

Module II

Remediation of Organophosphorus (OP) Pesticides

Exercise 1. Cloning of the gene encoding organophosphorus hydrolase (OPH), opd

Part 1.A. Isolation of Large Plasmid (> 30 Mdal) DNA

It has become evident over the past several decades that plasmids exist in virtually all bacterial species. These genetic elements are defined as autonomously replicating extrachromosomal DNA, and typically account for only a small fraction of a bacterial genome. While they generally range in size between 1 and 200 kilobases, large plasmids with sizes greater than 200 kb have been detected in several bacterial species. Plasmids of more than 50 kb might be characterized as large plasmids, whereas plasmids used as tools in molecular genetics are typically smaller than 10 kb. Plasmid isolation procedures are based on the typological difference between plasmids and genomic DNA, that is plasmids occur as covalently closed circular pieces of DNA. After cell lysis, all intracellular macromolecules have to be eliminated while the plasmid DNA is enriched and purified. In general, the smaller the plasmid, the easier is the isolation. DNA is very sensitive to mechanical stress, therefore the shearing forces of mixing or fast pipetting should be avoided. Gloves should be worn in order to prevent contamination with DNases. Typically, large plasmids are maintained at the level of only one copy per host cell chromosome. The large size and low copy number make recovery of significant quantities of good quality larger plasmids more challenging.

During plasmid isolate, effective lysis of the bacterial cell is probably the most important step, as it influences both the yield and quality of the harvested plasmid. Alkaline lysis is probably the most commonly used methods for cell lysis during a plasmid preparation, and it involves four basic steps: 1) resuspension, 2) lysis, 3) neutralization and 4) clearing of lysate. Alkaline lysis produces DNA of sufficient purity for uses such as in vitro transcription or translation or restriction digestion. However, for other applications or isolation of large plasmids, such as you are doing here, we will add an additional purification techniquesuch as phenol extraction, column purification, or silica-based purification. The basics of the alkaline lysis technique are as follows; your procedure has been adapted from these basic steps as required for isolation of large plasmids:

Resuspension:

- Spin down your cells. At this stage, the DNA is still in the cells, so it is in the pellet. Pay close attention to where the DNA is at all times. This can be tricky, as your DNA will move from supernatant to pellet to supernatant several times during the procedure.
 - Discard the supernatant. During growth, pieces of cell wall are released from the bacteria. These cell wall pieces can inhibit enzyme action on your final DNA, so it is important to get rid of all of the supernatant. This is accomplished by removing all traces of the supernatant by inverting the tube on a paper towel and wiping the lip with a Q-tip.
 - Resuspend the cells in buffer (often Tris), with EDTA and an osmolyte, such as glucose or sucrose. EDTA chelates divalent cations, which are primarily magnesium and calcium. Removal of these cations destabilizes the cell membrane and helps to inhibit any DNases which are present. The sugar is added to maintain osmolarity and prevent the buffer from bursting the cells.
- Lysis:
- The cells are lysed by the addition of a strongly alkaline solution of sodium hydroxide (NaOH) and sodium dodecyl sulfate (SDS) (hence the name “alkaline lysis”). Mix by gentle inversion and incubate on ice. Pay attention here, if you leave your sample too long at this step, your DNA will be irreversibly denatured. Three things are going on during this stage:

- SDS is a detergent found in many common items such as soap and shampoo. Here its function is to pop holes in the cell membranes.
- NaOH loosens the cell walls and releases the plasmid DNA and sheared genomic DNA.
- NaOH denatures the DNA. Genomic DNA becomes linearized and the strands are separated. Plasmid DNA is circular and remains topologically constrained.

Neutralization:

- Renature the plasmid DNA and get rid of the cellular debris by adding a salt such as sodium chloride (NaCl) or potassium acetate (KAc), which does three things:
 - The circular, or plasmid, DNA renatures. Sheared cellular DNA remains denatured as single stranded DNA (ssDNA).
 - The ssDNA is precipitated, since large ssDNA molecules are insoluble in high salt.
 - Adding sodium acetate to the SDS forms potassium dodecyl sulfate (KDS), which is insoluble. This allows for the easy removal of the SDS from your preparation.

Clearing of lysate:

- Centrifuge your sample to remove cell debris, KDS and cellular ssDNA. Note: the plasmid DNA is in the supernatant, while all of the debris is in the pellet. Separate the plasmid from the debris by transferring the supernatant to a clean tube.
- To further clean up the DNA, precipitate with alcohol (either ethanol or isopropanol) and a salt (such as ammonium acetate, lithium chloride, sodium chloride or sodium acetate) and spin this down. DNA is negatively charged, so adding a salt masks the charges and allows DNA to precipitate. Note: the DNA is now in the pellet
- Rinse your plasmid DNA (the pellet) in ice cold 70% EtOH and air-dry for about 10 minutes to allow the EtOH to evaporate.
- Resuspend your now clean DNA pellet in buffer and EDTA. You can may include RNases to remove any remaining RNA. Note: your DNA is now back in solution

[Pre-lab: Grow a 1 L culture of *Brevundimonas diminuta* for X hrs. Harvest by centrifugation, wash and dispense into 100 ml aliquots, pellet and freeze at -70 deg until just before use]

Option 1: Alkaline Lysis

Equipment:

Tubes

Vortex

Superspeed Centrifuge and GSA rotor (should I scale down so this is done in microfuge? Yield is ~10x less)

50 ml centrifuge tubes

Electrophoresis unit (gel box and power supply)

Reagents:

Lysis Buffer I

50 mM Tris, pH 8.0

1 mM EDTA

6.7 % Sucrose

Lysozyme

10 mg/ml in 25 mM Tris, pH 8.0

Lysis Buffer II

50 mM Tris, pH 8.0

250 mM EDTA

Sodium Dodecyl Sulfate (SDS)

50 mM Tris, pH 8.0
20 mM EDTA
20% wt/vol SDS
3.0 N NaOH (freshly prepared)
2.0 M Tris-HCl, pH 7.0
5.0 M NaCl
Saturated phenol solution
 Saturate phenol with 3% NaCl
Chloroform:isoamyl alcohol (42:1)
TE
 10 mM Tris, pH 7.5
 1 mM EDTA
Tris Acetate Buffer
 40 mM Tris, pH 8.1
 20 mM acetic acid
 2 mM Na₂EDTA
Agarose
 0.6% agarose in Tris Acetate buffer
Isopropanol

Procedure:

