Experimental Techniques in Bacterial Genetics

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Preface

The experiments in this manual use both *in vivo* genetic techniques and *in vitro* molecular techniques to study gene structure, function, and regulation in bacteria. Both approaches are useful for studying bacterial genetics: the function and regulation of a gene can be directly determined *in vivo* , and the detailed molecular structure of a gene can be determined *in vitro* . Both *in vivo* and *in vitro* techniques are also useful when cloning foreign genes in bacteria: in order to clone a foreign gene in bacteria it is sometimes necessary to genetically manipulate the host to stabilize the clone or to increase expression of the cloned gene (e.g., to eliminate host recombination functions, proteases, or other enzymes). Although the experiments in this manual use *S. typhimurium* and *E. coli* , many of the methods can be used for studying the molecular genetics of other organisms as well.

This manual is based on a course I have taught for four years at the University of Illinois, Urbana. The course is designed to give upper division undergraduates and graduate students hands-on experience with modern techniques in bacterial genetics and molecular biology. An enormous number of variations of molecular techniques are available. Every molecular geneticist likes some techniques and dislikes others, but no two molecular geneticists seem to have the same preferences. "New and improved" techniques are constantly being developed, but most of these techniques rely on a few basic skills and principles. The methods in this lab manual are not meant to demonstrate every possible technique, but to provide experience with a variety of basic genetic and molecular techniques. With this background a student should be able to quickly learn new techniques and develop personal likes and dislikes.

In addition to teaching experimental techniques, another goal of this course is to teach students how to design and interpret such experiments. The strategy for teaching these skills is to require students to think about their experiments instead of just following instructions. Students construct operon fusions with *S. typhimurium* genes, then use genetic and molecular techniques to characterize the regulation of the mutated gene and the nature of the gene products. Although each experimental technique is described in the lab manual, the students isolate different mutants, so they need to plan the specific experimental details required for their mutants. If an experiment does not work (as often happens in research labs), I consult with the students to try to figure out what went wrong and encourage them to try again. In order to interpret their results, I encourage students to look up references in the literature and compare their results with published results.

This format provides an intensive, individualized lab experience. Unlike many typical lab courses, it will not be possible to do everything during regular laboratory hours. Usually students will only have to drop by the lab for a few minutes on days the lab does not meet to check their plates or to stop an agarose

gel, etc., but a few experiments involve more lengthy procedures on odd-days which may require special arrangements.

In order to understand how an experiment works, it is important to know what reagents were used and why. The specific reagents required for each experiment are listed at the end of each experiment. In addition, for the convenience of the instructor all of the reagents are also listed alphabetically in the Appendix. I have mentioned specific brand names in some experiments because I have had satisfying experiences with those products -- it is not meant to imply that the generic equivalent will not work equally well.

Several of the experiments in this lab manual use radioisotopes. Some schools do not presently use radioisotopes in teaching laboratories. However, radioisotopes are used extensively in molecular biology, so I think use of radioisotopes is essential for a realistic laboratory experience. Relatively low levels of radioactivity are used in these experiments and only ^{35}S is used, an isotope with sufficiently low energy that shields are not required but high enough energy that it can be directly monitored with a thin-window Geiger counter. Nevertheless, most of the experiments in this manual do not use radioactivity. These experiments could be easily used for a "nuclear-free" laboratory including many techniques in bacterial genetics, cloning and restriction analysis.

This manual assumes that the students have some previous theoretical background in genetics and biochemistry. Short explanations of important concepts and the rationale for each experiment are included, but this lab manual is not meant to provide an extensive theoretical background in bacterial genetics or molecular biology. More details on the theory can be obtained from the references listed or from Microbiology, Genetics, Molecular Biology, or Biochemistry textbooks.

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Acknowledgments

All of the experiments done in this course are modifications of published protocols that are used in my lab. I learned many of these procedures in the Advanced Bacterial Genetics course taught at Cold Spring Harbor Laboratory by Tom Silhavy, Lynn Enquist, and Mike Berman, and while working in John Roth's lab. For many of these procedures the following excellent sources were my initial references:

Davis, R., D. Botstein, and J. Roth. 1980. *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory, NY.

