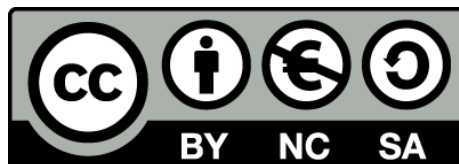




Electric polarization properties of single bacteria measured with electrostatic force microscopy

Theoretical and practical studies of Dielectric constant of single bacteria and smaller elements

Daniel Esteban i Ferrer



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UNIVERSITAT DE BARCELONA



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Daniel Esteban i Ferrer
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DOCTORAL THESIS

2 Bacterial cell Structure and Composition

It is supposed that there are more than 5×10^{30} bacteria on Earth [7] which make them the largest domain of living organisms. They are prokaryotic unicellular microorganisms. Bacteria were one of the first life forms to appear which make them able to live in most of the earth environments like ground, water, air, human (or other animal) bodies, vegetables and even in extreme conditions (high temperature, no-oxygen, high-salt concentration, etc) like the extremophile bacteria [8].

There is a vast number of bacterial species. Some of them can cause severe diseases and can be very virulent such as strains of *Bacillus anthracis* (Anthrax), *Corynebacterium diphtheriae* (Diphtheria), *Escherichia coli* (diarrhea, meningitis in infants, hemorrhagic colitis), *Listeria monocytogenes* (listeriosis-meningitis), *Mycobacterium tuberculosis* (Tuberculosis), *Salmonella typhi* (salmonellosis, typhoid fever), *Treponema palladium* (Syphilis), *Vibrio cholera* (Cholera), etc.

On the other hand there are many strains beneficial or even crucial for human health. Some of them help us in the digestion, avoid the proliferation of pathogenic bacteria (mainly in the intestinal tract), or boost the immune system, such as different strains of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Streptococcus thermophilus*, *Bifidobacterium animalis* or *Lactobacillus reuteri*. Other bacterial types can be helpful as food preservatives again avoiding pathogenic bacteria to proliferate like *Lactobacillus sakei* which inhibits the growth of *Listeria monocytogenes* or *Escherichia coli* in meat and fish products [9]. The way a bacterium reproduces is asexual [10]. A bacterial cell elongates and finally suffers a binary fission. Both daughter cells acquire a copy of the chromosomal DNA. Bacterial genetic variability is mainly influenced by horizontal gene transfer (HGT) . The different mechanisms of HGT make it possible that bacteria adapt to different environments. As an example, bacteria can become resistant to antibiotics by HGT

mechanisms (refs.) Their evolution comes from the DNA transmission by several kinds of horizontal gene transfer. By these DNA exchange bacteria can make resistance to antibiotics [11], among many other adaptations to hostile environments [12].

Whereas bacteria are very small living organisms, they can be quite complex in structure. They are prokaryotic unicellular cells, which mean that they do not have a nucleus separated by a membrane from the cytoplasm, and most of them do not have membranes around their internal compartments (organelles). They are in the range of the micrometer (μm) in size usually ranging from 0.5 to 5 μm although a few species can reach the millimeter range and can be seen by the naked eye. Viruses in comparison are usually in the nanometer (nm) range which makes them impossible to image with traditional optical techniques.

The basic structure of bacterial cell is a cell envelope which separates the cytoplasm (internal part of the bacteria) from the surrounding medium and acts as a barrier to get or retain nutrients and proteins, and to protect the cell from internal turgor pressure. It is composed by the cytoplasmic membrane (or plasma membrane), the cell wall, and some bacteria have an outer membrane. Inside the cytoplasm is where we can find the nucleoid (single circular chromosome with associated proteins and RNA) the ribosomes (that produce the proteins), and other organelles with different functions as storage, metabolism, regulation of buoyancy, etc. Some have an external layer termed capsule, a polysaccharide-containing structure (another shield that adds to their protection system), pili a hair like appendage that cross the entire cell envelope to the exterior (for gene transfer/attachment/movement) and/or flagella a lash-like appendage (for movement and/or sensing). An artistic representation of a model of bacterium with its labeled parts can be seen in Figure 2.1.

