Functional Genomics of *Chromobacterium violaceum*

Using Random Transposon Mutagenesis

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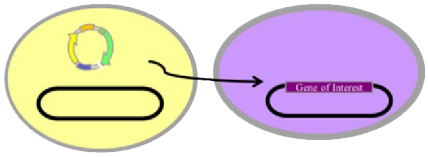
Edited and new protocols added by Sara Anderson 2013 & 2014

Ideas and protocols based on training from Brad Goodner and Kathleen (KT) Scott and others at the ASM/JGI Functional Genomics Institute, 2012

1. Isolation and identification

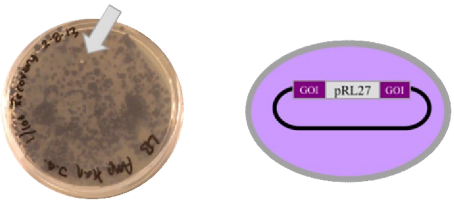
Bacteria isolated from pitcher plant fluid and identified as *Chromobacterium violaceum* via phylogenetic analysis of the 16s rRNA gene.

2. Conjugation with F+ *E. coli* carrying the Tn5 transposon

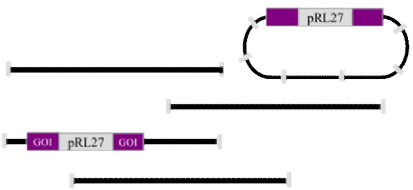


3. Screen for mutants with Kanamycin resistance

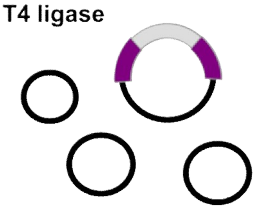
Transposon randomly disrupts genomic DNA. Non-pigmented mutants will have a transposon insertion in a gene related to pigment production.



4. Isolate unpigmented mutant genomic DNA and digest with SacII restriction enzyme

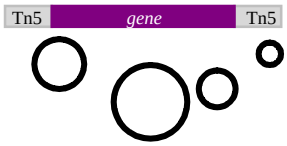


5. Circularize digested DNA with T4 ligase

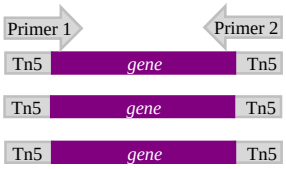


6. Linearize circular DNA with Tn5 on the ends

AvaII restriction enzyme cuts in the middle of the known pRL20-Tn5 sequence



7. Make many copies of unknown gene by PCR



8. DNA flanking transposon is sequenced and analyzed using genetic databases

DNA is sequenced using one of the two primers by the Sanger method. Sequences are compared to genome databases for gene identity and functional analysis.

Contents

Introduction

Goal and Overview

Functional genomics methods help us relate phenotypes to genotypes. Our project uses a form of gene knockout to identify genes that are responsible for particular phenotypes. A transposon is allowed to randomly mutate the genome of our study organism. We can screen this collection of mutant organisms to find individuals with phenotypes indicating disruptions of the genes that interest us. After the identification and isolation of mutant organisms with those particular phenotypes, we will extract their genomic DNA. Then, fragments of the genomic DNA containing the transposon and surrounding DNA can be cloned and sequenced. The gene(s) of interest may then be identified by comparing these sequences to the online databases of genome information. Our experiment can confirm a gene to phenotype (functional genomic) relationship. The cover figure shows a brief overview of the approach used in this project. Note that "step 1" has been done by other researchers and you will be joining the project at step 2.

To complete this project we will use a variety of common molecular laboratory techniques including DNA extraction, restriction digestion, gel electrophoresis and bioinformatics analysis. All of these methods will be discussed below in their respective steps of the procedure or by supplemental materials referenced by your instructor.

Chromobacterium violaceum and our strains

Chromobacteria violaceum (Fig. 1) is a common bacterium found in many different water and soil environments around the world. It has been studied most extensively for its signature dark-violet pigment. The pigment acts as an antibiotic that may also be useful in treating colorectal cancers (Kodach 2006).

Figure 1. An isolation plate of *C. violaceum*. Colonies are dark-violet with a metallic sheen.

A "pitcher plant" strain of *C. violaceum* was cultured by Paul Mechior's group of MSUM and NHCC students from the phytotelmata (fluid) of the carnivorous plant *Sarracenia purpurea*, commonly known as the northern pitcher plant. The phytotelmata was collected from plants found at Beckman Lake bog within the Cedar Creek Ecosystem Science Reserve just north of the Twin Cities in Minnesota. Cultured solutions revealed a large number and variety of bacteria living within the fluid of the plant.

Aided by their unique pigmentation, dark-violet colonies were isolated and identified by phylogenetic analysis of the 16S rDNA gene. Amie Beckel and John Wulkan isolated the bacterial DNA and amplified the 16S region with complementary primers. Once the 16S gene has been amplified, it was sent off for sequencing. The 16S rDNA gene was chosen because it codes for part of the 30S small sub-unit of prokaryotic ribosomes. Due to the importance of this structure in cell function, it is highly conserved and therefore serves as an effective method of identification among bacteria and archea. Beckel and Wulkan compared the sequence of the 16S rDNA gene to those in the NCBI databases, using BLAST (http://blast.ncbi.nlm.nih.gov/). The sequence had a 99.8% match with a *Chromobacterium violaceum* sequence in the database.

After the bacterium was identified, an ATCC (American Type Culture Collection) certified strain of *C. violaceum* (ATCC #12472) was also obtained from Carolina Biological (www.carolina.com). This strain was studied in parallel to the pitcher plant strain. Strain ATCC 12472's complete genome was published in 2003 (Brazilian National Genome Project Consortium 2003). Studying an organism whose genome is published aids in the final analysis of gene sequence.

Some aspects of culturing and differences between the pitcher plant strain of *C. violaceum* and the ATCC 12472 strain were discovered during work on protocol development. While the ATCC 12472 strain grows well on LB (Luria-Bertani) media in the range from room temperature (about 22°C) through 37 °C, the pitcher plant strain of *C. violaceum* is ideally cultured at room temperature on LB media. Both will grow on NA (nutrient agar) media, though at a slower rate. Growth in an incubator at 37°C will severely slow growth of the "pitcher plant" strain, though the ATCC 12472 strain seems to grow quickly at 37 °C. For both strains, it is important that a growing culture is established, be transferred to fresh media every few days, or it may lose viability on "old" plates. (If this happens, re-start by taking a scraped sample from frozen-in-glycerol stocks kept at -80°C.)

It was also discovered that the pitcher plant strain is "naturally" resistant to ampicillin at 22°C but sensitive to amp if grown at 37°C. The ATCC 12472 strain is not resistant to ampicillin, but our group created a derivative of this strain (by selection in ampicillin-containing media). This derivative of ATCC 12472 is resistant to ampicillin at room temp, but also retains this resistance at 37°C.

We exposed various samples of plasmid and lambda DNA to lysates of both *C. violoaceum* strains. The DNA's degraded condition (visible with agarose gel electrophoresis and ethidium bromide staining) was consistent with native restriction enzyme activity in the ATCC 12472 strain lysate. *CviI* restriction enzyme has been previously cited as produced in the strain ATCC 12472 (Roberts, 1985). However, we did not detect evidence of similar endonuclease activity in the pitcher plant strain.

