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Chapter 4

Incorporating Original Genomics Research into Genetics and Molecular Biology Courses

Brad Goodner & Cathy Wheeler

Department of Biology, Hiram College
Hiram, OH 44234
(330) 569-5260, (330) 569-5263
goodnerbw@hiram.edu, wheelerca@hiram.edu

Brad Goodner is an Associate Professor responsible for courses in genetics, cell and molecular biology, microbiology, advanced molecular biology, immunology, and bioinformatics. His academic background includes a B.S. in biochemistry from Texas A&M University, a Ph.D. in biology from Purdue University, and postdoctoral experience at University of North Carolina, as well as previous teaching and research experience with undergraduates at University of Richmond. He is a cofounder of the Hiram Genomics Initiative where he administers the overall initiative, incorporates research into the laboratory portions of all his courses, and disseminates best practices to colleagues at other colleges and universities. He is also program director for a Howard Hughes Medical Institute grant to expand the use of research within courses across the natural sciences at Hiram College. His research interests include using the genomic sequence of *Agrobacterium* to better understand its biology and involving undergraduates in all aspects of genome projects for strains of *Agrobacterium*, *Xenorhabdus*, *Chromohalobacter*, and *Sphingomonas*.

Cathy Wheeler is a Research Teaching Associate responsible for the laboratory portions of genetics, cell and molecular biology, and microbiology courses. Her academic background includes B.S. and M.S. degrees in biology from John Carroll University, and technical experience in both academic research and clinical lab settings. She is a cofounder of the Hiram Genomics Initiative where she incorporates research into the laboratory portions of all her courses and coordinates research collaborations with local high schools as an outreach component of the initiative.

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Introduction

Laboratory components of biology courses are usually justified in terms of providing students with hands-on experience with concepts, key lab skills, organisms, and biological processes. Good laboratory experiments do exactly that, but they always run the risk of becoming too "comfortable" for students and/or faculty such that they lose their ability to excite and challenge over multiple reiterations of a course. We would all agree that certain concepts and skills are essential, but we don't want faculty or students thinking "this has been done a million times." One possible solution is to incorporate original research into the laboratory component of courses in order to help teach basic scientific principles, keep students excited about biology, expose students to how research really works and encourage them to seek out more research opportunities, and to tie courses directly to the research interests of faculty. Such a strategy allows the lab component of a course to evolve over time and to make meaningful contributions to scientific knowledge.

Since its establishment in 2002, the Hiram Genomics Initiative (HGI) has incorporated genomic mapping, gap closure, bioinformatics, functional genomics, and comparative genomics projects, most of which are associated with larger collaborative genome projects, into several biology courses at Hiram College. During that time, over 135 undergraduates and 70 high school students have taken part in research within courses, most of the undergraduates receiving multiple exposures through projects in multiple courses. Many of these students have moved on to independent research projects, presentations at meetings, and post-baccalaureate training. We believe that this style of laboratory teaching is easily transferable to other institutions, can be used to formulate collaborations between colleagues and institutions, and benefits both students and faculty (Goodner et al., 2003). In this chapter, we provide three example projects that we have used in multiple iterations of either a genetics course or a molecular and cellular biology course. Each of these projects accomplishes the development of several skills in students while connecting the basic concepts to a larger research

goal. There is no doubt that each of these projects can be modified to fit an organism and/or a research question of interest to an individual instructor.

Project 1. Genetic & Physical Mapping of Bacterial Genomes

Student Outline

Introduction

As you already know, a gene is a segment of DNA that says "make a particular protein at a particular time and place." Over most of the last 50 years, scientists have studied genes and the proteins they encode one at a time. While this approach has been highly successful, there are questions that are best addressed by looking at many or all of the genes of an organism at the same time. All the genes of an organism (and the intervening sequences as well) are known collectively as its genome, and the study of genomes is called genomics. Genomics not only can answer questions about known genes, but the determination of the entire DNA sequence of an organism exposes hundreds to thousands of "new" genes and just as many new questions for future research.

So why figure out the complete genomic sequence for any particular organism, say for example the gamma-proteobacterium *Chromohalobacter salexigens* strain? What makes *C. salexigens* so interesting to study, compared to its better-known relatives *Escherichia coli* and *Pseudomonas aeruginosa*, is its ability to live in the presence of a lot of salt (Vreeland et al., 1980). *C. salexigens* requires at least 0.5 M NaCl to grow and can tolerate over 3 M NaCl, making it one of the most halophilic members of the Bacteria domain. Sodium ions, while incredibly abundant in many habitats, disrupt many biochemical functions at high intracellular concentrations. In order to live in salty habitats, organisms need special mechanisms. For example, in order to maintain osmotic balance, many halotolerant organisms accumulate compounds through uptake and/or synthesis that are more compatible with biochemical functions than NaCl (Csonka & Hanson, 1991). To better understand the halophilism of *C. salexigens*, it would be useful to know all of the proteins made by the organism and their potential functions.

Getting the entire genome sequence of *C. salexigens*, or any other organism, is just the first step, but it is not trivial (Figure 1). The genome has to be broken into manageable-size (1000-2000 bp) chunks and cloned, then tens of thousands of randomly selected clones have to be sequenced. Computer analysis will allow the small DNA chunks to be joined at sequence overlaps into larger contiguous pieces (contigs). The hard part is then figuring out how the large contigs go together to reconstruct the entire genome sequence, just like putting together a jigsaw puzzle. Having some reference points (e.g., printed design or shape of puzzle pieces) can help the assembly process tremendously. In this multiple-week research project, you and your fellow students will help construct a map of reference points in the form of genetic mutations and DNA restriction cut sites.

Our basic strategy will be to generate mutations in *C. salexigens* using a transposable element as the mutagen (Figure 2). The particular transposable element we will employ, Tn5-RL27, has several useful features (Larsen et al., 2002). First, it carries a gene that allows cells to be resistant to the antibiotic kanamycin. *C. salexigens* is naturally sensitive to kanamycin, so we can use kanamycin resistance as a selection for cells containing the transposon. Second, Tn5-RL27 carries cut sites for several rare-cutting restriction endonucleases, such as PacI. Cutting the genome of wildtype and mutant cells with such a restriction enzyme will allow us to determine the physical location of where the transposon had jumped. Third, once Tn5-RL27 jumps, it is stuck forever. This is due to the fact

that the transposase gene lies outside of the transposon – and the plasmid vector that carries the transposon and the transposase gene doesn't hang around very long (hence the name suicide vector). It can only replicate in certain engineered strains of *E. coli*. Fourth, the origin of replication that allows the suicide vector to replicate only in certain *E. coli* strains actually sits inside the transposon. The beauty of this feature is that you can recover the transposon later by turning it into a plasmid as we see later. Fifth, the transposase encoded by the suicide vector is a hyperactive variant so you get a higher than normal rate of transposition. Sixth, the suicide vector can be moved from *E. coli* to other gram-negative diderm bacteria (such as *C. salexigens*) in two-parent bacterial matings through the process of conjugation.

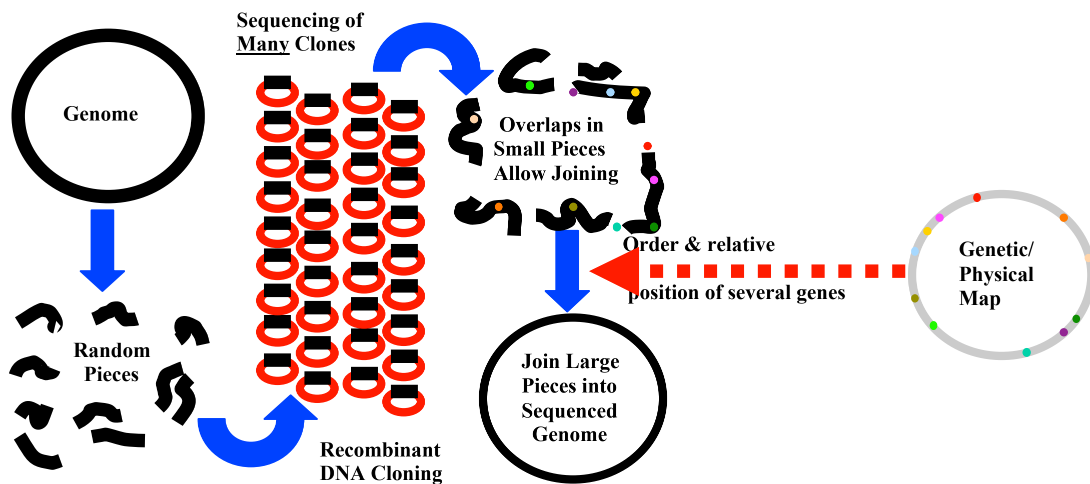

Figure 1. Basic steps of a genome sequencing project and the role of a combined genetic/physical map of the genome.