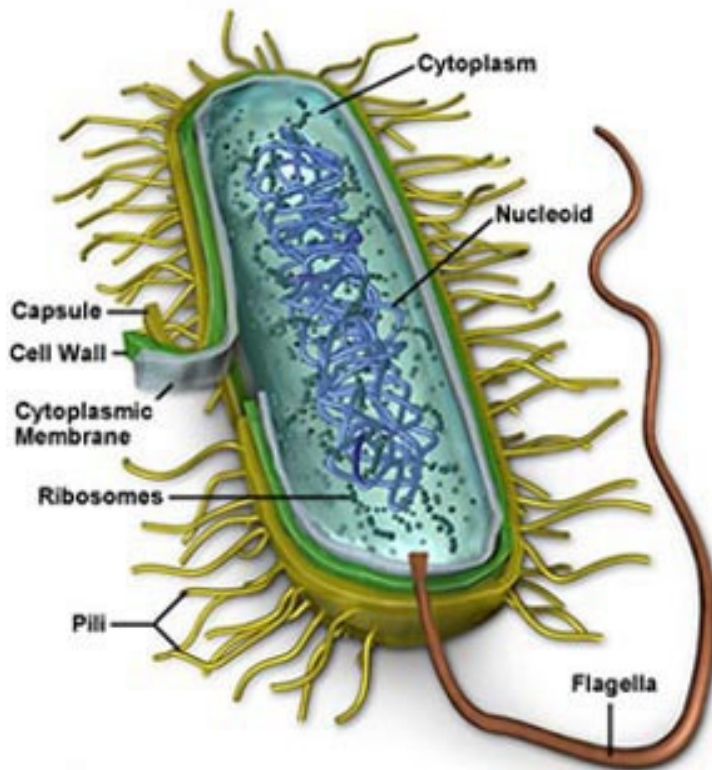


Figure 2.1 Artistic representation of a model bacterium with some of the most common parts labeled. Note that the cytoplasmic membrane and the cell wall compose the cell envelope, although other bacteria have an outer membrane between the cell wall and the capsule. (© McGraw-Hill).

The taxonomy of the bacteria relies on similarities on the basis of metabolism, structure, fatty acids composition, antigens, differences in cell components and others. Some bacteria though have very different morphologies within the same species, and modern techniques use gene sequencing as a way for classification and identification. Identification of bacterial species (and strains) is fundamental for several purposes, including identification of ethiological agents of infection.

One of the first methods for identification of bacteria was the Gram stain developed back in the 1884 by Hans Christian Gram. This

staining is based in one of the fundamental differences in two groups of bacteria, namely the cell wall. The thick layers of peptidoglycan in the Gram-positive cell wall stain purple while the thin Gram-negative cell wall appears pink. By combining gram staining with morphology (using an optical microscope), most bacteria can be classified as Gram-positive cocci, Gram-positive rod-shaped, Gram-negative cocci and Gram-negative rod-shaped (see Figure 2.2 as an example).

The main problems when facing the morphological identification of bacteria are the complex sample preparations and sample modification in the case of the use of staining, labeling and genome sequencing, and/or the low resolution of the classical optical microscopes which can hardly identify differences in morphology of the smallest bacteria. These problems are tried to overcome by the use of the Atomic Force Microscope (AFM).

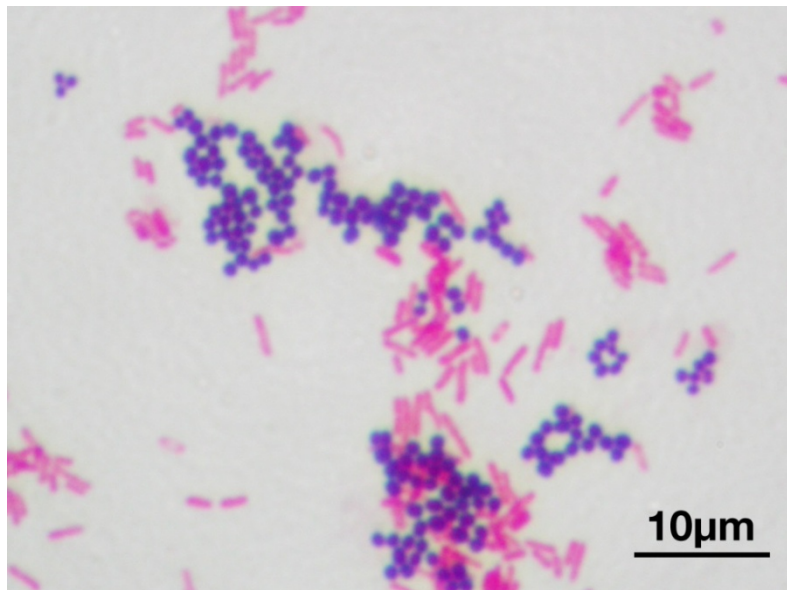


Figure 2.2 Gram-positive cocci bacteria (circular purple shapes) and Gram-negative rod-shaped bacteria (cigar-like pink shapes) as seen on an optical microscope (C.C. license).