PRL27-Tn5 Vector and Transposition

The pRL27 plasposon, a plasmid bearing a transposon (Fig 2), is used as a means to generate random mutations in the *C. violaceum* bacterial genome. A transposon is a genetic element that is able to integrate itself into new strands of DNA. The pRL27-Tn5 transposon functions through simple transposition. See your genetics textbook for more information about the mechanisms of transposition.

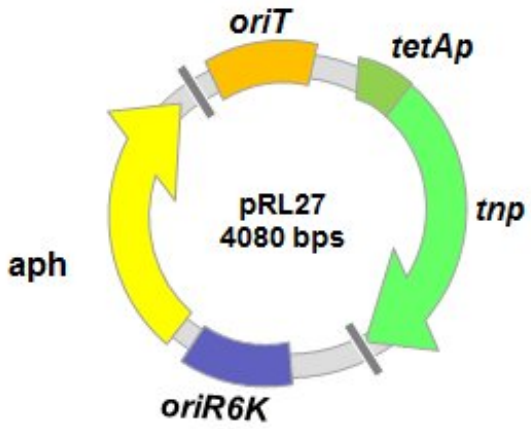


Figure 2. pRL27 is a suicide plasmid that contains a transposon. It is only able to transpose one time, and is not further replicated in the recipient cells. The plasmid also carries kanamycin resistance (aph) and an origin of replication (oriR6K) to allow later cloning of transposon insertion sites. The oriT origin allows for transfer from host to donor. TetAp is a promoter that is functional is a wide array of organisms. Tnp codes for transposase, the protein responsible for the transposition event. The small bars are modified Tn5 inverted repeats. (Plasmid created in the lab of Bill Metcalf)

During conjugation, the donor bacteria shares a copy of the pRL27-Tn5 transposon. During the transfer, when the plasmid is newly replicated by rolling-circle replication, the promoter responsible for transcription of functional transposase gene is active. The hyperactive transposon will be able to insert once randomly into the recipient's genome. Very soon after the transfer, methylation of the plasmid by its new host changes the production of transposase to an inactive form. Thus, the transposon has just one chance for action, and the resulting mutation is limited to a single site in the C. violaceum genome.

The plasmid carries various other elements, including a segment responsible for kanamycin resistance (hitches a ride during transposition and thus ends up in the recipient genomes), and a particular type of origin of DNA replication. The special origin of DNA replication is not functional without certain proteins (pi), which are produced bacteria such as our donor *E. coli*. However, these replication-enabling proteins are not available in our *C. violaceum* recipient cells, and thus the DNA of the plasmid that enters the recipient will not be further replicated in its new host. For this reason, the plasmid may be referred to as a "suicide plasmid."

Familiarize yourself with bacterial culture and sterile technique methods

Kathy Barker's book At the Bench: A Laboratory Navigator provides a very appropriate level of information about many laboratory methods used in this project. See chapters on "Bacteria," "Working Without Contamination," when you are beginning, plus others as relevant. Also consult with your instructor if you need more information about safe and effective techniques in microbial and molecular lab work.

Methods and Protocols

Conjugation of *Chromobacterium violaceum* with F+ *E. coli* carrying the pRL27 plasmid

Conjugation is one process that bacteria use as a mode of genetic transfer. See your Genetics textbook for a full explanation of this and other gene transfer methods in prokaryotes. Conjugation requires direct contact between a donor cell and a recipient cell. The donor cell contains a small circular segment of genetic material that is known as the F factor (fertility factor) that aids in construction of the pilus used as the passageway for DNA transfer. Conjugation is possible between bacteria of different species, resulting in "horizontal transfer" of genetic information. *E. coli* donor strain BW 20767, which produces the F factor, was provided from Bill Metcalf's lab at University of Wisconsin, Madison. This strain also expresses a π (pi) protein that allows maintenance of the transposon-bearing plasmid, pRL27, in low copy number. When these *E. coli* donor bacteria come in contact with *C. violaceum*, they can transfer the pRL27 plasmid to *C. violaceum* recipients.

There are two protocols for conjugation that have been used successfully by Moorhead students with *C. violaceum* strains. The co-streaking method, based on advice from Brad Goodner, worked well for our "pitcher plant" strain of *Chromobacterium violaceum*. A separate method, the biofilm method, based on advice from Kathleen (KT) Scott, works best for *Chromobacterium violaceum* strain ATCC 12472.

Co-Streaking Conjugation Method (to be used with *C. viol.* "pitcher plant" strain)

Materials Needed

- F+*E. coli* BW 20767 carrying pRL27 (kan resistant by gene on pRL27 transposon)
- Pitcher plant *C. violaceum strain* (naturally amp resistant at room temp)
- 0.9% NaCl (sterilized by autoclaving)
- LB agar plates, without antibiotic
- LB agar plates with 50µg/mL Kanamycin
- LB agar plates with 100µg/mL Ampicillin
- LB agar plates with 25µg/mL Kanamycin and 50µg/mL Ampicillin

Notes on antibiotic incorporation into agar plates:

1. Plates should be made in advance with antibiotics mixed into sterilized agar after its temp has been brought to 50- 55°C. Higher temps may damage the antibiotic.
2. The antibiotic concentrations of the plates with kan and amp combined are ½ of the strength, compared to the plates with single antibiotics. It was found that this was the ideal condition for optimal results.

Step 1 – Parent plates

Streak one colony of *C. violaceum* on a LB agar plate with ampicillin. Grow overnight at room temperature (~22°C). Streak one colony of the F+ *E. coli* on a LB plate with kanamycin. Grow overnight at 37˚C. Note that these two plates are NOT grown at the same temperature. Note: *this step completed by your instructors prior to lab*.

Step 2 - Mating plate

Using a disposable sterilized loop, pick one colony of the F+ *E. coli*. Streak this colony across an LB plate with no antibiotic (a "mating plate"). Spread thoroughly across the plate, rotating the plate while doing so. If necessary, use a second loop to pick up more cells from the parent plate to spread on the mating plate. Repeat this streaking procedure with a colony of *C. violaceum.* Make sure that the entire mating plate is covered and that the two strains overlap (see Fig 3). Allow to grow overnight at room temperature (~22°C). The growth may take 1-4 days before the next step can be completed. Plan to take a few minutes to check your plate over the next few days.

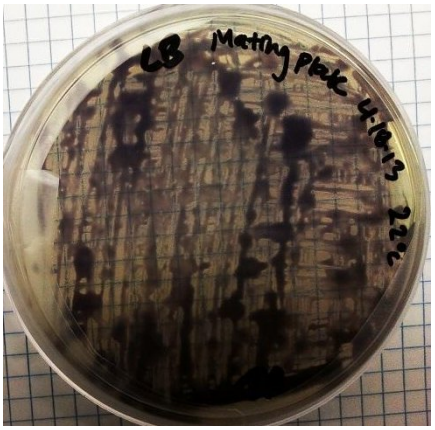


Figure 3. Example of what a mating plate should look like after spreading the *E. coli* and *C. violaceum* over the plate. Complete overlap and coverage is key to a successful conjugation. This picture was taken on the day after streaking, so the growth is visible.