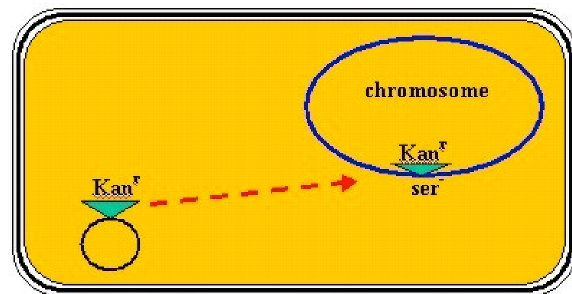

Figure 2. Replica plating of transposon mutants to screen for auxotrophs (grow on rich medium, but not on minimal medium).

To generate our map, we will first generate (select) thousands of mutants using Tn5-RL27 as our mutagen and then screen these mutants for auxotrophs, mutants unable to make a particular cellular building block (Figure 3). The selection for transposon insertion mutants involves plating the conjugation mix of *E. coli* harboring the suicide vector and *C. salexigens* onto rich medium containing kanamycin (only cells with the transposon live) and rifampicin (our *C. salexigens* strain is resistant, but the *E. coli* is not). The screen for auxotrophs is more laborious, as you would expect for a screen, in that *C. salexigens* mutants are patched onto rich and minimal media, with the auxotrophs only growing on the rich medium.

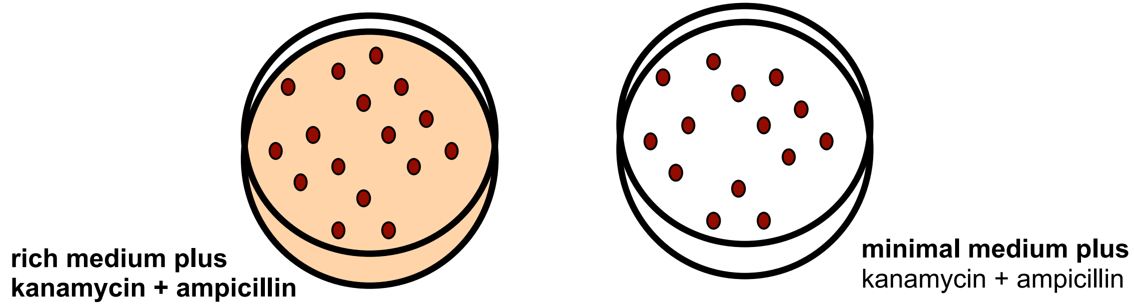

Figure 3. Replica plating of transposon mutants to screen for auxotrophs (grown on rich medium, but not on minimal medium).

Each auxotroph can be further characterized using nutrient pools and pathway intermediates to give a genetic name to that mutant (Figure 4.) Our next step will be to determine the location of the transposon in several randomly selected prototrophic mutants as well as in each of the auxotrophs we find using rare-cutting restriction enzymes and pulsed-field gel electrophoresis (Figure 5). This is known as restriction mapping, where we put the restriction fragments in the proper order. The DNA fragments we are dealing with here will be hundred of thousands of bp to millions of bp in size and such huge DNA fragments are very fragile, so the procedure for isolating DNA and cutting it are a little different from what we normally do and so is the electrophoresis for separating the large fragments. DNA molecules bigger than around 30,000 bp do not separate much at all on normal agarose gels because all of these biggies bump into the gel matrix at the same high rate so there is no sieving difference. However, it turns out that DNA molecules run through gels like a snake with their long axis parallel to the direction of the electric field. If the direction of the electric field changes, then the DNA molecule coils up and re-elongates in the proper orientation before running again. The bigger the DNA molecule, the longer it takes it to turn after a switch in the electric field orientation. Pulsed-field gel electrophoresis does exactly that with the aid of a microprocessor to control switch times. Finally, we can recover the site of transposon integration in order to directly identify the mutated gene through DNA sequencing (done off-site). This makes use of the fact that the transposon carries two features found in all plasmids – an origin of replication and a selectable marker (Figure 6).

Biochemical Pathway Intermediate	Growth or No Growth When Compound Added to Minimal Medium
3-phosphoglycerate	-
3-phosphohydroxypyruvate	-
3-phosphoserine	-
serine	+
glycine	+

Figure 4. Use of pathway intermediates to identify the step blocked in a given auxotroph (example from serine/glycine pathway).

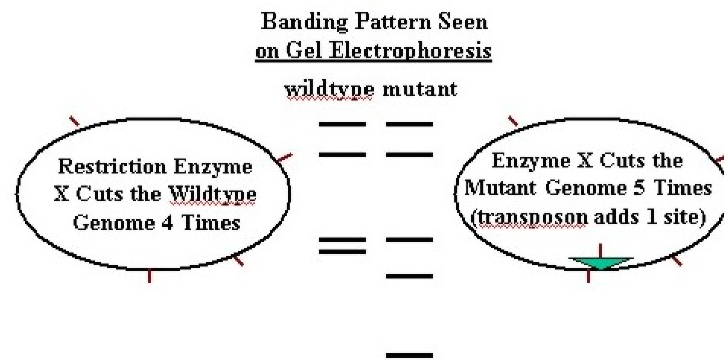

Figure 5. Use of rare-cutting restriction endonucleases to physically localize the position of a transposon insertion in a given mutant.

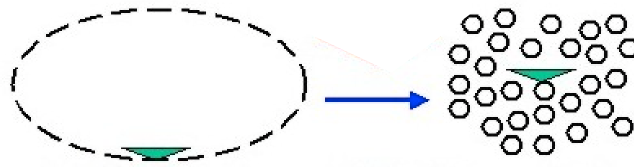

Figure 6. Recovery of a Tn5-RL27 insertion site for cloning as a plasmid and subsequent DNA sequencing. The genome is cut with a restriction enzyme that does not cut within the transposon, ligated at dilute DNA concentration to promote self-circularization, and transformed into an *E. coli* strain that allows for the selection of the transposon-based plasmid.

This is a real research project being carried out within this course, so once you learn the basic methods you and your fellow students will have to make decisions together, carry out the proper experiments, collect and store data in a usable format, and then analyze the data and draw conclusions. Our goal is to construct a genetic and physical map of the *C. salexigens* chromosome, but beyond that I cannot tell what results you will obtain. That is the challenge, the mystery, and the fun of research.

Methods Used Throughout the Project

Strains and growth conditions: For general growth, *C. salexigens* and *E. coli* cultures can be maintained at 37°C with aeration (shaking for liquid cultures), in LB for *E. coli* and in LB + 0.5M NaCl for *C. salexigens*. Where needed, the antibiotics kanamycin and rifampicin should be used at final concentrations of 50 μg/ml and 20 μg/ml, respectively.

Methods Used in Component I – Generation of and Screen for Auxotrophic Mutants

Bacterial matings:

1. To set up a mating, centrifuge down 1 ml each of overnight cultures of donor *E. coli* BW20767/pRL27 and recipient *C. salexigens* strains in sterile microcentrifuge tubes for 1 minute at maximum speed in a microcentrifuge, wash each pellet in 1 ml of 0.5 M NaCl, spin down again, and resuspend each pellet in 100 μl of 0.5 M NaCl.
2. Mix the strains together by spreading 100 μl of each strain on a LB + 0.5 M NaCl agar plate and incubate at 37°C overnight.
3. The next day, pipet 2 ml of 0.5 M NaCl onto the mating plate, gently scrape off the cell mixture into the buffer, collect 1 ml of the resuspended cell mixture, mix by vortexing or shaking for a

few seconds, dilute it 1/100, 1/1000, and 1/10,000 in 0.5 M NaCl, and plate 100 μl portions onto LB + 0.5 M NaCl + kanamycin + rifampicin plates (3 plates for each dilution). Incubate the plates for 1-3 days at 37°C until colonies appear.