1. Resuspend pelleted cells in 5 ml of Lysis Buffer I
2. Warm to 37°C
3. Add 1.25 ml of lysozyme
4. Incubate for 5 min at 37°C
5. Add 0.625 mls of Lysis Buffer II
6. Add 0.375 mls of SDS, mix immediately
7. Incubate for 5 to 10 min at 37°C to complete lysis
8. Vortex at highest setting for 30 s in an appropriate sized tube
9. Add 0.4 mls of freshly prepared 3.0 N NaOH; mix gently by intermittent inversion or swirling for 10 min in a 50 ml tube.
10. Add 0.65 mls of Tris-HCl, pH 7.0; continue gentle mixing for 3 min
11. Add 0.9 mls of 5.0 M NaCl
12. Add 9.3 mls of saturated phenol; mix thoroughly
13. Centrifuge for 10 min at 5,000 rpm in GSA rotor
14. Remove the upper phase and extract with 9.3 mls of chloroform-isoamyl alcohol solution
15. Remove the upper phase, add 1 vol of isopropanol, incubate at 0°C for >60 min

< Time estimate: 2 hrs Due to the 0°C incubation, this is effectively the end of this lab period.
Coordination of centrifuge runs could significantly impact time at these steps>

Questions:

- 1) relationship between Mdal and # bp
- 2) explain adaption of standard alkaline lysis to lg plasmid
- 3)

Option 2: Qiagen lg plasmid kit

Part 1.B. Restriction Digest

This exercise will pick up where you left off previously, completing the isolation of the DNA, followed by digestion into fragments and finally ligation into a plasmid vector. In total, this process is considered to be cloning, the process of making a genetically identical copy of, in this case, a gene or segment of DNA.

Restriction Digest

Restriction digestion is the process of cutting the sugar-phosphate backbone of DNA molecules with enzymes called restriction endonucleases. Most of the commercially available restriction enzymes have been isolated from bacteria where they are a part of a host defense system known as the restriction-modification system. This system is proposed to protect the cell from foreign or invading DNA, as for example an infecting phage (a bacterial virus), by "restriction" or digestion within the bacterium by these enzymes. The presence of restriction enzymes raises the question of why they do not chew up the genomic DNA. In most cases, a bacterium that makes a particular restriction endonuclease also synthesizes a companion DNA methyltransferase. Methyltransferases methylate the target sequence in the genomic DNA, thereby protecting it from cleavage. This combination of restriction endonuclease and methylase constitutes the restriction-modification system.

By convention, restriction enzymes are named after their host of origin. For example, EcoRI was isolated from *Escherichia coli*, PstI from *Providencia stuartii*, and BamHI from *Bacillus amyloliquefaciens* H. The sites of cleavage for restriction enzymes, called the recognition sequence, can be unambiguous or ambiguous (also called degenerate). The enzyme BamHI, for example, recognizes the sequence GGATCC and no others, and so is considered unambiguous. In contrast, SfiI recognizes a 6 bp degenerate sequence CTPuPyAG, where Pu is any purine nucleotide and Py is any pyrimidine nucleotide. Depending on the enzyme, the recognition sites vary from 4 bp sequences to recognition sequences greater than 13 bp in length. Simple statistics tells us that, in a random sequence of DNA, the length of the recognition sequence determines how frequently the enzyme will cut. For example, in a random sequence of DNA a 4 bp recognition site will occur roughly every 256 base pairs. The recognition sites are not necessarily exclusive to a single enzyme, and restriction enzymes that have the same recognition site are called isoschizomers.

<add discussion of blunt vs sticky ends>

Equipment:

- High Speed Centrifuge
- GSA rotor
- Temperature-controlled waterbath
- Microfuge with rotor
- Ice buckets
- P20 and tips
- Marking pens
- Electrophoresis chamber, gel casting tray, combs, dams, power supply and cords
- Gloves
- light table and camera (or scanner)

Reagents:

- TE Buffer
- 10 mM Tris, pH 7.5
- 1 mM EDTA
- pUC19

Lambda DNA (?)
Restriction enzymes and buffer: BamHI and PstI
Sterile microfuge tubes
Ice
Sterile ddH₂O
Loading dye with Syber green
1 kb ladder
1% Agarose – 25 ml per group

Note to MEW (quantitate the DNA? Add RE set-up worksheet)

Note to RI: I really struggled at this point between moving rapidly through each step or spending more time on design and data analysis. I think that I, finally, have settled on fewer techniques in each lab period and more time on getting the students to “design” the experiment itself. A more general version of this question is: we can do less wet work each lab and more worksheet/analysis. For example, DNA quantitation and worksheet, restriction digest design as well as implementation, etc. What do you think?

Plasmid Isolation.

Retrieve your samples from last lab period from the freezer. To recover the plasmid, do the following:

1. Centrifuge for 20 min at 8,000 rpm in GSA
2. Remove all traces of isopropanol and resuspend in 200 µl of TE Buffer

Restriction Digestion of Bc plasmid and pUC19.

Tips:

- All components involved in digestion should be kept on ice, and the order in which you add the components is critical!!
- Perform steps 1 – 3, and 4 – 6 concurrently.

1. Prepare a BamHI and PstI double digest of pUC19 DNA by adding the required components to a clean microtube in the following order:

14 µl distilled water
2 µl 10 X BamHI Buffer
2 µl 10 X Pst I Buffer
3 µl pUC19 DNA (uncut)
1 µl each of BamHI and PstI

Place your Group # (initials, group id) on this tube and label it pUC19/Bam-Pst

2. Flick the tube to mix well and then spin for 5 sec in the microfuge to bring all the components to the bottom.
3. Incubate this digest mixture at 37°C for 20 minutes in a 37°C waterbath.
4. Prepare a BamHI and PstI double digest your *Brevendomonas cepacia* plasmid DNA by adding the components to a clean microfuge tube in the following order:

10 µl distilled water
2 µl 10 X BamHI and PstI buffer
6 µl Bc plasmid DNA
1 µl each of BamHI and PstI

Place your initials on this tube and label it Bc/Bam-Pst

5. Flick the tube and spin for 5 seconds in the microfuge to bring all the components to the bottom.
6. Incubate at 37°C in an incubator or waterbath for 20 minutes.
7. When the incubation of the digest ends, transfer the tubes to ice.

Tip: At this point each group should have 2 tubes (2 digests) which contain loading dye. These tubes can be stored on ice overnight.