Maniatis, T., E. Fritsch, and J. Sambrook. 1982. *Molecular Cloning*. Cold Spring Harbor Laboratory, NY.

Roth, J. 1970. Genetic techniques in studies of bacterial metabolism. *Methods Enzymol. 17*: 1-35.

Silhavy, T., L. Enquist, and M. Berman. 1984. *Experiments with Gene Fusions*. Cold Spring Harbor Laboratory, NY.

Several reviewers made excellent suggestions, including Jon Beckwith (Harvard Medical School), Andrew Kropinski (Queen's University, Ontario), Paul Matsudaira (MIT), Charles Miller (Case Western Reserve University), and Valley Stewart (Cornell University). In addition, many useful suggestions were made by the previous teaching assistants for this course and my graduate students: Li-Mei Chen, Greg Deno, Don Hahn, Craig Kent, Eunhee Lee, Min-Ken Liao, Bob Lloyd, Rik Myers, Nick Santaros, Paula Spicer, and Ming Te Yang. Michele Beaudet and Joe Pogliano carefully proofread the final version of this manual.

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COURSE OUTLINE

A suggested outline for a lab course using this manual is shown below:

EXPERIMENTS

a. Isolate a Mud insertion mutant in *S. typhimurium* and determine its metabolic effect (e.g., the auxotrophic requirement).
b. Map the insertion mutation on the *S. typhimurium* chromosome.
c. Isolate regulatory mutants that affect expression of the Mud insertion mutant.
d. Construct a physical map of the chromosomal insertion mutation by Southern blotting.
e. Isolate a clone that complements the insertion mutation and characterize the cloned gene by mutagenesis, restriction mapping, and expression of the plasmid encoded proteins.
f. Subclone a fragment of the complementing gene into M13 and determine the DNA sequence of the cloned fragment.

NOTEBOOKS

Each student should keep a separate notebook. Do not count on your memory — write down your actions precisely in your notebook while you are doing the experiment. Your notebook should allow you to figure out why you did an experiment and exactly how you did it a long time after you did it. In addition, your notebook should be legible and thorough enough for someone else to read and understand what you did. The notebook should include:

a. *Rationale.* A short explanation of why you did the experiment.
b. *Protocol.* A detailed explanation of what you actually did. It is acceptable to cross-reference the lab manual, but you should record performance details that are not described in the lab manual (e.g., the genotype of mutants used and their auxotrophic requirements, any changes in the actual experiment compared to the protocol in the lab manual, and any unusual observations).
c. *Results.* The actual data (e.g., the number of colonies obtained, the OD determined, photos of agarose gels, etc.) should be included in your notebook. All plots and calculations should also be included. Show the equations used for all your calculations.
d. *Discussion.* A brief summary of the conclusions. (Did the controls work? What do the results mean?)

PROPOSALS

Since each group will be working with different mutants, sometimes each group will require different supplies. When this happens, I ask for a short description of the planned experiment (including controls) and the supplies needed. This allows us to obtain any special materials needed for the next experiments and to check for any weakness in the experimental design. Proposals should be turned in during the lab prior to when the supplies will be required.

PROGRESS REPORTS

I require each student to turn in a short report on each experiment. Progress reports should include a short summary and explanation of the experimental results. Progress reports should include the:

a. *Experiment number.*
b. *Purpose.* What did you expect to learn from the experiment?
c. *Results.* The results that should be included are outlined at the end of each experiment. When possible, the data should be presented in tables or figures. Indicate what controls were run and why.
d. *Conclusions.*

FINAL PAPER

I require a final paper summarizing the collective results of the experiments at the end of the course. It should be written up as a short paper suitable for submission to a scientific journal. You should compare and contrast your results with relevant references from the literature and cite the references in your paper.

LAB SAFETY

Safety is an important consideration for this type of course: the experiments involve bacteria, potentially harmful chemicals, and radioactivity. The instructor must thoroughly explain necessary safety precautions, demonstrate proper techniques, and carefully supervise the experiments.