Mutant isolation and characterization

1. Plating the diluted mating mixture onto LB + 0.5 M NaCl + kanamycin + rifampicin selects for *C. salexigens* cells containing the transposon. After 1-3 days, start picking well-isolated dull white (*C. salexigens*) colonies and patch them on M9 minimal glucose + 0.5 M NaCl + kanamycin + rifampicin plates and LB + 0.5 M NaCl + kanamycin + rifampicin plates to screen for auxotrophs.
2. Potential auxotrophs should be streaked for single colonies on LB + 0.5 M NaCl + kanamycin + rifampicin plates and then reconfirmed by patching single colonies on M9 minimal glucose + 0.5 M NaCl + kanamycin + rifampicin plates and LB + 0.5 M NaCl + kanamycin + rifampicin plates.
3. Further characterize auxotrophs using M9 minimal glucose + 0.5 M NaCl + kanamycin + rifampicin plates containing mixtures of various amino acids, bases, vitamins, and cofactors, and later with specific pathway intermediates (as per Davis et al., 1980).

Methods Used in Component II – Physical Mapping of Transposon Insertions

Pulsed field gel electrophoresis of intact and digested DNAs:

1. Grow wildtype and mutant *C. salexigens* strains in 2 ml LB or LB + 0.5 M NaCl + kanamycin + rifampicin broth respectively, for 48 hours at 37°C to ensure that the culture has reached stationary phase.
2. Transfer a culture to a sterile microcentrifuge tube, pellet the cells, and resuspend them in 400 μl "salty" cell suspension buffer.
3. Equilibrate cells at 50°C for 2 minutes, add 400 μl of molten 2% agarose (made up in sterile water, boiled, and kept at 50°C) and fill plug molds.
4. After plugs have harden, transfer plugs from each strain into separate tubes and digest overnight with Pronase E (2 mg/ml) in ES buffer at 50°C (Goodner et al., 1999).
5. Wash plugs 4 times in wash buffer and store plugs in 0.1X wash buffer at 4°C.
6. For enzyme digestions, cut 4 mm slivers of plugs and wash twice with cold sterile water for 5 minutes each, then equilibrate in 200 μl of the appropriate restriction buffer for 30 minutes. Replace buffer with fresh 200 μl of the appropriate restriction buffer with 2 μl BSA and 4 μl of the appropriate restriction enzyme(s). Digest genomic DNA plugs at the appropriate temperature for that enzyme (5-8 hours at 37°C cuts or overnight at 25°C).
7. Separate genomic fragments using a pulsed-field gel electrophoresis unit, using 0.5X TBE buffer, 1% agarose gels, and the following unit parameters: ramp of 50 to 90 sec, 22 hours run time, 5.4 V/cm.

Methods Used in Component III – Isolation and Characterization of Transposon Insertions

Genomic DNA isolation: Many protocols will work here, but we use the DNAEasy Tissue Extraction kit from QIAGEN.

Restriction enzyme digestion of genomic DNA & self-ligation:

1. Label a new microcentrifuge tube and add to it 26 ml of genomic DNA, 3 ml 10X buffer appropriate for SacII, and 1 ml SacII enzyme (SacII does not cut within Tn*5*-RL27, but it does cut the *H. elongata* genome quite often.) Incubate the digestion at 37°C for at least 3 hours.
2. To stop the restriction digestion, incubate the tube at 65°C for 20 minutes, then spin down very briefly (few seconds) in a microcentrifuge.
3. To the tube containing the SacII-digested genomic DNA, add 14 µl sterile water, 5 µl of 10X ligation buffer, and 1 µl T4 DNA ligase. Mix the solution very gently by moving your pipettor tip around, then spin down very briefly (few seconds) in a microcentrifuge. Incubate at 15°C overnight.

DNA purification & concentration: Many protocols will work here, but we use the QIAQuick kit from QIAGEN.

Transformation: Either chemical or electroporation transformation protocols for *E. coli* will work here, but we use electoporation with *E. coli* strain TransforMax EC100D pir-116 electrocompetent cells from EpiCentre. The only requirements of the competent *E. coli* strain are that it contains the λ pir gene (preferably the pir-116 allele) and that it does not already have a kanamycin resistance gene in its genome.

Isolation of plasmid DNA: Many protocols will work here, but we use the QIASpin kit from QIAGEN.

Restriction endonuclease digestion of DNA:

1. Choose 2 restriction enzymes for your analysis. For each of your plasmid clones, label 2 tubes with the clone name and the enzyme used.
2. For each of your plasmid clones, add 17 µl of the plasmid clone solution to both labeled tubes.
3. For each labeled tube, add 3 µl of the appropriate enzyme+buffer mix. Incubate all tubes at 37°C for 1 hour.

Gel electrophoresis:

1. Add 3 µl of loading buffer is added to each of your digested DNA samples, mix, and spin down briefly in a microcentrifuge. Loading buffer consists of three components: (1) EDTA, which chelates magnesium, stopping any reaction of DNA with enzymes, (2) glycerol, to make the density of the sample greater than the salt solution so that the sample will sink to the bottom of the hole, and (3) a negatively charged blue dye (bromophenol blue) which enables you to monitor the loading process and the progress of the electrophoresis.
2. Practice loading samples into wells using the gel marked "PRACTICE" and the solution marked "PRACTICE SAMPLE". Don't worry about loading under buffer – the loading buffer makes the sample dense enough to sink to the bottom of a well. Load 25 µl into each of a couple of wells. Try to hold the end of your pipettor tip just into the top of the well. Don't jam it into the bottom of the well. Gently expel the sample into the well. When finished, keep your thumb depressed on the pipettor while you lift your hand up.
3. Load each of your samples into separate wells on the gel. Obviously, it is essential to record carefully what sample went into what well! After everyone has loaded their samples, and DNA

standards have been loaded in additional wells, the gel is closed and the electricity is turned on. You won't be able to see the DNA move, but the blue dye will go toward the positive pole at about the speed of 300 bp DNA. When the dye has neared the end of the gel, it's done. The gel is then stained with the fluorescent dye, ethidium bromide, which binds to DNA and RNA. The gel is then ready to be photographed. The electrophoresis takes several hours and the staining another 10 or 15 minutes. You therefore will probably not be around to see what happened to your gel, but you will get a picture of your gel results.

Notes to Instructors

The project can be done as written over 6-8 weeks of lab with some outside time required, or one or more of the components can be done separately in 2-3 weeks each. In this regard, it is most likely that one would choose to do component I alone or do components I and III (skipping component II if one did not have access to pulsed-field gel electrophoresis).

This entire project was used as written at Hiram College for 3 reiterations of a genetics course with 15-24 students each semester split over two lab sections. We also used component I as an outreach collaboration with 2 local high school AP biology classes. Component I requires so little materials (mostly plates and toothpicks) that it is easy for us to supply the high school classes with what they need and handle the biohazardous waste easily. We have found that students take quick ownership of the project, often identifying with particular mutants that they have found and characterized. The project promotes team-building, group decision-making and delegating responsibilities, and yet still allows individuals to excel in their own niche. For some of our introverted, less-confident students, this project really allows them to blossom and we see more of them seeking out independent research opportunities after the course. Student feedback at different points in the project mirrors their level of comfort with the unique research-oriented format. Early on, they are hesitant and afraid because they are being asked to shoulder more of the burden of making decisions and because the specific outcomes are unknown (the very nature of research). However, as the project proceeds, students come around to greatly appreciate the day-to-day problem-solving, the mystery of not knowing what you will find, and the connections between many of the points we discussed in class coming together in one lab project.

Over 3 reiterations of this project in a genetics course at Hiram College, along with the efforts of 2 high school AP biology classes during one academic year, >5000 *C. salexigens* Tn*5*-RL27 insertion mutants were screened. So far, 44 auxotrophs have been isolated, characterized, and physically mapped with PacI+PmeI. These auxotrophs represent 16 different biosynthetic pathways. Transposon insertion sites have been recovered for all the auxotrophs and sequence information has been obtained for 12 up to date. Currently, the remaining auxotrophic transposon insertion sites are being sequenced and 50 randomly-chosen prototrophs are being physically mapped with PacI+PmeI and subjected to transposon insertion site recovery for later sequencing. Pulsed-field gel electrophoresis of uncut wildtype *C. salexigens* genomic DNA reveals one chromosome and one small (>100 kbp) plasmid. Three fragments result from a PacI+PmeI digestion of wildtype genomic DNA that together indicate a chromosome size of ~3.9 Mbp.

