Genetic and Physical Mapping of a Bacterial Genome

Resource Type: Curriculum: Laboratory

Publication Date: 1/5/2005

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Abstract

In this three-component project, students (i) use a transposon to generate mutants that they screen for auxotrophs and characterize through biochemical complementation, (ii) use rare cutting restriction enzymes and pulsed-field gel electrophoresis to physically map the transposon insertions, and (iii) recover the transposon insertions for restriction mapping and subsequent sequencing in order to generate a genetic and physical map of a bacterial chromosome. The focus is on student involvement in original research.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

INTRODUCTION

Learning Objectives.
By completing component 1 of the project, students will understand how a genome project works and where a genetic and physical map of a chromosome fits into the project, how transposons can be used to generate mutations, the nature of auxotrophy and usefulness of biochemical complementation, and how to use bacterial conjugation, transposon mutagenesis, genetic selections, and screens to search for auxotrophic mutants. By completing component 2 of the project, students will understand how the introduction of a new restriction enzyme cut site via an engineered transposon can be used to map a gene, what is different about electrophoresis of very large DNA molecules and how this impacts DNA isolation techniques and electrophoresis conditions, how to construct and use a standard curve for gel electrophoresis results, and how to make and use genomic DNA plugs for pulsed-field gel electrophoresis. By completing component 3 of the project, students will understand how an engineered transposon can allow one to recover the transposon insertion site easily; how isolation of total genomic DNA differs from isolation of plasmid DNA; the connection between a DNA sequence, a restriction map, and a DNA molecule; the basics of recombinant DNA cloning; and how to use restriction enzymes, gel electrophoresis, DNA isolation, DNA ligation, and transformation to isolate and characterize a plasmid.

Background.
For component 1 of the project, 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 making dilutions are all that are needed. For component 2 of the project, 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. For component 3 of the project, students need the same basic background and skills as for component 2 plus some understanding of how plasmids differ from chromosomes.

PROCEDURE

Materials.

For component 1 of the project:

- *Chromohalobacter salexigens* DSM3043 (7), rifampicin-resistant derivative available from authors or can be selected from wildtype stock
- *Escherichia coli* BW20767/pRL27 (5, 6)
- 0.5 M NaCl
- Luria broth (LB) and LB + 0.5 M NaCl broth (see recipes)
- LB + 0.5 M NaCl plates (see recipes)
- LB + 0.5 M NaCl + kanamycin (50 µg/ml) + rifampicin (20 µg/ml) plates (see recipes)
- M9 minimal glucose + 0.5 M NaCl + kanamycin (50 µg/ml) + rifampicin (20 µg/ml) plates (see recipes)
- Stocks and pools of essential building blocks and pathway intermediates (2); chemicals purchased from Sigma-Aldrich
- Sterile toothpicks
- Microbiological loops
- Pipettes
- Micropippetors and sterile tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Sterile test tubes
- Ethanol and "hockey stick" spreader

For component 2 of the project:

- Agarose (We use pulsed-field certified agarose, catalog #162-0137, purchased from Bio-Rad.)
- "Salty" cell suspension buffer (see recipe)
- Pronase E (catalog #P6911 purchased from Sigma-Aldrich)
- ES buffer (see recipe)
- Wash buffer (see recipe)
- PacI and PmeI restriction endonucleases (purchased from New England Biolabs; see possible modifications for further information on additional restriction enzymes)
- 0.5X Tris-borate-EDTA (TBE) (see recipe)
- Pulsed-field gel electrophoresis apparatus (We use the CHEF-DR II system, catalog #170-3612, from BioRad. Please note that this is the only costly item in this entire activity; see possible modifications for further information.)
- LB + 0.5M NaCl broth (see recipe)
- Microbiological loops
- Pipettes
- Micropippetors and sterile tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Sterile test tubes

For component 3 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 and 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 possible modifications for further information)
- LB + kanamycin (50 µg/ml) plates (see recipe)
- LB + 0.5 M NaCl broth (see recipe)
- SOC broth (see recipe)
- Microbiological loops
- Sterile toothpicks
- Pipettes
- Micropippetors and sterile tips
- Sterile microcentrifuge tubes
- Microcentrifuge
- Sterile test tubes
- Ethanol and "hockey stick" spreader
- Gel loading buffer (see recipe)
- 1X TBE (see recipe)
- Agarose
- Components for standard slab gel electrophoresis

Student Version.

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 DSM3043 (formerly called *Halomonas elongata* DSM3043)? 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 (7). *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 (Fig. 1). Pumping the sodium back out is one way, but by itself it leads to an osmotic gradient between the inside of the cell and the outside that causes other problems. Halotolerance involves the added mechanism of accumulating compounds, through uptake and/or synthesis, that are more compatible with biochemical functions in order to maintain osmotic balance (1). Halophilic ("salt loving") organisms have evolved even further mechanisms to make use of the inward-directed sodium gradient to drive the uptake of other needed compounds and may even require high osmotic conditions for normal biochemical functions. 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.

FIG. 1. Mechanisms involved in halotolerance and halophilism.

Getting the entire genome sequence of *C. salexigens*, or any other organism, is just the first step, but it is not trivial. The genome has to be broken into manageable-sized (1,000 to 2,000 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 (Fig. 2). The particular transposable element we will employ, Tn5-RL27, has several useful features (5). 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 has 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 does not 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.

FIG. 2. Mutagenesis using a transposon.

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 (Fig. 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. Each auxotroph can be further characterized using nutrient pools and pathway intermediates to give a genetic name to that mutant (Fig. 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 (Fig. 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 hundreds 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 reelongates 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 offsite). This makes use of the fact that the transposon carries two features found in all plasmids, an origin of replication and a selectable marker (Fig. 6).

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

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

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

FIG. 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, 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 1—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, pipette 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 at 1/100, 1/1,000, and 1/10,000 in 0.5 M NaCl, and plate 100-µl portions onto LB + 0.5 M NaCl + kanamycin + rifampicin plates (three plates for each dilution). Incubate the plates for 1 to 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 to 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 2).