BACTERIA

Many of the experiments in this course use *Salmonella typhimurium* LT2. Most natural isolates of *S. typhimurium* cause a serious bacteremia in mice and a less severe gastroenteritis in humans. Due to many years of maintenance in the lab, *S. typhimurium* LT2 is only weakly pathogenic for mice or humans, although very large doses may still cause an infection (see Sanderson and Hartman. 1978. *Bacteriol. Rev. 42*: 494). However, most of the strains used in this course contain plasmids or transposons that encode antibiotic resistance. In order to keep antibiotic resistant bacteria out of the environment (and you), all cultures and glassware that have come in contact with cultures should be sterilized after use and careful microbiological techniques should always be used when handling bacterial cultures.

RADIOACTIVITY

Several of the experiments in this course use radioactivity. Each step that involves radioactivity is indicated with an *. Proper procedures for the safe handling of radioactivity should be discussed in class and demonstrated. A few commonsense precautions should always be followed:

- Always wear gloves and a lab coat when handling radioactivity
- Never mouth-pipet a radioactive solution
- Only use radioisotopes in designated areas: no eating, drinking, or smoking is allowed in these areas
- Make sure all radioactive materials are labeled with a radiation sticker indicating the date, the isotope, and the activity
- If ^{32}P is substituted for ^{35}S in any of these experiments, always use ^{32}P behind a plexiglass shield
- Dispose of all contaminated materials and solutions in the appropriate radioactive waste containers
- Always check the work area for radioactivity when finished

CHEMICALS

Several chemicals used in this lab are hazardous. Precautions are noted in the lab manual when these chemicals are used. The following chemicals are particularly noteworthy:

- Phenol — can cause severe burns
- Acrylamide — potential neurotoxin
- Ethidium bromide — carcinogen

However, these chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipet them. If you accidentally splash any of these chemicals on your skin, *immediately rinse the area thoroughly with water* and inform the instructor. Discard the waste in appropriate biohazard containers. When in doubt, the toxicity of many chemicals can be looked up in the Merck Index.

ULTRAVIOLET LIGHT

Exposure to ultraviolet (UV) light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, *always wear appropriate eye protection* when using UV lamps.

ELECTRICITY

The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis and place a "High Voltage" sign in front of the electrophoresis setup while it is running. Always turn off the power supply and unplug the leads before removing a gel.

LABELS

Since you will use common facilities, everything stored in an incubator, refrigerator, etc. must be labeled. In order to limit confusion, each group should select a unique set of initials which will serve as identification. You can use these initials together with a number to designate your mutant strains (e.g., AB1, AB2, etc.). This method will prevent duplication of strain names later. Always mark plates on the back (not the lids) with your initials, the date, and relevant experimental data (e.g., strain numbers).

GENETIC NOMENCLATURE

STRAIN COLLECTIONS

The ease of rapidly accumulating a large number of mutants requires careful bookkeeping to avoid confusing one mutant with another. Each mutant should be assigned a strain number. Strain numbers usually consist of two capital letters designating their source and a serial numbering of the strains in the collection of that laboratory.

GENOTYPES

An extensive genetic map is available for *S. typhimurium* (Sanderson and Roth, 1988) and *E. coli* (Bachmann and Low, 1980). The genes are named using standard genetic nomenclature. Each gene is assigned a three-letter designation, usually an abbreviation for the pathway or the phenotype of mutants. When the genotype is indicated, the three-letter designation is written in lower case. (For example, mutations affecting pyrimidine biosynthesis are designated *pyr*). Different genes that affect the same pathway are distinguished by a capital letter following the three-letter designation. (For example, the *pyrC* gene encodes the enzyme dihydroorotase and the *pyrD* gene encodes the enzyme dihydroorotate dehydrogenase).

Each mutation in the pathway is consecutively assigned a unique allele number. (For example, *pyrC19* refers to a particular *pyr* mutation that affects the *pyrC* gene. In order to distinguish each mutation, no other *pyr* mutation, regardless of the gene affected, will be assigned the allele number 19). A separate series of allele numbers is used for each three-letter locus designation. The entire genotype is italicized or underlined (e.g., *pyrC19*).

