Practical Microbiology

Cihan University
Medical Laboratory Analysis

Lab 8: Differential staining: Acid-fast staining (Ziehl–Neelsen stain) & Spore staining techniques

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MSc: Microbiology

Second stage (1st semester)

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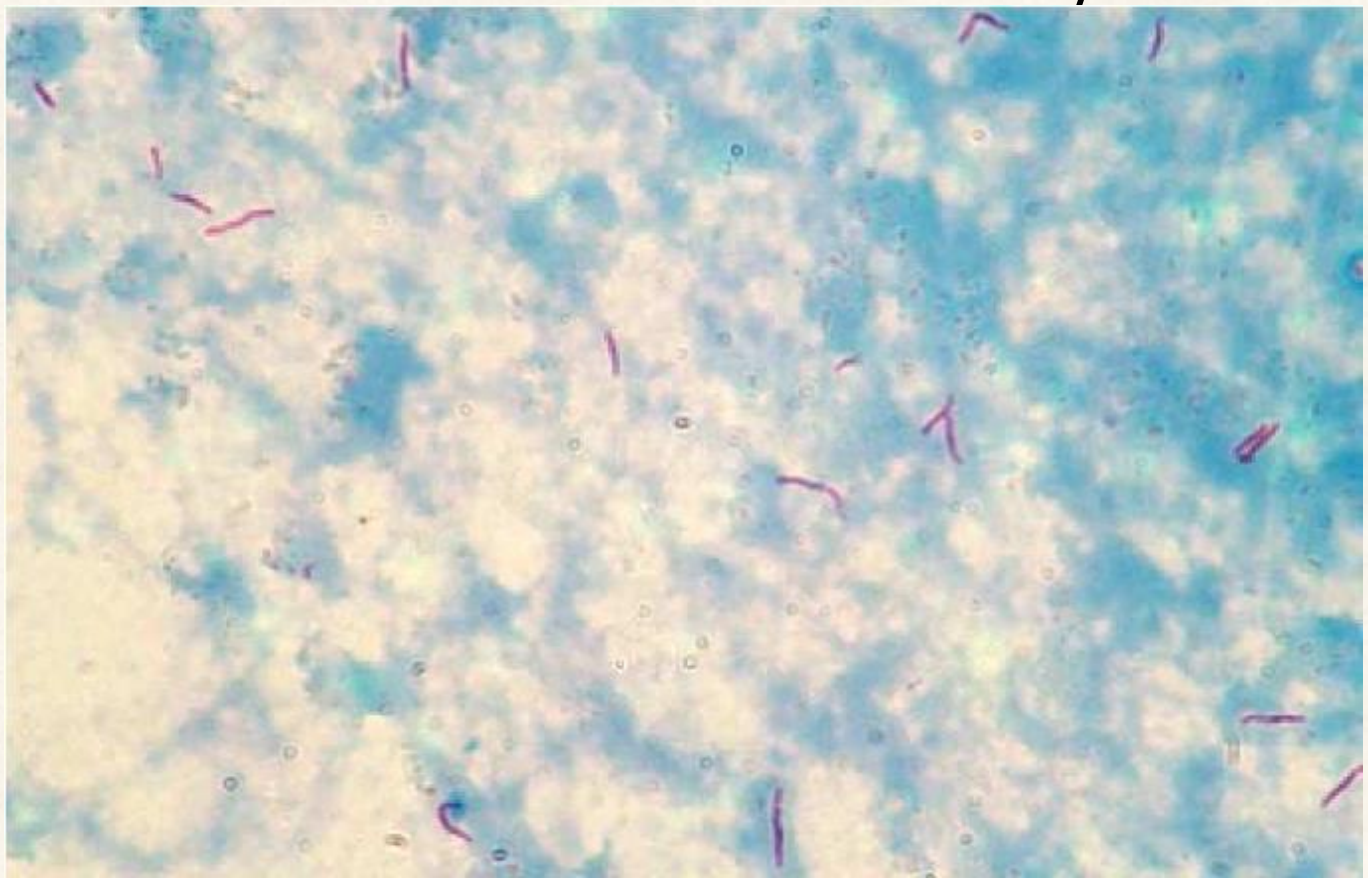
Objective

- ✓Each student should be able to understand:
- ✓Acid fast staining technique (for atypical cell wall).
- ✓Steps in acid fast staining.
- ✓Spore staining technique (for observing bacterial spore).
- ✓Steps in spore staining.

