The Ames Test

*** Note**: You will be writing up this experiment as **Scientific Research Paper #1** for the laboratory portion of Biology 222. Be sure you fully understand the background and fundamental biological principles of this experiment as well as why you are performing each step of the procedure. Take careful notes on your materials and methods and results as you will need these to prepare the paper.

Schedule

This week we will prepare the media and reagents needed for this experiment. Next week you will do a trial run of the experiment using control substances and you will design an independent investigation using this technique. The following two weeks will be used to carry out your investigation. ***Please complete the questions at the end of this handout before class time next week. You should also do some preliminary research on substances you may wish to test for your independent investigation and bring those ideas to class next week.***

Background

Humans and other animals are surrounded by a variety of chemical substances, both naturally occurring as well as synthetic, that have the potential to act as **mutagens**. Some of these substances are in the food we eat, others in the air we breathe, and still others can be absorbed through the skin or via other contact. Mutagens act in a variety of ways but they all have the ability to alter the DNA base sequence (e.g. recall point mutations, frameshift mutations, etc.) within the genome. Cancer researchers and clinical oncologists would likely agree that most (though not all) mutagens have the potential to act as **carcinogens** and can play a role in the induction of neoplastic cell growth seen in many cancers.

The procedure you will perform was developed by Bruce Ames in order to simply, quickly and cheaply screen substances (mainly commercial products) for their ability to act as mutagens in bacteria. Though not all mutagens identified by the Ames test turn out to be potent carcinogens, the procedure provides useful preliminary information that can be further investigated in more sophisticated (and time-consuming as well as more costly) animal model systems to assess the potential carcinogenicity of a substance that results in a positive Ames test.

The Ames test utilizes specific strains of the bacteria *Salmonella typhimurium* as tools to detect mutations. These strains of *S. typhimurium* used are known as **auxotrophs**. A bacterial strain is defined as an auxotroph if it is unable to produce a required nutrient (the test organism in this experiment cannot synthesize the amino acid histidine) and thus will not grow unless the nutrient is supplied in growth media. Auxotrophs are usually produced as a result of a mutation(s) that occurs in a **prototroph** (a bacteria that is able to synthesize the particular nutrient).

The mutant *S. typhimurium* strains TA1535 and TA1538 are histidine auxotrophs (denoted his^- in order to distinguish it from the original his^+ prototrophic strain they were derived from) due to a mutation in a gene encoding an enzyme required for histidine biosynthesis. These *S. typhimurium* strains also have other characteristics that enhance their ability to detect mutations. A mutation distinct from that present in the histidine

biosynthesis gene makes the cell wall of the bacteria more permeable to large molecules that normally would not enter the cells. They also possess a mutation in a gene responsible for proper excision and repair of DNA damage, thus increasing their sensitivity to mutagens. This last mutation also disrupts the gene for biotin, so these strains must be supplied with biotin as well as histidine.

The Ames test determines the ability of a tested substance to cause a reversal, also called a **back-mutation** of these auxotrophs to the original prototrophic state. During the test auxotrophs are grown on **glucose-minimal salts agar plates** that contain all required nutrients but only trace amounts of histidine (as well biotin). The auxotrophs are able to growth for several generations until the histidine in the media is exhausted, at which time they will stop growing unless they have sustained a back-mutation that has restored their ability to synthesize histidine (these his^+ cells can be described as **revertants**).

Suspected mutagenic substances are tested for their ability to stimulate back-mutations by placement onto paper discs which are set on the surface of the minimal agar plates previously inoculated with the auxotroph. The test substance diffuses into the surrounding media and, if mutagenic, will induce back-mutations which will then allow growth into visible colonies. The more revertants observed near the test substance, relative to experimental controls, the more mutagenic the substance is likely to be.

The trace amount of histidine included in the minimal media plates allows his^- auxotrophs to divide several times in the presence of the potential mutagen. This is critical since many if not most mutations occur due to errors made during DNA replication by DNA polymerase. Although bacteria are prokaryotic and therefore differ in significantly in cellular structure and metabolism from animal (eukaryotic) cells, the chemical nature of DNA is common to all organisms and thus permits the use of bacterial model systems in the preliminary evaluation of potential mutagens. Identified mutagens are presumably inducing many mutations throughout the *S. typhimurium* genome and not solely in the histidine biosynthesis genes. We are able to screen for reversion mutations to histidine prototrophy, however, therefore these are the only mutations 'observed' during this experiment.

Protocol

Preparation of media

Work in pairs to make the media to be used during both control and test runs of the experiment. You will make approximately **20 plates** of **glucose-minimal salts agar medium**.

a. You will make 500 ml of **glucose-minimal salts** in **1.5% agar**.
b. Calculate the amount of **agar** needed to make 500 ml of a 1.5% agar solution. Weigh out agar and add to flask containing 465 mls of ddH_2O.
   *Note: The agar you are using is not as highly purified as the type used for making agarose gels for running DNA. It will serve as a solidifying agent in your media.
c. As demonstrated by your instructor, sterilize the agar/water solution by autoclaving for 15-20 min. at 121ºC. Set up and label 20 sterile Petri plates.

d. Following sterilization, place flask in 45.5° C water bath to cool for 5 minutes, then take flask to your bench and carefully add 25 ml of sterile **40% glucose** and 10 ml of sterile **50X Vogel Bonner salts** solution using sterile glass pipettes. Swirl to mix well and pour approximately 20-25 mls per plate (stop pouring when plate surface is covered) into bottom of labeled Petri plates. Cover with the lid and allow agar to set. Leave plates alone on benchtop for at least 15 minutes or until they appear solid. Place the finished plates in a plastic bag in a refrigerator for next week.