This project was originally written for one particular organism, *C. salexigens*, as part of a larger genome project. However, the basic ideas behind the project came from earlier undergraduate work on another bacterium, *Agrobacterium tumefaciens* (Goodner et al., 1999), so please realize that this experimental strategy can be applied to a wide range of bacteria. If you wish to use the strategy on another bacterial species, the specific details you need to confirm in order for the project to work are:

1) the species of interest can undergo conjugation with *E. coli*, 2) the species of interest is not already kanamycin resistant (so you can select for the transposon), 3) the species of interest carries some other selectable marker (so you can counter-select against the *E. coli* parent after the mating; we usually use rifampicin-resistance which can be found as a rare spontaneous mutation in most bacteria), 4) the species of interest is prototrophic (so you can screen for auxotrophs).

Component I – Generation of and Screen for Auxotrophic Mutants

(1) Timelines of basic steps:
  Day 1 - Growth of donor and recipient cultures
  Day 2 - Bacterial matings
  Day 3 - Dilution of bacterial matings and selection for transposon mutants
  Day 5 and beyond - Screen for auxotrophic mutants
  Day 7 and beyond - Characterization of auxotrophic mutants using biochemical complementation

(2) Mutants may take from several days up to a week to appear on selective media once the matings have been plated. Do not panic if you don't see any colonies in the first couple of days.

(3) You can speed the process up, if needed or desired, by having some matings and some mutant colonies on selective plates already set up prior to day 1 so the students can set up matings, dilute and plate out matings, and start screening mutants for auxotrophs all on day one. They can repeat the protocols with their own matings on subsequent days.

(4) For testing auxotrophy we have the students use the same sterile toothpick to inoculate a mutant colony onto M9 and LB plates at the same time. The toothpicks are then autoclaved for reuse.

(5) Matings and the dilutions in 0.5% NaCl can be stored in the fridge for several days and then plated if more mutant colonies are desired.

(6) You might expect 1 auxotroph for every 50-100 colonies tested.

(7) Once you have mutants, permanent stocks can be made and frozen at -80°C. It may be useful to to have particular mutants available for further research. Grow a 24-48 h culture of the mutant in 1.0 ml LB + 0.5 M NaCl + kanamycin + rifampicin. Place 500 µl of the culture in a sterile microfuge tube and vortex. Add 500 µl 30% sterile glycerol and vortex. Store frozen at -80°C.

(8) This component can be repeated several reiterations of a course with new and different results expected each time. Students will get different auxotrophs or auxotrophs in the same pathway that map to different steps in the pathway.

(9) For characterizing auxotrophs, there is no need to test each pathway separately. Rather, one can use building block pool plates. Each pool plate contains a handful of building blocks (Davis et al., 1980). Growth of a mutant on only one or two pool plates identifies the blocked pathway. Once a pathway has been identified, one can often pin down the blocked step by using pathway intermediates. Many of these compounds, like the building blocks themselves, can be purchased from Sigma-Aldrich and added to minimal media (the exceptions are phosphorylated compounds which are not taken up easily by many bacteria). Quite often, a few grains of a compound sprinkled on the corner of a plate streaked with a mutant is sufficient to allow for complementation in a small area if the compound lies downstream of the pathway block.

(10) For this component, students need some basic knowledge of mutagenesis, transposons, conjugation, and gene-protein relationship in terms of biochemical complementation. Basic skills in handling bacteria with sterile technique, spread plating, using micropipettors, and dilutions are all that are needed.

Component II – Physical Mapping of Transposon Insertions

(1) Timeline of basic steps:
- Day 1 - Growth of mutant cultures
- Day 3 or 4 - Generation of genomic DNA plugs in agarose
- Day 5 – Washing of genomic DNA plugs
- Day 6 and beyond - Restriction digestion of genomic DNA plugs
- Day 7 and beyond - Pulsed-field gel electrophoresis
- Day 8 and beyond - Generation of standard curve and analysis of gel results

(2) It is very important that the cultures for making genomic DNA plugs be grown to stationary phase. In order to see the intact chromosome or restriction fragments of the chromosome clearly, the genome of most cells must not be actively replicated. If it is undergoing replication, then you will see a broad smear on the pulsed-field gel, even without restriction endonuclease digestion, and you will need to repeat the process.

(3) While making and using genomic DNA plugs lends itself easily to undergraduate students, it is vital for students to use good aseptic technique, handle their genomic plugs gently, and keep them cold at all times unless otherwise indicated. Genomic DNA plugs are usually good for up to 6 months and one plug has enough DNA in it for 5 or 6 gel lanes.

(4) The enzymes we used to cut the *C. salexigens* genome for pulsed-field gel electrophoresis are PacI + PmeI. PacI cuts within the transposon but doesn't cut within the genome. PmeI cuts the genome three times. Hence the wild type *C. salexigens* will show 3 bands on the pulsed-field gel and each transposon mutants will show 4 bands although the pattern will be unique depending on the site of transposon insertion.

(5) We typically use *Saccharomyces cerevisiae* chromosomes as linear DNA standards for pulsed-field gel electrophoresis because they span the range from 200 to 1900 kbp and be purchased already in agarose plugs (catalog #170-3605 from BioRad or catalog #N0345S from New England Biolabs).

(6) For this component, students need some basic knowledge of restriction enzymes and gel electrophoresis. Basic skills in handling bacteria, using micropipettors, and loading gels are all that are needed.

Component III – Isolation and Characterization of Transposon Insertions

(1) Timeline for basic steps:
- Day 1 - Growth of mutant cultures
- Day 2 - Isolation of genomic DNA from mutant cultures
- Day 3 - Digestion of genomic DNA, dilution, and self-ligation
- Day 4 - DNA purification, concentration, and transformation to recover transposon insertion sites
- Day 6 and beyond - Purification of transposon-based plasmids
- Day 6 and beyond - Characterization of plasmids by restriction mapping

(2) This component is listed third because it naturally follows the other components. However, once you have done component I in a previous reiteration of a course, any mutants generated can be used for component III at any time. You may find it useful to do this component before component I, depending on the order of lab skills you wish to stress, or even by itself in a particular reiteration of a course.

(3) We use SacII to digest the genomic DNA because it does not cut within the Tn5-RL27 transposon, so the fragment containing the transposon will also have arms of genomic DNA on

either side. Other restriction enzymes that do not cut the transposon are AccI, BamHI, BglII, EagI, EcoRI, EcoRV, FseI, HincII, HpaI, NaeI, NcoI, NdeI, NheI, NotI, PmeI, PstI, PvuII, SacII, SalI, SfiI, SpeI, SphI, and XmnI.

(4) Diluting the restriction digestion before ligation helps ensure that the fragments self-ligate to form circles. The only circle we are interested in is the one containing the transposon because it can act as a plasmid due to the origin of replication and kanamycin resistance gene present in the transposon.

(5) The copy number of the transposon-based plasmid in *E. coli* TAM1 lambda pir cells is fairly low, so using a 10 ml culture of LB + kanamycin broth to grow up each transformant will yield plenty of DNA for restriction analysis and subsequent sequencing, if desired.

(6) For restriction analysis of the plasmids, we typically use SacII to linearize each plasmid (students can then deduce the total size of the genomic fragment given that Tn5-RL27 is 1711 bp in size) and DraI to confirm the presence of the transposon since it cuts near each end of the transposon to release a fragment of 1575 bp.

(7) If you desire to get sequence from your transposon recovery plasmids to positively identify the site of transposon insertion (great lab in itself for teaching DNA sequence analysis), you can have the following primer made (we use Invitrogen primer synthesis service) and used for sequencing off one end of the transposon: 5'-AAcAAgccAgggATgTAAcg-3'. In your sequencing results, look for the end of the transposon (5'-GTGTATAAGAGACAG-3') – what follows it is the genomic sequence at the site of transposon insertion. You can use that sequence to look for open reading frames or look for sequence similarity in GenBank using BLAST. Students can then test whether their biochemical complementation data fits with the sequence identification of the gene hit by the transposon.