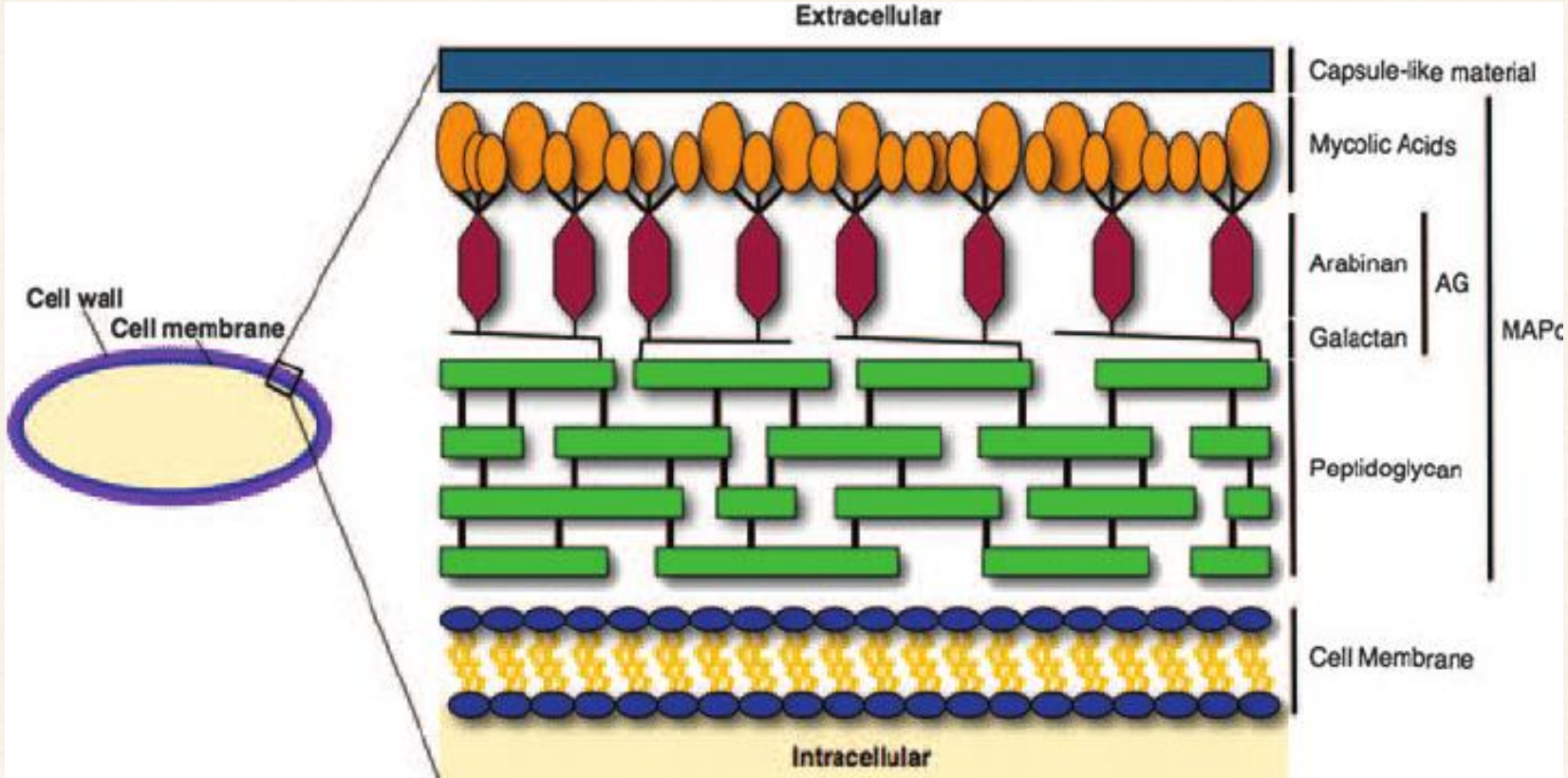
Acid fast staining

- The acid-fast stain is useful for differentiate acid-fast (waxy lipid cell wall) bacteria from non acid-fast bacteria.
- Most of these organisms are members of the genus *Mycobacterium.*
- One of the principal pathogen is ***Mycobacterium tuberculosis,*** which is the cause of tuberculosis in humans. *M. ulcerans* and *M. leprae* are other members.



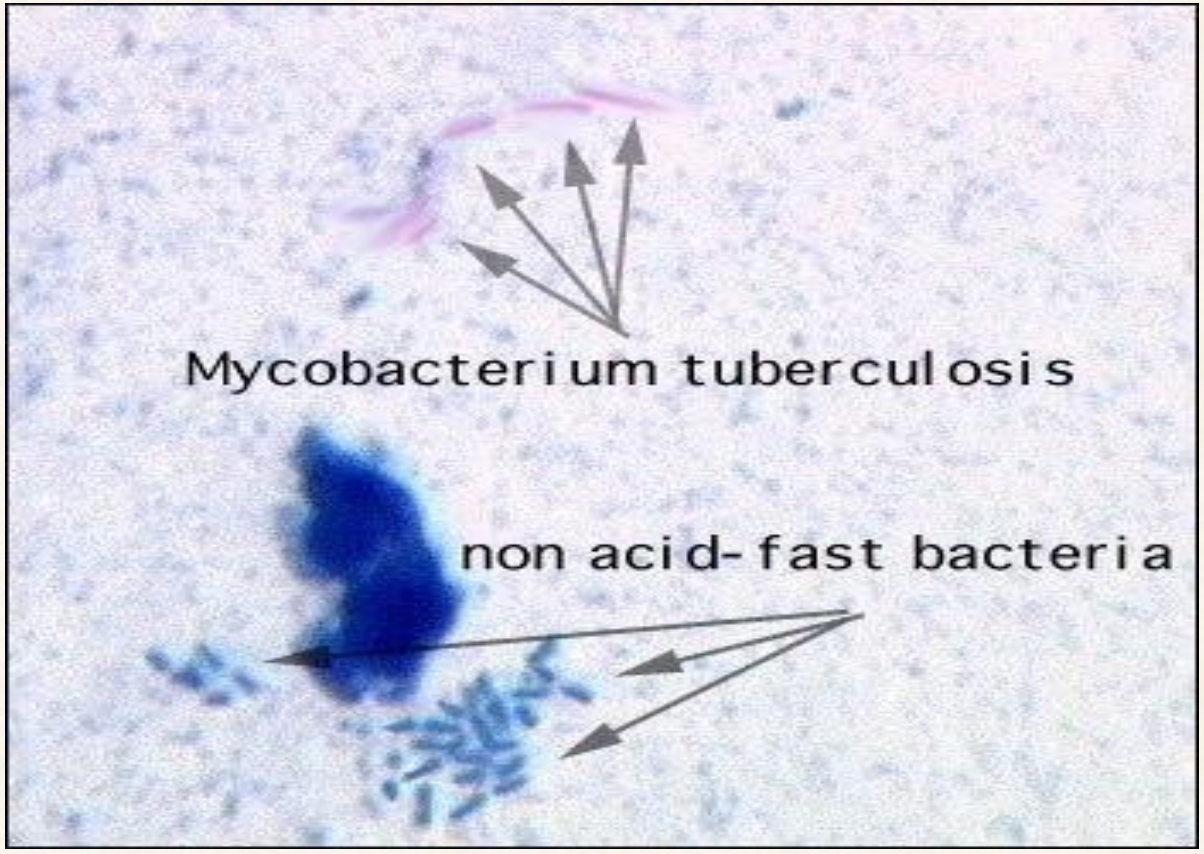
Acid fast staining

- These organisms have a Gram-positive cell wall structure, but the lipid in the cell wall prevents staining with the Simple or Gram-staining.
- The cell wall of Mycobacteria contains high concentrations of lipid making them waxy (Mycolic acid), hydrophobic, and impermeable to stains such as the Gram Stain. They are also resistant to acid and alcohol and are described as acid-fast bacilli (AFB) or acid alcohol fast bacilli (AAFB).