Step 3 – Recovery plates

After suitable growth (1-4 days; Figure 3), pipet 2 mL of 0.9% NaCl onto the mating plate (you may use the P1000 to transfer 1mL at a time). Using a sterilized loop, gently scrape the cells into the saline solution. Use a sterile pipet to collect 1000 µL of the saline/bacteria solution, squirt into a microcentrifuge tube and mix by repeated pipetting so the cells are floating in solution. Using a sterile disposable L-shaped spreader, evenly spread 100 µL of the bacterial solution on a new LB plate containing ampicillin and kanamycin (plates with the antibiotic pre-mixed are essential). This combination of antibiotics should select for *C. violaceum* (amp^r) that have taken up the pRL27 plasmid (kan^r). Incubate these plates for 2-4 days at room temperature.

Step 5 – Selection of mutants

Within two to four days, new growth will appear (Figure 4), if some F+ *E. coli* with the pRL27 plasmid conjugated with the *C. violaceum* during their time on the mating plates. *Take notes* on how many you got and what they look like. *Take pictures*! You will want them for your lab notebook and for figures in your final paper. For information on how to select specific mutants see the next section, Selection of mutants.

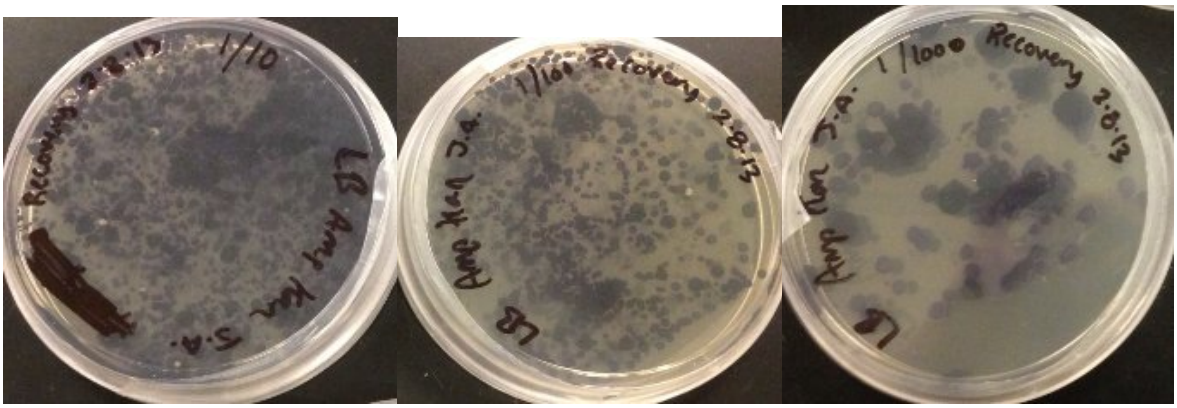


Figure 4. Example results of a sucessful conjugation of F+ *E. coli* with *C. violaceum*. Three recovery plates are shown each with a different dilution (1/10, 1/100 and 1/1000). Pigment mutants can easily be seen on the first two plates. Other mutants could be selected for through the use of restrictive growth conditions or biochemical tests.

Selection of mutants

At this point, any colonies that have survived on your recovery plates should be *C. violaceum* which carry the transposon within their genome. The recovery plate media contains both ampicillin and kanamycin. *Think about this*…After conjugation, there are three types of cells that may have been spread on the recovery plates: 1. Donor *E. coli* cells. 2. *C. violaceum* without pRL27. 3. *C. violaceum* pRL27 recipients. Why do you suppose that the first two types of cells are not surviving on the plate? Also, think about the pRL27 recipients. How do we know that the transposon actually moved from the plasmid into the genome? (See the section which mentions the term a "suicide plasmid" for a hint.)

Among the many colonies that you should have growing on your plates, each may carry the transposon in different sections of the genome. Depending on which gene sequence has been interrupted by the transposon, different phenotypes result. Some phenotypes may be easily visible in the color or shape/size of the colony. Many other phenotypes may not be detectible without including or excluding components in the media, employing special growth conditions, or biochemical tests.

Selection of non-pigmented mutants - *our focus!*

Some of the easiest genes and phenotypes to examine are related to pigmentation. Since our *C. violaceum* strains show a distinctive purple color, we can conclude that mutant colonies that are no longer producing purple pigment have a disruption in one of the genes responsible for violet pigmentation.

Colonies from the recovery plate should be streaked on fresh plates to isolate descendent colonies from their neighbors, and confirm that their phenotypes are stable. You may have to do this more than once to completely isolate the colony. Remember to *keep careful notes* and *take pictures*. You will want them for your lab notebook and for figures in your final paper.

Selection of auxotrophs and other mutants - *not our focus, but possible independent research project in the future*

For most mutants, it may not be possible to identify individuals with desired phenotypes "by eye" on one typical LB plate. For example, if you are hoping to identify genes involved in proline metabolism, you may need to compare growth of a colony on minimal media (no proline) and the same colony on minimal media plus proline. In this case, a mutant in proline production will not grow on minimal media, though it will be healthy on media that has been supplemented with proline.

In order to "screen" through many candidate mutant colonies to find a phenotype of interest such as this auxotroph example, you may need to pick colonies and make 2 or more organized replica plates, to be tested under your different growth conditions which you are comparing. A grid pattern which may be used to organize your replica plates c is in Kathy Barker's book At the Bench: A Laboratory Navigator. Just put your petri dishes above the grid, then pick and dot colonies into each of the separate 50 or 100 grid boxes. Repeat so that each plate replicates these positions. Incubate under your different conditions, then, determine which colonies are showing the phenotype that indicates loss of a gene of interest.

Step 6 - Extraction of genomic DNA from the selected mutant *C. violaecum*

After you have identified and isolated a mutant you will need to grow it overnight (1-2 days before your lab) in a liquid LB media so that you can extract its genomic DNA. The Tn5 transposon has transposed into your mutant's genome, and will be used as a marker to find the gene that has been disrupted in your organism. Your instructor will provide you with detailed instructions of how to perform a DNA extraction. There are many commercially available kits that make extraction quick and easy. All extractions during protocol development were done using UltraClean® Microbial DNA Isolation Kit. http://www.mobio.com/images/custom/file/protocol/12224.pdf

Note: It is important to have a high starting concentration of genomic DNA since the following steps will break down and dilute the sample with which you are working. For example, the kit mentioned above advises us to start with 1.5 mL of a culture grown overnight. To obtain higher yield, we suggest you fill a 15 mL conical tube with 10 mL (or more) of culture grown overnight. Spin the solution at 10,000 g for 1 minute and decant the supernatant. Then follow the guidelines of the remainder of the given procedure selected by your instructor.

The success of a DNA extraction can be assessed by visualization of DNA on an agarose gel and/or by spectrophotometric analysis. For gel analysis, make 1% agarose gels, following your lab's protocol (consult your instructor). Combine 1-5 µL of your DNA sample with 1µL of 6x DNA loading dye. Load 5 µL of a 1 kb or similar ladder in the first lane of the gel, and DNA sample or samples alongside. Run the gel at a voltage of 100-150, until the fastest-moving element of the dye has moved about ¾ of the distance across the gel. Stain and use your lab's method of visualizing the stained DNA. If you see a large single band, you likely have intact bacterial DNA. If your DNA appears as a longer smear, the DNA probably has some degradation, but will likely still be sufficient for the purposes of this project. If you do not see anything in your DNA lane, you need to re-examine your method of extraction.