(8) For this component, students need the same basic background and skills as for component II plus some understanding of how plasmids differ from chromosomes.

Materials & Equipment

For component I of the project

- *Chromohalobacter salexigens* DSM3043 (Vreeland et al., 1980; rifampicin-resistant derivative available from authors or can be selected from wildtype stock)
- *E. coli* BW20767/pRL27 (Metcalf et al. 1996; Larsen et al., 2002)
- 0.5 M NaCl
- LB and LB + 0.5 M NaCl broth (see Appendix)
- LB + 0.5 M NaCl plates (see Appendix)
- LB + 0.5 M NaCl + kanamycin (50 ug/ml) + rifampicin (20 ug/ml) plates (see Appendix)
- M9 minimal glucose + 0.5 M NaCl + kanamycin (50 ug/ml) + rifampicin (20 ug/ml) plates (see Appendix)
- Stocks and pools of essential building blocks and pathway intermediates (as per Davis et al., 1980; chemicals purchased from Sigma-Aldrich)
- Sterile toothpicks
- Microbiological loops
- Pipets
- Micropippetors and sterile tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Sterile test tubes
- Ethanol and "hockey stick" spreader

For component II of the project

- Agarose (we use Pulsed-Field Certified Agarose, catalog #162-0137, purchased from Bio-Rad)
- "Salty" cell suspension buffer (see Appendix)
- Pronase E (catalog #P6911 purchased from Sigma-Aldrich)
- ES buffer (see Appendix)
- Wash buffer (see Appendix)
- PacI and PmeI restriction endonucleases (purchased from New England Biolabs; see notes to instructors)
- 0.5X TBE (see Appendix)
- Pulsed-field gel electrophoresis apparatus (we use CHEF-DR II system, catalog #170-3612, from BioRad; please note that this is the only costly item in this entire activity; see notes to instructors)
- LB + 0.5M NaCl broth (see Appendix)
- Microbiological loops
- Pipets
- Micropippetors and sterile tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Sterile test tubes

For component III of the project

- *E. coli* S17-1 lambda-pir, TAM1 pir (purchased from Active Motif) or EC100 pir-116 (purchased from EpiCentre)
- Components for genomic DNA isolation (we use DNAEasy Tissue Extraction kit, catalog #69504, purchased from QIAGEN)
- Components for DNA purification & concentration (we use QIAQuick kit, catalog #69504, purchased from QIAGEN)
- Components for plasmid DNA isolation (we use QIAprep Spin Miniprep kit, catalog #27106, purchased from QIAGEN)
- SacII, other restriction endonucleases, and T4 DNA Ligase (purchased from New England Biolabs; see notes to instructors)
- LB + kanamycin (50 ug/ml) plates (see Appendix)
- LB + 0.5 M NaCl broth (see Appendix)
- SOC broth (see Appendix)
- Microbiological loops
- Sterile toothpicks
- Pipets
- Micropippetors and sterile tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Sterile test tubes
- Ethanol and "hockey stick" spreader
- Gel loading buffer (see Appendix)
- 1X TBE (see Appendix)
- Agarose
- Components for standard slab gel electrophoresis

Project 2. Functional Genomics (Protein Function)

Student Outline

Introduction

As a molecular biologist, you may be interested in studying how cells interact with their surroundings via cell receptors, or how certain biochemical reactions regulate cellular processes, or even how cells grow or divide. Ultimately, a big part of what determines how cells act is their genome, their genetic make-up. We hear a lot about genomes, sequencing genomes, and identifying putative genes within genomes. However, it is the *expression* of a genome (production of gene products, i.e. proteins) that gives a cell its unique qualities. Testing the function of putative genes is called functional genomics.

One way biologists study cellular processes is to alter the gene expression of cells. Let's say we are interested in how a pathogenic bacterium is capable of invading a host cell. What types of proteins (structural, enzymatic) are necessary for the process to occur? How many different proteins are involved? What is the role of each protein? One way to study the roles of various gene products involved in invasion is to see what happens when certain genes are not expressed. In other words, we could look at **mutant** forms of the bacteria in which a specific gene has been inactivated, and ask the question, how does the disruption of this gene affect invasion?

You are going to do something similar during this course. During the next three weeks, you will use PCR and recombinant DNA techniques to clone a DNA fragment that will be used to make a mutation in one predicted gene of the *Agrobacterium tumefaciens* C58 genome. Previous students helped determine the genomic sequence of this bacterial strain (Goodner et al., 2001) and now you will help test some of their functional predictions. Once you have verified that you have cloned an internal portion of your gene of interest, we will use that clone to generate a mutation back in *A. tumefaciens*. Amazingly, the bacterium will do the work for us through a process called homologous recombination. If two DNA molecules carry the same sequence, even if it is only a few hundred base pairs, then those two molecules can physically exchange segments through the shared sequence via a cut-and-paste mechanism that involves specific proteins. You have seen homologous recombination before - during prophase I of meiosis, but it also occurs to differing levels in various eukaryotic and prokaryotic microbes. Good enough, you say, but how does exchanging segments of identical DNA sequence lead to the generation of a mutation in a specific gene? Here is where the fact that you cloned an internal portion of the gene of interest comes into play. The plasmid you will use in your cloning cannot replicate in *A. tumefaciens*. Therefore, if you introduce your plasmid clone into *A. tumefaciens* (we will use an electric voltage to induce the cells to take up DNA), the only way the plasmid can stay around is if it becomes part of another DNA molecule that can replicate such as one of the chromosomes. The perfect sequence homology between the internal portion of the gene you cloned and the corresponding segment from the entire gene provides a site for homologous recombination to take place (Figure 7). Don't rely on your mind's eye, rather use your finger or a pencil to walk around the recombination event. Starting on the chromosome, you move right into the gene of interest, cross over into the plasmid clone, hit a breakpoint in the gene, go all the way around the plasmid clockwise, then reenter the gene of interest part way down its length and on to the rest of the chromosome. The plasmid has become integrated into the chromosome, but the gene of interest is disrupted into two nonfunctional parts. This is a rare event, but we can select for those rare events using the ampicillin/carbenicillin resistance gene carried on

the plasmid. Cells that did not take up the plasmid or took it up but did not recombine it into the chromosome will die in the presence of carbenicillin.

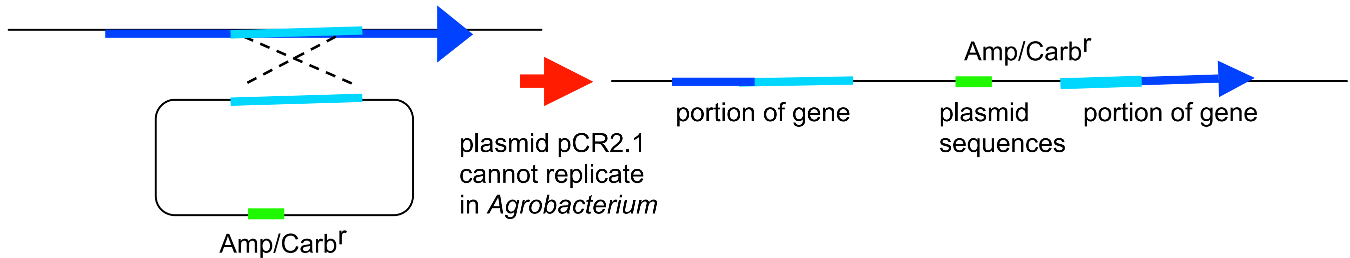

Figure 7. Schematic diagram of the construction of a gene disruption. Amp/Carb^r represents a gene conferring resistance to β-lactam antibiotics such as ampicillin and carbenicillin.