8. Prepare a 1% Agarose Gel (five well comb) and allow it to stand for 20 minutes
9. After removing the dams, add the chamber buffer. Then, after a few minutes to allow the gel to soften, remove the comb by pulling straight up.

(Note to MEW - time can be saved by doing steps 8 and 9 prior to class),

10. Load 20 µl of each sample (the one kilobase ladder and 3 digests) into the wells in the order indicated.

Lane 1 - one kb ladder

Lane 2 - leave empty or use for practice loading

Lane 3 - pUC19 digest

Lane 4 - Bc digest

Lane 5 – lambda digest (?)

11. After the samples have been loaded, close the lid, connect the power supply, and switch on. Set the voltage at 110 volts.
12. Electrophorese for about 30 minutes until the dye has reached midway between the second and third red stripes. Switch off the power supply and open the lid.
13. Wearing gloves, remove the gel and place the gel in a tray and bring to the light box.
15. The instructor will photograph your gel.
16. After photographing, discard the gel and gloves in the waste container for proper disposal.

<note: gel photography will probably have to occur after the lab>

Part 1.C. Ligation and Transformation

Ligation

Ligation is the process of joining linear DNA fragments together with covalent bonds. The enzyme used to achieve this is a DNA ligase from the T4 bacteriophage. This enzyme will join DNA fragments with either overhanging, cohesive ends or with blunt ends by creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. A ligation reaction requires that you have:

- Two or more fragments of DNA that have either blunt or compatible cohesive ("sticky") ends.
- ATP concentration of roughly 0.25 to 1 mM
- 0.01 (sticky ends) to 1 (blunt ends) units of T4 DNA ligase.

Transformation

In bacteria, competence refers to the state of being able to take up DNA. In nature, some bacteria carry sets of genes which specify the proteins required to bring DNA across the cell's membranes. These genes confer the ability to achieve a state of natural competence. While the evolutionary function of these genes is controversial, the two likely scenarios are that the ability to take up DNA and acquire new versions of genes imparts some adaptive function, or that the acquired DNA serves as a source of nucleotides. Once in the cell's cytoplasm, it may be degraded by nucleases (source of nucleotides) or recombined into the host chromosome (and so available for function). Natural transformation is very efficient for linear molecules such as fragments of chromosomal DNA, but not for circular plasmid DNAs.

Laboratory induced, or artificial competence, is a procedure in which bacterial cells are made passively permeable to DNA, using conditions that do not normally occur in nature. These procedures are relatively simple, and can be used to genetically alter the bacteria. The two most common methods include a chemically induced competence and electroporation. The chemical competence involves chilling the target cells in the presence of divalent cations, such as CaCl_2 , making the cell walls permeable to plasmid DNA. The cells are then incubated with the DNA and then briefly heat, which causes the DNA to enter the cell. This method works well for circular plasmid DNA, but not for linear molecules. Electroporation uses a brief electrical shock with an electric field of 100-200V to create pores, which allows the plasmid DNA to enter the cell. In both cases, natural mechanisms will repair the cell wall as the cell recovers. It should be noted, that in bacteria the term transformation is not applied to genetic changes which arise by transfer of DNA mediated by phages (transduction) or transfer of DNA mediated by conjugative plasmids (conjugation).

Equipment:

Temperature-controlled waterbath
Microfuge with rotor

Reagents:

TE Buffer
10 mM Tris, pH 7.5
1 mM EDTA
LB plates, +/- Amp and x-gal
Ampicillin
Agar
Yeast extract
Tryptone
NaCl
Sterile microfuge tubes
Ampicillin

CaCl₂
Sterile ddH₂O
Fermentas Rapid Ligation Kit

Ligation

1. To a microcentrifuge tube, add the following:

10 – 100 ng digested Vector DNA (pUC19)
X Bc DNA over vector
4µl 5X Rapid Ligation Buffer
Nuclease free water,

2. Bring up to 19 µl with nuclease free water
3. Add 1 µl of T4 DNA Ligase
4. Vortex and then spin briefly to collect all liquid in the bottom of the tube.
5. Incubate the mixture at 22°C for 5 minutes.
6. Use 2-5 µl of the ligation mixture for transformation

<Note to mew: potential stopping place; the reaction mixture can be stored at 0-4°C until used for transformation>

Transformation

While the restriction digestion is incubating, begin the preparation of chemically competent cells. (CaCl₂ Method)

Pre-lab

1. Grow single *E.coli* (DH5α) colonies on LB plates.
2. Transfer a single colony to 100 ml LB broth with the appropriate antibiotic in 500 ml flask. Incubate at 37°C at 300 rpm until culture reaches mid log phase (O.D. 600nm = 0.6).
3. Transfer the culture to ice cold 50 ml centrifuge tubes. Cool in ice for 10 min.

Lab

4. Pellet the cells at 3000 rpm at 4°C for 10 mins.
5. Decant the supernatant. Resuspend each pellet in ice cold 20 ml 0.1M CaCl₂.
6. Store on ice for 5-10 mins.
7. Pellet the cells again at 3000 rpm at 4°C for 10 mins.
8. Decant the fluid. Invert the tube on clean kimwipes till the fluid drains away.
9. Resuspend the pellet in 2 ml ice cold 0.1M CaCl₂.

Note to MEW: An alternative here is to provide competent cells.

10. Transfer 200µl of competent cells to a microfuge tube.
11. Add 10µl of plasmid (2 ng/µl) from your ligation mixture.
12. Mix the contents and store in ice for 30 mins.
13. Transfer the tubes to 42°C waterbath for 90 sec.
14. Transfer the tubes to ice and chill it for 1-2 mins.
15. Add 800 µl LB medium. Incubate at 37°C for 45 min.
16. Spread LB Amp (30µg/ml) plates with different amounts of transformants.
 - (i) 10µl from step 15 + 90 µl LB
 - (ii) 100µl from step 15.
17. Incubate the plates at 37°C overnight.

<NOTE to MEW: add plates labels>

Questions

1 What is the difference in number of sites which a RE with a six base recognition site will cut if it is unambiguous vs degenerate.

If we do the electrophoresis:

2. How many places does Bam/Pst cut pUC19? How many fragments are produced?
3. Approximate the size of the pUC19 fragment(s) using the kilobase ladder. (Note to MEW: Do we want to require graphing using semi-log paper?)
4. How many places does Bam/Pst cut Bc plasmid? How many fragments are produced? Approximate the sizes of these fragments.
6. How many fragments are produced by digestion of your Bc plasmid? Is this what you expected (refer to question 1 above)? Explain.