If you will use spectrophotometry to assess your DNA extraction, your instructor can give you information about your lab's available equipment (spectrophotometer, nanodrop, etc). In the "DNA, RNA, Protein" chapter of Kathy Barker's book At the Bench: A Laboratory Navigator, you will find an explanation of how to convert A260 readings into concentrations of DNA, and also how to use A260/A280 ratios to estimate purity of the sample. A goal for DNA concentration after your extraction is approximately 125-175 ng/µL or higher.

Step 7 - Creating smaller, circularized segments of genomic DNA

We will now prepare to "fish" for the mutated gene, among the whole genome in which it is now contained. We will do this by looking for "marker" elements that got incorporated into the mutant gene region when transposition happened: kanamycin resistance and a special origin of replication are there, along with the interruption of our gene of interest. We start by creating small "potential" plasmids, by cutting the genome in pieces and allowing them to form small circles. If we transfer these potential plasmids to a new bacteria, we can search among these bacteria for individuals which have become able to resist kanamycin and are replicating this circular segment of DNA, indicating that they carry the transposon and neighboring pieces of the gene it interrupted. We can also amplify this DNA directly using polymerase chain reaction (PCR) with the help of our known transposon sequence.

7.1 - Fragmentation of mutant genomic DNA with *SacII* restriction enzyme

If you have an adequate amount and quality of genomic DNA from your mutant, you should next break the DNA up into millions of short fragments. This is done with the help of a protein called a restriction enzyme. Restriction enzymes selectively cleave DNA at specific places in the DNA, depending on their recognition sequences.

Figure 5. *SacII* cleaves the phosphate backbone of DNA between the C and G bases as shown, in any DNA which has its exact recognition sequence.

For this step you will be using *SacII* restriction enzyme. *SacII* restriction enzyme is chosen because it will not cut within the pRL27 transposon; however, it will cut the rest of the genomic DNA frequently (Fig 5). After digestion, you will have millions of small fragments of DNA, each with *SacII* cleaved ends. These ends are "sticky," with single stranded regions that are complementary to each other, and we will take advantage of this aspect in the next step! Some of the pieces will contain the transposon and the neighboring pieces of the gene it interrupted.

1. Label a new 1.7ml tube and add the following solutions in the following order. [Caution! With these very small volumes you must remember to practice very good pipetting technique. Use the pipet tip to mix the reagents. Be careful not to draw out any of the reagent mixture when pulling the pipet out of the reaction tube. Change the pipet tip after each use to avoid cross-contamination.]

_26__ µl of genomic mutant DNA
_3__ µl 10X buffer appropriate for SacII
_1__ µl SacII enzyme
_30__ µl total volume

2. Incubate the digestion at 37°C for at least 10 min. The easiest way to do this is by setting a program on a thermocycler to run at 37°C. (Also note what happens in step 3 if you are setting up a program.) Pre-heated water baths also work well. Your instructor will direct you to the correct heating apparatus. Make sure that the caps are sealed tightly otherwise you may lose some volume due to evaporation.

3. Inactivate the enzyme by incubating the tube at 65°C for 20 minutes, then spin down very briefly (few seconds) in a microcentrifuge. Again, the easiest way to do this is to incorporate a 65°C step in the program you use in the thermocycler.

7.2 - Circularization of mutant genomic DNA with T4 ligase

After *SacII* restriction digestion created millions of fragments of *C.violaceum* genome, each with compatible sticky ends, the next step will be to circularize these fragments. DNA ligase can seal the broken phosphate backbones to each other.

1. To the tube containing the SacII digestion, add in the following order:

_4__ μl sterile water
_4__ µl 10X ligation buffer
_2__ µl T4 DNA ligase
_40__ µl total volume (including the 30 from the digest)

2. Mix the solution very gently by moving your pipette tip around, then spin down very briefly (few seconds) in a microcentrifuge.

3. Incubate at 15°C overnight in the PCR thermocycler or similar cooling block as indicated by your instructor.

Sort through the DNA to select the region containing the gene of interest

We now have many small circular DNA molecules, but only one type will have our gene of interest. By making a careful cut in the middle of the known transposon sequence, we can turn our circular molecule back into a linear fragment with the known sequences bracketing our unknown gene sequence. We'll use this feature in future steps to target the known DNA, which will allow us to get a sequence of the unknown DNA. What will happen to the circular DNA molecules without the transposon insertion?

Cleanup of circularized DNA

Prior to our next steps, DNA must be purified. Salts in the solution from the restriction digestion and ligation reactions can interfere with the following digest.

Cleanup can be achieved by use of a "PCR cleanup" kit. We used Qiagen's QIAquick during the development of this project. http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/DNA-Cleanup/QIAquick-PCR-Purification-Kit

Follow the instructions as provided by your instructor. *Elute in 30ul of elution buffer – let buffer sit on the filter for 1 min before centrifuging.*

Linearization of circular DNA fragments with *AvaII* restriction enzyme

Figure 6. *AvaII* cleaves the phosphate backbone of DNA between the C and G bases as shown, in any DNA which has its exact recognition sequence.

We have cut up our genomic DNA into fragments with the SacII enzyme, and then joined the ends of fragments together with T4 DNA ligase. One of these circular fragments contains our transposon insertion. Now, we will cut the circular DNA to make a linear fragment, because linear DNA is easier to amplify than circular DNA. We'll linearize in a way that controls where the ends of the transposon insertion occur in the fragment.

For this step you will be using *AvaII* restriction enzyme. *AvaII* restriction enzyme is chosen because it will cut once within the pRL27 transposon (Fig 6), leaving the ends of the transposon on either end of the DNA fragment. Fragments without the transposon insertion may or may not be cut by *AvaII*. After digestion, you will have linear fragments of DNA with pieces of the transposon on each end and part of the unknown interrupted *C. violaceum* gene in the middle. In our next step, we'll use these known transposon ends as targets to amplify our unknown gene.

1. Label a new 1.7ml (snap-cap) tube and add the following solutions in the following order. [Caution! With these very small volumes you must remember to practice very good pipetting technique. Use the pipet tip to mix the reagents. Be careful not to draw out any of the reagent mixture when pulling the pipet out of the reaction tube. Change the pipet tip after each use to avoid cross-contamination.]

Enzyme and buffer pre-mixed; just add DNA

26 µl of cleaned ligation product (circular DNA fragments)
3 µl 10X buffer appropriate for AvaII
1 µl AvaII enzyme
30 µl total volume

2. Incubate the digestion at 37°C for at least 10 minutes. Make sure that the caps are sealed tightly otherwise you may lose some volume due to evaporation.

3. Inactivate the enzyme by incubating the tube at 80°C for 20 minutes, then spin down very briefly (few seconds) in a microcentrifuge. [Note: skip this step if immediately cleaning reactions]

Cleanup of digested DNA

Prior to our next steps, DNA must be purified. Salts in the solution from the restriction digestion reaction can interfere with the following PCR.