Methods Used in Component 2—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 of 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 of "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 harden, transfer plugs from each strain into separate tubes and digest overnight with Pronase E (2 mg/ml) in ES buffer at 50°C (4).

5. Wash plugs four 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 a 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 to 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 3—Isolation and Characterization of Transposon Insertions

Genomic DNA isolation (instructions modified from DNAEasy manual)
1. Transfer 1.5 ml of bacterial culture into a microcentrifuge tube and spin down the cells for 1 minute at maximum speed (16,000 rpm).

2. Discard the culture supernatant by gently pouring it into a biohazard bag (keep the pellet on the high side of the tube to avoid losing cells). Resuspend the cell pellet in 180 µl of Buffer ATL. This is just a standard physiological buffer.

3. Add 20 µl of proteinase K solution and mix by vortexing briefly. Incubate the mixture at 55°C for 15 minutes. Proteinase K helps degrade the bacterial cell wall.

4. Vortex the tube for 15 seconds. Add 200 µl of Buffer AL to the sample and mix thoroughly by vortexing, then incubate the sample at 70°C for 10 minutes. Buffer AL contains guanidine hydrochloride, a chaotropic salt which disrupts membranes and solubilizes lipids and proteins. (If you see a white precipitate after adding Buffer AL, don't worry. Vortex the sample well and the material should dissolve during the incubation at 70°C. The precipitate does not interfere with genomic DNA isolation.)

5. Add 200 µl of ethanol (96% to 100%) to the sample, and mix thoroughly by vortexing. The ethanol helps with the solubilization of cellular materials that will allow the cell lysate to pass through the DNA-binding column easily.

6. Pipette the entire mixture into a DNAEasy spin column placed in a 20-ml collection
tube (make sure you label the top of the spin column). Centrifuge the tube at 8,000 rpm (~6,000 x g) for 1 minute. The spin column contains specially-treated tiny silica beads which will bind nucleic acids preferentially under high salt conditions (which we have here).

7. Discard the flow-through and collection tube, but DO NOT THROW AWAY THE SPIN COLUMN (IT HAS YOUR GENOMIC DNA). Place the spin column in a new 2-ml collection tube, add 500 µl of Buffer AW1, and centrifuge at 8,000 rpm for 1 minute. Buffer AW1 is a wash buffer containing guanidine hydrochloride, ethanol, and high salt to wash away unwanted proteins, etc., while allowing the genomic DNA to stay attached to the column material.

8. Discard the flow-through and collection tube, but DO NOT THROW AWAY THE SPIN COLUMN (IT HAS YOUR GENOMIC DNA). Place the spin column in a new 2-ml collection tube, add 500 µl of Buffer AW2, and centrifuge at 8,000 rpm for 3 minutes. Buffer AW2 is a high salt and ethanol wash buffer without guanidine hydrochloride. The extra spin time ensures that the wash buffer is removed completely before the elution step.

9. Very carefully, discard flow-through and collection tube (you don't want any liquid on the spin column). Place the spin column in a sterile microcentrifuge tube (properly labeled) and pipette 200 µl of Buffer AE directly onto the top surface membrane of the spin column. Buffer AE is a very low salt buffer. Incubate at room temperature for 1 minute, then centrifuge for 1 minute at 8,000 rpm to elute the DNA from the column. NOW YOU WANT TO KEEP THE SOLUTION, SO THROW AWAY ONLY THE SPIN COLUMN AND KEEP THE TUBE CONTAINING THE DNA SOLUTION.

Restriction enzyme digestion of genomic DNA and self-ligation
1. Label a new microcentrifuge tube and add to it 26 µl of genomic DNA, 3 µl of 10X buffer appropriate for SacII, and 1 µl of SacII enzyme (SacII does not cut within Tn5-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 of sterile water, 5 µl of 10X ligation buffer, and 1 µl of 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 and concentration (instructions modified from QIAQuick manual)
1. Prior to starting the transformation protocol, add the ligation mixture to 250 µl of Buffer PB in a sterile microcentrifuge tube and mix. The buffer adds enough salt so that the DNA will bind to the silica resin in the spin column.

2. Place a QIAQuick spin column (properly labeled) in a provided 2-ml collection tube. Apply the sample to the spin column and centrifuge for 30 to 60 seconds at 8,000 rpm.

3. Remove the spin column and hold in one hand, while discarding flow-through from collection tube. Place the spin column back into the same tube. Add 750 µl of Buffer PE (high salt and ethanol wash) to the QIAQuick column and centrifuge for 30 to 60 seconds at 8,000 rpm.

4. Remove the spin column and hold in one hand, while discarding flow-through from collection tube. Place the spin column back into the same tube. Centrifuge the column for 1 minute at 8,000 rpm to remove all residual liquid from the spin column.

5. Place the spin column in a sterile 1.5-ml microcentrifuge tube (properly labeled). Add 20 µl of sterile water to the center of the column membrane, wait for 1 minute, and centrifuge the column for 1 minute at 8,000 rpm. Throw away the spin column, BUT KEEP THE TUBE CONTAINING YOUR DNA SOLUTION.

Transformation (instructions modified from those for Active Motif TAM1 pir+ cells)
1. Thaw out a tube of competent *E. coli* TAM1 lambda pir+ cells on top of ice (not in the ice) until liquidy, then place the tube into the ice.

2. Add 5 µl of your cleaned up ligation mixture to the competent cells and mix very gently by gently tapping tube on bench top 2 to 3 times, not by pipetting up and down. Return cells to ice immediately and incubate on ice for 30 minutes.

3. Take your ice bucket over to the 42°C water bath. Transfer your transformation tube directly from the ice bucket into the water bath for 30 seconds (NO MORE) and then back into the ice for 2 minutes.