If we include a lambda digest, the following:

7. The digest of Lambda DNA by produces fragments of the following sizes; 21226 bp, 7421 bp, 5804 bp, 5643 bp, 4878 bp, 3530 bp. (?) The 5804 and 5643 fragments may appear as a single heavy band. Why? Using the graphing technique and the one kilobase ladder bands and their migration distances, approximate the sizes of the lambda fragments and compare these to the actual sizes listed above. Determine a percent error for each fragment measurement.

<Note to mew: add worksheet for platings and results>

Exercise 2. Screening of Library

Part 2.A. Selection and Screening

Probably the greatest strength of the discipline of molecular biology is the ability to manipulate the genetic material. Discrete fragments of DNA may be generated by the action of restriction endonucleases, isolated and rejoined in a new arrangement by the action of DNA ligase. This ability allows for the creation of novel genes and genetic constructs which can be used to isolate and study the function of genes or proteins. These “new genes”, typically located on plasmid DNA, are introduced into a host cell through the process of transformation. At least two outcomes are typically required of a transformation event: 1) that the transgenes are stably passed on through successive generations, and 2) that a new function is imparted to the organism. For our purposes, the new functions we want to confer onto the *E. coli* host are resistance to the antibiotic ampicillin and the ability to hydrolyze the OP compound, paraoxon.

These new functions allow for the identification of the transgenic organisms against a background of “wildtype” organisms. The transgenic *E. coli* can be identified either by *selecting* for the ability to withstand the antibiotic, or *screening* for the ability to hydrolyze paraoxon. Those two terms are very specific in their meaning, and there is generally a significant difference in the power to discriminate associated with the two. Selection provides a way to easily identify and select the transgenic cells from non-transgenic cells through the use of a selectable marker gene. In our case, the selectable marker is the *bla* gene, which encodes the protein β -lactamase (Figure 1—this will be a plasmid map). β -lactamase is an enzyme that is responsible for resistance to β -lactam antibiotics, such as ampicillin. Structurally, the lactams all have a four-atom ring known as a β -lactam. These antibiotics stop bacterial growth by interfering with cell wall synthesis. The lactamase is secreted into the surrounding medium, where it destroys the antibiotic by opening the ring structure. This allows the bacterium to synthesize a cell wall and thus grow, conferring the phenotype of growth in the presence of ampicillin. By plating the population of transformed cells onto media containing ampicillin, only those cells which are expressing the *bla* gene will be able to resist the antibiotic and grow into visible colonies. This is a powerful method which allows for the identification of even a handful of transgenic organisms from a population of 10^9 – 10^{11} cells.

With the advent in the past decade of high throughput methods, screening is rapidly becoming as powerful as selection. Screening involves the identification of a desired phenotypic characteristic uniquely associated with the transgenic cells. In our case, the ability to hydrolyze the OP pesticide paraoxon can be used to identify the transgenic colonies. Paraoxon hydrolysis can be monitored by the appearance of the yellow p-nitrophenol anion. Colonies incubated in the presence of paraoxon will hydrolyze the substrate, turning the incubation medium yellow. Although this is a useful method for the identification of cells transformed with the *opd* gene, it can be labor intensive as it requires the harvesting and evaluation of hundreds, possibly even thousands, of individual colonies.

<note to mew: add discussion on blue/white screening, and add discussion of extinction coefficient adjustment for pathlength>

Equipment:

Plate reader, visible wavelength

Supplies and Reagents:

96-well plates, flat bottom

Diethyl p-nitrophenyl phosphate (Paraoxon)

MW 275.21

ChemService cat#PS-610

LD50 = 2 mg/kg

CHES (2-[cyclohexylamino]ethanesulfonic acid), 99%

MW 207.3
Sigma cat # C-2880
CoCl₂ • 6H₂O
MW 237.9
Sigma cat # C-3169

Pre-lab preparation:

Reaction Buffer:

Stock 1 M CoCl₂, sterile

23.79 g CoCl₂ per 100 ml ddH₂O
can be filter sterilized or autoclaved

Stock 200 mM CHES, pH 9.0, sterile

4.15 g + 80 ml ddH₂O
adjust pH to 9.0 with NaOH
bring to 100 ml total volume with ddH₂O
can be filter sterilized or autoclaved

Assay Buffer

20 mM CHES, pH 9.0
50 μM CoCl₂

Paraoxon preparation:

1. Paraoxon will arrive as an oily liquid in 100 mg aliquots. Add 200 mgs of paraoxon to 50 mls ddH₂O.
2. Let stir in the cold for 2-3 days to be sure it is fully dispersed and dissolved.
3. The [paraoxon] should be 14.5 mM; due to loss during pipetting, solubility, etc. the solution rarely reaches this concentration.
4. To determine the [paraoxon], make the following dilutions:

1:100 10 μl paraoxon stock:990 μl ddH₂O
1:500: 2 μl paraoxon stock:998 μl ddH₂O
1:1000: 10 μl (1:100) paraoxon:990 μl ddH₂O

5. Read O.D. at 274 nm; typical readings are:

1:100 = 1.1
1:500 = 0.5
1:1000 = 0.130

6. The extinction coefficient of diethyl p-nitrophenyl phosphate (paraoxon) is 8,900 M⁻¹cm⁻¹

7. Sample calculations:

$(1.1/8,900) \times 100 = 0.0123 \mu\text{mol}/\mu\text{l}$
 $(0.0123 \mu\text{mol}/\mu\text{l}) \times (1,000,000 \mu\text{l}/\text{l}) \times (\text{mm}/1000 \mu\text{moles}) = 12.3 \text{ mM concentration of paraoxon}$

Note to mew: Have students do steps 4 – 7?

Note to mew: for selection and blue/white screening develop a worksheet.

Activity screening:

1. Using a P200, add 150 μ l of reaction buffer to the first 6 rows of a 96 well plate.
2. Using sterile toothpicks, pick 48 colonies from your transformation plate into each well of rows 1 - 4. Swirl your toothpick to leave as much biomass in the well as possible. Select only those colonies which meet the selection and screening criteria to be good candidates for having the *opd* gene.
3. Using a new, sterile toothpick, go back to the same colony on your transformation plate, and transfer to a new LB + Amp plate.
4. Using similar technique, pick 12 colonies as negative controls into each well of row 5.
5. Add 2 μ l of prepared substrate to each well, rows 1 - 6.
6. Cover the plate, labeling the side with your group # and put the plate in the 30° incubator
7. Every 15 min (?) remove the plate, and determine the absorbance at 410 nm. Record the readings.

