

Searching for microbes Part II.

Microscopical diagnostics II Gram staining + capsulla

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A Tale (based on real story, but still more fiction than truth 😊)

- There was a man from Denmark, named **Christian Gram**. He stained bacteria and was angry. Sometimes he stained a patient's sample, but beside bacteria, epitheliae were also stained. „Awful epitheliae, they hide my bacteria!“, said he.
- So he started a research. He wanted to find something that would stain him bacteria, but not epitheliae...

To be continued

Tale continues

- He found, that when the sample is stained by gentiane or crystalline violet, and then binding of the dye to the cell wall is supported by Lugol Solution, bacteria do not decolorize even by alcohol. Epitheliae, on the other hand, decolorize. „Great!“
- But soon he saw, that with epitheliae, part of bacteria decolorize, too. „Stupid staining“, said he, drunk remaining decolorizing alcohol, and throught his work into the corner of the room.

And end of the tale...

- Some twenty years later, one young researcher found in a corner of a laboratory the dusty work of Mr. Christian Gram.
- As he read it, he thought – it is not bad, it only needs a bit more to be added.
- And so, to the end of the processus, he added counterstain of **safranin** (or **Gabbet = carbolfuchsin**). Not only bacteria, but also epitheliae stained red, but he said – why not? To know, if epitheliae are present, may be quite usefull!
- **And so Gram staining of today was developped.**

Survey of methods in medical microbiology

Survey of methods

- **Direct methods: We search for a microbe, its part or its product** (e. g. a bacterial toxin)
 - **Direct detection in specimen** – we use the whole specimen (blood, urine, CSF etc.)
 - **Strain identification** – isolate determination
- **Indirect methods: We search for antibodies.** An antibody is neither a part nor a product of a microbe – it is a macroorganism product, after being challenged by a microbe

Survey of direct methods

Method	Specimen examination	Identification
Microscopy	yes	yes
Cultivation	yes	yes
Biochemical identificat.	no	yes
Antigen detection	yes	yes
Animal experiment	yes	usually not
Molecular methody	yes	usually not*

*but in molecular epidemiology – detection of simillarity of strains - yes

Microbiological laboratory



Foto O. Z.

Microscopy: review

What we can see in a microscope

- **When we work with a strain,** we can see one type of microbial cells
- **When we work with a specimen,** we can see
 - **microbes** – sometimes no microbes, sometimes more than ten various species of organisms
 - **cells of host organism** – usually epitheliae, WBCs, sometimes RBCs and other cells
 - **other structures,** e. g. fibrin fibers, cellular detritus etc.

Types of microscopy

- **Electron microscopy** – in viruses, rather research than routine diagnostics
- **Optical microscopy**
 - **Wet mount** – large and/or motile organisms
 - **Wet mount – dark field** (mostly spirochets)
 - **Fixated and stained preparations**, e. g.
 - Gram staining – most important bacteriological stain
 - Ziehl-Neelsen staining – e. g. for TB bacilli
 - Giemsa staining – to some protozoa
 - Fluorescent staining – for better visualisation

What we already know

- Microbes have various **size**. Yeasts are larger than bacteria, and bacteria than viruses
- Bacteria have various **shape** (cocci, coccobacilli, bacilli of various shapes, spirochets)
- Bacteria have various **arrangement** (clusters, chains, couples); cocci in chains should not be named „streptococci“, because they could be for example enterococci.
- Some bacteria form **endospores**, that do not stain

Comparison of size: yeast of genus *Candida* and bacterium *Staphylococcus*

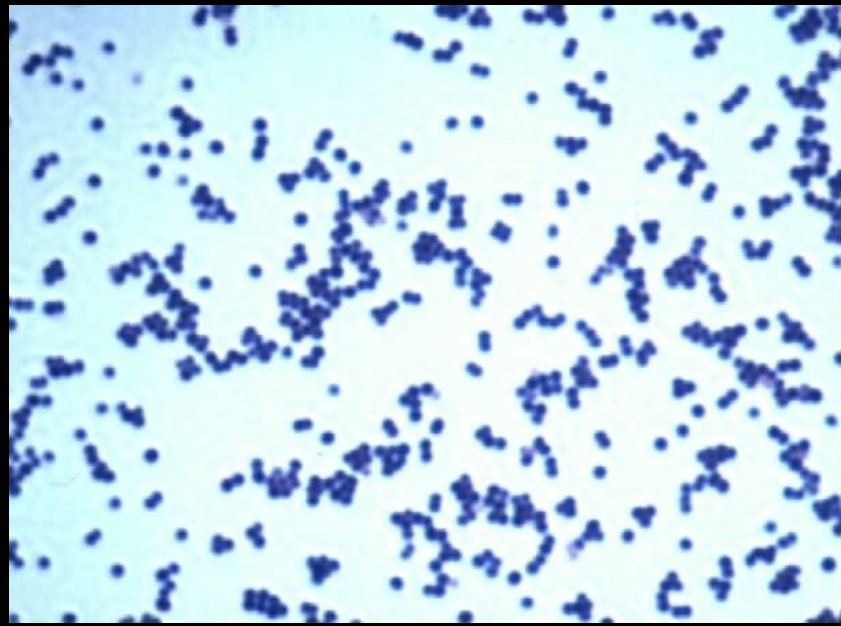
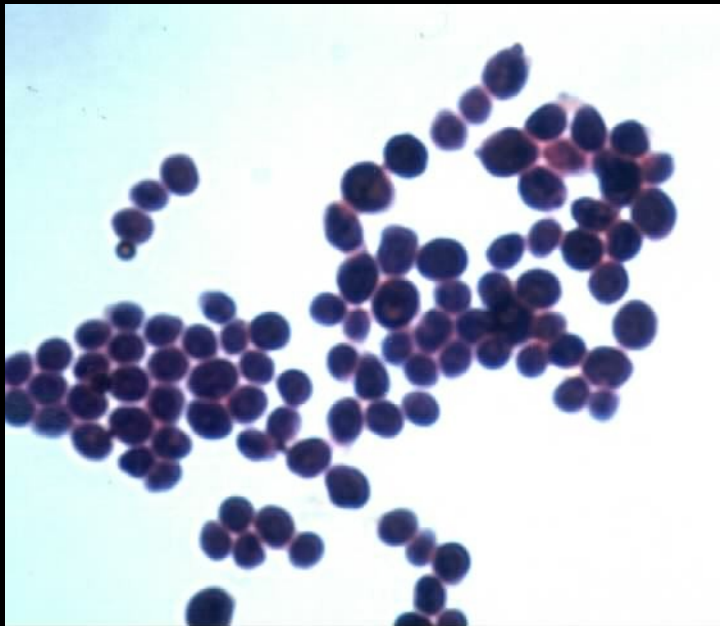