Once you have cloned a portion of a gene of interest and used it to generate a gene disruption mutation in that gene within *A. tumefaciens*, your next job later in the semester will be to use that mutant strain to determine the function of the protein encoded by the gene of interest. In other words, how has the biology of *A. tumefaciens* been affected by its inability to produce a specific protein? You will work in small groups to design an experiment or set of experiments to test your mutant strain (usually with wildtype as a control) and determine whether or not gene function has been truly knocked out. These experiments do not have to come out of thin air. Why not make use of information gleaned by other scientists studying proteins like yours in other organisms. Where do you find such information? Journal articles in the primary scientific literature. In addition to knowledge from work done in other organisms, you can and should make use of what we know about the biology of *A. tumefaciens*

A. tumefaciens is a Gram$^-$ bacterial species found in temperate soils worldwide. It was identified at the turn of the century as the causal agent of a tumor-like disease, called crown gall, on many plants (Smith & Townsend, 1907). It was later found that the bacterial cells did not have to be around all the time in order for the disease to occur. If the bacterial cells were allowed to infect a wound on the plant for a few hours, one could kill the bacterial cells and a tumor would still form weeks later at the wound site (Braun, 1958). The bacteria had somehow transformed the plant cells at the wound site! What was the transforming principle? (Where have we heard that before and what did it mean then?). In 1977, it was finally proven that *A. tumefaciens* transfers a piece of DNA from itself into a plant cell and it is the expression of genes within the transferred DNA that leads to the disease state (Figure 8) (Chilton et al., 1977). We know of no other example of such a virulence mechanism. The transformed plant cells grow out of control and produce lots of nutrients that the bacterium parasitizes. Lots of work has been done on how *A. tumefaciens* transfers DNA into plant cells, but there is still much to learn about other parts of the interaction with plants and other aspects of the life of this soil bacterium. Here is your chance to make a significant contribution.

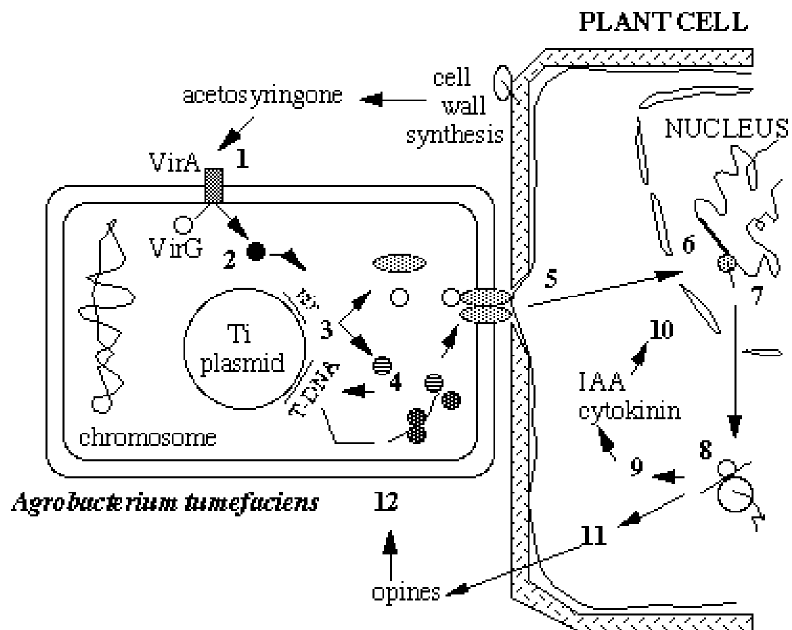

Figure 8. Schematic model of the pathogenic interaction between *Agrobacterium tumefaciens* and a plant cell in a wound site. Steps: 1, the bacterium detects compounds released by the plant cells in the wound; 2, a transcription factor, VirG, is activated; 3, VirG turns on the transcription of several virulence genes; 4, the virulence gene products help replicate a short piece of the plasmid DNA (T-DNA) and transport it into the plant cell; 5, the T-DNA is integrated into one of the plant cell's chromosome; 6, genes on the T-DNA are transcribed; 7, T-DNA transcripts are translated; 8, T-DNA gene products catalyze the synthesis of plant growth hormones; 9, T-DNA gene products catalyze the synthesis of strange compounds that the bacterium can use as food.

Methods

Genomic DNA isolation: Each pair of students will isolate total genomic DNA from an overnight culture (in LB broth) of *A. tumefaciens* strain C58. Many protocols will work here, but we use the DNAEasy Tissue Extraction kit from QIAGEN.

PCR amplification of gene of interest:

1. Each group of students will be assigned a particular gene of *A. tumefaciens* C58. A pair of PCR primers has been designed for each gene to amplify out an internal portion of the gene. Obtain the primers for your gene of interest and set up the following reaction in a PCR thermocycler-compatible tube. Be sure to add the reagents in the specified order and to put all the reagents into the bottom of the tube.
   a. 1 Ready-to-Go PCR Bead (purchased from Pharmacia)
   b. 22 µl sterile distilled water
   c. 1 µl primer #1
   d. 1 µl primer #2
   e. 1 µl *Agrobacterium* genomic DNA

Adding the reagents in the order above helps prevent cross-contamination of primers with target DNA, a crucial weakness for a technique as powerful as PCR. Place your tube on ice until all groups are ready to go.

2. With the instructor's help (this may have been done for you), set up the PCR thermocycler for the following run and carry it out for all the PCR reactions set up in the class:
   - 1 cycle of 94°C for 3 minutes
   - 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute
   - 1 cycle of 72°C for 5 minutes
   - hold at 4°C until ready for use

Gel electrophoresis (Standard slab agarose gel electrophoresis)

Confirm amplification of the target DNA by mixing 5 µl of the PCR reaction mixture with 2 µl of loading dye (blue juice), loading it onto a 1% agarose gel, and running the gel at 70V for 1-2 hours.

Ligation of PCR product to prepared vector

1. To a sterile microcentrifuge tube, add:
   - 4 µl from remaining PCR reaction mixture
   - 1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$)
   - 1 µl pCR2.1TOPO plasmid vector (purchased from Invitrogen)
2. Mix the solution very gently by moving your pipettor tip around, not by pipetting up and down.
3. Incubate at room temperature for 30 minutes.

Transformation

1. Thaw out a tube of competent *E. coli* cells on top of ice (not in the ice), then place the tube into the ice.
2. Add 3 µl of ligation mixture to competent cells and mix very gently by moving your pipettor tip around, not by pipetting up and down.
3. Incubate transformation mixture on ice for 15 minutes.
4. Walk your ice bucket over to the heat block set at 42°C. Transfer your tube from the ice to the heat block for exactly 30 seconds and then place the tube back in the ice for 2 minutes.
5. Add 250 µl SOC broth medium to the tube and shake the tube at 37°C for 1 hour.
6. Obtain 2 LBAmpXgal plates and label them on the bottom with your initials and the date.
7. Pipette 50 µl onto one plate and the remainder of the transformation mix onto the other plate. Sterilize a hockey stick spreader and spread the liquid evenly over each plate surface.
8. Incubate the plates at 37°C overnight.
9. The day before the next lab period, find 5 well-isolated large white colonies (they have an insert in the plasmid). For each colony, use a sterile toothpick to pick up a portion of the colony and inoculate a tube of LB broth containing 50 µg/ml ampicillin (already prepared on a rack in the refrigerator). Incubate the tubes in the 37°C incubator.

Isolation of plasmid DNA

Many protocols will work here, but we use the QIASpin Plasmid DNA Isolation kit from QIAGEN. Each group of students will isolate plasmid DNA from each of the 5 transformants they inoculated into culture. Be sure to elute or resuspend each isolated plasmid DNA sample in sterile water (important for use in electroporation later).

Restriction endonuclease digestion of plasmid DNA samples

Students need to set up EcoRI digestions of each isolated plasmid DNA sample and check these digestions by agarose gel electrophoresis to confirm the presence of a PCR insert in the plasmid clones.