4. Add 250 µl of sterile SOC medium to the cells. Incubate the transformed cell mixture at 37°C for 1 hour with shaking.

5. Obtain a LB + kanamycin plate and label it on the bottom with your initials and the date.

6. Pipette 200 µl of the transformation mix onto the plate and spread. Incubate the plate upside down at 37°C overnight.

Isolation of plasmid DNA (instructions modified from QIASpin manual)
1. Find three well-isolated colonies on your transformation plate. For each colony, use a sterile toothpick to pick up a portion of the colony and inoculate a tube of LB + kanamycin broth. Incubate the tubes in the 37°C incubator.

2. For each culture, label a sterile microcentrifuge tube (1 through 3). Transfer the cultures into the microcentrifuge tubes and pellet the cells by spinning the tubes in a microcentrifuge for 1 minute at full speed (16,000 rpm).

3. Pour the supernatant into an autoclave bag. Resuspend each pellet in 250 µl of Buffer P1. Buffer P1 is a weak buffer at physiological pH (7.5). It also contains RNase which will degrade RNAs once the bacterial cells are broken.

4. Add 250 µl of Buffer P2 to each tube and mix by gently inverting the tubes several times. The cell suspension should become viscous. The high pH and sodium dodecyl sulfate (SDS) detergent in this buffer disrupt membranes and bacterial cell walls, and the detergent solubilizes proteins and lipids. The broken pieces of bacterial chromosome will denature at high pH and the strands will float away from each other. The two strands of the intact circular plasmid molecules cannot separate from each other very far (topologically linked).

5. Add 350 µl of Buffer N3 to each tube and mix by gently inverting the tubes several times. The quanidinium salts in Buffer N3 and the lowered pH (back to approximately 7.5) lead the SDS-solubilized material to form insoluble complexes and fall out of solution, accounting for the snowstorm going on in your microcentrifuge tubes. The huge single-stranded pieces of the bacterial chromosome will get caught up in the precipitating complexes, but the much smaller intact plasmid molecules will stay in solution and renature to a double helix conformation.

6. Spin the tubes in a microcentrifuge at full speed for 10 minutes to pellet the unwanted cell debris (proteins, lipids, and chromosome fragments). While tubes are spinning, label five spin columns and place each in a catch tube.

7. Carefully pour each supernatant into a spin column (sitting in a catch tube). The double-stranded plasmid DNA will bind to the tiny glass beads in the spin column under the high salt conditions of Buffer N3, while RNA, ssDNA, proteins, and other cell components will not. Spin in a microcentrifuge at full speed for 30 seconds. Discard the flow-through from the catch tube.

8. Add 500 µl of Buffer PB to each spin column. This high salt wash helps clean away any contaminants from the bound plasmid DNA. Spin in a microcentrifuge at full speed for 30 seconds. Discard the flow-through from the catch tube.

9. Add 750 µl of Buffer PE to each spin column. This high salt and ethanol wash further cleans the bound plasmid DNA. Spin in a microcentrifuge at full speed for 30 seconds. Discard the flow-through from the catch tube.

10. Without adding any additional buffer, spin the tubes in a microcentrifuge at full speed for 1 minute. Throw away the catch tube and any flow-through it contains and place the dried spin column in a labeled sterile microcentrifuge tube.

11. Add 50 µl of Buffer EB to each spin column and let stand for at least 1 minute. The low salt conditions of Buffer EB bump the plasmid DNA off of the glass beads. Spin in a microcentrifuge at full speed for 1 minute. Throw away the spin column and close the microcentrifuge tube. You now have purified plasmid DNA!

Restriction endonuclease digestion of DNA
1. Choose two restriction enzymes for your analysis. For each of your plasmid clones, label two 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 and buffer mix. Incubate all tubes at 37°C for 1 hour.

Gel electrophoresis
1. Add 3 µl of loading buffer to each of your digested DNA samples, mix, and spin down briefly in a microcentrifuge. Loading buffer consists of three components: EDTA, which chelates magnesium, stopping any reaction of DNA with enzymes; glycerol, which makes the density of the sample greater than the salt solution so that the sample will sink to the bottom of the hole; and bromophenol blue, a negatively charged blue dye 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 of practice sample solution 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 which well! After everyone has loaded their samples and DNA standards have been loaded in additional wells, close the gel and turn the electricity 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 is 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.

Instructor Version.
There is no doubt that genomics and bioinformatics have revolutionized biology over the past few years. However, their impact on education has lagged and a new revolution is possible in the undergraduate curriculum. For example, the publication of the complete genome sequence of the bacterial pathogen *Agrobacterium tumefaciens* strain C58 had 11 undergraduates as authors (3) and that work built upon a genetic and physical map of *A. tumefaciens* C58 constructed by eight undergraduates (4). This multiweek lab project builds on this framework and strives to place students in an original research setting within the confines of a course. The project was originally written for students in a genetics course to construct a combined genetic and physical map of the genome of *Chromohalobacter salexigens* strain DSM3043. Please note that this project could also be done in a microbiology, molecular biology, or biotechnology course and that the experimental strategy would apply to a wide range of bacteria. Also note that this project provides a great outreach mechanism for high school students in Advanced Placement

biology courses to collaborate on the research efforts.

The project can be done as written over 6 to 8 weeks of lab with some outside time required, or one or more of the components can be done separately in 2 to 3 weeks each. In this regard, it is most likely that one would choose to do component 1 alone or do components 1 and 3 (skipping component 2 if one did not have access to pulsed-field gel electrophoresis). The methods in the student version involve several commercially available kits, but these could be substituted with kits from other manufacturers or home-grown protocols that accomplish the same tasks. The method section is repeated below *with explanations for instructors in italics* and additional notes and hints at the end of each section.