Photo: archive of the institute, from www.medmicro.info



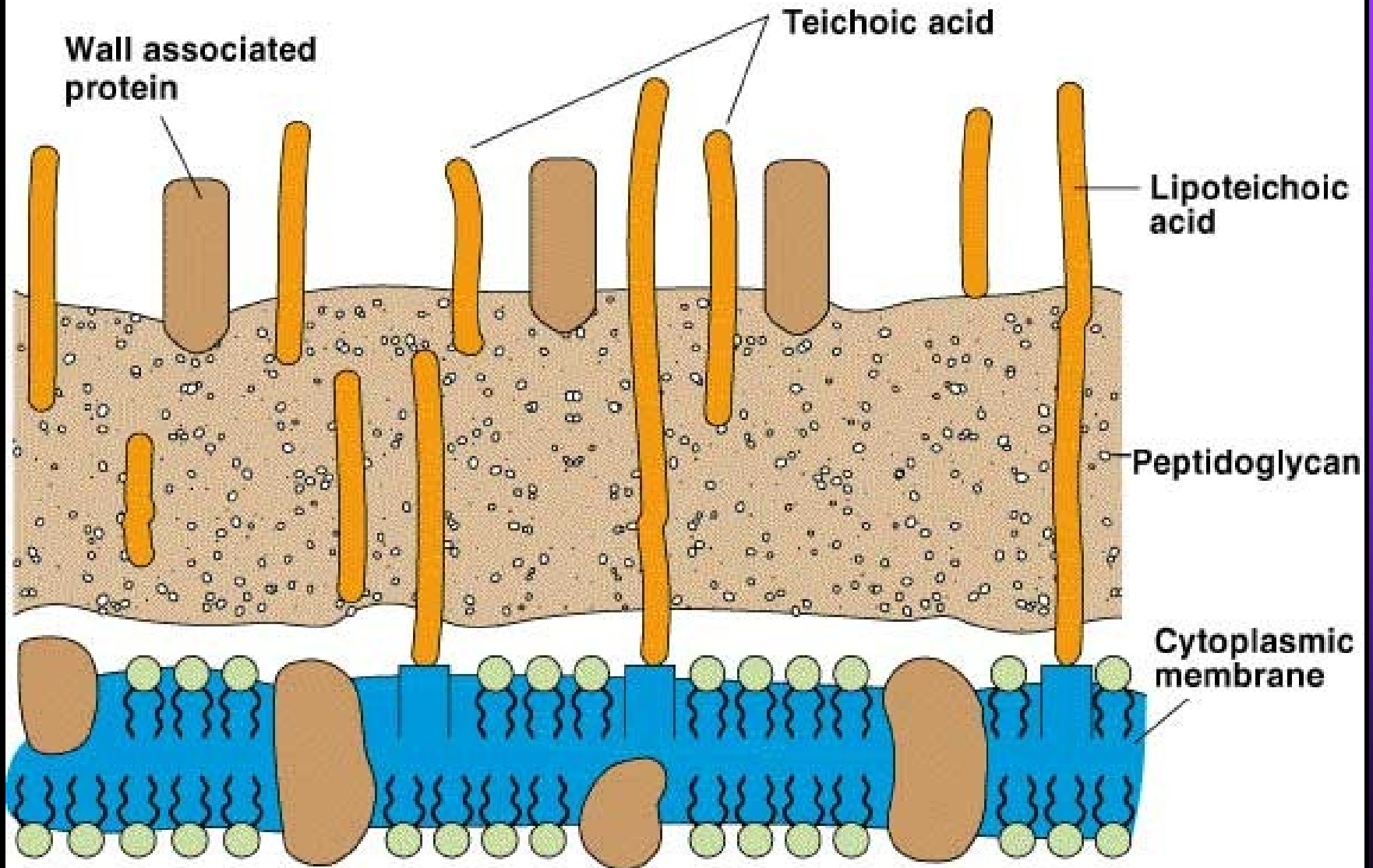
Gram stain: principle

Bacterial cell wall

- There are bacteria, that are **mechanically strong**, their cell wall is **thick and simple**. They are called **gram-positive bacteria**.
- There are other bacteria, that are rather **chemically strong**, their cell wall is **thinner, thin, but more complex**. They are called **gram-negative bacteria**.
- Besides these and those, there are also so named **Gram non-staining bacteria**.

