Experiment 3
(Lab Periods 3 and 4)

Extraction of DNA from Bacteria

The first demonstration that pure DNA could transmit genetic information from one kind of cell to another was reported in l944 by Avery, McCarty and MacLeod, who investigated the chemical identity of the "transforming principle". Although their proof was biologically and chemically rigorous, it was not immediately accepted by biologists, biochemists and geneticists because nucleic acids were regarded as too simple to carry information. Gradually, other kinds of investigations provided reasons to believe that genes were, indeed, composed of DNA, and when the double-helical structure of DNA and the triplet nature of its information content were elucidated, it became clear how DNA molecules could serve as genes.

In this experiment, you will attempt to extract DNA from a species of bacteria (*Bacillus subtilis*) and observe the stringy nature of DNA molecules. The DNA you lift out of the tube at the end would be suitable for introducing heritable information into another kind of bacterium. You will then use spectrophotometry to calculate the amount of DNA that you have extracted from the cell by resuspending the DNA in solution and measuring A_{260}. Finally, this will allow you to calculate your yield of DNA per bacterium.

You will be provided with l0 ml of a culture of *Bacillus subtilis* from which you will plate a sample in order to calculate the concentration of cells. You will collect the rest of the cells by centrifugation and wash the cells in Lysis Medium. The washed cells will then be treated with an enzyme (lysozyme) that dissolves their cell walls, a detergent (SDS) that dissolves their cell membranes, and a second enzyme (pronase) that dissolves their proteins. The only large molecules left intact will be nucleic acids. These will be "salted out" and mildly dehydrated with ethanol so that the long DNA molecules will become entangled with each other and will stick to a glass rod. Once you have isolated the DNA you will measure the amount you have isolated by using spectrophotometry.

Procedure

DNA Extraction (Lab Period 3)

As you work through the procedure, record observations of the macroscopic appearance (turbidity and viscosity, in particular) of the mixtures. To begin each group will be provided with an Extraction Sample. In addition there will be a Measurement Sample for the entire section.

1. Using a pipet, the first group will take 0.1 ml of the Measurement Sample and mix it with 0.9 ml of saline solution. After vortexing, remove 0.1 ml of the diluted sample and mix with 0.9 ml of saline to produce a 10^{-2} dilution.

2. The first group will plate 0.1 ml of the 10^{-2} dilution following the plating procedure from Experiment 2. The 10^{-2} dilution will then be passed to group two which will repeat step 1 to produce a 10^{-4} dilution, from which 0.1ml will be added to a plate. This 10^{-4} dilution will be passed on to group three and the procedure will continue through all groups to produce 10^{-6}, 10^{-8} etc. dilutions, each of which is plated by a different group. (The plating can be done anytime during the procedure – the best time is during the centrifugation steps or during the incubation in step 7. To plate, add 50ul of the dilution you are working with to a plate labeled with your group number, lab period and the dilution factor, and spread it following the procedure you used in Experiment 2.)

3. Record the volume of your Extraction Sample and then place your tube of Extraction Sample in the rotor of the centrifuge across from another student's tube; tubes must be placed in the rotor so that it is balanced across its diameter at every position. Centrifuge at 5,000 rpm for 8 min.

4. When the rotor has stopped, remove your tube. Using a Pasteur pipet, lift off and discard the clear liquid (the supernatant).

5. Add 2 ml of Lysis Medium (LM) to the pellet of bacteria at the bottom of the centrifuge tube and resuspend the bacteria in the liquid by alternately slurping the mixture up and down in the pipet and hand-vortexing it. Add a further 8 ml of LM, mix well, and centrifuge again at 5,000 rpm for 8 min.

6. Lift off and discard the clear supernatant as in step 2. Resuspend the pellet in 2 ml of LM as in step 3. Add a further 3 ml of LM and mix well. Note the appearance of the suspension.

7. Add 0.5 ml of lysozyme solution, mix well, then place the tube in the rack in a water bath at 37°C. Incubate the mixture at 37°C for 30 min. Two or three times during this period, gently mix the contents of the tube.

8. At the end of the 30 min., add 0.5 ml of l0 % SDS solution, mix gently, but thoroughly, and transfer the tube to the rack in a 50°C water bath. Incubate at 50°C for l0 min.

9. At the end of the l0 min., add l ml of pronase solution, mix gently, but thoroughly (slurp up and down in the pipet), then continue to incubate at 50°C for 30-50 min. During this time, divide your glass rod in two by heating the center of the rod in a flame until it softens. While it is soft, pull it out gently to create two points that then separate. Fuse just the tip of each point so that a bead of fused glass not more than l mm in diameter forms at the tip. One of these will be your spooling rod for step 13. The other will be used to push DNA off the spooling rod.

10. At the end of the 30-50 min. (longer is better, but allow about 30 min. to complete the remaining steps), remove the tube to room temperature.

IMPORTANT: Place the tube containing 2 ml SSC close to the tubes containing the lysis mixture, and lay a microscope slide beside it. The next steps must be done without hesitation because both treatments--NaCl and ethanol additions--will withdraw water of hydration from the DNA and cause it collapse. Successful purification of the DNA requires just the right amount of dehydration; excessive loss of water from the DNA will cause it to collapse into a rubbery mess.

11. Add 0.8 ml of 5M NaCl. To mix, pour back and forth into the larger centrifuge tube, finally leaving the mixture in the larger tube.

12. Very slowly, pour l5 ml ethanol down the wall of the tube onto the mixture; two layers should form.

13. Insert the pointed end of the rod prepared in step 7 into the viscous, lower, layer and gently rotate the rod in one direction. As you continue to rotate the rod, tilt the tube and pull the rod slowly out of the tube through the ethanol layer, using the wall of the tube to prevent the DNA from sliding off the rod. (This technique is known as "spooling" DNA.) Transfer the spooled DNA to the tube containing 0.5ml of TE buffer. Seal the tube with parafilm, label it with your group number, and give this to your instructor.

Calculations (Lab Period 4)

C1. During the week your DNA sample should be fully resuspend in the TE buffer. To determine the amount of DNA you isolated you will use spectrophotometry. Vortex you sample very well and transfer 10 ?l to a tube containing 990ul of water. Mix this well and with your instructor measure the absorbance of the dilution at 260nm (the peak of the DNA absorption spectrum). Since this is not in the visible region (DNA is not a pigment) you must use a special spectrophotometer than can emit UV light. To determine the amount of DNA you isolated:

1. Conc. in ?g/?l (diluted sample) = (A_{260} / E) * (1?g/1000ng)

where E for DNA at 260nm is 1 / (50 ng/?l)

2. Conc. of original sample (?g/?l) = Conc (diluted sample) * 100 (the dilution factor)

3. Amount of DNA (?g) = Concentration of original solution * 500?l (volume you resuspended your DNA in)

C2. Determine which plate has a suitable concentration of colonies and count the number on this plate. Calculate the concentration of cells in the original culture as follows:

1. Cells/ml (diluted sample) = # colonies * 10 (dilution factor for plating)

2. Conc. of original culture (cells/ml) = # colonies/(dilution for that plate)

3. Number of cells = Conc. of culture * (Volume of the Extraction Sample)

4. Amount of DNA (?g) per cell = Amount of DNA / Number of cells.

Lab Report

There is a lab report due for this experiment. Following the general outline, write a lab report that presents your findings concerning the amount of DNA present in a single cell of *Bacillus subtilis*.