<note to mew: develop table for data collection>

Note to RI: This one is still pretty muddled in my mind. I think it best to do the following, in three different labs:

- 1) selection and blue/white screening + set-up PCR screening (including primer selection)
- 2) gel of PCR and data analysis,
- 3) activity screening

Exercise 3. Expression and partial purification of OPH

<Note to RI: How much of the growth, which is 40 hrs, do we want the students to participate in>

The organophosphorus hydrolase enzyme is purified by contacting an aqueous solution of cell free bacterial proteins with a strong cation exchange resin, the aqueous solution comprising soluble organophosphorus hydrolase enzyme, washing the strong cation exchange resin with a washing buffer to remove unbound proteins from the strong cation exchange resin, eluting proteins that remain bound to the strong cation exchange resin by washing the resin with an eluting buffer comprising salt in a concentration that starts at about zero and is raised during the eluting process to about 0.5M, and detecting and collecting eluate comprising a protein having organophosphorus hydrolase enzyme activity. All processing steps are conducted at a pH that is less than the isoelectric point of the organophosphorus hydrolase enzyme

Note to RI: we have three different options here for lysis, depending on what equipment is available. They are all briefly outlined here.

Pre-lab

Reagents

TB

ampicillin

LB

Purification Buffer

10 mM KPO, pH 6.7

50 μ M CoCl₂

CoCl₂

Equipment

fernbach flasks

autoclave

culture tubes

roller drum or tube rack at 37° C.

shaking incubator at 30°C

superspeed centrifugation

centrifuge bottles

Freezer, -20 °C

Procedure

Day -4:

1. Prepare 4 fernbach flasks with 1 L of TB per flask and autoclave to sterilize.
2. Add 5 μ l of ampicillin to four culture tubes containing 5ml of LB
3. Inoculate the culture tubes containing LB+amp with colonies from your streak plate.
4. Place the overnight tubes in either a roller drum or tube rack to agitate overnight at 37° C.
5. Prepare:
8L of 10mM KPO, 50 μ M CoCl₂, pH 6.7

Day -3:

1. Add 1ml of CoCl₂ and 1ml of ampicillin to each fernbach flask.
2. Inoculate each flask with 1 overnight tube.
3. Place the four inoculated flask in a shaking incubator heated to 30°C at 4pm.
4. 12-15 hr later, add another 1ml of ampicillin to each flask

Day 1:

1. Approximately 40 hours later, harvest the cultures by centrifugation. Weigh the bottles first so have the biomass weight. Pour off the spun down media
2. Weigh the bottles containing the cell pellets, you should obtain ~25 g of wet cell pellet per liter. Add 4-5x the weight of the pellet in 10mM KPOi, 50 μ M CoCl₂, pH 6.7.
3. Agitate until the pellet is resuspended.
4. Collect cells again by centrifugation. pour off wash supernatant, freeze pellets until ready for use.

Cell harvest. <note to MEW: add section on growth of culture>

Reagents

Equipment

superspeed centrifugation
centrifuge bottles
double balance

Procedure

1. Obtain 60 mls of the prepared bacterial culture.
2. Transfer 500 μ l of this culture to a microfuge tube. Label with your initials, group id, sample #1. Store on ice.
3. Fill two centrifuge tubes with 30 mls each.
4. At the double balance, transfer culture from one centrifuge tube to the other until the tubes are balanced. Make sure to place the caps on the balance as well as the centrifuge tubes. Dispose of the dirty pipettes in the autoclave waste.
5. Pellet the bacteria by centrifugation at 8,000 rpm for 10 min.
6. Decant the supernatant from each tube into a waste bucket, taking care not to disturb the pellet.

Cell lysis (note to RI: we have 4 options here, depending on available equipment>

Option 1. enzymatic lysis

Reagents

Lysosyme
Lysis buffer
50 mM Tris-HCl pH 7.5
5% glycerol (v/v)
1 mM DTT
1 mM PMSF

Stock solutions

1 mg/ml DNase in water
100 mM PMSF (phenylmethylsulfonyl fluoride) in isopropanol (?)
1M MgCl₂

Equipment

Ultra or superspeed centrifuge
Rotors (Ti24 or SS34)

Procedure

1. Resuspend the cells in chilled lysis buffer in a ratio of 1 g cell wet weight to 1 ml lysis buffer.
2. Add the PMSF (10 μ l PMSF (100 mM) per ml of cell suspension) at this point.

3. Add lysosyme to a final concentration of 300 $\mu\text{g/ml}$ and incubate the cell suspension at 4°C for 4 h. (Note: this will effectively end this lab period)
4. Add 5 μl MgCl_2 (1 M) and 1 μl DNase solution (1 mg/ml) per ml of cell suspension and incubate the solution at 4°C for 30 min.
5. Remove cell debris by ultracentrifugation at 4°C for 30 min at 45 000 rpm using a 45Ti rotor (I think we can get by with a superspeed at 10,000 – 12,000 rpm, but will be a longer spin)

Option 2. sonication

Reagents

Lysis buffer

50 mM Tris-HCl pH 7.5

5 mM DTT

1 mM PMSF

Stock solution

100 mM PMSF (phenylmethylsulfonyl fluoride) in isopropanol (?)

Equipment

Sonicator and tip

Ultra or superspeed centrifuge

Rotors (Ti24 or SS34)

Procedure

1. Resuspend the cells in chilled lysis buffer. Normally ratios of cell wet weight to buffer volume of 1:1 to 1:4 are used.
2. Cool the cell suspension on ice for 10 min.
3. Add 10 μl PMSF (100 mM) per ml of cell suspension after this step.
4. Sonicate the cell suspension with 6-7 short burst of 50 sec followed by intervals of 50 sec for cooling. Tips: Keep the suspension at all times on ice, avoid foaming. Don't go away while the sonicator is in operation. It is possible that the beaker breaks or turns in the melting ice.
5. Remove cell debris by ultracentrifugation at 4°C for 30 min at 45 000 rpm using a 45Ti rotor (45 min at 13,000—we use a SLA rotor)

Option 3. French press

Reagents

Lysis buffer

50 mM Tris-HCl pH 7.5

1 mM MgCl_2

5 mM DTT

1 mM PMSF

Stock solutions

1 mg/ml DNase in water.

100 mM PMSF in isopropanol.