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. *Liquid cultures can be grown in a temperature-controlled shaker incubator while plate cultures can be grown in a standard incubator. If temperature-controlled conditions are unavailable, growth at room temperature is okay but you should expect longer growth times.*

Methods Used in Component 1—Generation of and Screen for Auxotrophic Mutants

Timeline 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

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. *The wash gets rid of any antibiotics used to maintain selection on the parental strains during growth and resuspending each strain in 0.5 M NaCl is essential to maintaining the viability of C. salexigens during the mating.*

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. *By mixing the strains on the plate, we are allowing conjugation to occur whereby the suicide plasmid vector carrying the transposon can be transferred into C. salexigens cells.*

3. The next day, pipette 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 at 1/100, 1/1,000, and 1/10,000 in 0.5 M NaCl, and plate 100-µl portions onto LB + 0.5 M NaCl + kanamycin + rifampicin plates (three plates for each dilution). Incubate the plates for 1 to 3 days at 37°C until colonies appear. *You can use a variety of sterilized tools to scrape the mating mixture off of the plate: rubber policeman, hockey stick-type spreader, or even a pipette tip. Tilting the plate helps concentrate the scraped mixture for easier resuspension and recovery.*

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 to 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. *Rarely will you see a colony of E. coli break through the double selection.*

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. *You need to reconfirm the putative auxotrophs to weed out false positives due to insufficient transfer of colonies to the minimal medium in the first round of patching.*

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 2). *This is the very biochemical complementation that we discuss with students when we talk about the classic experiments of Beadle and Tatum. For more information on the makeup of these nutrient mixtures and the concentrations used for various nutrients, see note (h) below and Tables 1 and 2. Tables 1 and 2 should be handed out to students to help them understand the results of their pool plates. For example, Table 2 can be used by students to determine the auxotrophy of particular mutants. If a mutant only grows on the minimal pool plates 1 and 6, then that mutant requires adenosine. Table 1 helps students understand that cells have different quantitative requirements for different nutrients and it can be used when students help prepare solutions.*

Notes and hints:

(a) 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.

(b) 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 1. They can repeat the protocols with their own matings on subsequent days.

(c) 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.

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

(e) You might expect one auxotroph for every 50 to 100 colonies tested.

(f) Once you have mutants, permanent stocks can be made and frozen at -80°C. It may be useful to you to have particular mutants available for further research. Grow a 24 to 48 hour 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 of 30% sterile glycerol and vortex. Store frozen at -80°C.

(g) This component can be repeated during several iterations 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.

(h) 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 (2; Tables 1 and 2). 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.

TABLE 1. Stock solutions for building blocks used in the first step of biochemical complementation of auxotrophs[a,b]

Building block	Stock solution (%)
Adenosine	2.67
Alanine	0.84
Arginine	2.53
Asparagine[Fc]	0.84
Aspartate[F]	1.0
Biotin	0.49
Cysteine[F]	0.73
Diaminopimelate (DAP)	0.38
Glutamate[F]	14.6
Glutamine	14.6
Glycine	0.2
Guanosine	1.7
Histidine	0.31
Isoleucine	0.79
Leucine	0.79
Lysine	1.1
Methionine	0.9
Nicotinic acid	0.25
Pantothenate	0.48
Phenylalanine[d]	0.99
Proline	4.6
Pyridoxine HCl	0.41
Serine	8.4
Thiamine	0.34
Threonine	0.71
Thymine	0.81
Tryptophan[F]	0.41
Tyrosine[F]	0.36
Uracil	0.22
Valine	0.7

[a] Table modified from data prepared by Davis, Botstein, and Roth (2).

[b] To supplement a given building block, add 5 ml of the appropriate stock solution to one liter of minimal medium.

[c] The superscript F denotes a stock solution that must be filter sterilized rather than autoclaved.

[d] The phenylalanine stock should be made up in 0.01 N HCl.

TABLE 2. Pool plate compositions for the first step of biochemical complementation of auxotrophs[i]

Pools	1	2	3	4	5
6	Adenosine[a,b,c]	Guanosine[d]	Cysteine	Methionine	Thiamine[b]
7	Histidine[c]	Leucine[e]	Isoleucine[e]	Lysine[g]	Valine[e]
8	Phenylalanine[f]	Tyrosine[f]	Tryptophan[f]	Threonine	Proline
9	Glutamine[h]	Asparagines	Uracil[d]	Aspartate	Arginine[d]
10	Thymine	Serine	Gluatamate	DAP[g]	Glycine
11	Pyridoxine, Nicotinic acid, Biotin, Pantothenate, Alanine				
12	Homoserine, Vitamin B12 (cobamalmin), Myo-inositol				

[a] Some purine auxotrophs can use adenosine or guanosine and can grow on pools 1, 2, and 6.

[b] Some purine auxotrophs require adenosine + thiamine and can grow only on pool 6.

[c] Some purine auxotrophs require adenosine + histidine and can grow only on pool 1.

[d] Auxotrophs with mutations in pyrA require uracil + arginine and can grow only on pool 9.

[e] Auxotrophs with early blocks in the branch chain amino acid pathway can grow only on pool 7.

[f] Auxotrophs with early blocks in the aromatic amino acid pathway can grow only on pool 8.

[g] Auxotrophs with early blocks in the lysine pathway can grow only on pool 4.

[h] It is possible to distinguish low-glutamine-requiring auxotrophs from high-glutamine-requiring ones if pool 9 is made with 4x the normal amount of glutamine stock solution.

[i] Table modified from data prepared by Davis, Botstein, and Roth (2).

Methods Used in Component 2—Physical Mapping of Transposon Insertions

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

Pulsed field gel electrophoresis of intact and digested DNAs

1. Grow wildtype and mutant *C. salexigens* strains in 2 ml of LB or LB + 0.5 M NaCl + kanamycin + rifampicin broth respectively, for 48 hours at 37°C with shaking aeration to ensure that the culture has reached stationary phase. *The cultures have to reach stationary phase in order to have replication events finished and chromosomal DNA intact (no Okazaki fragments).*

2. Transfer a culture to a sterile microcentrifuge tube, pellet the cells, and resuspend them in 400 µl of "salty" cell suspension buffer. *The addition of 0.5 M NaCl to the cell suspension buffer ensures the integrity of the C. salexigens cells until the proper time for cell lysis within the genomic plugs.*

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. *Equilibrating the cell suspension allows one more time to mix in the agarose and fill the plug molds. However, there might be situations (e.g., thermal induction of prophages) that prevent one from using the equilibration before adding the molten agarose.*

4. After plugs have hardened, transfer plugs from each strain into separate tubes and digest overnight with Pronase E (2 mg/ml) in ES buffer at 50°C (4).

