrigerator until the class is ready to proceed.

Experiment 3:
Testing for the Virus

When bacterial cells are spread on a solid growth medium, individual cells begin to divide, and the population rapidly increases. If, for example, 30 bacterial cells are distributed on a nutrient agar plate, they will, within a day, develop into 30 well-isolated colonies. If, however, thousands or millions of cells are spread on a similar plate, the resulting population growth will resemble a confluent mass of cells known as a *lawn*. Since viruses are not alive and have no independent metabolism, they will only multiply inside living cells. Thus virus colonies will not appear on a nutrient agar medium the way bacterial colonies will. However, if thousands of bacteria are first spread on a nutrient agar plate, followed by the introduction of one drop of the enriched phage prep in the center of the plate, the bacterial lawn will grow only on the periphery of the plate, where no virus has infected the cells. In the center of the plate there will be a clear circle, almost devoid of bacteria. This is the region of coliphage destruction of *E. coli*. The growth of the lawn and the development of the zone of destruction is evident after 24 hours of incubation. When such a region of destruction has been caused by the progeny of a single virus particle, it is known as a *plaque*. Since the region of clearing each student sets up has been caused by the application of many viruses from the drop of the enriched phage prep, we call it a "macroplaque." The macroplaque zone of clearing is generally about the size of a dime.

After each student has completed this part of the experiment, we have available for further experiment a number of genetically different strains of *E. coli* and a number of genetically different strains of coliphage. The number of bacterial and viral strains coincides with the number of students in the laboratory. The stage is now set for determining the host range of the virus isolates. Another way of saying this is that we can now determine the susceptibility of each bacterial strain to each virus.

Experiment 4:
Testing The Host Range of Isolated Coliphages

Determining the number of bacterial isolates a given virus will infect can be done in 24 hours. Suppose there are 20 students in the laboratory. Each student spreads thousands of his or her own *E. coli* cells on 21 nutrient agar plates. One of these plates is incubated without any virus added. This is a bacterial control plate, which, after incubation at 37°C should contain a solid lawn of bacterial grown. Another plate is for the student to test that bacterial strain against his or her own virus. This is another kind of control, which shows that the bacterial strain that was used for that virus isolation can, indeed, be infected and destroyed by that homologous virus.

The issue that remains to be explored is how sensitive that bacterial strain is to coliphages isolated from all the other *E. coli* strains recovered from the class. Of the remaining 19 plates, therefore, one is given to each member of the class. After all the students exchange plates in this fashion, each student should have a plate of bacteria from every other student. The final step is for each student to place one drop of his or her enriched phage prep in the center of each of the 19 plates. After 24–48 hours of incubation, the macroplaques will be evident wherever the virus can infect the particular cells on the plate.

If, for example, coliphage from student number 1 can infect bacterial strains 1, 4, 8, 14, and 15, then those five bacterial strains constitute the host range of that phage. Phage from student 2 may

infect bacterial strains 2, 9, and 19. Phage 3 may infect only phage 3. It is likely that almost every phage isolated in this manner will have a unique host range (see Appendix A).

Experiment 5:
Recovering Virus-Resistant Mutants

If the plates with the macroplaques are allowed to incubate for a few days, several small, isolated bacterial colonies may eventually appear within the circular region of destruction we have been calling the macroplaque. These colonies are phage-resistant mutants of the *E. coli* host strain being tested. Although resistant to one phage strain, however, they may still be sensitive to other virus strains. These phage-resistant mutants can be recovered and purified, and can be tested against the other coliphages that had been shown to infect the parent bacterial strain, as described in Experiment 3. This step will clarify whether the mutation to resistance is specific for one particular coliphage or whether it extends to other viruses as well. Obviously this can provoke a substantial amount of discussion.

Besides the techniques that are developed along the way, these experiments also show that though a particular species of bacteria can be isolated from each person, each bacterial isolate has a unique genetic history and has had ample opportunity to accumulate mutations that make it subtly different from other strains of the same species. The differences are apparent in their differential susceptibility to various viruses. Likewise, the viruses are all coliphages, but it is unusual for any two coliphages isolated in this way to have identical host ranges.

