A. Chemical Mutagenesis of E. coli using Nitrosoguanidine

The chemical mutagen N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG) is a powerful and commonly used mutagen. It is a member of an important class of mutagens called alkylating agents. Alkylating agents induce mutations by adding methyl or ethyl groups (ie. alkyl groups) to DNA at positions that alter the pairing properties of the bases. Thus, mutagenesis by alkylating agents involves mispairing during DNA replication, and this leads primarily to base pair substitution mutations.

Nitrosoguanidine (MNNG)

Alkylating agents have long been recognized as animal carcinogens. In fact, the link between DNA damage and carcinogenesis was first clearly demonstrated for these agents. Alkylating agents are present in the environment as a consequence of industrial processes. There are probably non-anthropogenic alkylating agents, but their identities are not established. There a some reports of non-enzymatic methylation of DNA by endogenous S-adenosylmethionine (SAM). The infamous "nitrosamines" formed by cooking meat products preserved with nitrites are also alkylating agents.

Base pair substitution following treatment with NTG involves methylation of guanine at the oxygen of carbon #6. During DNA replication, the O6-methylguanine adduct pairs with thymine (rather than cytosine), leading ultimately to substitution of an AT pair for the original GC pair. Thus, NTG is an example of a unidirectional base pair substitution-inducing mutagen.

Alkylated bases may be repaired or replaced prior to the DNA replication when mispairing would occur. In this case, no mutation is induced. The widespread occurrence of specific enzymes capable of repairing alkylated DNA suggests that alkylating agents are prevalent mutagens in the natural environment.

An example of such a repair enzyme is O6-methylguanine methyltransferase, the product of the ada gene in E. coli. This enzyme recognizes O6-meG in DNA and removes the methyl group by transferring it permanently to one of its constituent amino acids. Expression of the ada gene in E. coli is induced by exposure to NTG.
Mutagenesis invariably leads to some extent of cell killing. After all, many of the introduced mutations will have lethal phenotypes. Therefore, we cannot hope to increase our yield of viable mutants by simply increasing the exposure to the mutagen because we will simply end up killing all the cells. Mutagenesis, then, requires finding a happy medium between mutagenesis and killing.
PROCEDURE: NTG Mutagenesis of E. coli

HAZARD

Nitrosoguanidine is a powerful mutagen and known carcinogen.

The procedure will be performed as a demonstration through Day 1, Step 8.

SCHEDULE

Day 1: Tuesday 4/13
Day 2: Wednesday 4/14
Day 3: Thursday 4/15
Day 4: Friday 4/16
Day 5: Tuesday 4/20

Pre-Lab: Prepare flowsheet for Day 3, step 1 before class.
Day 1  Tuesday 4/14

1. Dilute 1.0 ml of an overnight culture (strain MG 1655) into 30 ml LB and incubate 1.5 hr. at 37°C. (This will be done before lab begins.)

2. Harvest cells by centrifugation and re-suspend in an equal volume of Tris-Maleate Buffer (TM).

3. Harvest cells again and re-suspend in an equal volume of TM.

4. Split the cell suspension into two equal portions (15 ml each). One portion is treated with NTG, the other is the control.

5. Add 5 ml of NTG [1 mg/ml] to one cell suspension and 5 ml of diH2O to the control.

6. Incubate cell suspensions at 37°C for 20 minutes.

7. Harvest the cells by centrifugation and re-suspend in an equal volume of TM.

   Supernatant is hazardous waste.

8. Harvest cells again and re-suspended in an equal volume of LB.

   Supernatant is hazardous waste.

9. Each pair of students will get a 2 ml sample of cell suspension from the mutagenized culture and a 2 ml sample from the control population.

10. Dilute and plate 50 µl samples of the cell suspensions as follows:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>10^0</th>
<th>10^-3</th>
<th>10^-4</th>
<th>10^-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUTAGEN</td>
<td>LB+Rifampicin</td>
<td>LB</td>
<td>LB</td>
<td>LB</td>
</tr>
<tr>
<td>CONTROL</td>
<td>LB+Rifampicin</td>
<td>LB</td>
<td>LB</td>
<td>LB</td>
</tr>
</tbody>
</table>

See the dilution flowsheet below for details.
Incubate plates at 37°C for 18-24 hrs.

11. Incubate the remaining cell suspension overnight in a shaker bath at 25°C.

12. Have instructor or TA review your flowsheet for Day 3.
Day 2  Wednesday 4/14 (any time between 8:00 AM – 5:00 PM)
1. Remove Day 1 plates from the 37°C incubator, and store at 4°C until Day 3.
2. Remove overnight culture tube from the 37°C shaker and store at 4°C until Day 3.

Day 3  Thursday 4/14
1. Remove overnight culture from the refrigerator and set up dilutions as follows:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>10^0</th>
<th>10^-1</th>
<th>10^-5</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUTAGEN</td>
<td>LB+Rifampicin</td>
<td>LB+Rifampicin</td>
<td>LB</td>
<td>LB</td>
</tr>
<tr>
<td>CONTROL</td>
<td>LB+Rifampicin</td>
<td>LB</td>
<td>LB</td>
<td></td>
</tr>
</tbody>
</table>

Incubate the plates at 25°C for 48 hours (i.e. until Thursday), then seal with parafilm and transfer to 4°C.
2. Count colonies on Day 1 plates.

Day 4  Saturday 4/17
1. Remove Day 2 plates from the 37°C incubator, and store at 4°C until Day 5.

Day 5: Tuesday 4/20
1. Begin part B. of the exercise (SCREENING)
2. Count colonies on the plates from Day 2.
**Results**

Each group will contribute the following data based on their colony counts:

- Names
- Section (AM/PM)
- Mutagen (+/-)
- Day 1 Total cell conc.
- Day 1 Freq. Rif\(^R\) Mutants
- Day 2 Total cell conc.
- Day 2 Freq. Rif\(^R\) Mutants

Cell concentrations are reported in "colony-forming units" per ml (cfu · ml\(^{-1}\)) to 2 significant figures in scientific notation.

Frequency values are unitless. Note that it will not be appropriate to report a frequency value as 0 (zero).

You will enter your data in an Excel spreadsheet and the class data will be posted. Microsoft Excel uses an idiosyncratic format for scientific notation, thus 2.6 \( \times 10^7 \) = 2.6E+7.

**REPORT**

1. Does the class data show NTG-induced cell killing?

2. Why did NTG treatment kill cells?

3. Compare the frequency of Rif\(^R\) mutants in:
   - the control cells
   - the treated cells immediately after mutagenesis
   - the treated cell population after they had grown overnight

   Does the class data show efficient mutagenesis?

4. Why was the frequency of Rif\(^R\) mutants in the mutagenized cell population higher in the Day 2 plating than the Day 1 plating?

5. Why do we grow the treated cells overnight in LB before plating them to obtain mutants (screening)? Wouldn't it have been faster and easier to screen colonies from the Day 1 plates, and to eliminate the Day 2 plating altogether?