Acid fast staining (Ziehl–Neelsen stain)

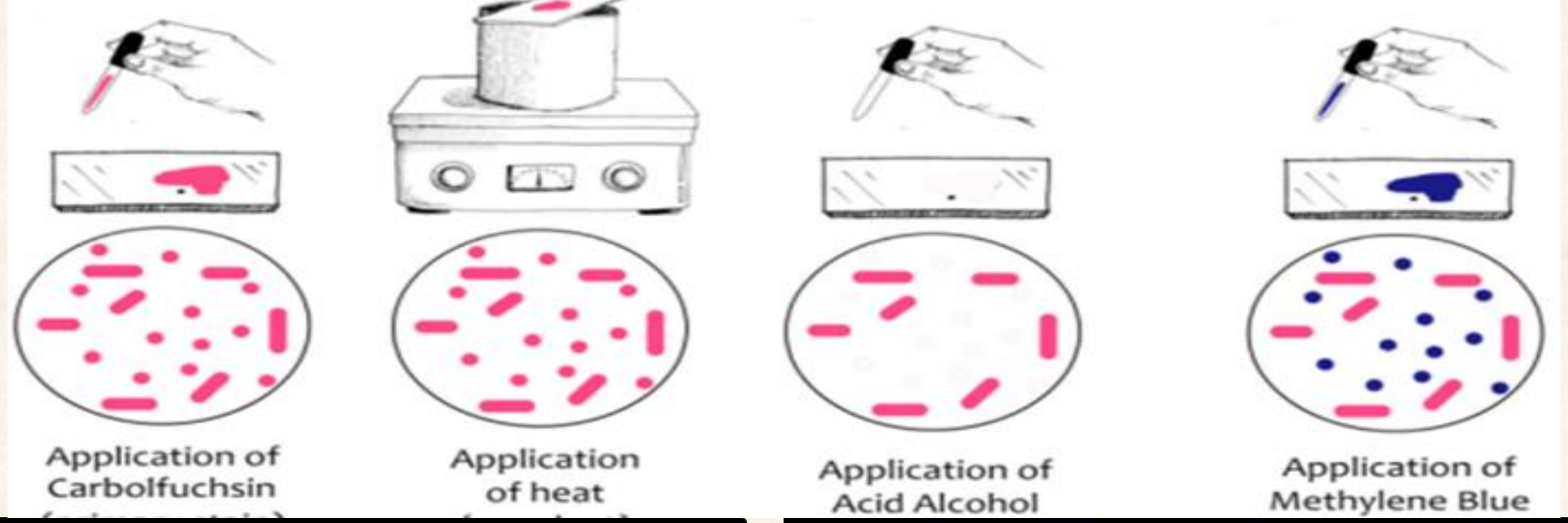
Principle: In this technique two stains are used. It distinguishes bacteria based on the wax content of their cell wall. These microbes can be stained by heating them with **Carbolfuchsin**. Once the microbes have taken up the **Carbolfuchsin**, they are not decolorized by acid-alcohol, and hence are termed acid-fast. This acid-fastness is due to the high lipid content (Mycolic acid). Non acid-fast bacteria are those without wax content, so lose **Carbolfuchsin** when decolorized and take up the counter stain **methylene blue**.

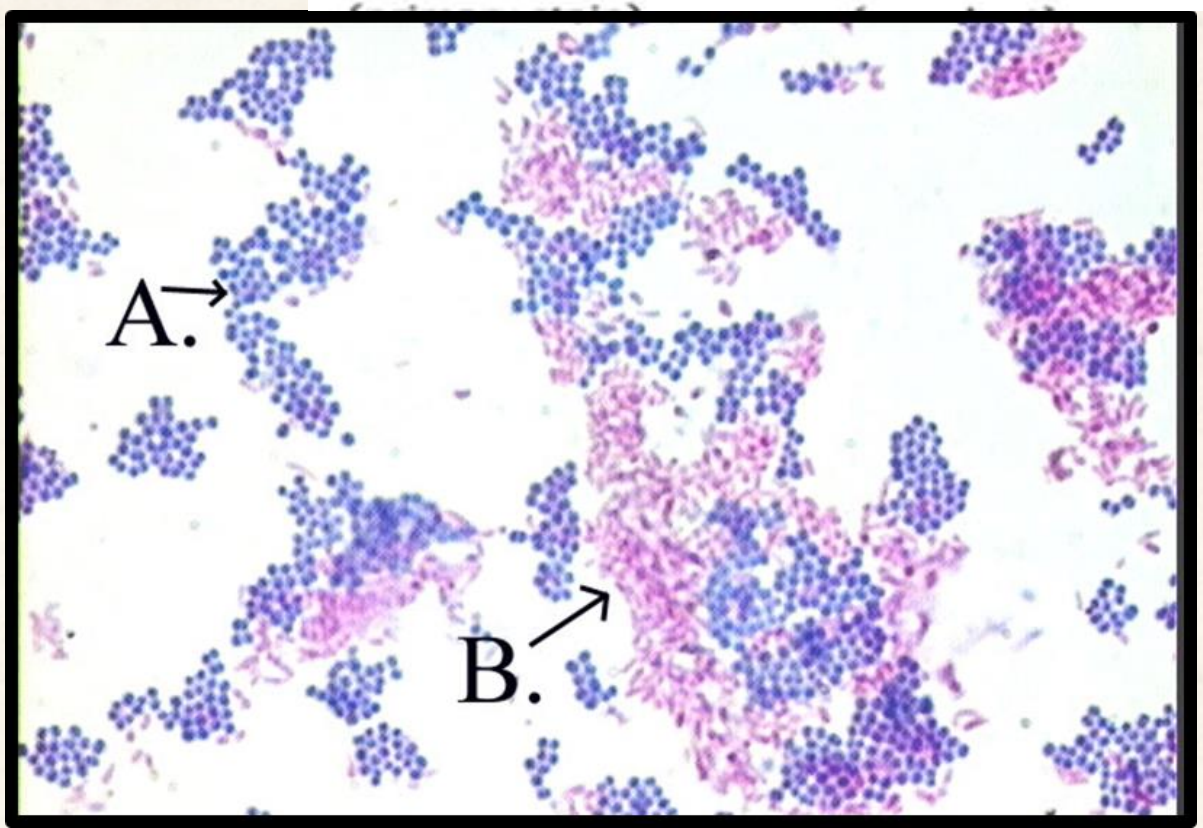


Procedure

- ✓Perform a bacterial smear, and fix it.
- ✓Place **carbolfuchsin** over the slide, heat (hot steam) the slide gently for 5 minutes (avoid boiling or drying).
- ✓Pour it off then wash with water.
- ✓Decolorize the slide with (20% H_2SO_4 or 3% HCL in 95% alcohol) acid-alcohol.
- ✓Rinse the slide gently with water.
- ✓Place counterstain with **methylene blue** for 2 minutes.
- ✓Rinse the slide gently with water.
- ✓Observe the slide under the microscope (4x-100x power).
- ✓Acid-fast appears **red,** straight or slightly curved rods, occurring singly or in small groups, may appear beaded.
- ✓Non acid-fast appears **blue.**

	Acid-fast rod	Non-acid-fast rod
Cells prior to staining are colorless.		
Cells are colored red by hot carbolfuchsin.		
The decolorizing agent, acid-alcohol, removes the red from non-acid-fast cells; acid-fast cells retain the stain.		
Non-acid-fast cells take up the counterstain, methylene blue, and are colored blue; acid-fast cells remain red.		