PHENOTYPES

It is often necessary to distinguish the phenotype of a strain from its genotype. The phenotype is usually indicated with the same three-letter designation as the genotype but phenotypes start with capital letters and are not underlined. (For example, strain TR251 [*hisC527 cysA1349 supD*] has a Cys^+ His^+ phenotype because the *supD* mutation suppresses the amber mutations in both the *cysA* and the *hisC* genes.)

TRANSPOSON INSERTIONS

Transposable elements can insert in known genes or in a site on the chromosome where no gene is yet known. When an insertion is in a known gene, the mutation is given a three-letter designation, gene designation, and allele number as described above, followed by a double colon then the type of insertion element. (For example, *pyrC691*::Tn*10* designates a particular insertion of the transposon Tn*10* within the *pyrC* gene).

When a transposon insertion is not in a known gene, it is named according to the map position of the insertion on the chromosome. Such insertions are named with a three-letter symbol starting with z. The second and third letters indicate the approximate map position in minutes: the second letter corresponds to 10-minute intervals of the

genetic map numbered clockwise from minute 0 (a = 0-9; b = 10-19; c = 20-29, etc.); the third letter corresponds to minutes within any 10-minute segment (a = 0; b = 1; c = 2; etc). For example, a Tn*10* insertion located near *pyr*C at 23 minutes is designated *zcd* ::Tn*10* . Allele numbers are assigned sequentially to such insertions regardless of the letters appearing in the second and third positions, so if more refined mapping data suggests a new three-letter symbol, the allele number of the insertion mutation is retained. This nomenclature uses *zaa* (0 min) to *zjj* (99 min). Insertion mutations on extrachromosomal elements are designated with zz, followed by a letter denoting the element used. (For example, *zzf* is used for insertion mutations on an F ' plasmid.)

References

Bachmann, B., and K. Low. 1980. Linkage map of *Escherichia coli* K-12, Edition 6. *Microbiol. Rev.* 44: 1-56.

Davis, R., D. Botstein, and J. Roth. 1980. *Advanced Bacterial Genetics*, pp. 2-4. Cold Spring Harbor Laboratory, NY.

Sanderson, K., and J. Roth. 1988. Linkage map of *Salmonella typhimurium*, Edition VII. *Microbiol. Rev.* 52: 485-532.

BACTERIAL STRAINS USED

Strain	Genotype	Experiment
S. typhimurium		
LT2	Wild type	1,3,5
MS1063	*hisD9953* ::MudJ *his-9941* ::Mud1	1
MS1973	*del(his)640* / F'42(ts) *lac*$^+$ *zzf-20*::Tn*10 finP301*	2
MS2	*thrA9 rpsL1*	2
MS4	*proA36 rpsL1*	2
MS5	*pyrC7 rpsL1*	2
MS6	*pyrF146 rpsL1*	2
MS8	*hisO1242 del(his)2236 rpsL1*	2
MS10	*ysA533 rpsL1*	2
MS12	*serA13 rpsL1*	2
MS13	*cysG439 rpsL1*	2
MS14	*metA53 rpsL1*	2
MS100	*ilv-508 rpsL1*	2
MS1974	*del(his)640* / F'42 *lac*$^+$ *zzf-20*::Tn*10 finP301*	8
E. coli		
EM257	*del(recA-srl) srl* ::Tn*10 zfi* ::Tn10dCam *supF supE hsdR galK trp A metB lacY tonA* /pBR328 (*Hin* dIII fragment of MudJ)	5
EM158	*del(recA-srl) srl* ::Tn*10 zfi* ::Tn*10*dCam *supF supE hsdR galK trpR metB lacY tonA*	9
EM383	*hsd-5 del(lac-proBA) supE thi* /F' *proB*$^+$ *proA*$^+$ *lacI*^{q} *del(lac)M15*	10,11
EM258	*hsd-5 del(lac-proBA) supE thi* /F' *proB*$^+$ *proA*$^+$ *lacI*^{q} *del(lac)M15*/M13mp18	10
EM259	*hsd-5 del(lac-proBA) supE thi* /F' *proB*$^+$ *proA*$^+$ *lacI*^{q} *del(lac)M15* /M13mp19	10