5. Wash plugs four times in wash buffer and store plugs in 0.1X wash buffer at 4°C. *Washing involves pouring off the previous solution without pouring out the plugs (the tube cap can be used to retain the plugs), then adding the next wash solution.*

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 a fresh 200 µl of the appropriate restriction buffer with 2 µl of BSA and 4 µl of the appropriate restriction enzyme(s). Digest genomic DNA plugs at the appropriate temperature for that enzyme (5 to 8 hours at 37°C cuts or overnight at 25°C). *As with any restriction enzyme digestion, the enzymes should be kept on ice or in the freezer until needed.*

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. *The gels are made using 1 g of agarose, 50 ml of TBE buffer, and 50 ml of deionized water. Microwaving the solution for 4 to 5 minutes at half power will lead to full melting of the agarose solution without boiling over. The molten agarose solution can be maintained in a 50°C waterbath until needed while the gel bed is prepared and the plugs loaded onto the comb. In this type of gel electrophoresis, the comb simply acts as a support for the gel plug slivers. Using a small spatula, transfer each sliver to the bottom of the comb teeth as the comb sits horizontally. Before setting up the comb vertically, use a Kimwipe to absorb some of the excess liquid from the slivers. Once the comb is set up vertically within the gel bed, pour the molten gel slowly but continuously into one bottom corner of the gel bed.*

Notes and hints:

(a) 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 replicating. 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.

(b) 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.

(c) The enzymes we used to cut the *C. salexigens* genome for pulsed-field gel electrophoresis are PacI and PmeI. PacI cuts within the transposon but does not cut within the genome. PmeI cuts the genome three times. Hence the wild type *C. salexigens* will show three bands on the pulsed-field gel and each transposon mutant will show four bands, although the pattern will be unique depending on the site of transposon insertion.

(d) We typically use *Saccharomyces cerevisiae* chromosomes as linear DNA standards for pulsed-field gel electrophoresis because they span the range from 200 to 1,900 kbp and can be purchased already in agarose plugs (catalog #170-3605 from BioRad or catalog #N0345S from New England Biolabs).

Methods Used in Component 3—Isolation and Characterization of Transposon Insertions

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

Genomic DNA isolation (instructions modified from DNAEasy manual)

1. Transfer 1.5 ml of bacterial culture into a microcentrifuge tube and spin down the cells for 1 minute at maximum speed (16,000 rpm). *The bacterial cultures can be started up to 1 to 2 days before this procedure.*

2. Discard the culture supernatant by gently pouring it into a biohazard bag (keep the pellet on the high side of the tube to avoid losing cells). Resuspend the cell pellet in 180 µl of Buffer ATL. This is just a standard physiological buffer provided in the kit.

3. Add 20 µl of proteinase K solution (provided in the kit) and mix by vortexing briefly. Incubate the mixture at 55°C for 15 minutes. Proteinase K helps degrade the bacterial cell wall.

4. Vortex the tube for 15 seconds. Add 200 µl of Buffer AL (provided in the kit) to the sample and mix thoroughly by vortexing, then incubate the sample at 70°C for 10 minutes. Buffer AL contains guanidine hydrochloride, a chaotropic salt which disrupts membranes and solubilizes lipids and proteins. (If you see a white precipitate after adding Buffer AL, don't worry. Vortex the sample well and the material should dissolve during the incubation at 70°C. The precipitate does not interfere with genomic DNA isolation).

5. Add 200 µl of ethanol (96% to 100%) to the sample, and mix thoroughly by vortexing. The ethanol helps with the solubilization of cellular materials that will allow the cell lysate to pass through the DNA-binding column easily.

6. Pipette the entire mixture into a DNAEasy spin column placed in a 2-ml collection tube (provided in the kit; make sure you label the top of the spin column). Centrifuge the tube at 8,000 rpm (~6,000 x g) for 1 minute. The spin column contains specially-treated tiny silica beads which will bind nucleic acids preferentially under high salt conditions (which we have here).

7. Discard the flow-through and collection tube, but DO NOT THROW AWAY THE SPIN COLUMN (IT HAS YOUR GENOMIC DNA). Place the spin column in a new 2-ml collection tube, add 500 µl of Buffer AW1 (provided in the kit), and centrifuge at 8,000 rpm for 1 minute. Buffer AW1 is a wash buffer containing guanidine hydrochloride, ethanol, and high salt to wash away unwanted proteins, etc., while allowing the genomic DNA to stay attached to the column material.

8. Discard the flow-through and collection tube, but DO NOT THROW AWAY THE SPIN COLUMN (IT HAS YOUR GENOMIC DNA). Place the spin column in a new 2-ml collection tube, add 500 µl of Buffer AW2 (provided in the kit), and centrifuge at 8,000 rpm for 3 minutes. Buffer AW2 is a

high salt and ethanol wash buffer without guanidine hydrochloride. The extra spin time ensures that the wash buffer is removed completely before the elution step.

9. Very carefully, discard flow-through and collection tube (you don't want any liquid on the spin column). Place the spin column in a sterile microcentrifuge tube (properly labeled) and pipette 200 µl of Buffer AE (provided in the kit) directly onto the top surface membrane of the spin column. Buffer AE is a very low salt buffer. Incubate at room temperature for 1 minute, then centrifuge for 1 minute at 8,000 rpm to elute the DNA from the column. NOW YOU WANT TO KEEP THE SOLUTION, SO THROW AWAY ONLY THE SPIN COLUMN AND KEEP THE TUBE CONTAINING THE DNA SOLUTION.