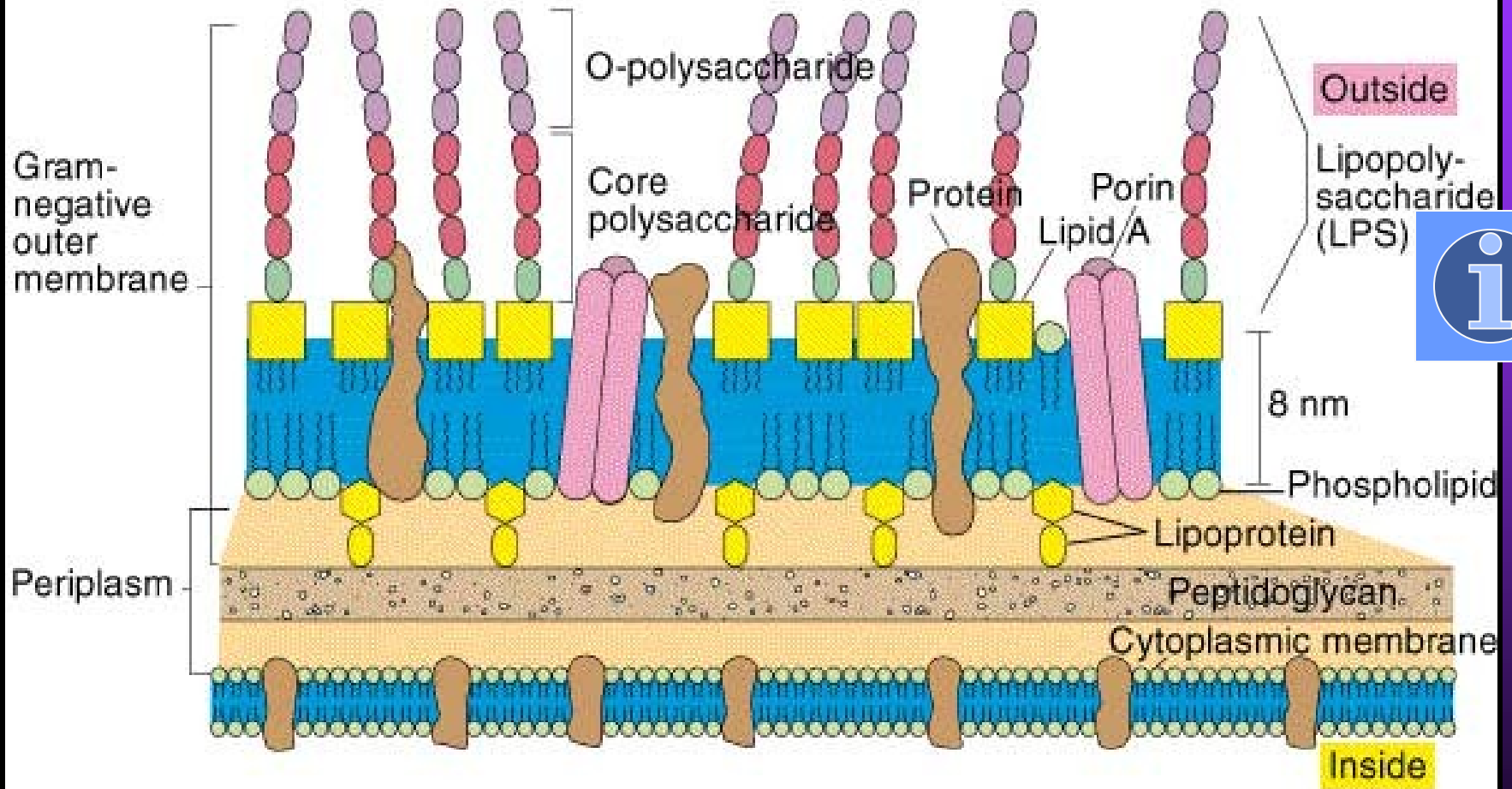
Gram-positive cell wall

www.arches.uga.edu





Gram-negative cell-wall

www.arches.uga.edu



Gram staining – principle 1

- **Gram -positive bacteria** have a thick peptidoglycan layer in the cell wall.
 - So, gentiane/crystallin violet binds more firmly to them, and...
 - ...after confirmation of this bound by Lugol  iodine solution...
 - ...even alcohol is not able to decolorize them.
- **Gram-negative bacteria** are decolorized by alcohol and then stained pink by safranin.

Gram staining – principle 2

Chemical	Gram-positive	Gram-negative
Crystal. violet	Staining violet	Staining violet
Lugol iodine	Confirmation	Less confirm.
Alcohol	Not decolorized	Decolorized
Safranin	Remain violet	Stain to red

Gram non staining bacteria do not stain in the first step, because of **lack of any cell wall (*Mycoplasma*)** or a very **hydrophobic type of the cell wall (*Mycobacterium*)**.

Spirochetes would stain gram-negative, but they are very thin, so they, too, use to be often considered to be „Gram non-staining“ and Gram staining is not used in diagnostic.

Other structures than bacteria: how do they stain?