Experiment 6:
Antibiotic Sensitivity Testing

For a different kind of experiment, I provide bulk samples of some of the more common antibiotics, such as ampicillin, streptomycin, tetracycline, bacitracin, and nalidixic acid, and I ask the students to devise an experiment to determine an antibiotic sensitivity profile for their own *E. coli* and for any other bacterial species they might have isolated (such as a species of *Bacillus* from soil). In other words, I want them to determine the killing concentration of each of those drugs for each of their bacterial isolates. I tell them nothing else, and I leave the lab for about 20 minutes while they discuss what they are going to do. Obviously, they don't have a clue about whether it will take a gram, a milligram, or a microgram of, say, streptomycin to kill their cells, but I let them discover that they don't know that, and I give them time to work out a plan. When I return to the room, we discuss their rudimentary experimental design, and I am available to answer questions about procedure. Within the boundaries of safety and sanity, I try to make available to them whatever they need to do their experiment, no matter how bizarre it may sound to me. This, I believe, is the key to this kind of laboratory experience: even if their plan yields meaningless results, they will at least have learned where they went wrong, and together we can refine the experimental procedure and re-do the experiment after we "iron the wrinkles out of it."

Eventually, perhaps after some false starts, each student is able to construct the antibiotic sensitivity profile I requested, usually by inoculating a million or more cells from their bacterial species onto a series of nutrient agar plates containing various dilutions of filter-sterilized antibiotic. These plates not only determine the information I requested, but also set up Experiment 7, below.

Clearly, it seems a luxury to be able to repeat experiments that have not yielded the desired information. But this is, after all, the way science is done, and this is what makes science exciting.

Experiment 7:
Recovering Antibiotic-Resistant Mutants

Although a given concentration of a particular antibiotic may kill almost all of the cells exposed to it, a few might have mutated to antibiotic resistance. Thus a population of a million bacterial cells might contain one, or dozens, or even hundreds of mutants resistant to the particular antibiotic to which they are being subjected. All cells except these mutants will be killed. The surviving mutants will be able to grow in the antibiotic- containing medium, and resistant mutant colonies can easily be recovered after several days growth on the plates. In this way, the students can build a mutant library. They can, for example, recover a strain resistant to penicillin, another resistant to streptomycin, and a third resistant to both penicillin and streptomycin. These genetically-marked strains might be useful later on, should the students want to attempt a bacterial genetics experiment.

APPENDIX A
Host Range Results

Phage strains, identified by lowercase letters, were each isolated after growing in the presence of bacterial strains with the corresponding uppercase letter. Some phages, such as *f* and *o*, were quite specific, killing only the original host. Other, such as *d*, *h*, and *p*, killed almost every bacterial strain tested.

Phage strain

Bacterial strain	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p
A	k			k	k			k		k		k		k		k
B		k		k				k				k				k
C			k		k		k	k								k
D				k	k			k	k	k	k	k		k		k
E	k			k	k			k	k	k						k
F						k			k							k
G				k			k	k				k				
H				k	k			k	k	k		k	k	k		k
I	k	k		k					k				k			k
J	k			k	k		k	k		k			k			k
K	k			k	k			k			k	k	k			k
L	k			k			k	k				k				k
M				k	k		k	k			k		k			
N				k			k	k			k		k	k		k
O															k	
P	k	k	k	k	k			k	k	k	k	k	k	k		k

*k=phage kills bacteria

APPENDIX B
Further Reading

Fenner, F., and A. Gibbs (Editors). 1988. Portraits of viruses: A history of virology. Karger, Basel, Switzerland, 344 pages. [Major concepts in virology in the context of the historical development of the fields.]

Fraenkel-Conrat, H,. P. C. Kimball, and J. A. Levy. 1988. Virology. Second edition. Prentice-Hall, Englewood Cliffs, New Jersey, 440 pages. [A rather short and comfortably readable virology textbook.]

Watson, J. D., N. H. Hopkins, J. A. Steitz, and A. M. Weiner. 1987. Molecular biology of the gene. Fourth edition. Benjamin-Cummings, Menlo Park, California, 2 volumes. [Each edition is a classic, with considerable information about the reproduction of bacteriophage.]