Cleanup can be achieved by use of a "PCR cleanup" kit. We used Qiagen's QIAquick during the development of this project. http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/DNA-Cleanup/QIAquick-PCR-Purification-Kit

Follow the instructions as provided by your instructor. *Elute in 40ul elution buffer – let buffer sit on filter for one min before centrifuging.*

Amplification of target Gene of Interest by PCR

In order to study the specific fragment of DNA that is from the gene interrupted by our transposon, we will need to make billions of copies of that fragment while excluding all of the other DNA fragments in our solution. We will achieve this by using the polymerase chain reaction (PCR). PCR was developed in 1985 by Kary Mullis, and it is a method of using heat, enzymes, and targeting molecules (primers) to make many copies of a specific gene location. In the decades since Mullis' original invention, many improvements and specializations have been made to PCR methods. The DNA Learning Center has an informative set of animations that can help you understand all of the different steps of PCR: http://www.dnalc.org/resources/animations/pcr.html

1. Label a 0.2ml PCR tube (very tiny tube!) with your initials on two sides of the tube. These are very small tubes so be careful with your labeling. Add the following solutions to this tube:

   5ul of your cleaned DNA fragments
   20ul of the PCR master mix
   25 ul total

2. Put your tube into the thermalcycler as directed by your instructor. The thermalcycler will run the following program:

   30s @ 95 C
   30s @ 95 C
   15s @ 45 C
   2.5m @ 68 C — 30X cycles
   5m @ 68 C
   Hold @ 10 C

3. Your tubes will be stored in the freezer until the following lab. The program above runs for about 2.5 hrs.

Assess PCR products by agarose gel electrophoresis

Before we sequence our amplified PCR products, we will assess whether or not the reaction worked by visually inspecting the fragments after they are separated by agarose gel electrophoresis. The sequencing reaction will be conducted at an outside lab that has sensitive capillary electrophoresis equipment. Because we will be paying for each sequencing reaction, we will only send the PCR products that produced a clean band.

1. Transfer 10ul of the reaction product into a new tube and combine it with 2ul loading dye. This allows us to save ~15ul of product for sequencing. Note any deviations from this protocol.

2. Load your mixed sample into the gel and set to 100 v. Record the volts and start/end time of the gel run.

3. Visualize the gel on the gel documentation system and sketch your photo here. This will allow you to include the important information with your gel photo in your written document. You should include
   - Sizes of ladder fragments (size standards)
   - Estimated size of your fragment (if visible) with your lane/band indicated

4. Should your sample be sent for sequencing? Discuss with your instructor and decide based on band clarity and intensity. If your sample is going to be sequenced, put it in the location indicated by your instructor and record the sample number (see master sample sheet form). If your sample is not going to be sequenced, the remaining portion does not need to be kept.

Sequencing and analysis of gene using online databases

After you have confirmed the presence of the pRL27-Tn5 transposon within a DNA region of interest, we may wish to determine the sequence of that DNA surrounding the transposon.

Figure 7. This diagram shows how specific primers are used to sequence a portion of the transposon DNA and the genomic DNA flanking either side to reveal the interrupted gene that caused the mutation. Primer sequences may be found in the appendix.

Sequencing facilities will accept samples in a variety of forms, ranging from purified plasmid DNA to agar plates with isolated colonies. The facilities take the DNA (or isolate it themselves from colonies on the plates or from liquid cultures), and use primers which the customers specify, to perform a sequencing reaction. See your textbook for much more information about DNA sequencing reactions. The primers we will ask them to use are complementary to DNA inside our transposon (See Figure 7.). This sequence we know. But the primers will get extended during the sequencing reaction into regions outside of the transposon. This is the sequence we don't know yet, but we'd love to find out! We expect that the sequence around the known transposon contains the gene which relates to the mutant phenotype.

After the sequence data is returned to you, you can analyze it to find the gene of interest.

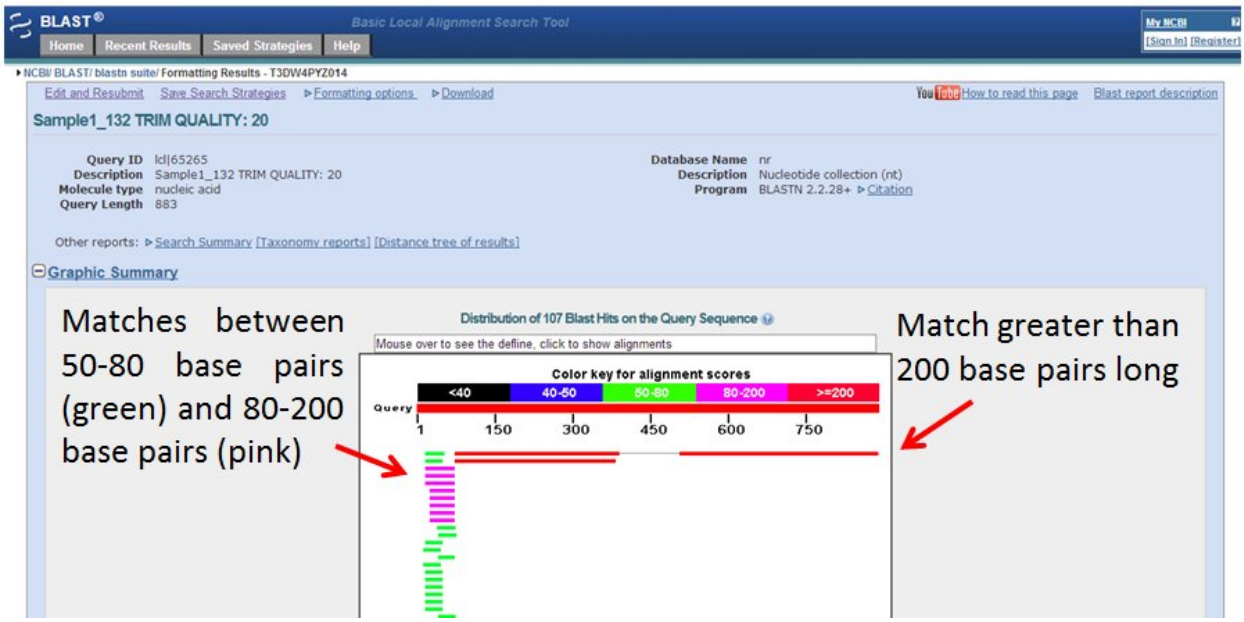
1. The text files may be opened in any major text editing software (i.e. notepad, Word, etc.). Once opened, you will see DNA sequence information (below). The first line displays information about the sample; the name given to the DNA sequenced and the trim quality, which is a method for determining the certainty of a given sequence. Capital letters signify high levels of certainty of at given base while lower case letters indicate a lower level of confidence.

