Chapter 10

Plasmids

Plasmids are small, circular pieces of DNA that replicate independently of the host chromosome. Plasmids have revolutionized molecular biology by allowing investigators to obtain many copies of custom DNA molecules. In this lab, you will isolate plasmids from non-pathogenic strains of *Escherichia coli*, which you will use in subsequent experiments to transform *Saccharomyces cerevisiae met* strains.

Objectives

At the end of this laboratory, students should be able to:

- describe the structure of plasmids and their mechanism of replication.
- identify functional elements that have been engineered into laboratory plasmids.
- explain how the physical properties of plasmids are used in their purification.
- isolate plasmids from transformed strains of *Escherichia coli*.
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Plasmids are the workhorses of molecular biology. Plasmids are small, circular DNA molecules that replicate independently of the chromosomes in the microorganisms that harbor them. Plasmids are often referred to as vectors, because they can be used to transfer foreign DNA into a cell. The plasmids used in molecular biology have been constructed by researchers, who used recombinant DNA technology to incorporate many different functional elements into naturally-occurring plasmids. Plasmids have been engineered to carry up to 10 kb of foreign DNA and they are easily isolated from microorganisms for manipulation in the lab. For the next few labs, your team will be working with yeast overexpression plasmids. Your team will work with three plasmids: a plasmid carrying an *S. cerevisiae* MET gene, a plasmid carrying its *S. pombe* homolog, and a plasmid carrying the bacterial lacZ gene, which will act as a negative control. In this lab, you will isolate plasmids from bacterial cultures. In the next few weeks, you will characterize the plasmids and then use them to transform mutant yeast strains, testing whether MET gene function has been conserved between *S. cerevisiae* and *S. pombe*.

**Plasmids are composed of functional elements**

**Plasmid replication depends on host cell polymerases**

Plasmids are found naturally in many microorganisms. Plasmids can be transferred between species by transformation or conjugation, but they generally have a restricted host range. When you think of plasmids, you probably also think of bacteria, but plasmids are not restricted to bacteria. In fact, most *S. cerevisiae* strains carry a large plasmid known as the 2 micron or 2 µm plasmid. Multiple copies of the 2 µm plasmid are usually present in the nucleus of a yeast cell, and the plasmid number is stable through many rounds of cell division.

Although plasmids replicate independently of the chromosomal DNA, they rely on host enzymes to catalyze their replication. Host DNA polymerases bind to an origin of replication (*ori*) sequence in the plasmid. Plasmids that replicate in bacteria have *ori* sequences that bind bacterial DNA polymerase, while plasmids that replicate in yeast have distinct *ori* sequences that bind yeast DNA polymerase. The plasmids that we are using are sometimes referred to as “shuttle vectors,” because they are able to replicate in more than one kind of cell. Our plasmids contain the *ori* of plasmid, pBR322, which is replicated in *E. coli* to a copy number of 30-40. The plasmids also contain the *ori* of the *S. cerevisiae* 2 µm plasmid described above. In this class, we will propagate the shuttle vectors in bacteria, because bacteria grow more rapidly than yeast and because the yield of plasmid from bacteria is higher than the yield from yeast. We will harvest the plasmids from bacteria and then use them to transform yeast cells.

**Laboratory plasmids carry selectable markers**

Plasmids place a toll on the host cell’s metabolism, and they would normally be lost from their host cells if they did not confer some selective advantage to the host. The plasmids used in molecular biology therefore carry genes for selectable markers, which allow transformed cells to grow under non-premissive conditions, conditions where untransformed cells are unable to grow. Our plasmids contain the β-lactamase (*ampR*) gene, which allows *E. coli* to grow in the presence of ampicillin.
of ampicillin, an antibiotic that interferes with bacterial cell wall synthesis. The plasmids also contain the *S. cerevisiae* URA3 gene, which allows *ura3* mutants like BY4742, the parent strain of our mutants, to grow in the absence of uracil only after they are transformed with plasmids (Chapter 12).

**Promoters control transcription of plasmid sequences**

The plasmids that we will use this semester contain MET genes that have been cloned into plasmids directly downstream of the promoter sequence for the yeast GAL1 gene (Johnston, 1987). Transcription from the GAL1 promoter is normally controlled by regulatory proteins that sense glucose and galactose levels in yeast (Chapter 13). In the plasmids, the GAL1 promoter has been placed at the 5'-ends of protein coding sequences for *S. cerevisiae* Met proteins, their *S. pombe* orthologs or bacterial LacZ. The presence of the GAL1 promoter will allow you to manipulate expression of the Met proteins or LacZ in transformed yeast cells.