Application of Carbolfuchsin
Application of heat
Application of Acid Alcohol
Application of Methylene Blue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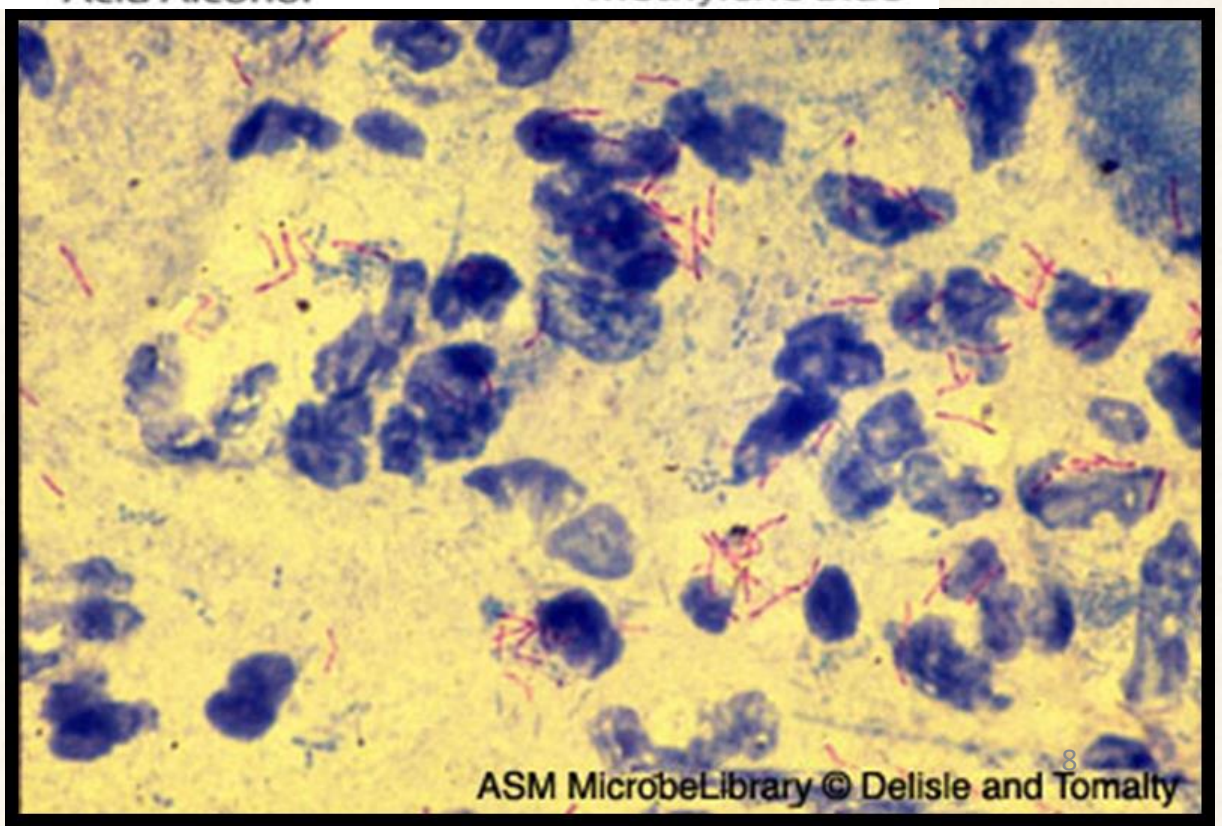
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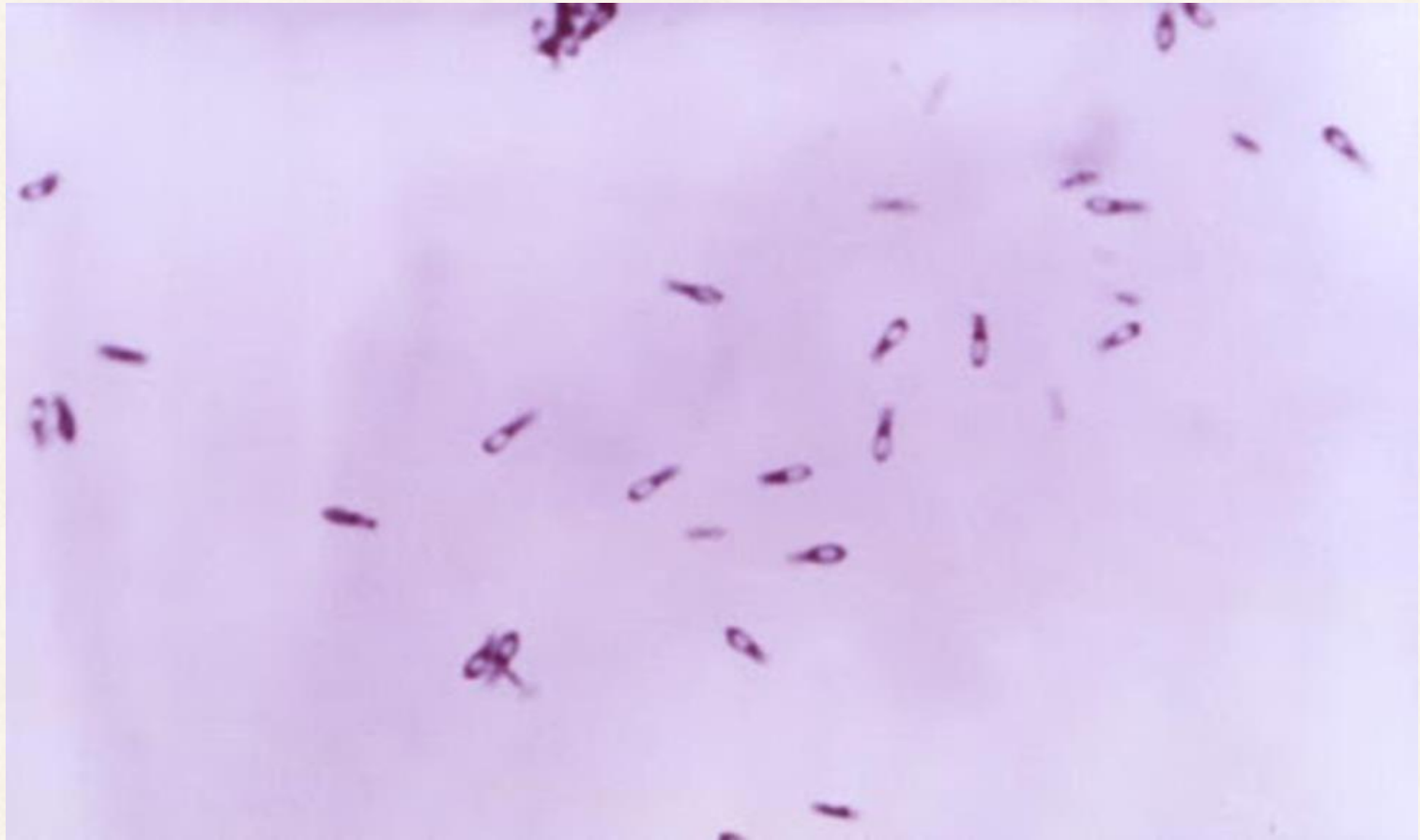
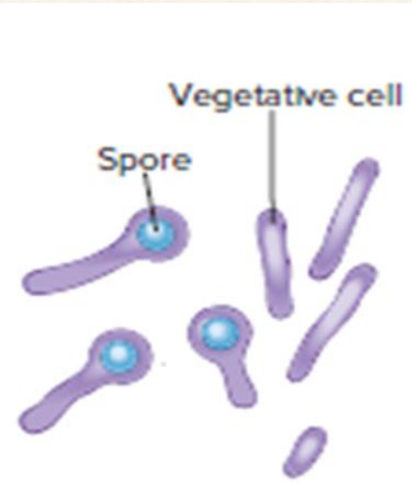
Spore staining