- **Yeasts** mostly stain violet like G+ bacteria. They have a cell wall of their own type, but in Gram staining it works similarly like that of Gram positive bacteria
- **Human cells** stain mostly red, although nuclei may be partially blue

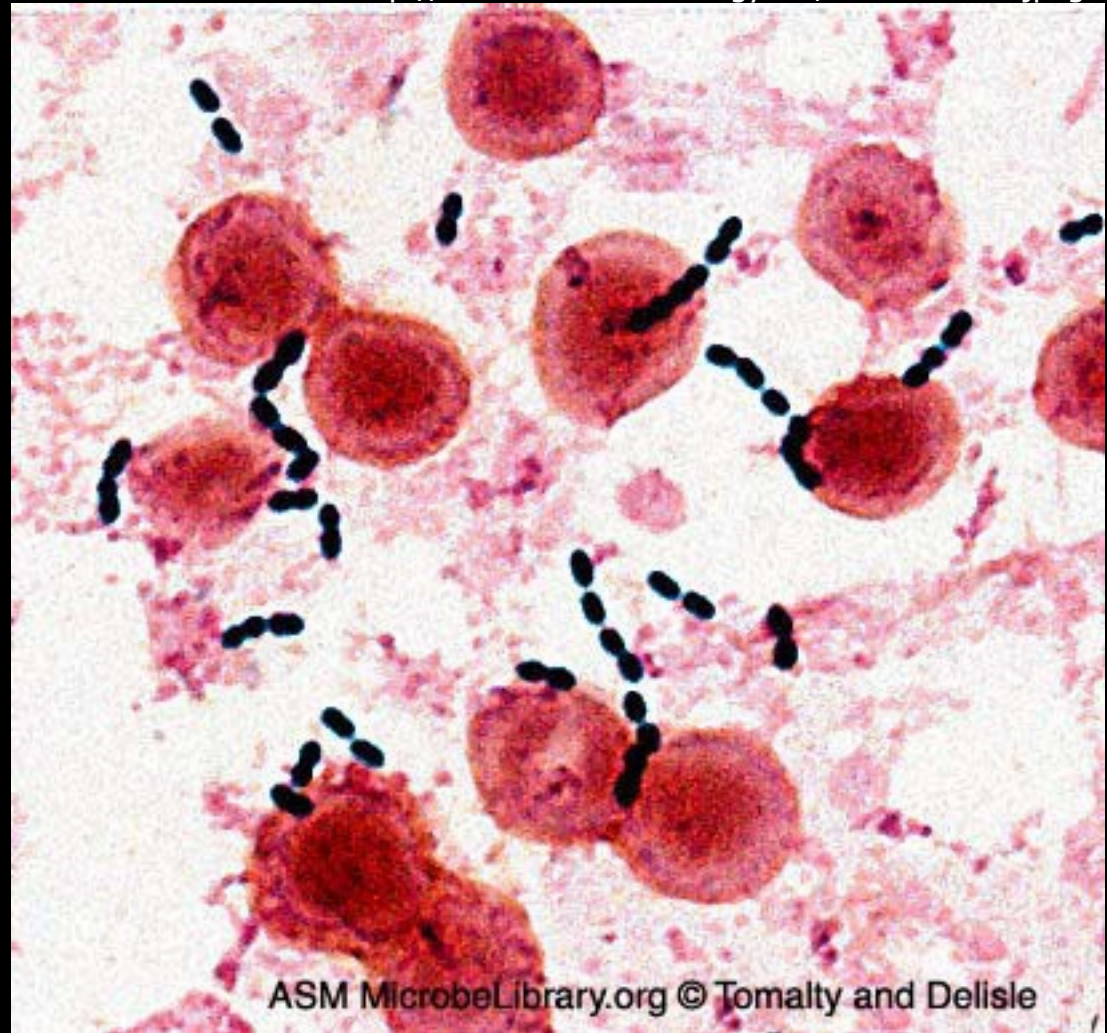
Do not forget, that preparations of specimens might contain various fibers, cell detritus etc., and all preparations may contain staining artifacts, too. Sometimes they are very confusing and may be mistaken for bacteria by a non-experienced observer!