>Sample1_132 TRIM QUALITY: 20
AGCTCTTATTAATTTAAATCTAGAGTCGACCTGCAGGCATGCAAGCTTCA
GGGTTGAGATGTGTATAAGAGACAGGTCCATTGCTGCGACGCGGTGAGTC
CGGCCGGGAGCGGTCTTGCATTGATGGGTTTTGAAGTCACCATGAGCGGG
TCTTCCTTGTTCTTGGTTCTGTGTGCCGCAGATAGTACCCCACATGCTTG
TTGCATGTAAGACCCTGCGGATTTGAATTCATATGAATTGTTGCTTTTTC
GTCATTGAGTATACCAAATTTGTAAGAGCATGAATCCTAGGAAAATTACT
GATAATTCATCATTTTGTTTGTCATAACGGCATTCGTTACAAATAGAAAC
ATGCCGGGAGGGGAAAAGAATTTGACTTTTTTATAATTGTAAAAACGATG
AAATTGATTTGCATCTGCGATGACAAGGGCATGATTTGTCTGCAAATAAT
GCTTTGCTTATATTTATTTTGCAAGATGCGCCTGACGTTGCGATGAGTGA
GGATGTCGCATTTTACCTTCACTTCAATGTCATAAATTGTAAGCAATGAC
TAATAAATTCTGGCAAGCGAACGCTTTAAAGCGCATAATTGGATCGCATA
AAATAATTGATAAGCCAGCGGTGCCCGTTCGAGGACGCGGCTGTCAAACA
TCGGGGATGTAGGGTAGGCAGTCGACGCGGCCAAAGTGGTGGATAGTATA
TGAGATTAAACTATATTCTTTAATATCATGCAGAATTGATGTAAGCTGTT
TTAATAAATAGCTTCAATTCTGTGAACGATCCGACTGCTCGCCAGACCAA
TCAATCangCaAAACAGCATGGATCGCTTCAGGAATCGccccgcTTATGG

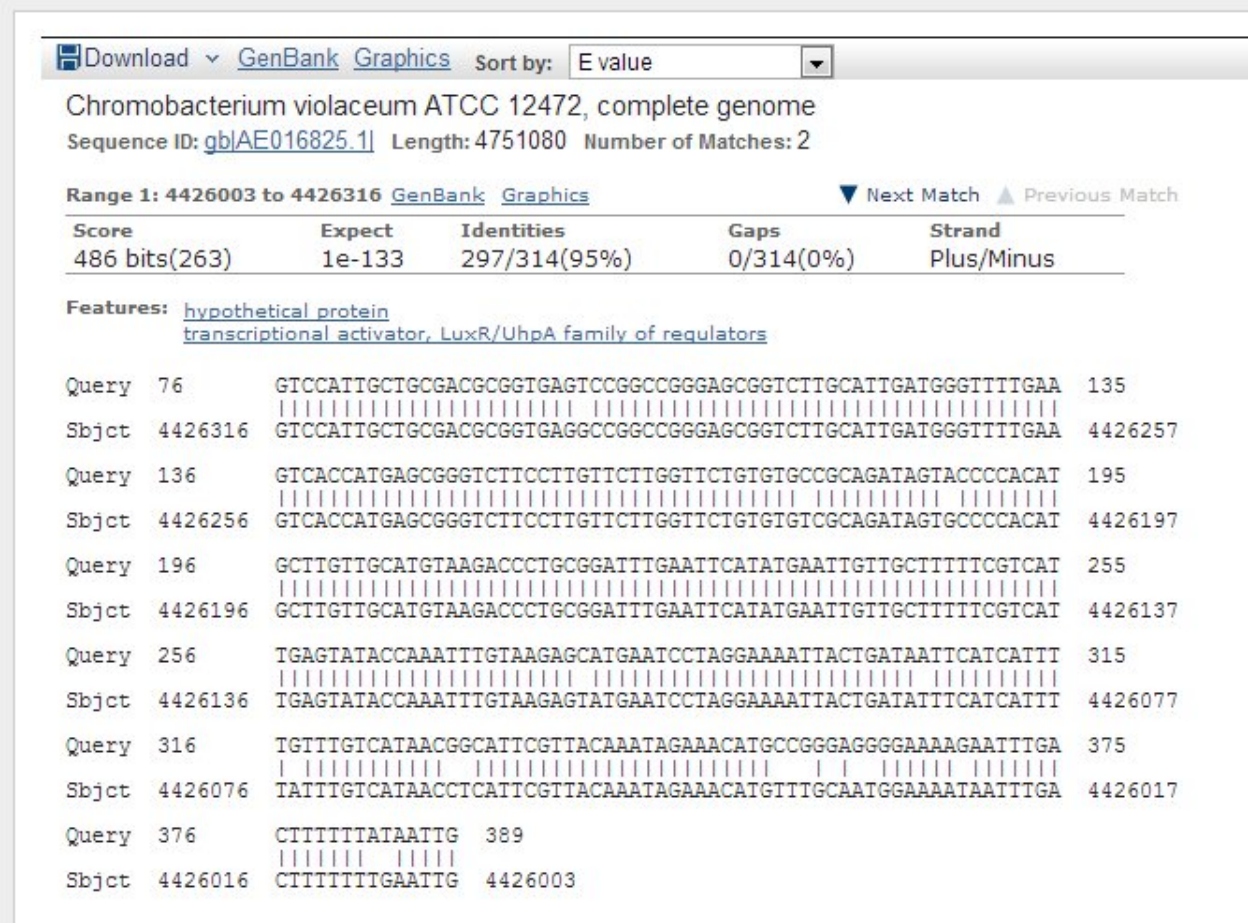
ATACCTTAAAAGCACTCATGGtctTCATGACcA

2. Open up a web browser and go to the NCBI Blast website. http://blast.ncbi.nlm.nih.gov/ Once there, click on the link that says, "nucleotide blast." Copy your sequence (similar to that above) and paste it into the text box where it says "Enter Query Sequence." Leave all other settings at their defaults. Scroll down and hit the button that says, "BLAST."