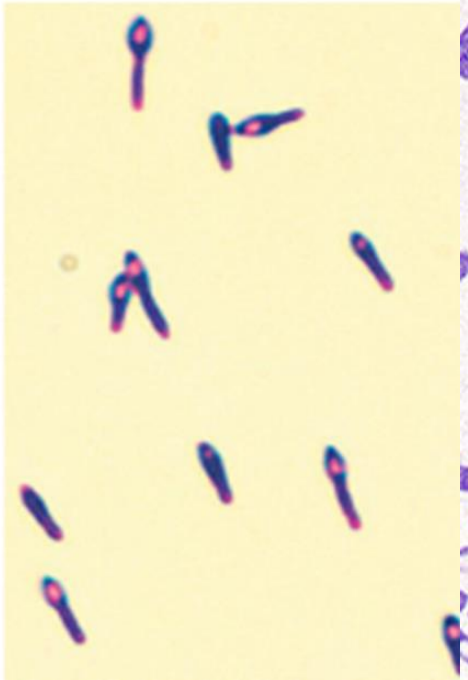
- Endospore staining differentiate spore-forming from nonspore-forming bacteria.
- It stains the spores and distinguished from the vegetative part of the cells.
- Spore is a special resistant dormant structure formed within a vegetative cell by process called sporogenesis, protect a bacterium from adverse environment.
- Resting bodies, remain dormant for a long period.
- Resistant to:
- Heat 200 °C.
- Chemical agent.
- Stains.
- Depletion of nutrient.
- Desiccation.