Restriction enzyme digestion of genomic DNA and self-ligation

1. Label a new microcentrifuge tube and add to it 26 µl of genomic DNA, 3 µl of 10X buffer appropriate for SacII, and 1 µl of SacII enzyme (SacII does not cut within Tn5-RL27, but it does cut the *H. elongata* genome quite often). Incubate the digestion at 37°C for at least 3 hours. *As with any restriction enzyme digestion, the enzymes should be kept on ice or in the freezer until needed.*

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 of sterile water, 5 µl of 10X ligation buffer, and 1 µl of 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. *The dilution of the digested genomic DNA helps ensure that self-ligation is more probable than ligation of two fragments together.*

DNA purification and concentration (instructions modified from QIAQuick manual)

1. Prior to starting the transformation protocol, add the ligation mixture to 250 µl of Buffer PB (provided in the kit) in a sterile microcentrifuge tube and mix. The buffer adds enough salt so that the DNA will bind to the silica resin in the spin column.

2. Place a QIAQuick spin column (provided in the kit; be sure to properly label the spin column) in a provided 2-ml collection tube. Apply the sample to the spin column and centrifuge for 30 to 60 seconds at 8,000 rpm.

3. Remove the spin column and hold in one hand, while discarding flow-through from collection tube. Place the spin column back into the same tube. Add 750 µl of Buffer PE (provided in the kit; a high salt and ethanol wash) to the QIAQuick column and centrifuge for 30 to 60 seconds at 8,000 rpm.

4. Remove the spin column and hold in one hand, while discarding flow-through from collection tube. Place the spin column back into the same tube. Centrifuge the column for 1 minute at 8,000 rpm to remove all residual liquid from the spin column.

5. Place the spin column in a sterile 1.5-ml microcentrifuge tube (properly labeled). Add 20 µl of sterile water to the center of the column membrane, wait for 1 minute, and centrifuge the column for 1 minute at 8,000 rpm. Throw away the spin column, BUT KEEP THE TUBE CONTAINING YOUR DNA SOLUTION.

Transformation (instructions modified from those for Active Motif TAM1 pir+ cells)

1. Thaw out a tube of competent *E. coli* TAM1 lambda pir+ cells on top of ice (not in the ice) until liquidy, then place the tube into the ice.

2. Add 5 µl of your cleaned-up ligation mixture to the competent cells and mix very gently by gently tapping tube on bench top 2 to 3 times, not by pipetting up and down. Return cells to ice immediately and incubate on ice for 30 minutes.

3. Take your ice bucket over to the 42°C water bath. Transfer your transformation tube directly from the ice bucket into the water bath for 30 seconds (NO MORE) and then back into the ice for 2 minutes.

4. Add 250 µl of sterile SOC medium to the cells. Incubate the transformed cell mixture at 37°C for 1 hour with shaking.

5. Obtain a LB + kanamycin plate and label it on the bottom with your initials and the date.

6. Pipette 200 µl of the transformation mix onto the plate and spread. Incubate the plate upside down at 37°C overnight.

Isolation of plasmid DNA (instructions modified from QIASpin manual)

1. Find three well-isolated colonies on your transformation plate. For each colony, use a sterile toothpick to pick up a portion of the colony and inoculate a tube of LB + kanamycin broth. Incubate the tubes in the 37°C incubator.

2. For each culture, label a sterile microcentrifuge tube (1 through 3). Transfer the cultures into the microcentrifuge tubes and pellet the cells by spinning the tubes in a microcentrifuge for 1 minute at full speed (16,000 rpm).

3. Pour the supernatant into an autoclave bag. Resuspend each pellet in 250 µl of Buffer P1. Buffer P1 (provided in the kit) is a weak buffer at physiological pH (7.5). It also contains RNase which will degrade RNAs once the bacterial cells are broken.

4. Add 250 µl of Buffer P2 (provided in the kit) to each tube and mix by gently inverting the tubes several times. The cell suspension should become viscous. The high pH and sodium dodecyl sulfate (SDS) detergent in this buffer disrupt membranes and bacterial cell walls, and the detergent solubilizes proteins and lipids. The broken pieces of bacterial chromosome will denature at high pH and the strands will float away from each other. The two strands of the intact circular plasmid molecules cannot separate from each other very far (topologically linked).

5. Add 350 µl of Buffer N3 (provided in the kit) to each tube and mix by gently inverting the tubes several times. The quanidinium salts in Buffer N3 and the lowered pH (back to ~7.5) leads the SDS-solubilized material to form insoluble complexes and fall out of solution, accounting for the snowstorm going on in your microcentrifuge tubes. The huge single-stranded pieces of the bacterial chromosome will get caught up in the precipitating complexes, but the much smaller intact plasmid molecules will stay in solution and renature to a double helix conformation.

6. Spin the tubes in a microcentrifuge at full speed for 10 minutes to pellet the unwanted cell debris (proteins, lipids, and chromosome fragments). While tubes are spinning, label five spin columns and place each in a catch tube.

7. Carefully pour each supernatant into a spin column (sitting in a catch tube). The double-stranded plasmid DNA will bind to the tiny glass beads in the spin column under the high salt conditions of Buffer N3, while RNA, ssDNA, proteins, and other cell components will not. Spin in a microcentrifuge at full speed for 30 seconds. Discard the flow-through from the catch tube.

8. Add 500 µl of Buffer PB (provided in the kit) to each spin column. This high salt wash helps clean away any contaminants from the bound plasmid DNA. Spin in a microcentrifuge at full speed for 30 seconds. Discard the flow-through from the catch tube.

9. Add 750 µl of Buffer PE (provided in the kit) to each spin column. This high salt and ethanol wash further cleans the bound plasmid DNA. Spin in a microcentrifuge at full speed for 30 seconds. Discard the flow-through from the catch tube.

10. Without adding any additional buffer, spin the tubes in a microcentrifuge at full speed for 1 minute. Throw away the catch tube and any flow-through it contains and place the dried spin column in a labeled sterile microcentrifuge tube.