Gram stained preparation

<http://textbookofbacteriology.net/Enterococcus.jpeg>

Cocci in chains

~~Streptococci~~, genus
Enterococcus



ASM MicrobeLibrary.org © Tomalty and Delisle



Gram stain: procedure

Part One: fixated preparation

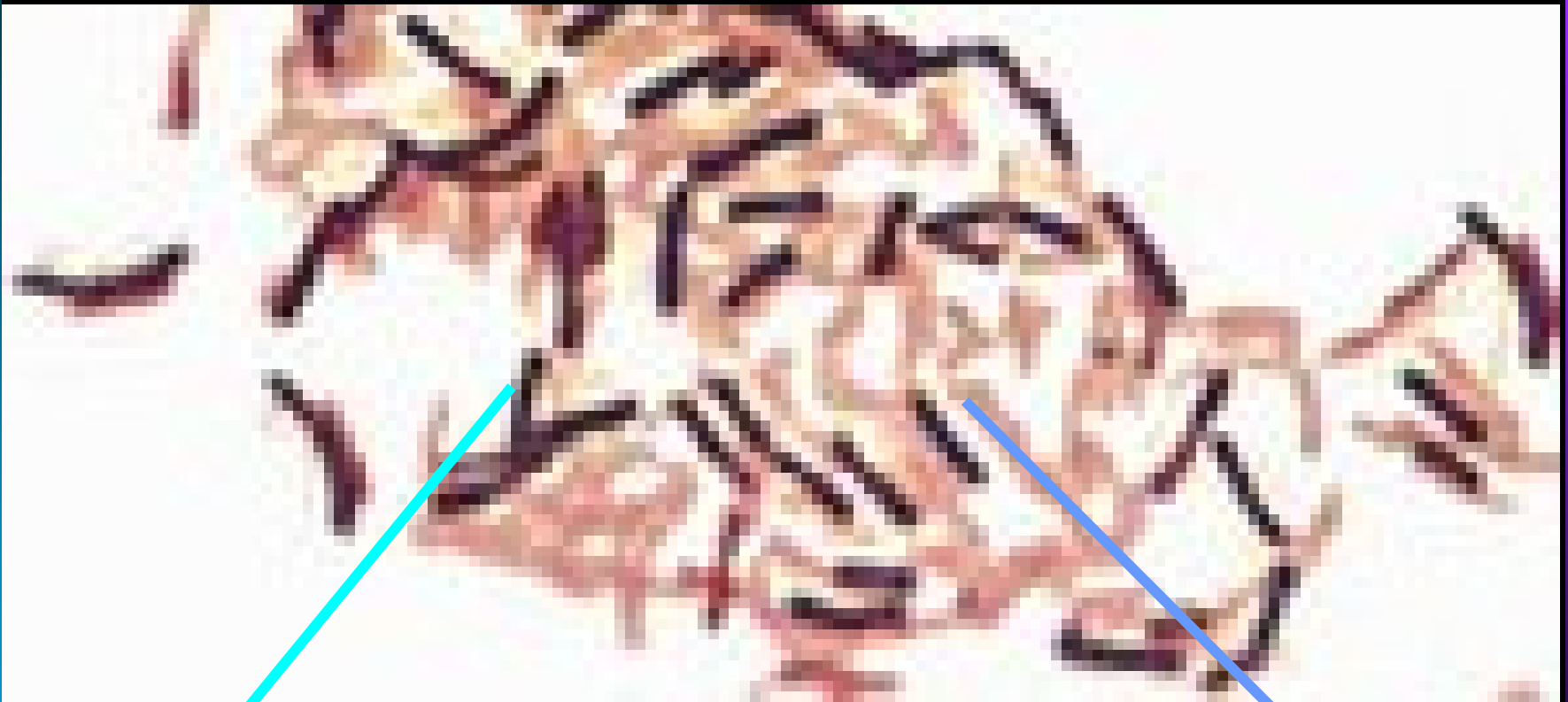
1. We make a small saline drop
2. We sterilize your loop and wait until it stops to be too hot
3. We take some mass of microbes by your loop (stains A to E)
4. We mix in the drop
5. We sterilize your loop again and place back
6. We let the drop dry, or dry AROUND your burner
7. We fixate the slide by passing it THOUGH the flame of the burner
8. We move the slide to the sink for staining

Part Two: proper Gram staining

- **Gentian/crystalline violet (20 –) 30 sec.**
- (rinse by tap water – not necessary)
- **Lugol (20 –) 30 sec.**
- (rinse by tap water –not necessary)
- **Alcohol 15 (– 20) sec.**
- **rinse by tap water!!! important!**
- **Safranin 60 – 120 sec.**
- rinse by tap water
- dry by filtration paper
- microscopy as in Task One

Mixture of gram-positive and gram-negative bacteria

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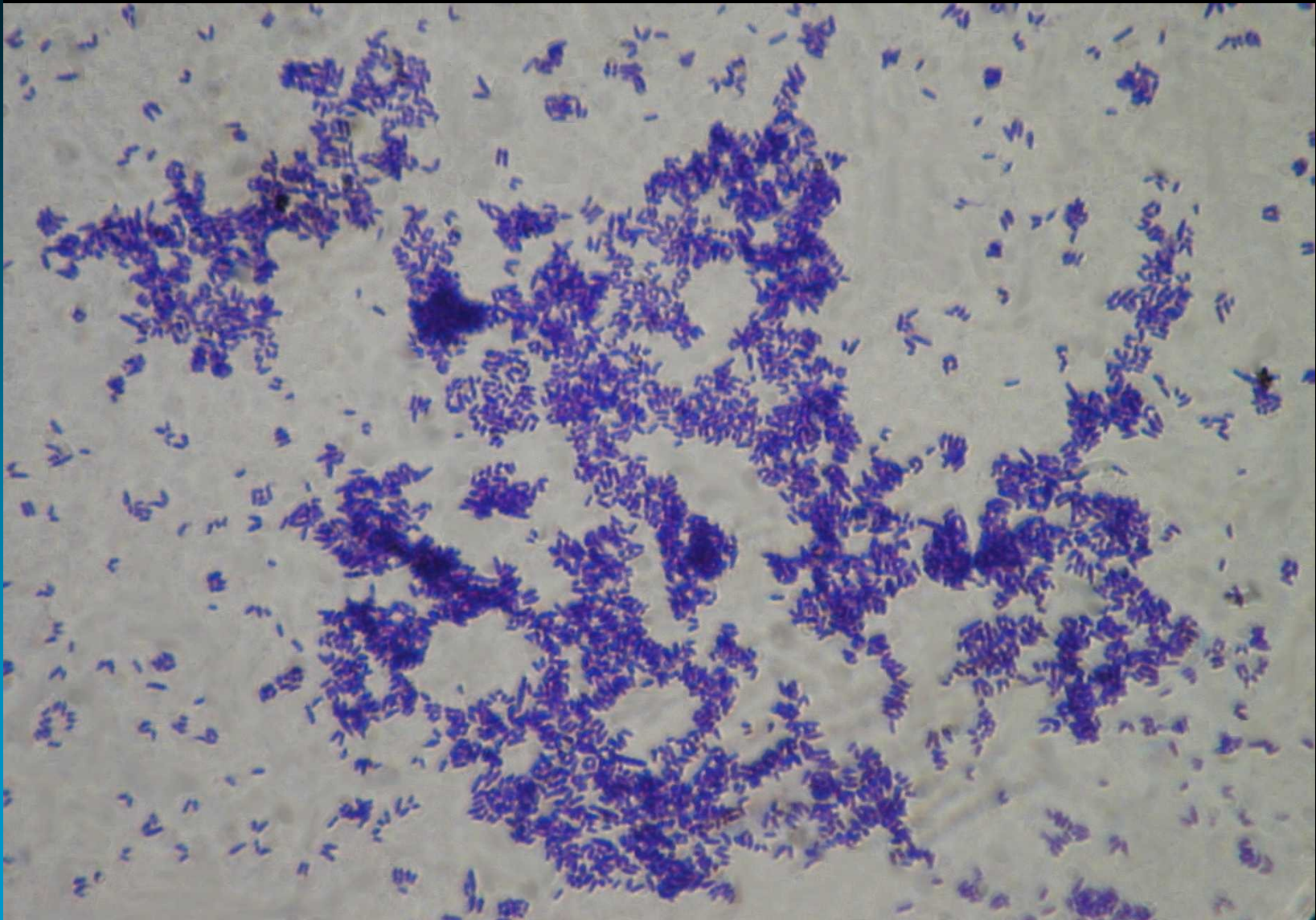