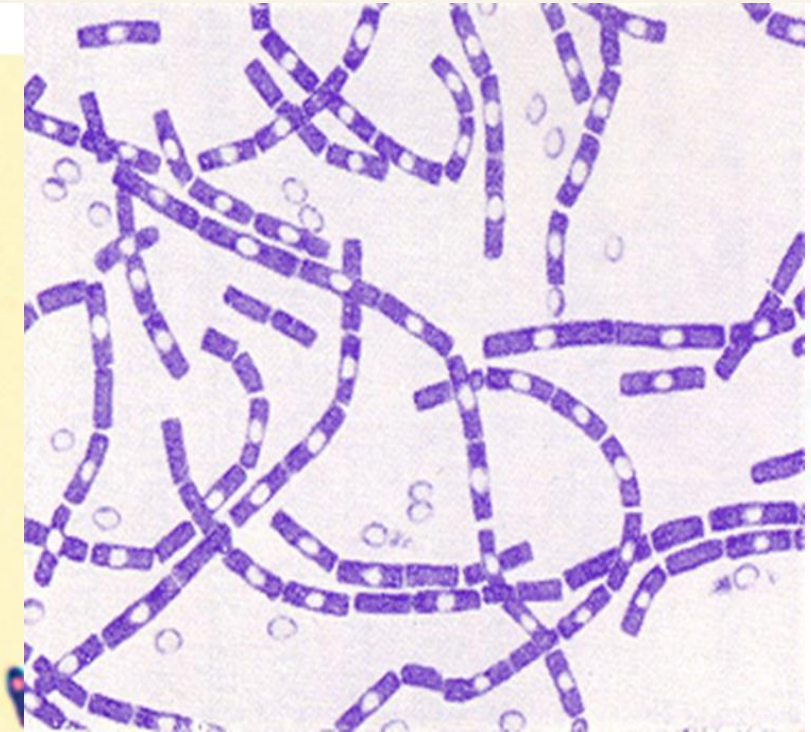


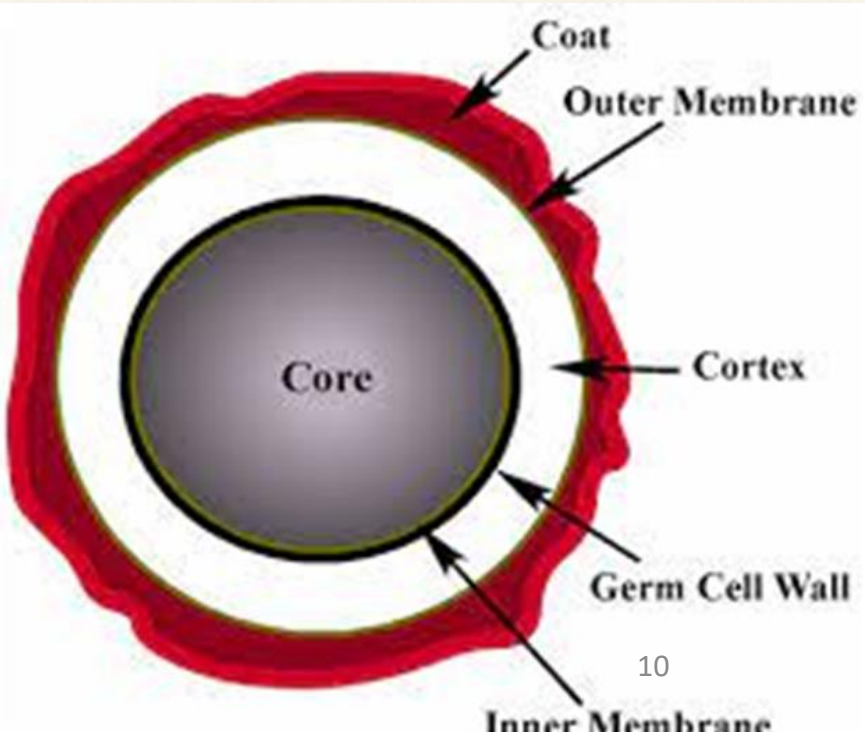
Spore staining

- Endospores are produced by a few genera of Gram-positive bacilli such as *Bacillus* and *Clostridium.*
- When spores are detected in bacteria, their size, shape, and location are useful in identification.









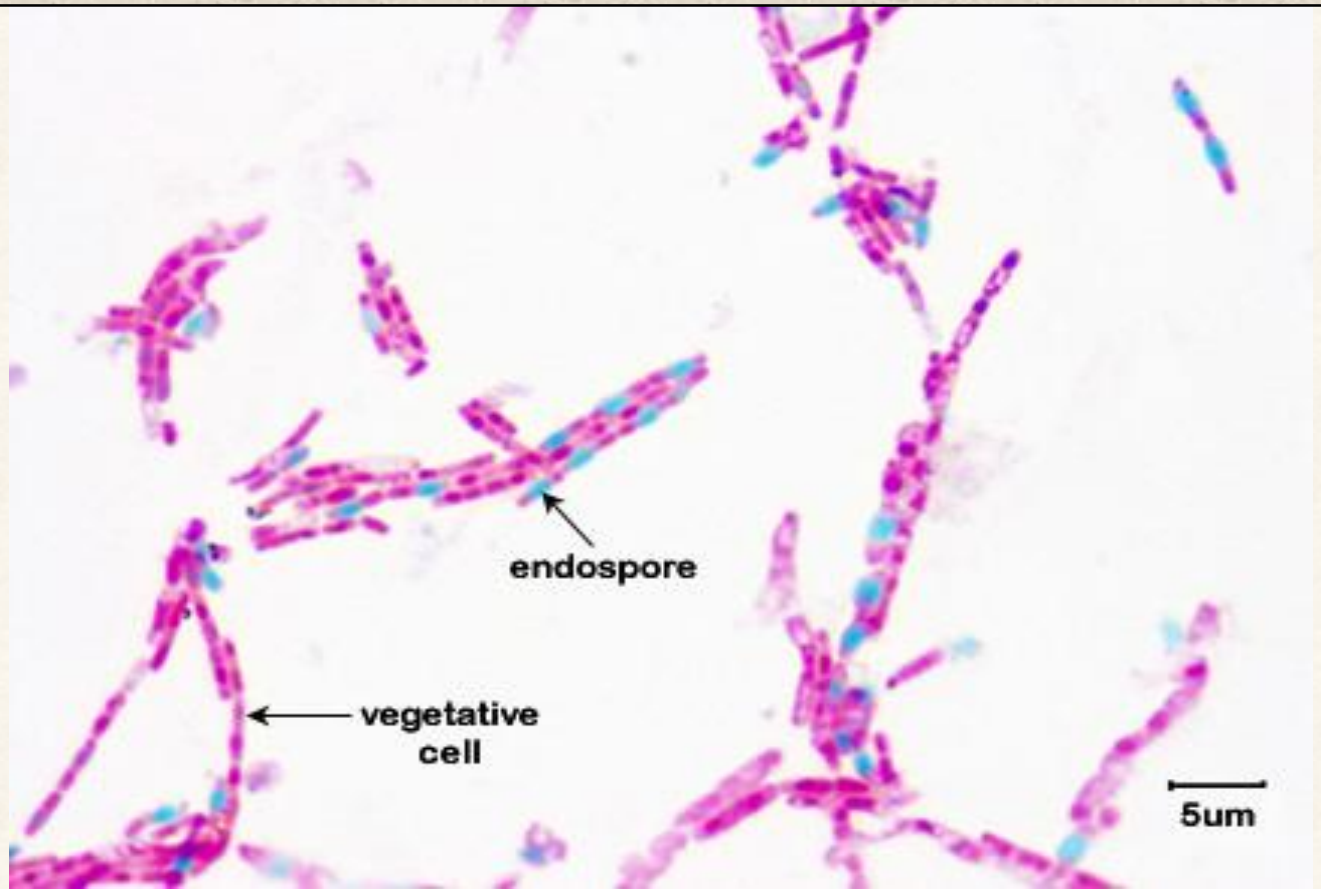
Spore staining

- There are different methods for endospore staining, the most common are:

- ✓Schaeffer-Fulton stain technique.
- ✓Dorner’s methods.
- ✓Modified Zeihl-Nelson’s method.
- ✓Barthelomew-Mittwar’s method.
- ✓Abott method.
- ✓Moller stain technique.