Electroporation of plasmid DNA into electrocompetent A. tumefaciens C58

1. Thaw out a tube of electrocompetent *A. tumefaciens* C58 cells on top of ice (not in the ice) until liquidy, then place the tube into the ice.
2. Add 3 ul of purified plasmid DNA (from a clone confirmed to contain a PCR insert) to the competent cells, then transfer the entire mixture to an electroporation cuvette with a 0.1 cm gap.
3. Transform the competent cells by electroporation (Charles et al., 1994; McCormac et al., 1998).
4. Quickly add 1 ml of MG/L broth to the electroporation cuvette to recover the transformed cells, then transfer the mixture to a sterile test tube. Incubate the tube at 25°C with shaking for 1-3 hours.
5. Obtain 2 LBCarb plates and label them on the bottom with your initials and the date.
6. Pipette 100 μl onto one plate. Spin down the remainder of the transformation mixture, pour most of the liquid into an autoclave bag, resuspend the cell pellet in the remaining liquid, and pipette the remainder of the transformation mix onto the other plate. Sterilize a hockey stick spreader and spread the liquid evenly over each plate surface.
7. Incubate the plates at 25°C for 4-7 days until tranformants appear.
8. Verify the carbenicillin resistance of 2-3 transformants by streaking onto fresh LBCarb plates. Once cultures grow up, they can be used for student experiments and for the preparation of glycerol permanents for long-term storage.

Student-driven experimentation with A. tumefaciens C58 gene disruption mutants

1. You and your partners need to spend some time thinking about how to test the putative function of your gene of interest. Don't reinvent the wheel – look in the primary research literature and find out others have tested the function of this gene or related genes in other organisms.
2. Develop a basic experimental design and a list of the materials you need. These need to be approved by your instructor.
3. You and your partners will have 1-2 weeks to carry out your experiments and report on them to the class.

Notes to Instructors

This project can be done as written as two separate units with 3-4 weeks for the PCR and cloning and later 2-3 weeks for the student-driven experiments on their constructed mutants. A proposed timeline for the project goes as follows.

Week 1: During lab: isolation of genome DNA, PCR amplification of gene of interest
Outside lab: gel electrophoresis of PCR samples

Week 2: During lab: ligation of PCR products into prepared vector, transformation into *E. coli*

Week 3: Outside lab: pick transformants and inoculate cultures
During lab: isolation of plasmid DNA, restriction endonuclease digestion
Outside lab: gel electrophoresis of restriction digestions

Week 4: During lab: electroporation of plasmid clone into *A. tumefaciens* C58

Weeks 9-11: Outside lab: students search literature and brainstorm on possible experiments, develop experimental design and list of needed materials
During lab: students carry out experiments to test putative function of gene of interest

Over the past few years, students in the molecular & cellular biology course at Hiram College have studied the function of over 20 different genes in *A. tumefaciens* C58 using this project strategy. Students have used a wide variety of experiments: enzyme assays (e.g., measuring breakdown of hydrogen peroxide by catalase), growth curves and assays (e.g., testing nitrate reductase, nitrite reductase, and nitric oxide reductase mutants for growth with nitrate as a sole N source and for growth anaerobically with nitrate as electron acceptor), and motility and chemotaxis assays. In most cases, groups had enough time to run an experiment, then make adjustments and redo the experiment or do a followup experiment based on the results of the first experiment. Several students requested the opportunity to continue working on "their" mutant after the course through independent research. There are several final things to note about this project strategy:

- It can be used on a wide variety of genes, as long as there is some possible direct or indirect assay for the function of interest.
- It can be used on a wide variety of organisms or for comparative studies between organisms.
- It can take place after a complete genome sequence becomes available or can occur earlier (once the sequence of the gene of interest becomes available).
- It works great for genes with predicted functions, but it can also be used for hypothetical genes. For example, a common set of growth assays and microscopic studies could be run to categorize the functional role (e.g., cell division, building block biosynthesis, not essential) of each hypothetical gene.
- If one picks the right plasmid vector and cloning strategy, then the generation of the gene disruption mutant can simultaneously generate a *lac*Z fusion that allows for gene expression studies as well with the mutant strain.
- Once a mutant has been made, the possible follow-up studies are numerous and not limited to just one course.

Materials & Equipment

- Genomic DNA Source - Overnight culture of *Agrobacterium tumefaciens* C58.
- Genomic DNA Isolation Reagents – There are many protocols for isolating total bacterial DNA. We use a commercial kit that is very fast, convenient, and without dangerous chemicals (DNAEasy Tissue Extraction Kit purchased from QIAGEN).
- PCR reagents - To control contamination and conserve reagents, we use Ready-to-Go PCR Beads purchased from Pharmacia. We design primers using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/) and order primers from Invitrogen.
- Cloning Vector – We use plasmid pCR2.1TOPO, purchased from Invitrogen, that comes previously cut in order to have a 3' overhang of a single T residue on each end. Topoisomerase I from vaccinia virus, which has ligase activity, has already been added to the cut plasmid. pCR2.1 carries a ampicillin/carbenicillin resistance gene and the cloning site is situated within a lacZ gene to allow for blue-white screening.
- Competent *E. coli* cells (any genotype that will allow for blue-white screening; we use TOP10 cells from Invitrogen)
- LB + Ampicillin (50 μg/ml) + Xgal plates (may have to add IPTG if *E. coli* strain is $lacI^+$) (see Appendix)
- LB + Ampicillin (50 μg/ml) broth (see Appendix)
- Plasmid Isolation Reagents – There are many protocols for plasmid DNA isolation. We use the QIASpin MiniPrep Plasmid Isolation Kit purchased from QIAGEN.
- Restriction Enzymes
- Agarose Gel Electrophoresis
- Electrocompetent *A. tumefaciens* C58 cells (made per instructions in Charles et al., 1994 or McCormac et al., 1998)
- Electroporator
- MG/L broth (see Appendix)
- LB + Carbenicillin (20 μg/ml) plates (see Appendix)
- Materials and protocols requested by students for their independent experiments to test their mutant strains.

Project 3. Functional Genomics (Gene Expression)

Student Outline

Introduction

You know from our discussions in class and our previous lab on the regulation of the lac operon in *E. coli* that the availability of useful β-galactosides, such as ONPG, X-gal, and IPTG, as well as ease of the β-galactosidase enzyme assay make *lac*Z a great gene to study. Yet, most genes we want to study do not have such great tools ready for use. How can we determine when such genes are expressed? We could isolate total RNA at different times or under different conditions, separate the various RNAs on a gel, blot the gel, and hybridize the blot with a DNA strand from the gene of interest. The amount of DNA-RNA hybrid would indicate the level of gene expression (this is called a Northern blot). Unfortunately, this takes a lot of work. Luckily, there is an alternative and it makes use of *lac*Z itself (or one of a small set of other genes for which there are easy enzyme assays). It is called a reporter gene fusion. If we place a promoterless *lac*Z gene in the proper orientation behind the promoter of the gene of interest, then β-galactosidase will be made at the time that our gene is normally turned on!

There are two types of reporter gene fusions. In translational fusions, the promoterless *lac*Z ORF is not only in the same orientation as the gene of interest but it is also in frame with the gene of interest with no intervening stop codon. The protein made is therefore a chimera encoded by a fusion of two genes. β-galactosidase will tolerate large additions to its amino end without affecting its activity. The extra advantage of a translational fusion is that β-galactosidase is not only made when the protein of interest is made, but it is subject to many of the protein-level regulatory mechanisms that might impact the protein of interest (e.g., protein half-life, cellular localization). However, the big problem is the precision required to generate a translational fusion – *lac*Z must be in frame with the gene of interest. In the other type of reporter gene fusion, called transcriptional fusions, the promoterless *lac*Z ORF is inserted in the same orientation as the gene of interest, but it carries its own ribosome binding site and stop codons in all three frames just upstream of the *lac*Z start codon. In this way, transcription of the gene of interest makes an artificial operon – multiple ORFs on one mRNA. The *lac*Z ORF is then translated on its own. Transcriptional fusions are much easier to make, just get the *lac*Z ORF in the same orientation within the gene of interest or just behind it.

In this project, you and your partners will be given a *lac*Z transcriptional fusion derivative of *Agrobacterium tumefaciens* C58. For some groups, the *lac*Z fusion is in a known gene (e.g., *vir*G). In other cases, the *lac*Z fusion was inserted via a transposon insertion and all we know is that the resulting fusion does generate some β-galactosidase activity. Either way, your job will be to carry out an experiment to determine the expression profile of your assigned gene fusion. Before you can carry out your experiment, you need to find out what you can about the gene you are studying, brainstorm on how best to study its expression, and prepare the necessary materials for the experiment. We will provide all the basic components of the assay and standard growth media for *A. tumefaciens*, but you need to let us know what specialty items you want to use. IF we have the necessary components, you can make your specialty items and have them ready in time for the actual experiment.