11. Add 50 µl of Buffer EB (provided in the kit) to each spin column and let stand for at least 1 minute. The low salt conditions of Buffer EB bump the plasmid DNA off of the glass beads. Spin in a microcentrifuge at full speed for 1 minute. Throw away the spin column, close the microcentrifuge tube. You now have purified plasmid DNA!

Restriction endonuclease digestion of DNA

1. Choose two restriction enzymes for your analysis. For each of your plasmid clones, label two 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 to each of your digested DNA samples, mix, and spin down briefly in a microcentrifuge. Loading buffer consists of three components: EDTA, which chelates magnesium, stopping any reaction of DNA with enzymes; glycerol, which makes the density of the sample greater than the salt solution so that the sample will sink to the bottom of the hole; and bromophenol blue, a negatively charged blue dye 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 of practice sample solution 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 which well! After everyone has loaded their samples and DNA standards have been loaded in additional wells, close the gel and turn the electricity 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 is 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. *If you use ethidium bromide to stain your gels, please use standard precautions for handling a mutagen-carcinogen: wearing gloves and disposing of liquid and solid waste in labeled containers for proper disposal later. However, nontoxic alternatives to ethidium bromide, such as SYBR Green, can be used to visualize DNA in agarose gels.*

Notes and hints:

(a) This component is listed third because it naturally follows the other components. However, once you have done component 1 in a previous iteration of a course, any mutants generated can be used for component 3 at any time. You may find it useful to do this component before component 1, depending on the order of lab skills you wish to stress, or by itself in a particular iteration of a course.

(b) 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, SalI, SfiI, SpeI, SphI, and XmnI.

(c) 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.

(d) 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.

(e) 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 1,711 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 1,575 bp.

(f) If you desire to get a 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.

(g) If you wish to have students know more about the transposon Tn5-RL27, the sequence of the suicide plasmid pRL27 which carries the transposon is shown below (sequence courtesy of Dr. Bill Metcalf, University of Illinois; 5). The plasmid is 4,080 bp in length and the transposon goes from position 1793 to 3504.

GAATTCTCTAGAATGATTCTCCGCCAGCATGGCTTCGGCCAGTGCGTCGAGCAGCGCCCGCTTGTTCCTGAAGTGCCAGTAAAGCGCCGGCTGC
TGAACCCCCAACCGTTCCGCCAGTTTGCGTGTCGTCAGACCGTCTACGCCGACCTCGTTCAACAGGTCTAGGGCGGCACGGATCACTGTATTCG
GCTGCAACTTTGTCATGCTTGACACTTTATCACTGATAAACATAATATGTCCACCAACTTATCAGTGATAAAGAATCCGCGCGTTCAATCGGACC
AGCGGAGGCTGGTCCGGAGGCCACATATGATAAACTTCTGCTCTTCATCGTGCGGCCGACTGGGCTAAATCTGTGTTCTCTTCGGCGGCGCTGGGT
GATCCTCGCCGTACTGCCCGCTTGGTTAACGTCGCCGCCCAATTGGCAAAATATTCTGGTAAATCAATAACCATCTCATCAGAGGGTAGTAAAGC
CGCCCAGGAAGGCGCTTACCGATTTATCCGCAATCCCAACGTTTCTGCCGAGGCGATCAGAAAGGCTGGCGCCATGCAAACAGTCAAGTTGGCT
CAGGAGTTTCCCGAACTGCTGGCCATTGAGGACACCACCTCTTTGAGTTATCGCCACCAGGTCGCCGAAGAGCTTGGCAAGCTGGGCTCTATTCA
GGATAAATCCCGCGGATGGTGGGTTCACTCCGTTCTCTTGCTCGAGGCCACCACATTCCGCACCGTAGGATTACTGCATCAGGAGTGGTGGATGC
GCCCGGATGACCCTGCCGATGCGGATGAAAAGGAGAGTGGCAAATGGCTGGCAGCGGCCGCAACTAGCCGGTTACGCATGGGCAGCATGATGA
GCAACGTGATTGCGGTCTGTGACCGCGAAGCCGATATTCATGCTTATCTGCAGGACAAACTGGCGCATAACGAGCGCTTCGTGGTGCGCTCCAAG
CACCCACGCAAGGACGTAGAGTCTGGGTTGTATCTGTACGACCATCTGAAGAACCAACCGGAGTTGGGTGGCTATCAGATCAGCATTCCGCAAAA
GGGCGTGGTGGATAAACGCGGTAAACGTAAAAATCGACCAGCCCGCAAGGCGAGCTTGAGCCTGCGCAGTGGGCGCATCACGCTAAAACAGGGG
AATATCACGCTCAACGCGGTGCTGGCCGAGGAGATTAACCCGCCCAAGGGTGAGACCCCGTTGAAATGGTTGTTGCTGACCAGCGAACCGGTCGA
GTCGCTAGCCCAAGCCTTGCGCGTCATCGACATTTATACCCATCGCTGGCGGATCGAGGAGTTCCATAAGGCATGGAAAACCGGAGCAGGAGCCG
AGAGGCAACGCATGGAGGAGCCGGATAATCTGGAGCGGATGGTCTCGATCCTCTCGTTTGTTGCGGTCAGGCTGTTACAGCTCAGAGAAAGCTTCA
CGCCGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAAT
GTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGC
GGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGGAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCC
GCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCCGTCGACTGTCTCTTATACACATCTCAACCATCATCGATGAATTCGAGCTCGGTACCC
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TTTTATTGTTCAAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTTAGCCATGAGAGCTTAGTACGTTAGCCATGAGGGTTTAGTTCGT
TAAACATGAGAGCTTAGTACGTTAAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTACTATCAACAGGTTGAACTGCTGATCTTCAG