Equipment

French Press cell (40 ml)

Superspeed or ultra centrifuge

Ti24 or SS34 rotors

Procedure

1. Prechill the French Press cell at 4°C.
2. Resuspend the cells in chilled lysis buffer. Normally ratios of cell wet weight to buffer volume of 1:1 to 1:4 are used. Add 1 µl DNase solution (1 mg/ml) per ml of cell suspension to avoid viscosity problems.
3. Add 10 µl PMSF (100 mM) per ml of cell suspension at this point.
4. Apply the sample to the French pressure cell and bring the cell under the desired pressure (7000 to 10,000 psi).
5. While maintaining the pressure, adjust the outlet flow rate to about one drop every second. Collect the cell lysate in a flask that is kept on ice.
6. Repeat steps 3 and 4.
7. Remove cell debris by ultracentrifugation at 4°C for 30 min at 45 000 rpm using a 45Ti rotor

Option 4. Freeze-thaw (this is probably our best option, although not a widely used technique)

Reagents

Lysis buffer

50 mM phosphate, pH 7.5

5 mM DTT

Lysozyme

1 mM PMSF

Stock solutions

1 mg/ml DNase in water.

100 mM PMSF in isopropanol.

Equipment

Dry ice/ethanol bath

Superspeed or ultra centrifuge

Ti24 or SS34 rotors

Procedure

1. To each tube, add 2.5 ml of phosphate buffer and resuspend the pellet by repeated pipetting up and down.
2. Once the pellets in both tubes are resuspended, combine the contents into one conical tube, keeping the empty tubes for step 6 below.
3. Freeze the cells by incubating in the dry ice/ethanol bath until the cells are completely frozen (3 -5 minutes).
4. Thaw the cells in a 37°C water bath until they are completely thawed.
5. Repeat steps 9 and 10.
6. Transfer the lysed bacteria back to a single centrifuge tube, using the second one as a balance (fill with water).
7. Centrifuge at 15,000 rpm for 10 minutes.
8. The supernatant should turn a murky amber color as protein is released from the cell. If the supernatant is clear, repeat steps 9 and 10. If lysis has occurred, carefully decant the supernatant into a clean calibrated conical tube, measure the volume. The supernatant fraction is the “cell free extract”, or CFX.

<note to mew: this is a potential stopping place>

Partial purification

Reagents

10% streptomycin sulfate solution in 10 mM Tris, pH 7.0

Ammonium sulfate

Purification Buffer:

10 mM KPOi, pH 6.7

50 μ M CoCl₂

Equipment

Stir plates

Stir bars

Large flasks (> 2L)

1. Add 10 ml of 10% streptomycin sulfate solution per 100 ml of supernatant.
2. Stir the solution for 15-20 minutes on ice. The solution should be cloudy.
3. Spin in the SLA1500 rotor for 30-40 minutes at 12,000rpm.
4. Carefully decant the supernatant into your calibrated tube, record the volume and remove 500 μ l to an epp tube. Label with your initials, group id, sample #2. Store on ice.
5. Pour the supernatant back into the glass beaker. Weigh out 25.8 g ammonium sulfate per 100 ml of supernatant. Crush any clumps so that you have a fine crystalline powder.
6. Add the ammonium sulfate very slowly while stirring. When all has been added, the solution is now 45% ammonium sulfate.
7. Stir the ammonium sulfate solution for one hour.
8. Spin down the ammonium sulfate solution in the SLA1500 rotor at 15,000 rpm for 70 minutes
9. Decant the supernatant and add x ml of 10 mM KPOi, 50 μ M CoCl₂ pH 6.7 to the pellet
10. Shake the tubes gently to resuspend the pellet. Remove 500 μ l to an epp tube, label with your initials, group id, sample #3. Store on ice.
11. Transfer the solution to a dialysis bag and dialyze for 2 hrs against 2 L of 10 mM KPOi, 50 μ M CoCl₂ pH 6.7
12. Exchange the buffer for 2 L of fresh 10 mM KPOi, 50 μ M CoCl₂ pH 6.7.
13. Dialyze for another 2-3 hours.

<Note to MEW: need to shorten the procedure, or split between 2 lab periods>

Exercise 4 Enzymatic Assay of Organophosphorus Hydrolase (OPH)

Equipment

U.V. Spectrophotometer
U.V. 1 cm pathlength cuvettes
Pipettes

Reagents

Diethyl p-nitrophenyl phosphate (Paraoxon)

ChemService cat# PS-610

CAS# 311-45-5

MW 275.22

LD50_{oral-rat or mouse} = 0.7-2 mg/kg

LD50_{dermal-rat or mouse} = 6 mg/kg

Specific gravity = 1.2683 g/ml

Water solubility_{20 deg C} = 3.64 g/L

Vapor Pressure_{pxn @ 20 deg} = 0.0000011 mm Hg (Vapor Pressure_{H2O @ 20 deg} = 17.5 mm Hg)

CHES (2-[cyclohexylamino]ethanesulfonic acid), 99%

MW 207.3

Sigma cat # C-2880

CoCl₂ • 6H₂O

MW 237.9

Sigma cat # C-3169

Pre-lab

Reaction Buffer Preparation:

- 1 M CoCl₂, (sterile)
add 23.79 g CoCl₂ to 100 ml ddH₂O
can be filter sterilized or autoclaved
- 200 mM CHES, pH 9.0 (sterile)
add 4.15 g CHES to 80 ml ddH₂O
adjust pH to 9.0 with NaOH
bring to 100 ml total volume with ddH₂O
can be filter sterilized or autoclaved
- Assay Buffer
20 mM CHES, pH 9.0
50 μM (micromolar) CoCl₂

Paraoxon preparation:

1. Paraoxon will arrive as an oily liquid in 100 mg aliquots. Add 200 mgs of paraoxon to 50 mls ddH₂O.
2. Let stir in the cold for 2-3 days to be sure it is fully dispersed and dissolved.
3. The [paraoxon] should be 14.5 mM; due to loss during pipetting, solubility, etc. the solution rarely reaches this concentration.
4. To determine the [paraoxon], make the following dilutions:

1:100: 10 μl paraoxon stock:990 μl ddH₂O

1:500: 2 μl paraoxon stock:998 μl ddH₂O

1:1000: 100 μl 1 (1:100) paraoxon:900 μl ddH₂O

5. Read O.D. at 274 nm; typical readings are:

$$1:100 = 1.1$$

$$1:500 = 0.5$$

$$1:1000 = 0.130$$

- The extinction coefficient of diethyl *p*-nitrophenyl phosphate (paraoxon) is $8,900 \text{ M}^{-1}\text{cm}^{-1}$
- Sample calculations:
 $(1.1/8,900) \times 100 = 0.0123 \text{ } \mu\text{mol}/\mu\text{l}$
 $(0.0123 \text{ } \mu\text{mol}/\mu\text{l}) \times (1,000,000 \text{ } \mu\text{l}/\text{l}) \times (\text{mm}/1000 \text{ } \mu\text{moles}) = 12.3 \text{ mM concentration of paraoxon}$

Paraoxonase assay

This is typically an extremely fast reaction. You will determine activity by monitoring the production of *p*-nitrophenol at Abs_{405} , using its extinction coefficient of $17,000 \text{ M}^{-1}\text{cm}^{-1}$.