While the basics of the assay remain the same for *A. tumefaciens* as you have previously used for *E. coli*, there are some key changes that reflect the biology of *A. tumefaciens*. The modified assay listed below is based on that of Stachel et al. (1985) as organized by Gelvin and Karcher (1996).

Methods

Modified β-galactosidase assay:

1. Regrow cells: You will be given a plate culture of your *A. tumefaciens* C58 *lac*Z fusion strain. Two days before your assay, start up a 2 ml overnight culture of your strain in LB broth + kanamycin (40 ug/ml), and incubate overnight at 25C with shaking.
2. Prepare and inoculate experimental conditions: You and your partners will try 6 conditions, so the day before your assay you should label 6 sterile test tubes accordingly for your conditions. Then prepare 2 ml of the appropriate medium for each test tube. Vortex the overnight culture of your *Agrobacterium lac*Z fusion strain to insure a homogenous culture, then inoculate each test tube with 20 ul of the overnight culture and incubate at 25C (or experimental temperature if you wish) overnight with shaking.
3. Pellet cells for assay: Label 6 microcentrifuge tubes to match your 6 experimental conditions. Pipet 1 ml of the appropriate culture into each microcentrifuge tube, spin down for 1 minute, pour off the supernatant, and resuspend each pellet in 1 ml of Z buffer. Transfer the resuspended cultures to fresh test tubes containing 3 ml of additional Z buffer (total of 4 ml).
4. Adjust and record OD_{600}: Blank a spectrophotometer at 600 nm with Z buffer as a blank. Read each tube, and adjust with additional Z buffer (mixing each time) until OD_{600} is between 0.1 and 0.25. Write this final reading down for each culture.
5. Start assay: Transfer 2 ml of each adjusted culture and transfer to a fresh test tube. Add 2 drops of 0.1% SDS and 4 drops of chloroform to each tube, then vortex for 3 seconds. Incubate the tubes in 30C waterbath for 10 minutes, then add 400 ul of ONPG stock (4 mg/ml in Z buffer) to each tube, vortex for 3 seconds, return to 30C waterbath, and start timing the assay.
6. Monitor and terminate reaction: Check your assay tubes every 1-2 minutes for a yellow color (same as last week). If a tube shows a noticeable yellow color, then terminate the reaction in that tube by adding 1 ml of 1M sodium carbonate and vortexing for 3 seconds, then note the time. If any tubes have not turned yellow in 30 minutes, then terminate the reaction and note the time.
7. Redo assay if necessary: If the color developed within several seconds for any of the assay tubes, that is too fast to be accurately measured. In that case, go back to the adjusted culture, dilute it by 1/5, and redo that assay starting at step 5.
8. Measure β-galactosidase activity and scatter from cell debris: Blank a spectrophotometer at 420 nm using a tube set up exactly like your assay tubes, minus any culture (just use Z buffer), as a blank. Read each of your assay tubes. Then, repeat the blank and read process at 550 nm.
9. Calculate β-galactosidase specific activity: For each assay, use the following equation to calculate the specific ß-galactosidase activity.

$$\text{Specific activity (in modified Miller units)} = \frac{1000 \times [OD_{420} - (1.75 \times OD_{550})]}{\text{time (in minutes)} \times OD_{600}}$$

Notes to Instructors

This project can be run for 1-2 weeks depending on how much freedom you wish to give students to follow up their initial experiments. At Hiram College, we do this project directly after a 1-week lab on regulation of the *lac* operon in *E. coli*. The transition to using *lac*Z as a reporter gene is very easy for the students. Over the past 3 years, we have tested various changes in environmental conditions (e.g., salt concentration) on the induction of virG by acetosyringone at pH 5.5, and have tested several random Tn5*lac*Z1 insertions. Some of these gene fusions have shown temperature regulation (i.e., gene expression was greater at lower temperatures than at the optimal growth temperature, while most of these fusions have shown fairly constitutive expression across a range of environmental conditions.

(1) You could precede this lab by having the students themselves generate the Tn5*lac*Z1 random transposon insertions or these can be generated by the instructor well ahead of time. After mobilization of the transposon into *A. tumefaciens* C58, we select for Kan^r colonies on an appropriate medium of interest containing X-gal (40 *m*g/ml).

(2) There are many other *lac*Z fusions in known genes of *A. tumefaciens* C58. You can find some by literature search and request the appropriate strain from the authors.

Materials & Equipment

- *A. tumefaciens* C58 derivatives:
  - C58[*vir*G::*lac*Z translational fusion; Kan^r] (Kalogeraki & Winans, 1997)
  - C58[*pic*A::lacZ transcriptional fusion; Kan^r] (Rong et al., 1990)
  - C58[Unknown *lac*Z fusions due to Tn*5lac*Z1 insertions; Kan^r] (Wheeler & Goodner, unpublished)
- LB broth (see Appendix)
- M9 salts pH 7 (see Appendix)
- M9 salts pH 5.5 (see Appendix)
- Various sugars as C sources: 20% stocks, filter sterilized
- Acetosyringone (inducer of *A. tumefaciens* vir genes): 100mM stock in dimethylformamide
- P Buffer (see Appendix)
- Z buffer: 50 mM ß-mercaptoethanol in P Buffer
- SDS: 0.1% stock
- Chloroform
- ONPG: 4 mg/ml *ortho*-nitrophenyl-ß-D-galactoside, in P buffer (made fresh)
- Sodium carbonate: 1 M Na_2CO_3 stock
- Sterile and nonsterile glass test tubes
- Test tube racks
- Sterile microcentrifuge tubes
- Microcentrifuge
- Spectrophotometer (visible range is all that is needed; we use a digital Spectronic20)

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Appendix

This appendix provides recipes for the media and solutions needed for the 3 projects described in this chapter.

(1) LB broth (per liter): 10 g tryptone, 5 g yeast extract, and 5 g NaCl, then autoclave.

(2) LB + 0.5 M NaCl (per liter): 10 g tryptone, 5 g yeast extract, and 29.2 g NaCl, then autoclave.

(3) LB agar for plates (per liter): Same as LB broth with 15 g agar added, then autoclave. Add antibiotics, if needed, after autoclaving and mix before pouring plates.

(4) LB + 0.5 M NaCl agar for plates (per liter): Same as LB + 0.5 M NaCl broth with 15 g agar added, then autoclave. Add antibiotics, if needed, after autoclaving and mix before pouring plates.

(5) M9 + 0.5 M NaCl minimal agar for plates (per liter): Part A = 6 g sodium phosphate dibasic, 3 g potassium phosphate monobasic, 29.2 g NaCl, and 1 g ammonium chloride in 500 ml water. Part B = 2 g glucose and 15 g agar in 500 ml water. After autoclaving, add part A to part B plus 1 ml of sterile 1 M magnesium sulfate and 0.1 ml sterile 1 M calcium chloride. Add antibiotics, if needed, after autoclaving and mix before pouring plates.

(6) SOC (per liter): 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, and 10 ml 250 mM KCl, then pH to 7 and autoclave. After autoclaving, add 20 ml of sterile 1 M glucose and 5 ml of sterile 2 M magnesium chloride.

(7) 1000X kanamycin stock: 50 mg kanamycin sulfate per ml water. Filter sterilize and store frozen, then add to media as needed at 1 μl per ml of medium.

(8) 1000X carbenicillin stock: 50 mg carbenicillin, sodium salt, per ml water. Filter sterilize and store frozen, then add to media as needed at 1 μl per ml of medium.

(9) 1000X rifampicin stock: 20 mg rifampicin per ml methanol. Filter sterilize and store frozen in the dark, then add to media as needed at 1 μl per ml of medium.

(10) "Salty" cell suspension buffer:10 mM Tris, 0.5 M NaCl, 50 mM EDTA, pH 7.2

(11) ES buffer: 100mM EDTA, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, pH 8

(12) Wash buffer: 20mM Tris, 50mM EDTA, pH 8

(13) MG/L (per liter): To 500 ml LB, add 10 g mannitol, 2.32 g sodium glutamate, 0.5 g KH_2PO_4, 0.2 g NaCl, 0.2 g $MgSO_4$, and 480 mL dH_20. Adjust pH to 7.0, then adjust final volume to 1 liter and autoclave.