**Comparison of yeast overexpression vectors**

The figure below compares the plasmids that you will be using to overexpress *S. cerevisiae* and *S. pombe* proteins. The plasmids have many similarities, but some significant differences. ORFs for the *S. cerevisiae* proteins were cloned into the plasmid pBG1805 cloning vector in a genome-wide experiment (Gelperin *et al.*, 2005). ORFs for the *S. pombe* proteins were individually cloned into the pYES2.1 plasmid by the BIOL2040 staff. In all cases, the ORFs were cloned downstream of the GAL1 promoter (element 5 in the diagram). Both plasmids contain the yeast URA3 gene (element 1) and the bacterial β-lactamase gene (element 2). Both plasmids also contain the pBR322 ori sequence for bacterial replication and the 2 µm ori sequence for yeast replication. The placement of the functional elements in the two plasmids is quite distinct, consistent with their independent development by different laboratories.
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As you can see from the figure on the previous page, the plasmids also differ in the C-terminal tags that are added to overexpressed proteins. The proteins expressed from both plasmids are fusion proteins that are longer than the natural coding sequences. During the cloning processes used to construct the overexpression plasmids, researchers deleted the natural stop codons of the ORFs so that transcription would continue into plasmid-encoded sequences. The plasmid sequences encode tags that can be used for procedures such as western blots (Chapter 15) or protein purification. The pBG1805 plasmid adds a 19 kDa extension (element 6) to expressed proteins, while the pYES2.1 plasmid adds a smaller 5 kDa extension (element 7). The tags will be discussed in greater detail in Chapter 13.

Plasmid nomenclature

Proper nomenclature is important for distinguishing plasmids. The “p” in pBG1805 denotes that it is a plasmid, while the remainder of the plasmid name is a code used by the researchers who constructed the plasmid. Often, the letters in a plasmid’s name contain the initials of the researcher who performed the final step in its construction. In this case, “BG” refers to Beth Greyhack, who was one of the senior authors on the paper (Gelperin et al., 2005). The pYES2.1 plasmid was developed by researchers working for a commercial source. In this course, we will follow the convention of denoting the plasmid backbone in normal font, followed by a hyphen and then the name of the cloned ORF in italics. In our experiments:

- pBG1805-MET1 would designate the *S. cerevisiae MET1* gene cloned into pBG1805.
- pYES2.1-Met1 would designate the *S. pombe Met1* gene cloned into pYES2.1. (Recall from Chapter 6 that *S. cerevisiae* genes are unusual in using 3 capital letters for their names.)
- pYES2.1-lacZ+ would designate the bacterial LacZ gene cloned into pYES2.1. 
  (Note: bacterial gene names are not capitalized. Plus sign indicates wild type gene.)

*NOTE: The *S. pombe* ortholog of an *S. cerevisiae* gene may not share the same gene number.* Many of the *S. pombe* genes received their names due to their homology to previously identified *S. cerevisiae* genes, but some *S. pombe* genes had been named before their DNA sequences were known. For example, the ortholog of *S. cerevisiae MET2* in *S. pombe* is *Met6.*

Plasmids are easily isolated from bacterial cells

Plasmid isolation takes advantage of the unique structural properties of plasmids. Plasmids are small, supercoiled circular pieces of DNA. Unlike the much larger bacterial chromosome (which is also circular), plasmids are quite resistant to permanent denaturation. Today, most laboratories use commercial kits for plasmid isolations, because the kits are convenient and relatively inexpensive. The kits give good yields of high-quality DNA, while avoiding the need for organic denaturant. A variety of less expensive, but somewhat more time-consuming, procedures have been described for investigators who want to make their own reagents. These latter procedures generally give good yields of DNA, but the DNA is often less
pure than DNA isolated with kits. Whatever the isolation procedure, the general principles of plasmid isolation are the same. The figure and paragraphs below summarize the steps and general principles used for plasmid isolation.

1. **Lysis and denaturation** - Strong denaturing conditions are used to weaken the tough bacterial cell wall. The most common procedures use a combination of strong base and a detergent. The detergents help to solubilize lipids in the cell wall, allowing the denaturants to enter the cell. Proteins, because of their fragile structures, are irreversibly denatured. The treatment also breaks the hydrogen bonds holding together the two strands of DNA helices.

2. **Neutralization** - Neutralization allows complementary DNA strands to reanneal and causes proteins to precipitate. Plasmids renature because they have supercoiled structures that have held the two strands of the helix together during denaturation. Chromosomal DNA is not able to renature, however, because its longer strands have become mixed with denatured proteins. **Samples must be mixed gently at this step to prevent fragmentation of the long, chromosomal DNA into pieces that might be able to reanneal and co-purify with the plasmids.**

3. **Centrifugation** - Plasmid DNA is separated from large aggregates of precipitated proteins and chromosomal DNA by centrifugation.