- For a 1.0 ml assay, determine the necessary enzyme dilution (see Appendix) to yield a linear change in absorbance for your reaction time, generally 0.5-1.0min.
- In a 1.5 mL cuvette add the following:

790 μl ddH₂O
 100 μl 200 mM CHES
 10 μl enzyme dilution

- Add 100 μl of 10 mM paraoxon to start the reaction, mix by inverting.
- Record the absorbance at 405 nm (A_{405}), blanking against CHES buffer + paraoxon. (Under these conditions, there will be a small amount of hydrolysis of paraoxon without enzyme present).
- To do a substrate saturation assay, perform the assay as described in steps 2-4, using the amounts indicated in the following table:

[Paraoxon] (mM)	10 mM PXN (μl)	ddH ₂ O	Enzyme	200 mM CHES
0.00	0.0	890	10 μl	100 μl
0.01	1	889	↓	↓
0.05	5	885		
0.1	10	880		
0.2	20	870		
0.3	30	860		
0.5	50	840		
0.7	70	820		
1.0	100	790		
1.5	150	740		
2.0	200	690		

Data Analysis

Calculate the initial velocities for the recorded slopes.

$$\frac{\Delta Abs \cdot \text{min}^{-1}}{\epsilon} \cdot \frac{1}{60 \text{ sec}} \cdot 1 \text{ cm} \cdot \frac{1 \text{ L}}{1000 \text{ ml}} \cdot \frac{1 \text{e}^6 \mu\text{mol}}{\text{mol}} = \frac{\mu\text{mol}}{\text{sec}}$$

The extinction coefficient (ϵ), will be different depending on the product that is monitored. For *p*-nitrophenol use $17,000 \text{ M}^{-1}\text{cm}^{-1}$. The pathlength may vary (for example, if assay is performed in a 96 well plate), this is for a 1 cm cuvette.

Plot v_0 versus $[S]$ and fit to the Michaelis equation to determine V_{max} and K_M .

$$v_0 = \frac{V_{\text{MAX}} \cdot [S]}{K_M + [S]}$$

Then calculate k_{cat} :

$$k_{\text{cat}} = \frac{V_{\text{max}}}{[E_T]}$$

DemetonS Assay

The hydrolysis of demeton-S by OPH yields a product that contains a free sulfhydryl. The assay procedure utilizes the reaction of 2,2'-dithiodipyridine (2,2TP, 1 mM final concentration in the reaction) with thiols, which gives the corresponding 2-thiopyridone. The ultraviolet absorption of the thiopyridone formed is measured at 343 nm in 0.5X tripart buffer (2X tripart: 50 mM MES, 25 mM N-ethyl morpholine, 25 mM diethanolamine), pH 8.0 at 25°C. This product is colorless, with a molar extinction coefficient of $7,060 \text{ M}^{-1}\text{cm}^{-1}$. (Grassetti & Murray, 1967)

Reagents:

Demeton-S (3.87 M)

ChemService cat# PS-662

CAS Number: 126-75-0

MW 258.36

2,2'-Dithiodipyridine (5 g from Sigma, cat # D-5767)

MW 220.3

2-N-morpholinoethanesulfonic acid (MES from Sigma, cat # M2933)

MW 195.2

Diethanolamine (from Sigma, cat # D8885)

MW 105.1

4-ethylmorpholine (from Sigma, cat # E0252)

MW 115.2

Reaction Buffer Preparation:

2X Tripart:

7.808 g MES

2.58 ml N-ethylmorpholine

2.15 ml diethanolamine

Add to 180 ml ddH₂O and titrate to pH 8.0 with KOH; add to 200 ml volumetric flask and adjust to final volume of 200 ml. Store at 4°C.

8 mM Stock of DemetonS (50 mls)

- a) Weigh out 0.011 g of 2,2'-Dithiodipyridine in a microfuge tube and add 500 :l of 100% methanol and vortex to dissolve it.
- b) Add 30 mls of ddH₂O, 12.5 mls of 2X tripart buffer to a beaker with a stir bar. Add 103 :l of ChemServ DemetonS stock directly to the beaker, cover, and let stir for a while.
- c) Add the dissolved 2,2'-Dithiodipyridine, and let the solution stir.
- d) Titrate to pH 8.0 if necessary with KOH
- e) Bring to a final volume of 50 mls using a volumetric flask.
- f) Store in a screw cap tube closed tightly to reduce evaporation and store at 4°C until use (which should be soon).

Reaction Buffer (0.5X Tripart) (50 mls)

- a) Weigh out 0.011 g of 2,2'-Dithiodipyridine in a microfuge tube and add 500 :l of 100% methanol and vortex to dissolve it.
- b) Add 30 mls of ddH₂O, 12.5 mls of 2X tripart buffer to a beaker with a stir bar.
- c) Add the dissolved 2,2'-Dithiodipyridine, and let the solution stir.
- d) Titrate to pH 8.0 if necessary with KOH
- e) Bring to a final volume of 50 mls using a volumetric flask.
- f) Store in a screw cap tube closed tightly to reduce evaporation and store at 4°C until use (which should be soon).

Assay

This assay utilizes the reaction of 2,2'-dithiodipyridine with the free thiol of hydrolyzed demeton-S to yield the corresponding 2-thiopyridonem which can be monitored at 343 nm in 0.5X tripart This product is colorless, with a molar extinction coefficient of 7,060 M⁻¹cm⁻¹.

