Experiment 2
(Lab Periods 2 and 3)

Determining the Number of Bacteria in a Colony

Bacteria are found throughout the biosphere, inhabiting places that eukaryotes find uninhabitable, sharing all the places where eukaryotes live, and living in and on the bodies of eukaryotes. Most of the time, however, their presence is not obvious, and detection of live bacteria requires the employment of special techniques that must achieve two ends at once:

(l) Allowing bacteria in a given sample to multiply until a number sufficient for them to be observable is attained, while
(2) Avoiding allowing bacteria from any other material to grow and multiply.

A commonly-used technique for the first purpose is the viable count, in which a sample of bacteria is mixed with a liquid (and diluted further in liquid if necessary) then spread out on the surface of a growth- supporting medium, usually agar in a petri plate. The bacteria are then allowed to grow and reproduce until each bacterium has produced so many offspring that the accumulated mass is macroscopically visible. Such a mass of bacteria is a colony, and the cell or cluster of cells that initiated development of the colony is a colony-forming unit (cfu). (The container of growth medium plus growing bacteria is a culture, whether it is liquid or solidified with agar.) The number of colonies on the plate's surface tells you how many bacterial cells were in the solution you added.

The methods for achieving the second purpose are collectively known as aseptic technique, or asepsis (literally, without infection). The first basic rule for asepsis is that all materials that come into contact your sample must be sterile. With reasonable care, this is possible except for air, which contains bacteria and fungi. Therefore, the second basic rule of asepsis is that all containers must be kept closed except when the experimenter must work within the containers, e.g., to inoculate (introduce the bacteria into) the medium or to remove some of the culture for one purpose or another.

In this experiment, you will perform a viable count aseptically in order to determine how many live bacteria (cfu) are in a colony that you scrape off of an agar plate. Petri plates containing colonies different microorganisms (one organism per plate) will be distributed to the class. The colonies formed by different species of bacteria look different--in shape, size (diameter), color, whether they are shiny or matte in appearance, and the smoothness of the edge of the colony. At some time during the laboratory period, examine the colonies of all the organisms and record a description (including maximum diameter, in mm) of a typical colony of each organism; your description should be adequate to help you recognize the colonies of each organism.

Each group of four students will be assigned to work with one of the organisms. Each student in the group will conduct the entire experiment with one colony to provide a four-fold replication of the determination of the number of viable units in a colony of the organism assigned.

Procedure

Before you begin, prepare your materials as follows.

a. Label two tubes containing l0 ml of sterile saline: "-2" and "-4".
b. Label two petri plates of nutrient agar medium "0.05" and your name.
c. Label two petri plates of nutrient agar medium "0.0l" and your name.

l. Using a sterile applicator stick, pick up an entire colony and suspend the cells in l ml sterile saline; return to the colony, if necessary, to transfer all visible bacterial mass to the saline. Mix the cells into the saline by rubbing the stick along the walls of the tube and stirring thoroughly. Discard the stick as directed by the laboratory instructor.

2. Mix the saline suspension by spinning it into a vortex. Hold the top of the tube firmly with the thumb and forefinger of one hand while slapping the side of the tube with the other hand. This is what "mix well" means in the following steps.

3. Dilute the suspension through an exponential series as follows.

a. Take 0.l ml of suspension into a l-ml pipet, and deliver the 0.l ml to 9.9 ml of sterile saline in the tube labeled "-2"; mix well. The dilution factor of this sample is 0.1 in 10, or 1 in 100 which is expressed as 1 x $l0^{-2}$.

b. Using a fresh pipet, transfer 0.l ml of the $l0^{-2}$ dilution to the tube of sterile saline labeled "-4"; mix well. Dilution factor = $l0^{-2}$ of 10^{-2} = $l0^{-4}$ or 1 in 10,000.

4. Follow the **Directions For Use Of The Finn Micropipetter** to plate samples from the $l0^{-4}$ dilution, as follows:

a. Turn on your Bunsen burner flame.
b. Place 50 ?l of the $l0^{-4}$ dilution onto each of the two plates of nutrient agar labeled "0.05." Lay the micropipetter down and immediately:
c. Dip a bent glass spreading rod into a beaker of alcohol. Ignite the alcohol on the spreader by passing the spreader through the Bunsen flame. DO NOT: hold the spreader in the flame; drip flaming alcohol into the beaker of alcohol; or tilt the spreader upward so that flaming alcohol drips down onto your hand.
d. Spread the 50 ?l droplet as evenly as possible over the surface of one of the plates, then of the other. (You do not need to flame the spreader between uses, since these are duplicate samples.) When the samples on both plates have been spread, place the spreader in the beaker of alcohol.
e. Remove and discard the used pipet tip. Reset the micropipetter to l0 ?l, place a fresh tip on the pipetter, and deliver l0 ?l of the $l0^{-4}$ dilution to each of the plates of nutrient agar labeled "0.0l."

f. Spread these samples as in steps (c) and (d). Be sure to ignite the alcohol on the spreader before using the spreader; when finished spreading the samples, dip the spreader in the alcohol and flame it once again before returning it to the instructor.

5. Submit your four plates to the instructor for incubation.

6. Return to the plate from which you removed a colony for dilution and plating. From the same kind of colony, prepare two samples for microscopical examination, as follows.

a. Place a droplet of water on each of two CLEAN slides. Touch the tip of a wooden applicator stick to one of the colonies, pick up only a small sample of the colony and suspend it in one of the drops. Make a very slightly cloudy suspension, smearing the bacteria into the water and over an area about 2 cm in diameter. Let this smeared slide dry.

b. Suspend more of the bacteria in the second droplet, making a just-barely-cloudy suspension, cover the droplet with a cover slip and examine it immediately. Use the oil immersion lens and observe the shape of the cells; look around within the mount to determine whether, in any area, the cells are swimming. Swimming (motile) cells will move in every direction, each cell following its own path.

Two other kinds of motion will be seen, whether or not the cells are motile:

i. Brownian motion, which is vibratory and due to bombardment of the cells by molecules in the water; bacteria are tiny, and molecules can push them around.

ii. Drifting, which is due to microcurrents in the water; it will tend to carry all the cells in the same direction.

If you find motile cells, record the organism as motile. If not, record simply that motile cells were not observed.

c. When the smear has dried completely, pick up the slide with forceps or a test tube holder and pass it through (don't hold it in) the Bunsen flame three or four times to "heat fix" the cells to the glass slide.

d. Stain the dried, heat-fixed smear by flooding the slide with crystal violet solution, allowing the stain to remain for 30 to 60 seconds, then rinse the stain solution off the slide with a gentle stream of water (direct the water perpendicular to the face of the slide at one end while tilting the slide so that the water runs over the smear sideways). Blot (don't rub) the excess water from the slide.

e. Examine the stained smear under oil immersion and record cell shape (rods or cocci) and cell arrangement (chains, or clusters, or only unicells and pairs or dividing cells).

At the next laboratory period perform the calculations below:

C1. Count the number of colonies on each of your four plates. Calculate the mean number for the two plates labeled "0.05" and the mean for the two plates labeled "0.01".

C2. Using the mean value that is closer to l00 (if both values are < l00 or > l00), calculate the number of cells in the original colony that was suspended in l ml of saline, as follows:

$$\text{Number of cfu/colony} = N / V \times 1 / D$$

where:
N = Average number of colonies on the plates of a specific dilution factor.
V = Volume plated in ml
D = Dilution factor of plates counted.

Example: If the mean number of colonies on your plates spread with 50 ?l of the $l0^{-4}$ dilution is 70 then:

$$(70/0.05\text{ml}) \times (l/10^{-4}) = 1400 \times l0^{4} = l.4 \times l0^{7} \text{ cfu/colony}$$

C3. Compare your results with those of other students who performed the experiment with the same bacterium that you used. Combine your results and calculate a mean value for the number of cfu per colony for that organism.

a. Compare the results for that organism with the results obtained for other bacteria, used elsewhere in the class.

b. Is there any correlation between cfu per colony and the size or shape of the colony?

c. Is there any correlation between the number of cfu per colony and any of the traits that were determined by microscopical examination of the different kinds of bacteria used (motility, cell shape, cell arrangement)?

C4. Assuming that the initial cfu was a single cell, calculate the number of binary fissions that occurred in the colony before you suspended it in saline. The calculation is based on the growth equation:

$$N_t = N_o \times 2^n$$

N_t = Number of cells in the colony at the point in time you count.
N_o = Initial number of cells
n = the number of binary fissions that occurred

To solve for n:

$$N_t/N_o = 2^n$$

$$n = \log_2(N_t/N_o)$$

Then, assuming $N_0=1$, (that is the initial cfu was a single cell.)

$$\mathbf{n = \log_2(N_t)}$$

C5. Assuming that binary fissions occurred throughout the incubation period as the colony developed, calculate the average generation time during growth of the colony.

Generation time = Total time/# generations = t/n.

ASEPSIS

Asepsis in the laboratory is much more difficult that avoiding catching an infectious disease; when germs get into your body, you have plenty of ways to defend yourself against their growth and multiplication. In the laboratory, when unwanted microbes enter sterile materials or a microbial culture, they will be fed and warmed, and they will grow and multiply. Every motion, every manipulation performed with aseptic materials is likely to introduce bacteria and fungi from your hands, from microscopic droplets of saliva you expel while talking, from your clothing, from the bench top, from the lid of the aseptic container that you removed and laid on the bench top (a NO NO), from a used pipet or pipet tip, from the air, etc., etc., etc.

To prevent the entry of unwanted microorganisms into your aseptic materials, attentively follow the following guidelines and proceed to handle your materials as demonstrated by the laboratory instructor.

l. Do not share an aseptic transfer, with one student holding the culture tube and another holding its cap and a third dipping into the culture with a pipet tip placed on a pipetter by a fourth student, while a fifth waits with another container to receive the sample from the third student. Asepsis is a personal responsibility, not a communal operation.

2. Pick up and open only one aseptic container at a time, holding its lid or cap in the hand that holds the sampling instrument (pipet, pipetter), using the small finger of that hand. As soon as the sample is removed, replace the lid or cap, place the first container in a stable position (e.g., in a test tube rack), and then pick up the next container.

For example, to place a measured sample of diluted bacterial suspension aseptically onto an agar medium:

a. Place the sterile pipet tip on the pipetter.
b. Pick up the diluted suspension.
c. Remove the cap of the tube with the small finger of the hand that is holding the pipetter, introduce the pipet tip into the liquid and draw the sample into the tip.
d. Withdraw the pipetter, replace the cap on the tube. Set the tube down.
e. With the hand that manipulated the tube, lift the lid of the petri plate, expel the sample onto the agar surface, replace the lid. Repeat steps (b) through (e) to inoculate the duplicate plate. Then set the pipetter aside.
f. Dip the glass rod in alcohol and ignite the alcohol. Lift the lid of one of the petri dishes, spread the sample over the agar surface, then close the petri dish. Lift the lid of the duplicate petri dish, spread the sample over the agar surface, then replace the lid.
g. Dip the spreader in the alcohol, ignite the alcohol, then set the spreader aside.
h. NOW go back and remove and discard the pipet tip from the pipetter.

3. If you doubt whether any article is aseptic, assume it is not.

4. Believe in the ubiquity of bacteria.

DIRECTIONS FOR USE OF THE FINN MICROPIPETTER

The Finn micropipetter has five parts to be operated: the long red section (on which the tips are mounted when in use), a black locking collar, a transparent volume-setting cylinder attached to a black collar below it (this rotating cylinder sets the volume to the value indicated by the inner, fixed scale plus the value indicated on the rotating scale), a black handle, and a plunger at the end of the handle. Examine the pipetter and identify each part.

l. Loosen the locking collar and rotate the cylinder to set the volume desired (in microliters), then retighten the locking collar.

2. Holding the red section of the pipetter, place a sterile pipet tip firmly on the pipetter without touching the point of the pipet tip.

3. To obtain a measured sample of liquid: depress the plunger to the first stop, insert the pipet tip just below the surface of the liquid and release the plunger to draw liquid into the tip.

4. To deliver a measured sample of liquid: Insert the pipet tip just below the surface of the receiving liquid or lightly touching the agar surface (for delivery to a solid medium) and expel the liquid from the pipet tip by depressing the plunger. As you withdraw the tip from the liquid being delivered, depress the plunger past the first stop, to the second stop. (This uses is the "blow-out" volume of air in the pipetter. Withdrawing the tip from the receiving liquid or lifting it away from the agar surface while blowing out should be done in one motion so that the liquid is completely expelled from the tip.)