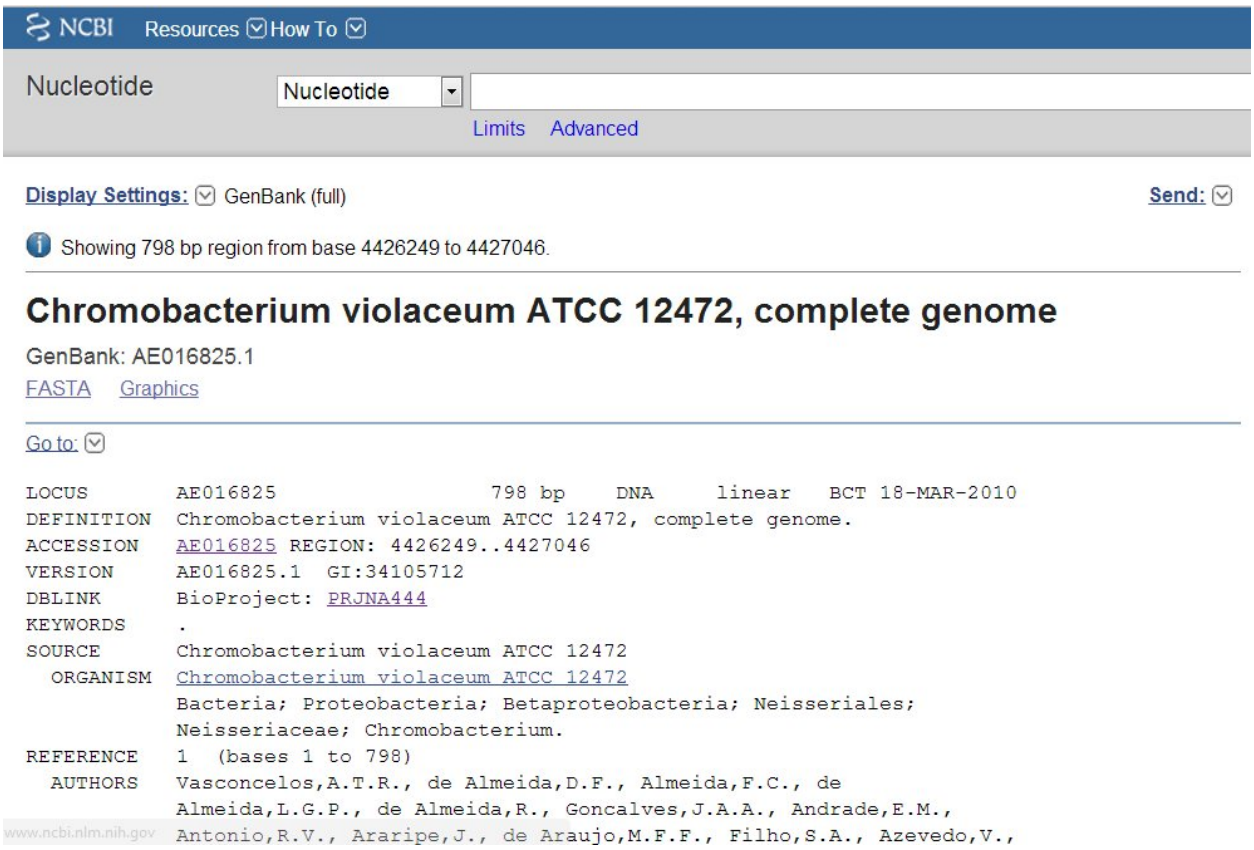
3. Depending how many other people are using the database, it may take as long as a couple minutes to retrieve the information you are searching. Once the sequence has been analyzed in the NCBI database, it will display the number of hits found that contained a significant base pair alignment. The graphic below should be similar to what is seen on your BLAST search.



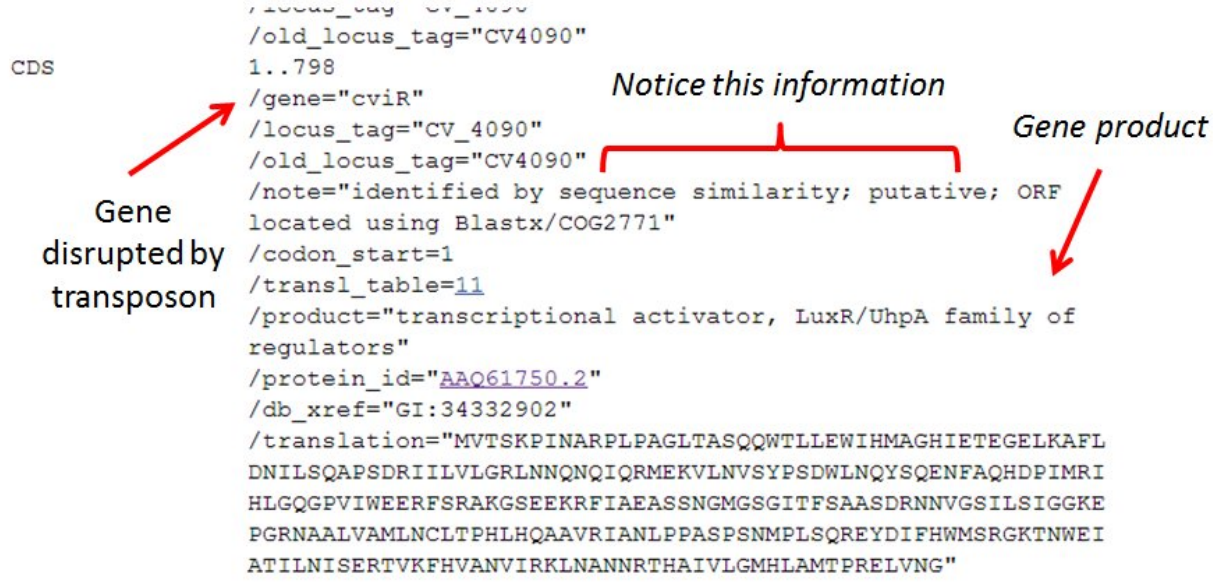
4. Click on the line with the longest match of base pairs. It will bring you further down the page where more information is given about the sequences that were successfully aligned. You should see something similar to the image below. The section that lists "Features" gives information about the sequences. The example below says that the sequence is a hypothetical protein and a transcriptional activator in the LuxR/UhpA family of regulators. Your sequence will most likely describe some other gene or gene product. This page also gives us information about where in the bacteria's genome the interrupted gene is found. Query represents the bases sequenced from the mutant bacteria and the sbjct represents the section of the genome where the gene is found in *Chromobacterium violaceum* ATCC 12472. The short lines between the bases represent a match. The absence of a short line indicates that the bases do not match.



5. If there is a clickable link after the features information, do so. The link will bring you to a new page (example below) with more detailed information about the genes identified (if available). Search the page for the features attached to the sequence that was aligned (in this example: LuxR/UhpA family of regulators).



6. Once you have found more information further down the page you will be able to learn more about the gene that was affected by transposon mutagenesis. In this example we see that the gene was identified by sequence similarity and that its function is putative based on previous analysis of the *C. violaceum's* genome. Just below that there is a description of the protein product of the gene. The name of the gene is also listed. We now know that the gene disrupted by the transposon has been previously identified and named *cviR*. The gene interrupted in your mutant may or may not be annotated in NCBI.



7. At this point it is necessary to do a little investigative work. If the gene you disrupted is known, then you may do a simple search with the gene's name and *Chromobacterium violaceum* within the pubmed database. http://www.ncbi.nlm.nih.gov/pubmed The picture below displays results of a search for cviR and *Chromobacterium violaceum*. The second link down leads to a paper that discusses the role of *cviR* in quorum sensing and its other roles in the cell. With your own gene and sequence you will have to do some of your own searching and hunting. If the gene is listed in any papers, read those papers to help you understand the role and function of the gene.

References

Brazilian National Genome Project Consortium (2003). "The complete genome sequence of Chromobacterium violaceum reveals remarkable and exploitable bacterial adaptability". Proc Natl Acad Sci USA 100 (20): 11660–5

Kodach, LL, Bos, CL, Durán, N, Peppelenbosch, MP, Ferreira, CV, Hardwick, JC (2006). "Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells". Carcinogenesis 27 (3): 508–16.