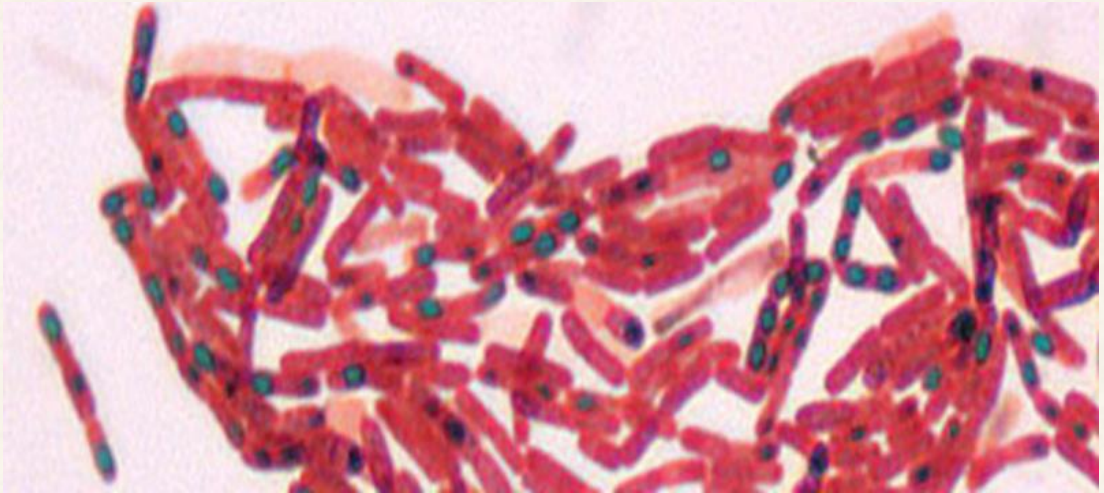
Schaeffer-Fulton stain technique

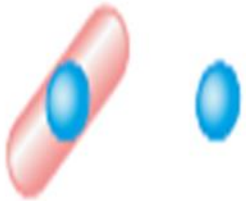
Principle: heat-fixed smear is flooded with **malachite green** solution and steamed, the heat assists the stain to penetrate through the spore. Once the endospore has absorbed the stain, it is resistant to decolonization, but the vegetative cells are easily decolorized with water. When stained with **safranin**, the vegetative cells take the color of **safranin** and appear red or pink, in contrast to the **endospores that appear green.**

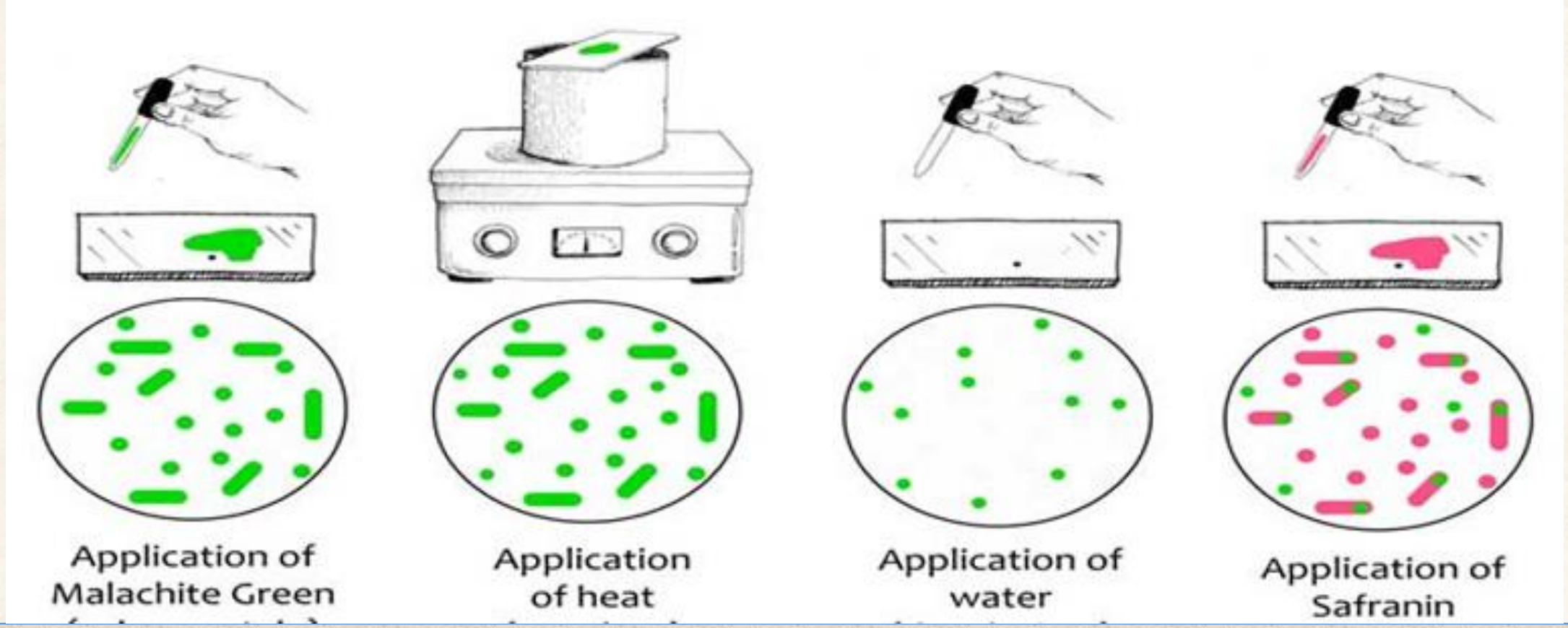


Procedure

- ✓Perform a bacterial smear and fix it.
- ✓Place **malachite green** over the slide, Heat (hot steam) the slide gently for 5 minutes.
- ✓Rinse the slide gently with water.
- ✓Place counterstain with **safranin** for 2 minutes.
- ✓Rinse the slide gently with water.
- ✓Observe the slide under the microscope (4x-100x power).
- ✓Spores are **green** (oval or spherical) free or inside **pink** vegetative cells.

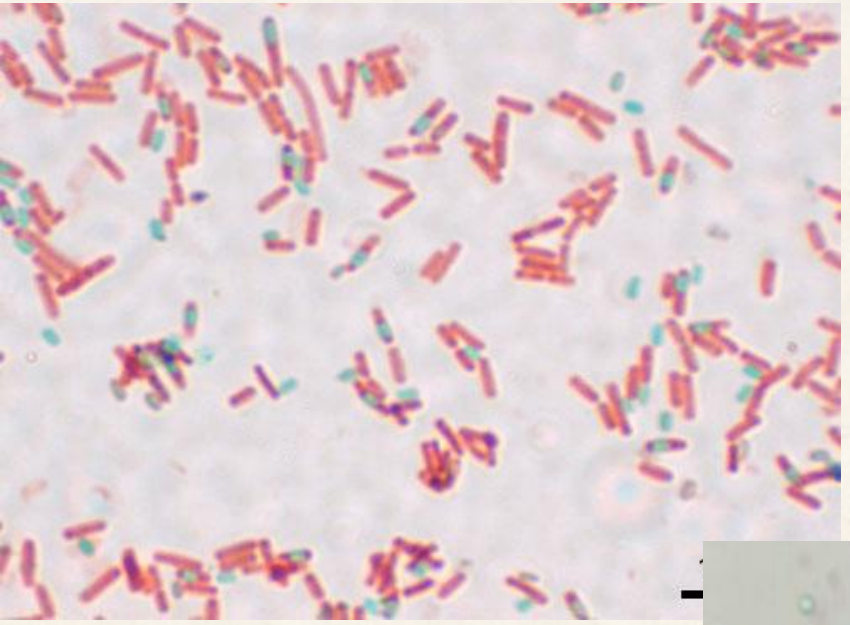


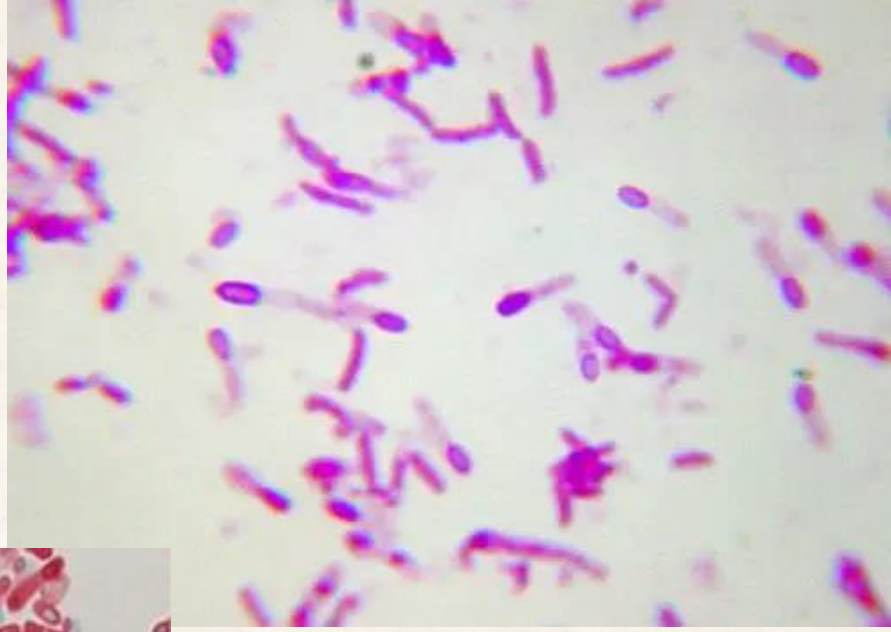
	Spore-forming rod	Non-spore-forming rod
Cells and spores are colorless prior to staining.		
Cells and spores are colored green with hot malachite green.		
The decolorizing agent, water, washes the malachite green from cells; spores retain the stain.		
Cells are colored red with the counterstain, safranin.		

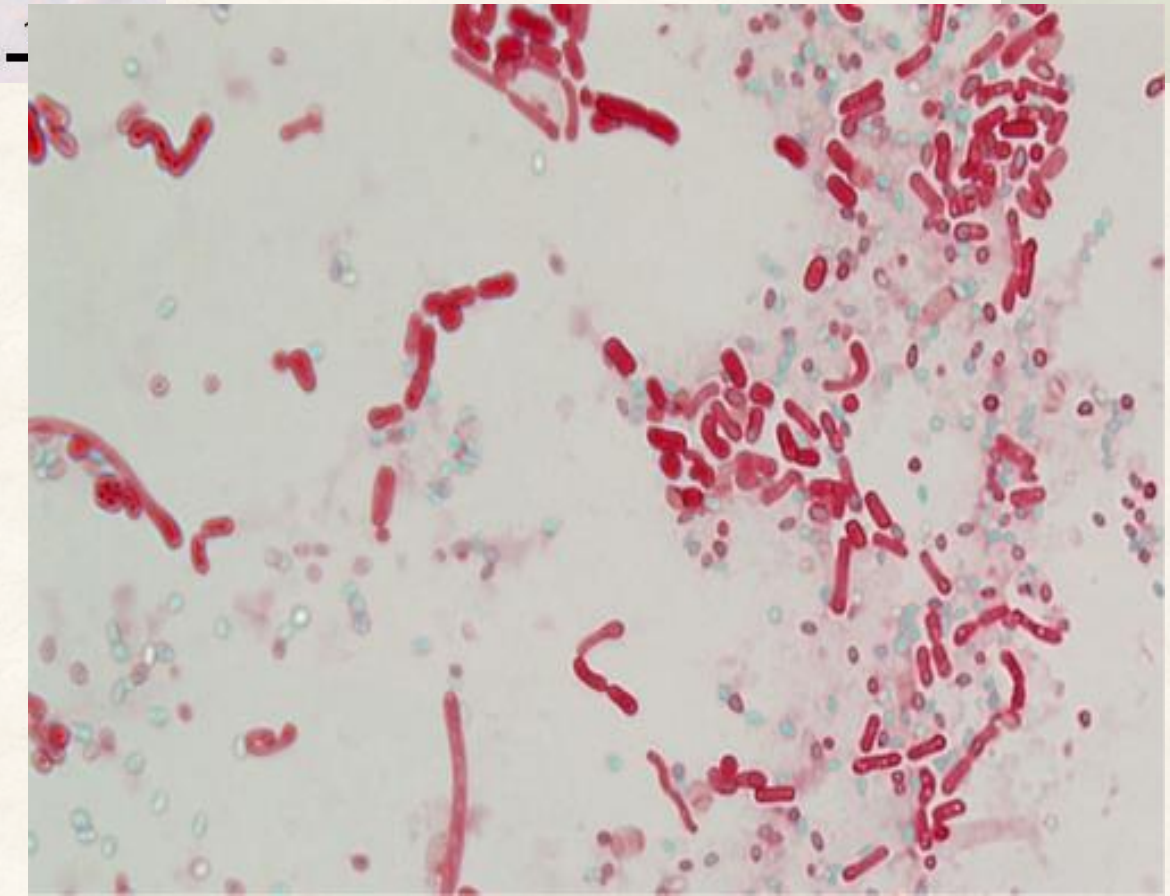


Spore-former bacteria

(*Clostridium perfringens, C. botulinum, C. tetani, Bacillus anthracis, Bacillus cereus, Desulfotomaculum* spp, *Sporolactobacillus* spp, *Sporosarcina* spp.)







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