G+

G-

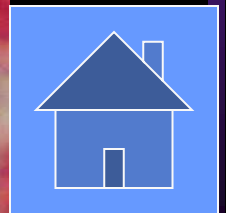
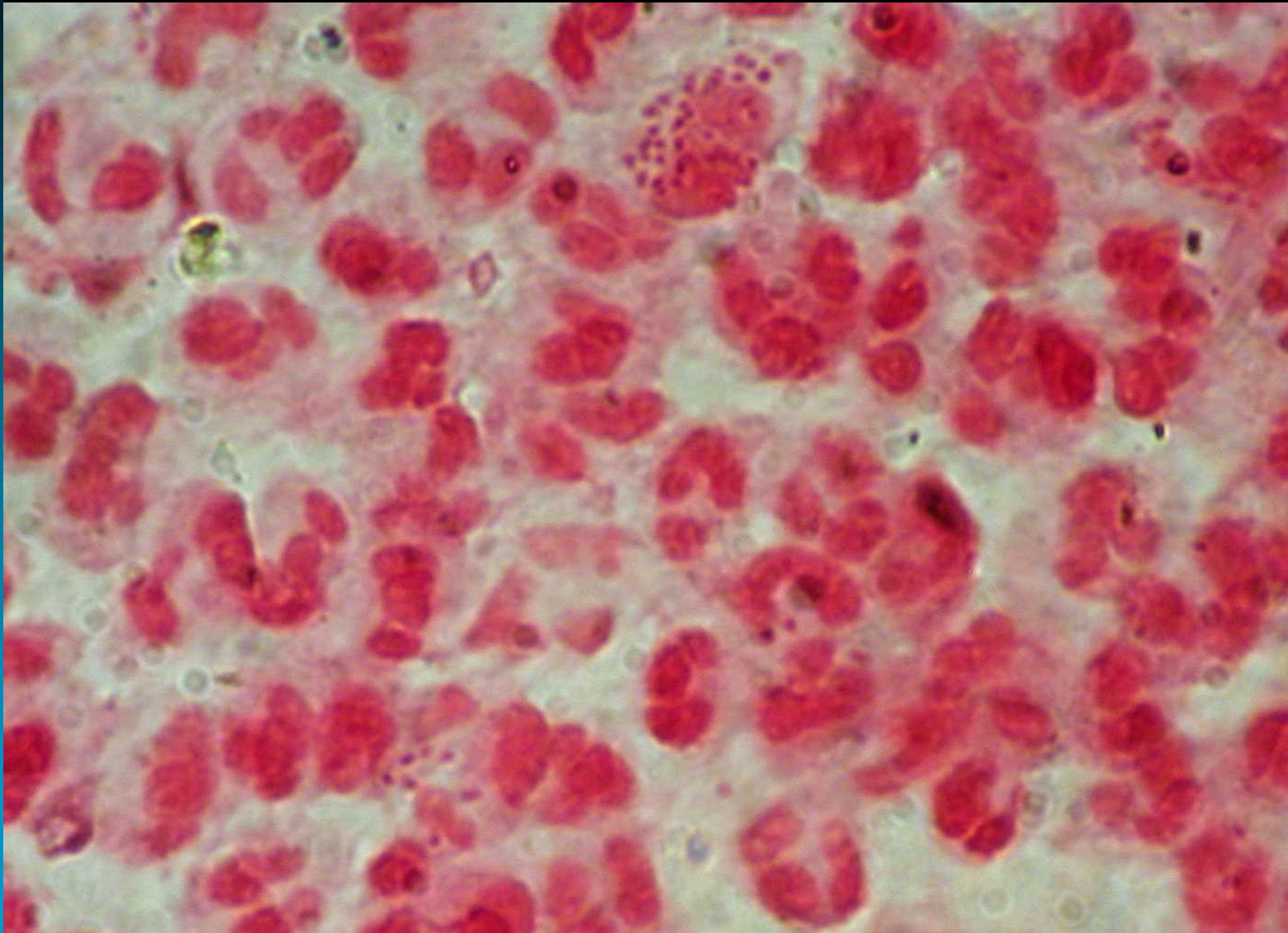
Strain microscopy (Gram positive bacilli)

Foto O. Z.