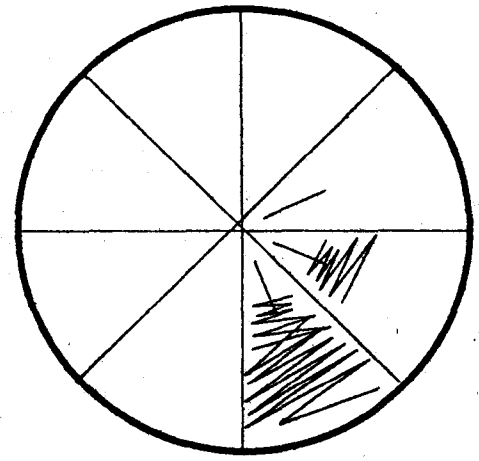
[1]The *rpsL* mutation causes resistance to streptomycin (Str^r).

BASIC TECHNIQUES FOR BACTERIAL GENETICS

STREAKING PLATES

When picking and streaking lots of bacterial colonies it is often quicker to use sterile toothpicks and sticks instead of using a wire loop that must be sterilized by passing it through a flame between each colony. Using the technique shown below, eight colonies can be streaked for isolation on a single plate. (Save the used toothpicks and sticks to be reautoclaved).

Divide the plate into 8 sectors.



In each sector:

(1) Pick a single colony with a sterile toothpick and streak a single short line.

(2) Streak half way with a sterile stick.

(3) Finish streaking with a new sterile stick.

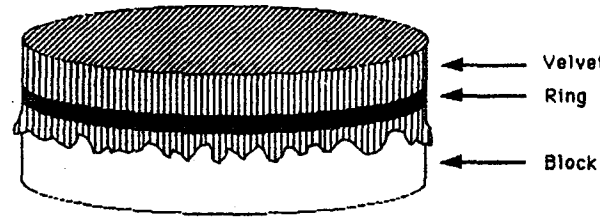
PICKING AND PATCHING COLONIES

When checking out bacterial strains, it is often useful to patch many colonies on a single petri plate so they can be tested simultaneously by replica printing onto a series of media. To do this, place a fresh master plate over a "patching grid." (A replica patching grid is supplied at the back of the manual). Touch the top of each colony with a sterile toothpick and draw a small x on the new master plate. (Only use each toothpick once. Save the used toothpicks to be reautoclaved). Many patches can be placed on a single plate. After patching incubate the plate overnight to let the patches grow. The next day this plate can be used as a master for replica printing. Always mark each plate at the top of the patch grid since the patch grids are symmetrical.

REPLICA PRINTING

This technique transfers cells from an array of colonies (or patches) on one plate, to a series of "replica" plates. Thus, each of the replica plates is inoculated by cells in the same arrangement as on the original ("master") plate. The transfer is done as follows:

1. Mark the top of the master plate and each replica plate. Always make the last plate replicated a control that all the colonies can grow on. This insures that failure of a colony to grow is not simply due to inefficient transfer.
2. Place a sterile velvet over the replica-printing block (fuzzy side up) and push a ring down over the velvet to hold it in place (see the figure below).



3. Press a plate containing an array of bacterial colonies (or patches) onto the surface of the sterile velvet. Press just hard enough that the fabric pattern become visible in the agar. Carefully lift the plate straight up and remove it to avoid smudging the print. Most of the cells on the plate will be transferred to the surface of the velvet.
4. Once a replica of the master plate has been formed on the velvet, press each of the fresh plates to be inoculated onto the surface of the velvet which carries cells. Many replicas can be made from a single master. (Save the velvets. If the velvets are washed and autoclaved, they can be used many times.)

PLATE MATINGS

1. Grow fresh overnight cultures of the donor (F^+) and recipient (F^-) strains. (If the F-factor is unstable it may be necessary to grow the donor in selective media. In addition, if the F-factor is temperature sensitive, then the donor must be grown at 30°C.)
2. Plate on a medium that selects for the phenotype of the exconjugants. Divide the plate into 3 sections.