Inoculation and pouring of soft agar overlay

You will be working with small volumes of soft agar into which bacteria as well as histidine/biotin will be added. It is important to work quickly once you begin additions to the tubes of soft agar and to use glucose-minimal agar plates that have been warmed to room temperature or warmer (up to 35°C). Pipetting, mixing and pouring agar as an overlay on your plates should take no more than 20-30 seconds. Delay in pouring or spreading on plate will result in ripples on plate surface and will make your results more difficult to interpret.

a. Find 2 tubes containing 3 mls of molten soft (top) agar in a 45.5°C water bath. Using pipettors and tips, pipette 150 µl of *Salmonella typhimurium* strain TA1538 and 300 µl of histidine/biotin solution into each tube.
b. To mix, roll tubes between hands or use a vortex mixer. Pour immediately on top of glucose-minimal media agar plates. Tilting and rotating plates, distribute the melted top agar evenly over the surface. Allow the inoculated soft agar to set for several minutes.

Trial experiment:

Setting up plates for exposure to mutagens

You and your partner will use a 'spot test' in which controls and test substances are absorbed onto sterile filter paper disks. The discs are then placed on the surface of the plates to allow substances to diffuse into the agar and contact dividing *Salmonella* cells.

a. One plate will serve as the location of a negative control: sterile water. A second plate will serve as the location of two positive controls: 4-nitro-o-phenylenediamine (4-NOPD) at 100 ug/ml in 95%EtOH, and 2-nitrofluorene at 100 ug/ml in 95%EtOH.
   ***Note**: Remember that next week you will be testing substances that you supply on your own at this stage.
b. Dip a set of forceps in ethanol, flame to sterilize, then pick up a sterile paper disk and touch it to the surface of distilled H_2O. Allow the water to just moisten the disk (it should not be dripping wet). Touch disk to side of the container to remove excess liquid. Place the disk on your negative control plate and tap gently to adhere to agar surface. Label as the negative control. Repeat procedure on the positive control plate for 4-NOPD and 2-nitrofluorene.
c. Incubate both plates inverted at 35°C for 48 hours.

Observations after 48 hours

You should notice the appearance of a light 'lawn' or haze of bacterial growth throughout the medium on both plates. Why did all the auxotrophic mutants grow in this manner?

Observe, count and record the number of revertant (i.e. back-mutated) colonies appearing on both plates (Are the cells in these colonies his^+ or his^-?). Are there more colonies associated with the positive controls when compared to the negative control? Devise a way to quantify the relative mutagenicity of a test substance that accounts for the background of spontaneous mutations (which are nearly always seen) observable in the negative control experiment. You can develop a scale of high, moderate, low and none to judge the relative potency of the test substance as a mutagen when compared to controls.

Test Protocol:

This experiment will be performed individually and is the basis for the first scientific research paper. Now that you have experience setting up an Ames test, you will design your own test(s) using controls and test substances of your choosing.

You will have available:

1) Glucose-minimal agar plates made during week 1 (should be warmed to at least room temperature prior to pouring soft agar overlay).
2) Soft agar in 3 ml batches at 45.5ºC (maximum of 9/student)
3) Overnight culture of *Salmonella typhimurium* (two tester strains will likely be available; TA1535 and TA1538)
4) Histidine/biotin solution
5) Negative and positive controls, sterile paper disks, forceps, incubators, etc.

You will need to bring:

Substances to test for mutagenicity. They can be as straightforward or as creative as you like, but remember that you are going to write up this experiment as a formal scientific paper. Put some thought into just what you would like to test. Think about substances that you encounter on a routine basis in your diet, lungs or through direct skin contact. You have 9 plates available and multiple disks (up to 3 or 4) can be placed on a plate. You could therefore try different concentrations of your test substances during your experiment and/or test effects on both tester strains of *Salmonella*.

Criteria for testable substance:

1) Must be liquid or able to be made into a liquid or paste for application to disks
2) Must be available to yourself or through asking your instructor about availability. Many commercial products are testable using the Ames test.
3) Make sure you bring or arrange to have access to your test substances next week during laboratory.

You will set up the experiment, collect data, describe your results, form conclusions and relate your experiment in a scientific paper.

Questions

1) Describe the purpose of an Ames test.

2) Define the following terms:
    a. Mutagen

    b. Carcinogen

    c. Auxotroph

    d. Prototroph

    e. Back-mutation

3) Name three characteristics of the *Salmonella* tester strains used that make them useful for mutagenesis studies.

4) Explain the relationship (if any) between mutagens and carcinogens.

5) What is the purpose of the histidine/biotin solution in the experiment?

6) What are some advantages of the Ames test over tests for carcinogens that use rats or mice?