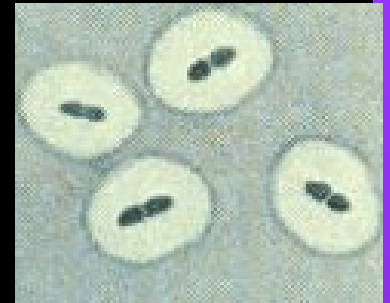
Specimen microscopy (WBCs, G- cocci)

Foto O. Z.



Capsule.

Burri staining



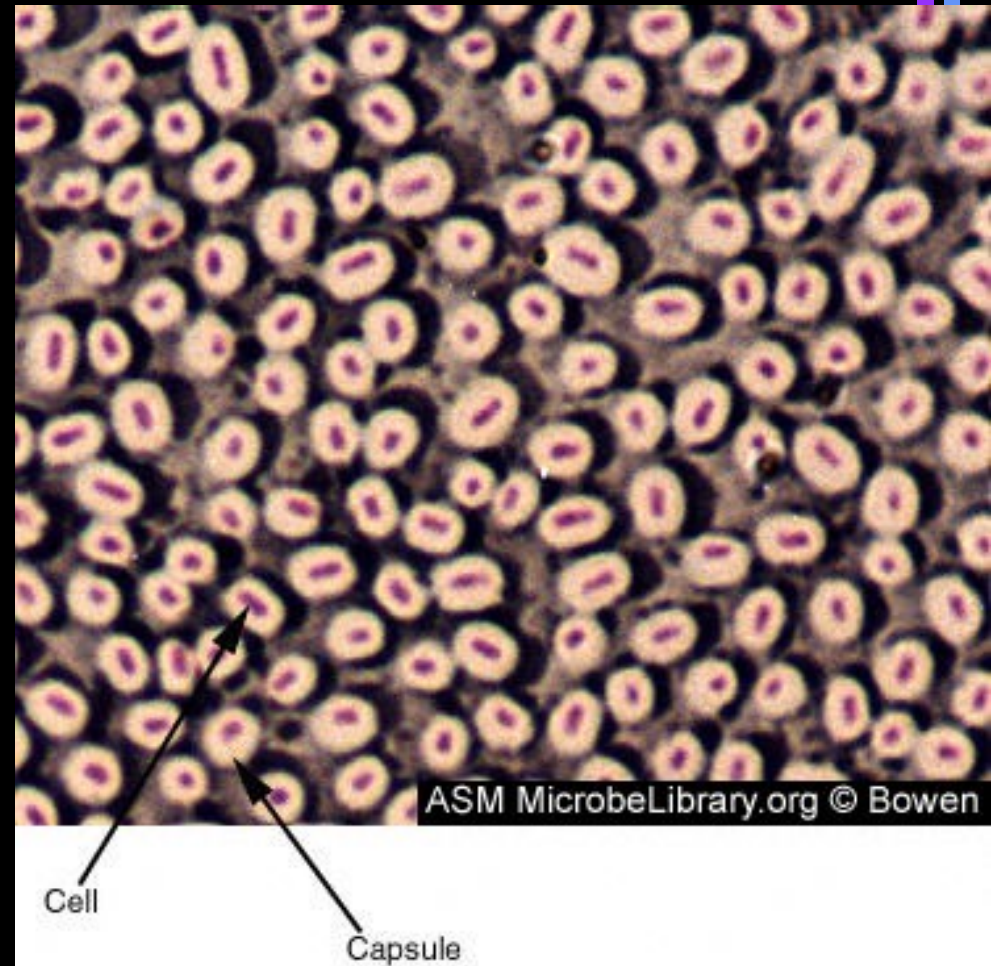
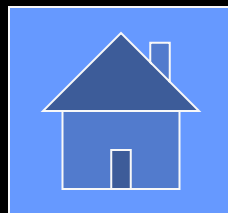
Capsule and biofilm

- **Capsule** surrounds an individual bacterium or a couple of bacteria. It is not an integral part of a bacterial cell, rather a layer of molecules (mostly polysaccharides) that protect the cell. Usually negative staining is used (capsule is an unstained place on a stained background)
- **Biofilm** is a complex layer, composed of bacteria, their capsulae and other material. Biofilm is much stronger than individual bacteria, living in so named planktonic form.

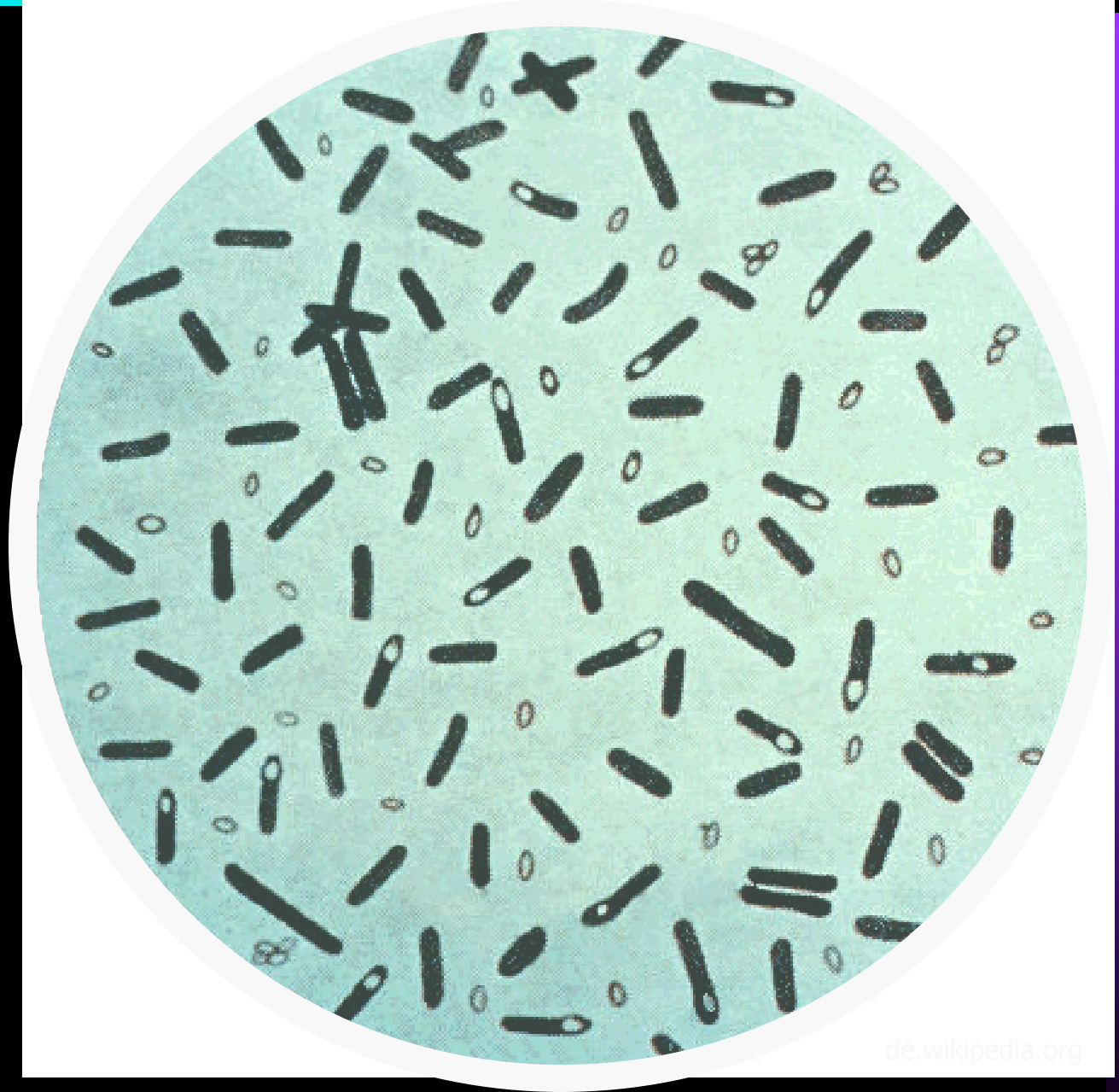
Burri capsular staining

pathmicro.med.sc.edu

In Burri staining, bacteria were stained red and the background by black ink. Capsule is the unstained place between the red bacterium and the black ink.



The End



Lugol iodine = mixed I_2 + KI

Jean Guillaume Auguste Lugol (18 August 1786 – 16 September 1851) was a French physician. He was born in Montauban. He studied medicine in Paris and graduated MD in 1812. In 1819 he was appointed acting physician at the Hôpital Saint-Louis a post he held until he retired. Lugol was interested in tuberculosis and presented a paper to the Royal Academy of Science in Paris in which he advocated the use of fresh air, exercise, cold bathing and drugs. He also published four books on scrofulous diseases and their treatment (1829, 1830, 1831, 1834). He suggested that his iodine solution could be used to treat tuberculosis. This assertion attracted much attention at the time. Although not efficacious in treating tuberculosis, Lugol's iodine was successfully used to treat thyrotoxicosis by Plummer.

http://en.wikipedia.org/wiki/Jean_Guillaume_Auguste_Lugol



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Prof. Christian Gram



Hans Christian Joachim Gram (September 13, 1853 - November 14, 1938) was a Danish bacteriologist. Gram studied botany at the University of Copenhagen and was an assistant in botany to the zoologist Japetus Steenstrup. He entered medical school in 1878 and graduated in 1883. In Berlin, in 1884, he developed a method for distinguishing between two major classes of bacteria. In 1891, Gram became a lecturer in pharmacology, and later that year was appointed professor at the University of Copenhagen. In 1900 he his Chair in Pharmacology to become Professor of Medicine.

en.wikipedia.org/wiki/Hans_Christian_Gram.

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Lipopolysaccharide of G- cell wall

- It contains **lipid A**. This lipid is also called endotoxin. It is released when the cell is broken. It is an important factor of virulence.
- It also contains the **polysaccharide part**. It is a virulence factor; it also contains body antigens (called also O-antigens). These antigens are often important in diagnostics (especially in enterobacteria like *Escherichia coli* or *Salmonella*)

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