4. **Additional purification** - Plasmids are further purified by organic extraction or adsorption to a resin.
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The plasmids that we are working with in this lab have been maintained in a laboratory strain of *Escherichia coli*. *E. coli* is a proteobacterium that normally inhabits the intestinal tract of warm-blooded mammals. The virulent strains of *E. coli* that appear in the news have acquired, often by lateral gene transfer, pathogenicity islands containing genes for virulence factors, toxins and adhesion proteins important for tissue invasion. Pathogenicity islands are not present in lab strains, which are derivatives *E. coli* strain K12. K12 is a debilitated strain that is unable to colonize the human intestine. It has been propagated and used safely in the laboratory for over 70 years. The *E. coli* strains used in molecular biology have also been selected to tolerate large numbers of plasmids.

We will use the Zyppy™ (Zymo Research) kit to purify plasmids from the transformed *E. coli* strains. The final purification step in the procedure involves a spin column of silica resin. Nucleic acids bind tightly to silica in the presence of high concentrations of salt. Following a wash step that removes any residual proteins, the plasmids are eluted with a low salt solution. Plasmid solutions are very stable and can be stored for long periods of time in the refrigerator or freezer.

**Exercise 1 - Plasmid isolation with the Zyppy™ kit**

**Obtain the plasmid-bearing bacterial cells**

1. Collect the three bacterial cultures that your group has been assigned. The bacteria have been transformed with plasmids containing either an *S. cerevisiae MET* gene, its *S. pombe* ortholog or bacterial *LacZ*. Each culture contains 0.6 mL Luria Bertani (LB) medium with 100 µg/mL ampicillin. Cultures were grown overnight at 37 degrees C, and the cell density is expected to be 3-4 X 10⁹ cells/mL.

   *What is the purpose of the ampicillin? How does it work?*

2. Add 100 µL of 7X Blue Zyppy Lysis buffer to the tube. Mix the buffer and cells by gently inverting the tube 4-6 times. Be gentle! Too much mechanical stress could fragment the bacterial chromosomal DNA and contaminate your plasmid preparation. The solution should turn from a cloudy blue to a clear blue.

   **NOTE: This step is time-sensitive!! Proceed to the next step within 2 minutes.**

3. Add 350 µL of cold Yellow Zyppy Neutralization buffer (w/RNAse A) to the tube, and mix the contents thoroughly by inverting several times. The solution will turn yellow when neutralization is complete, and a yellowish precipitate will form. Invert the sample an additional 3-4 times to ensure complete neutralization. **Be sure there is no more blue color.**
4. Centrifuge the mixture at maximum speed for 3 minutes to remove denatured proteins and chromosomal DNA. Notice that the tube contains a white precipitate that has collected on one side of the tube. The pale yellow supernatant contains the plasmid DNA.

**Purify plasmid DNA by adsorption to a silica resin.**
5. Using a pipette, carefully transfer the pale yellow supernatant (~900 µL) onto a Zyppy spin column. Be careful not to transfer any of the yellow precipitate! *Label the column - NOT the collection tube that you will use in the next step.*
6. Place the column with the collection tube attached into a centrifuge and spin at maximum speed for ~1 minute.
7. Remove the column and discard the flow through in the collection tube.
8. Place the column back into the collection tube and add 200 µL of Zyppy Endo-Wash solution. (Endo-Wash contains guanidine hydrochloride and isopropanol, which will remove denatured proteins from the resin.)
9. Centrifuge for ~1 minute and discard the flow through.
10. Place the column back into the collection tube then add 400 µL of Zyppy Column Wash buffer. (This steps removes contaminating salts.) Centrifuge for ~1 minute. Empty the collecting tube.
11. Repeat the centrifugation step to remove any residual ethanol.

**Elute the plasmid DNA**
12. Transfer the Zyppy column to a clean (and appropriately labeled) 1.5 mL centrifuge tube, leaving the lid of the tube open.
13. Carefully, add 100 µL of TE buffer directly on top of the white column bed. Place the pipette tip as close as you can to the white column bed without poking it. Slowly dispense the buffer on top of the resin bed.
14. Allow the buffer to percolate into the column by letting the column sit in the microcentrifuge tube for 1 minute.
15. Centrifuge the column at maximum speed for 30 seconds. Again, it's fine to leave the cap open during this spin.
16. Remove the column, cap the tube and place it on ice. This tube should now contain plasmid DNA. *Label the tube. SAVE the DNA for future experiments.*
References