Larsen, RA, Wilson, MM, Guss, AM, Metclaf, WW (2002). "Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria". Arch Microbiology 178:193-201

Roberts, RJ (1985). "Restriction and modification enzymes and their recognition sequences". Nucleic Acids Research (supplement) 13:r165-r200

Appendix

pRL27-Tn5 plasmid sequence

The region in caps is the transposon. The highlighted underlined letters correspond to primer regions.

gaattctctagaatgattctccgccagcatggcttcggccagtgcgtcgagcagcgcccgcttgttcctgaagtgcc
agtaaagcgccggctgctgaacccccaaccgttccgccagtttgcgtgtcgtcagaccgtctacgccgacctcgttc
aacaggtctagggcggcacggatcactgtattcggctgcaactttgtcatgcttgacactttatcactgataaacat
aatatgtccaccaacttatcagtgataaagaatccgcgcgttcaatcggaccagcggaggctggtccggaggccaca
tatgataacttctgctcttcatcgtgcggccgactgggctaaatctgtgttctcttcggcggcgctgggtgatcctc
gccgtactgcccgcttggttaacgtcgccgcccaattggcaaaatattctggtaaatcaataaccatctcatcagag
ggtagtaaagccgcccaggaaggcgcttaccgatttatccgcaatcccaacgtttctgccgaggcgatcagaaaggc
tggcgccatgcaaacagtcaagttggctcaggagtttcccgaactgctggccattgaggacaccacctctttgagtt
atcgccaccaggtcgccgaagagcttggcaagctgggctctattcaggataaatcccgcggatggtgggttcactcc
gttctcttgctcgaggccaccacattccgcaccgtaggattactgcatcaggagtggtggatgcgcccggatgaccc
tgccgatgcggatgaaaaggagagtggcaaatggctggcagcggccgcaactagccggttacgcatgggcagcatga
tgagcaacgtgattgcggtctgtgaccgcgaagccgatattcatgcttatctgcaggacaaactggcgcataacgag
cgcttcgtggtgcgctccaagcacccacgcaaggacgtagagtctgggttgtatctgtacgaccatctgaagaacca
accggagttgggtggctatcagatcagcattccgcaaaagggcgtggtggataaacgcggtaaacgtaaaaatcgac
cagcccgcaaggcgagcttgagcctgcgcagtgggcgcatcacgctaaaacaggggaatatcacgctcaacgcggtg
ctggccgaggagattaacccgcccaagggtgagaccccgttgaaatggttgttgctgaccagcgaaccggtcgagtc
gctagcccaagccttgcgcgtcatcgacatttatacccatcgctggcggatcgaggagttccataaggcatggaaaa
ccggagcaggagccgagaggcaacgcatggaggagccggataatctggagcggatggtctcgatcctctcgtttgtt
gcggtcaggctgttacagctcagagaaagcttcacgccgccgcaagcactcagggcgcaagggctgctaaaggaagc
ggaacacgtagaaagccagtccgcagaaacggtgctgaccccggatgaatgtcagctactgggctatctggacaagg
gaaaacgcaagcgcaaagagaaagcaggtagcttgcagtgggcttacatggcgatagctagactgggcggttttatg
gacagcaagcgaaccggaattgccagctggggcgccctctgggaaggttgggaagccctgcaaagtaaactggatgg
ctttcttgccgccaaggatctgatggcgcaggggatcaagatctgatccGTCGACTGtctcttatacacatctcaac
catcatcgatgaattcgagctcGGTACCCCCCCATGTCAGCCGTTAAGTGTTCCTGTGTCACTCAAAATTGCTTTGA
GAGGCTCTAAGGGCTTCTCAGTGCGTTACATCCCTGGCTTGTTGTCCACAACCGTTAAACCTTAAAAGCTTTAAAAG
CCTTATATATTCTTTTTTTTCTTATAAAACTTAAAACCTTAGAGGCTATTTAAGTTGCTGATTTATATTAATTTTAT
TGTTCAAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTTAGCCATGAGAGCTTAGTACGTTAGCCAT
GAGGGTTTAGTTCGTTAAACATGAGAGCTTAGTACGTTAAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGT
ACGTACTATCAACAGGTTGAACTGCTGATCTTCAGATCCTCTACGCCGGACGCATCGTGGCCGGGGTTCGAAATCGA
TGAGCTCGGGGGGGGGGGGGAAAGCCACGTTGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATAT
CATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAAACG
TCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGG
GCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTA
GCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAG
CATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGA
AGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTT
GTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGAT
GCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATT
CTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTT
GTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTTT
TCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTT
GATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGG
ACGGCGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACC
GTTCCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTCCCTC
ACTTTCTGGCTGGATGATGGGGCGATTCAGGCCTGGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCG
CCCCCCCCCCCCCGAGCTCTTAATTAATTTAAATCTAGAgtcgacctgcaggcatgcaagcttcagggttgagatgt

gtataagagaCAGTCGACctgcaggatcgatggcgcgccgacgtgctcatagtccacgacgcccgtgattttgtagc
cctggccgacggccagcaggtaggccgacaggctcatgccggccgccgccgccttttcctcaatcgctcttcgttcg
tctggaaggcagtacaccttgataggtgggctgcccttcctggttggcttggtttcatcagccatccgcttgccctc
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cagtgaagaaggaacacccgctcgcgggtgggcctacttcacctatcctgcccggctgacgccgttggatacaccaa
ggaaagtctacacgaacccttttggcaaaatcctgtatatcgtgcgaaaaaggatggatataccgaaaaaatcgctat
aatgaccccgaagcagggttatgcagcggaaaagcgctgcttccctgctgttttgtggcgcgccatcgatccgccg

Primers for sequencing off the ends of the transposon

tpnRL17-1 AACAAGCCAGGGATGTAACG
tpnRL13-2 CAGCAACACCTTCTTCACGA

Restriction Map of Transposon Tn*5*-RL23 (courtesy of New England Biolabs)

New England Biolabs' virtual restriction mapping page was used to generate the following information for Tn*5*RL23, 1711 bp in length (Larsen et al., 2002).

Enzymes that do not cut

Enzyme	**Specificity**
AccI	GTMKAC
BamHI	GGATCC
BglII	AGATCT
EagI	CGGCCG
EcoRI	GAATTC
EcoRV	GATATC
FseI	GGCCGGCC
HincII	GTYRAC
HpaI	GTTAAC
NaeI	GCCGGC
NcoI	CCATGG
NdeI	CATATG
NheI	GCTAGC
NotI	GCGGCCGC
PmeI	GTTTAAAC
PstI	CTGCAG
PvuII	CAGCTG
SacII	CCGCGG
SalI	GTCGAC
SfiI	GGCCNNNNNGGCC
SpeI	ACTAGT
SphI	GCATGC
XmnI	GAANNNNTTC

Enzymes that cut once

Enzyme	Specificity	Cut positions (blunt - 5' ext. - 3' ext.)
KpnI	GGTACC	5/1
PacI	TTAATTAA	1696/1694
PsiI	TTATAA	156
PvuI	CGATCG	*1002/1000
SmaI	CCCGGG	*876
StuI	AGGCCT	#1626
SwaI	ATTTAAAT	1702
XbaI	TCTAGA	1706/1710
XhoI	CTCGAG	*600/604

Enzymes that cut twice

Enzyme	Specificity	Cut positions (blunt - 5' ext. - 3' ext.)
ClaI	ATCGAT	*437/439, *693/695
DraI	TTTAAA	127, 1702
HindIII	AAGCTT	121/125, 1120/1124
SacI	GAGCTC	446/442, 1690/1686