1. For a 1.0 ml assay, determine the necessary enzyme dilution (see Appendix) to yield a linear change in absorbance for your reaction time, generally 0.5-1.0min. (For purified OPH, [protein] of approximately 1 x 10⁻⁵ μmol in a 1.0 ml assay usually gives good results)
2. In a 1.5 mL cuvette, mix the following:

990 μl 8 mM demetonS

3. Add 10 μl of enzyme dilution to start the reaction, mix by inverting.
4. Record the absorbance at 343 nm (A₃₄₃), blanking against Rxn buffer + D-S.
5. To do a substrate saturation assay, perform the assay as described in steps 2-4, using the amounts indicated in the following table:

DemetonS (mM)	8 mM D-S (μl)	Reaction Buffer	Enzyme
0.00	0.0	990	10 μl
0.1	12.5	977.5	↓
0.25	31.25	958.75	
0.5	62.5	927.5	
1.0	125.0	865	
2.0	250.0	740	
4.0	500.0	490	
6.0	750.0	240	
8.0	990.0	0	

Data Analysis

Calculate the initial velocities for the recorded slopes.

$$\frac{\Delta Abs \cdot \text{min}^{-1}}{\epsilon} \cdot \frac{1}{60 \text{ sec}} \cdot 1 \text{ cm} \cdot \frac{1 \text{ L}}{1000 \text{ ml}} \cdot \frac{1e^6 \mu\text{mol}}{\text{mol}} = \frac{\mu\text{mol}}{\text{sec}}$$

The extinction coefficient (ϵ), will be different depending on the product that is monitored. For 2-TP use $7,060 \text{ M}^{-1}\text{cm}^{-1}$. The pathlength may vary (for example, if assay is performed in a 96 well plate), this is for a 1 cm cuvette.

Plot v_0 versus $[S]$ and fit to the Michaelis equation to determine V_{max} and K_M .

$$v_0 = \frac{V_{\text{MAX}} \cdot [S]}{K_M + [S]}$$

Then calculate k_{cat} :

$$k_{\text{cat}} = \frac{V_{\text{max}}}{[E_T]}$$

Divide the k_{cat} by the K_M .

Reference:

Grassetti, D.R., and Murray, JR., J.F., Determination of Sulfhydryl Groups with 2,2'- or 4,4'-Dithiopyridine. *Archives of Biochemistry and Biophysics* **119**, (1967) 41-49.

Exercise 5 Structure and Kinetic Characteristics of Organophosphorus Hydrolase

PyMOL is one of several freeware visualization and modeling software; a tool which is essential to understanding structural biology today. Visualization is key part of understanding the nature of life at the molecular level.

<note to mew: expand intro to mol graphics>

Equipment

Windows 2000 or XP

A 3D OpenGL compatible graphics accelerator card from nVidia, ATI, 3Dlabs or similar

512 MB RAM (768 MB or 1 GB preferred)

3 Ghz Pentium 4 processor or similar

Three button mouse

Getting Started:

1. Click on the **Start** menu, follow it to **Programs** (or **All Programs** on Windows XP), and then release the mouse on **PyMOL**.
2. PyMOL normally starts with three windows: The Viewer Window and the External GUI Window.

<note to mew: add pic of pymol windows>

The **Viewer Window** represents the heart of the PyMOL visualization system. This is a single OpenGL window where all 3D graphics are displayed and where all direct user interaction with 3D models takes place. The Internal GUI contained within this window allows you to perform actions on specific objects and specific atom selections. From top to bottom, it contains an object list, a mouse button configuration matrix, a frame indicator, and a set of "VCR"-like controls for working with movies. The Viewer also contains a command line (bottom) which can be used to enter PyMOL commands. It is also possible to view PyMOL text output in the Viewer window. You can hit the ESC key anytime to toggle between text and graphics mode inside the Viewer window.

PyMOL comes with a single **external GUI window** which provides a standard menu bar, an output region, a command input field, and a series of buttons. One important advantage of the external GUI window is that standard "cut and paste" functions for text will only work within the External GUI, and not within in the PyMOL Viewer. Furthermore, you must use Ctrl-X, Ctrl-C, and Ctrl-V to cut, copy, and paste because a standard Edit menu has not yet been implemented.

3. Loading structural files from the Protein Data Bank (PDB files).
Files can be loaded for viewing either using the external GUI window or Command line entry. We will be loading the pdb file for OPH, and both options will work. There are currently x structural files for OPH currently in the Protein Database. We will use XXXX as our initial example, but you are free to experiment with any of the files which interest you.
 - The external GUI provides a standard "Open" in the File menu which you can use to select the file you wish to open.
 - The syntax for using the Command option is load <filename>, for example load test/dat/pept.pdb
4. After loading a PDB file, you will need to become familiar with some of the commands and views.
 - The mouse is the primary control device for manipulating the view of your molecule. Keyboard modifier keys (SHIFT, CTRL, SHIFT+CTRL) are used to modulate mouse behavior.

- A table of the **basic mouse button/keyboard combinations** for view manipulation is provided below. There is an abbreviated version of this table, called the Mouse Matrix, displayed in the Internal GUI to help you remember which key and mouse button performs which action.

<note to mew: insert command table>

- **Virtual Trackball Rotation** works as if there is an invisible ball in the center of the scene. When you click and drag on the screen, it is as if you put your finger on the sphere and rotated it in approximately the same manner. If you click outside the sphere, then you get rotation about the Z-axis only. Generally, the view will be easiest to control by either clicking in the center of the scene and moving outwards (mostly XY-rotation), or by clicking and dragging around the edge of the screen and moving in a circular fashion (Z-rotation).
 - **The Clipping Planes** control is somewhat unusual and may take a few minutes to get used to. Instead of having separate controls for the front and back clipping planes, controls are combined into a single mode where up-down mouse motion moves the front (near) clipping plane and left-right mouse motion controls the back (far) clipping plane. After you get used to it, the clipping plane control becomes easy through the diagonal motions shown below. (see the graphics below). Alternatively, you can also use the "clip" command to control the clipping planes.
5. When visualizing molecules, it is frequently necessary to change the origin of rotation so that you can inspect a particular region of the molecule. The fastest way to do this is to Control-Shift-Middle-Click on a visible atom in the scene.

In the next section, we will step through a typical PyMOL session, introducing typed commands and describing how PyMOL responds to them. At this point, however, I recommend that you spend five or ten minutes getting comfortable with the controls described thus far. Specifically, you should be able to accomplish the following tasks:

- Load a PDB file into PyMOL
- Rotate, translate, and zoom the camera
- Adjust the front and back clipping planes to clearly view a slice of the molecule
- Change the origin of rotation about any particular atom of interest