ATCCTCTACGCCGGACGCATCGTGGCCGGGGTTCGAAATCGATGAGCTCGGGGGGGGGGGGGAAAGCCACGTTGTGTCTCAAAATCTCTGATGTTA
CATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAA
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GTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGAT
AACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACT
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ATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTT
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AGTCGACCTGCAGGATCGATGGCGCGCCGACGTGCTCATAGTCCACGACGCCCGTGATTTTGTAGCCCTGGCCGACGGCCAGCAGGTAGGCCGACA
GGCTCATGCCGGCCGCCGCCGCCTTTTCCTCAATCGCTCTTCGTTCGTCTGGAAGGCAGTACACCTTGATAGGTGGGCTGCCCTTCCTGGTTGGCTTG
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AAGGGACAGTGAAGAAGGAACACCCGCTCGCGGGTGGGCCTACTTCACCTATCCTGCCCGGCTGACGCCGTTGGATACACCAAGGAAAGTCTACAC
GAACCCTTTGGCAAAATCCTGTATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGCTATAATGACCCCGAAGCAGGGTTATGCAGCGGAAAA
GCGCTGCTTCCCTGCTGTTTTGTGGCGCGCCATCGATCCGCCG

Safety Issues.
There are no special safety issues associated with this project. Standard lab safety in terms of dealing with biohazardous wastes (bacterial cultures) and waste from ethidium bromide staining of DNA are all that are needed.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.
We have used quizzes at the beginning of lab periods to assess student's preparation for that day's lab and understanding of past lab activities and results. The biochemical complementation data from component 1 and the gel results from components 2 and 3 lend themselves nicely to homework assignments for analysis and interpretation. Component 3 of the project alone can be used for a very nice lab write-up in the form of a scientific report. It requires the students to generate a standard curve of their gel electrophoresis results and interpret their gel results. Since the project can span multiple iterations of a course without reaching completion, no one set of students will see the same data sets so you can keep quizzes and homework assignments fresh.

Field Testing.
This entire project was used as written at Hiram College for three iterations of a genetics course with 15 to 24 students each semester split over two lab sections. Component I was also used as an outreach collaboration with two local high school Advanced Placement biology classes. Component 1 requires so few materials (mostly plates and toothpicks) that it is easy to supply high school classes with what they need and handle the biohazardous waste. 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 they will find, and the connections between many of the points we discussed in class coming together in one lab project.

Student Data.
Over three iterations of this project within a genetics course at Hiram College, along with the efforts of two high school Advanced Placement biology classes during one academic year, over 5,000 *C. salexigens* Tn5-RL27 insertion mutants were screened. So far, 44 auxotrophs have been isolated, characterized, and physically mapped with PacI and PmeI. These auxotrophs represent 16 different biosynthetic pathways (representative data in Table 3). Transposon insertion sites have been recovered for all the auxotrophs and sequence information has been obtained for 12 to date. Currently, the remaining auxotrophic transposon insertion sites are being sequenced and 50 randomly-chosen prototrophs are being physically mapped with PacI and 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 and PmeI digestion of wildtype genomic DNA that together indicate a chromosome size of ~3.9 Mbp (Fig. 7).

TABLE 3. Representative data on characterization of *C. salexigens* auxotrophs

Mutant	Auxotrophy	Additional biochemical complementation tests (+) indicates complementation	PacI + PmeI map location	Mutated gene based on sequence adjacent to transposon insertion
EF	Glutamate		Fragment C	Phosphogluconate dehydratase
3E	Valine	Alpha-ketoisoyalerate + alpha-keto-beta-methylyalerate (+)	C	*ilv*C
4A	Adenosine	Hypoxanthine (+)	A	*pur*K
4F	Luecine	Ketoisocaproate (+)	B	*leu*D
BB3	Unknown (did not grow on any pool plate)		C	2-dehydro-3-deoxyphosphooctonate aldolase (LPS synthesis?)

BB4	Unknown		B	Acyltransferase family (lipid synthesis?)
M4BC	Adenosine + histidine		B	*his*H

FIG. 7. Representative pulsed-field gel results. The right lane shows PacI and PmeI digestion of a wildtype (no transposon insertion) *C. salexigens* genomic plug. The three fragments are in decreasing size: A, ~1,540 kbp; B, ~1,410 kbp; C, ~970 kbp. The left lane shows the largest of the *Saccharomyces cerevisiae* chromosomes used as linear DNA size standards. The standards are in decreasing size: 1,900 + 1,640, 1,120 + 100, 945, 915, 815 (barely visible at bottom).

SUPPLEMENTARY MATERIALS

Possible Modifications.
1. 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* (4), 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: i) the species of interest can undergo conjugation with *E. coli*, ii) the species of interest is not already kanamycin resistant (so you can select for the transposon), iii) 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), and iv) the species of interest is prototrophic (so you can screen for auxotrophs).
2. A combined genetic and physical map of a genome by itself is a worthwhile research goal, let alone as a tool within a larger genome project. Further, the mutants generated can be used in a wide range of other projects in other courses.
3. One could easily carry out screens for other types of mutants (e.g., salt-sensitive), characterize the mutants further, map the mutations, and recover the transposon insertion sites for sequencing and bioinformatics analysis.
4. As new rare cutting restriction enzymes become available, you could try to find additional enzymes or combinations of enzymes that cut the *C. salexigens* genome just a few times and also cut with Tn5-RL27. Once found, you can map your collection of auxotrophic and prototrophic mutants with these additional enzymes. We need mapping information for at least two different cuts in order to generate an unambiguous map of the chromosome.

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

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

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

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

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

SOC (per liter): 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, and 10 ml of 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.

1,000X kanamycin stock: 50 mg of kanamycin sulfate per ml water. Filter sterilize and store frozen, then add to media as needed at 1 µl per ml of medium.

1,000X rifampicin stock: 20 mg of 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.

"Salty" cell suspension buffer: 10 mM of Tris, 0.5 M of NaCl, 50 mM of EDTA; pH 7.2.

ES buffer: 100mM of EDTA, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine; pH 8.

Wash buffer: 20mM of Tris, 50mM of EDTA; pH 8.

1X TBE: 10.8 g of Tris base, 5.5 g of boric acid, and 4 ml of 0.5 M EDTA (pH 8).

6X gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol.