Option B – Practical 2

Gram staining of bacteria

Safety

- Alcohol is flammable. Ensure that alcohol is not used near a naked flame.
- Gram's iodine is harmful.
- Wear eye protection and a laboratory apron. Work in a well-ventilated area.
- Wash any chemicals off the skin promptly and flush the skin with water.
- The bacteria are a potential hazard and should be used and disposed of using normal laboratory procedures.

Apparatus and materials

- cultures of *E. coli* and *S. albus* in nutrient broth in McCartney bottles
- bacteriological loop
- Bunsen burner or spirit lamp
- microscope slides
- a few drops of crystal violet stain (≤5 cm^3)
- a few drops of Gram's iodine (≤5 cm^3)
- a few drops of basic fuchsin (≤5 cm^3)
- laboratory alcohol (95% ethanol) (approximately 10 cm^3)
- distilled water
- tissue or blotting paper
- microscope with ×400 magnification and oil immersion lens
- lens tissue
- antiseptic wipes and hand soap for cleaning benches and hands after the practical

Introduction

Gram staining is a technique, developed by Danish biologist Hans Christian Gram, which is used to distinguish two major groups of bacteria: Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan, which is stained purple by crystal violet, whereas Gram-negative bacteria have a thinner peptidoglycan layer, so they do not retain the crystal violet colour, and are instead stained pink by a counter-stain, basic fuchsin.

The test has four main steps:

i First, the bacterial cells are treated with crystal violet.

ii Next, iodine is added, which traps the crystal violet in the cell.

iii Then the cells are decolourised using alcohol (or acetone).

A Gram-positive cell is dehydrated by the alcohol treatment. Crystal violet becomes trapped within the Gram-positive cell due to the several layers of peptidoglycan present, so Gram-positive cells retain the purple colour. By contrast, in a Gram-negative bacterium the alcohol interacts with the lipids of the cell membrane, causing it to lose the outer lipopolysaccharide layer, so the inner peptidoglycan layer is exposed. Crystal violet is washed from the gram-negative cell along with the outer membrane, so Gram-negative cells lose the purple colour.

The decolourisation treatment must be timed carefully because crystal violet stain is removed from both Gram-positive and Gram-negative cells if the alcohol is left on for more than 30 seconds.

iv Finally, the counter-stain basic fuchsin is added, which stains decolourised Gram-negative cells pink.

Procedure

1. Place a ‘loopful’ of one of the bacterial cultures supplied onto the surface of a microscope slide.
2. Leave the slide to dry completely in air.
3. Pass the slide once or twice through the flame of a Bunsen burner. This is called heat fixing, and is done in order to fix the bacteria to the slide, so that they are not rinsed off during the next stages of the procedure.
4. Cover the bacteria with crystal violet stain and leave for 30 seconds.
5. Wash the stain away with Gram’s iodine and leave the slide covered with the iodine for 60 seconds.
6. Rinse the slide with distilled water.
7. Wash with 95% ethanol until no further blue/purple dye washes out.
8. Rinse again with distilled water.
9. Cover the slide with basic fuchsin and leave for 30 seconds.
10. Wash the slide again with distilled water, and blot gently with tissue paper.
11. Wash your hands carefully.
12. Leave the slide to dry thoroughly in air and then observe under the microscope. Focus on low and medium powers first, then under ×400 and oil immersion.
13. Note the colour of the bacteria and identify whether they are Gram-positive or Gram-negative.
14. If time permits, repeat the whole procedure using the other bacterial culture provided. Otherwise, observe other students’ results using that culture, and compare them with your own.
15. At the end of the practical, wipe down laboratory surfaces with antiseptic wipes.

Questions and further work

1. Use reference sources to investigate the structure of bacterial cell walls.
2. Sketch the arrangement of layers in a Gram-positive and a Gram-negative bacterium.