2.1 Gram-negative bacteria

As it has been commented above Gram-negative bacteria have a thinner cell wall than the Gram-positive bacteria. Its envelope is composed by a cytoplasmic (or plasma) membrane and an outer membrane. Both define a periplasmic space between them. The cell wall is in the periplasm. The Gram staining procedure is as follows i) a crystal violet dye is applied to the bacteria and ii) an alcoholic solution removes the outer membrane and decolorizes the exposed peptidoglycan layer previously stained by the crystal violet to finally iii) counterstain it with a pink dye which gives its characteristic color after the procedure.

The cytoplasmic membrane is basically composed by a phospholipid-bilayer whereas the outer membrane contains lipopolysaccharides in the outer leaflet and only phospholipids in the inner leaflet. There are also partly embedded or integral proteins (like porins) across the inner (cytoplasmic) and outer membranes respectively. The space between both membranes is called periplasmic space where the cell wall composed by peptidoglycan is accommodated. Some Gram-negative bacteria contain lipoproteins that link the outer membrane and the peptidoglycan chain by a covalent bond. Gram-negative bacteria can also have pili (for gene transfer/attachment/movement) and/or flagella (for movement) as well as some can have a capsule for protection. A schematic representation of the cell envelope of a gram-negative bacterium can be seen in Figure 2.3

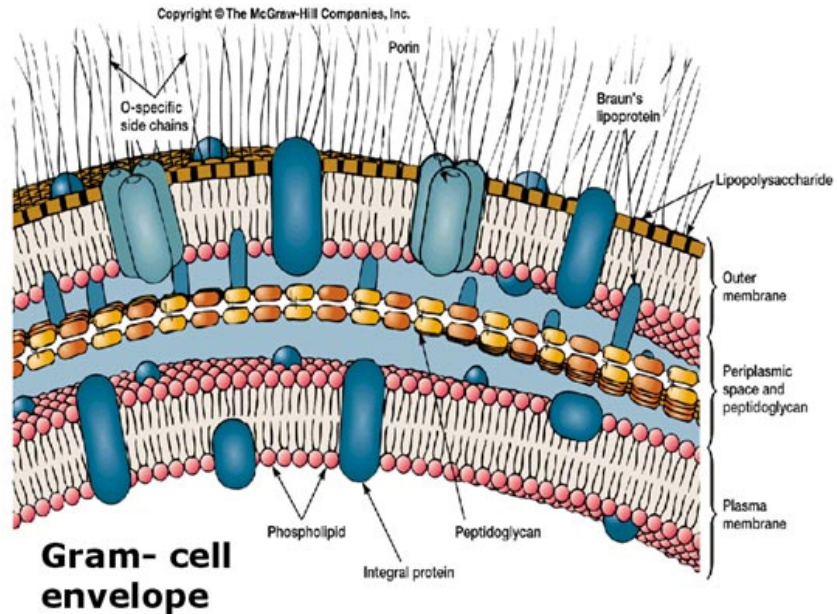


Figure 2.3 Schematic of the cell envelope of a Gram-negative bacterium with the labels of the main parts (copyright McGraw-Hill).

2.1.1 *Salmonella typhimurium*

Salmonella typhimurium (*S. Typhimurium*), is a pathogenic Gram-negative, rod-shaped, aerobic bacteria found some mammal animal gastric tract including humans. Its outer membrane is largely composed of lipopolysaccharides (LPS) which protect the bacteria from agressions of the lumen. The LPS is made up of an O-antigen, a polysaccharide core, and lipid A, which connects it to the outer membrane. Predominantly motile. Its diameter when in physiological media is around 0.7 to 1.5 μm and its lengths from 2 to 5 μm (as obtained by electronic microscope or atomic force microscope). They obtain the energy from oxidation and reduction of organic compounds.

In Figure 2.4a an atomic force microscope (AFM) topographic image in 3D of a *S. Typhimurium* cell is shown. They were deposited over a graphite substrate and left to dry (see dielectric characterization of

single bacteria chapter for details) at ambient conditions which made them to slightly collapse although the cytoplasm volume is conserved. The height is around 200 nm (Figure 2.4b and Figure 2.4c) which is lower than the diameter when in suspension although its width (1.3 μm Figure 2.4c) is larger. The length is about 2.2 μm within the range of the bacteria in liquid medium (Figure 2.4b).

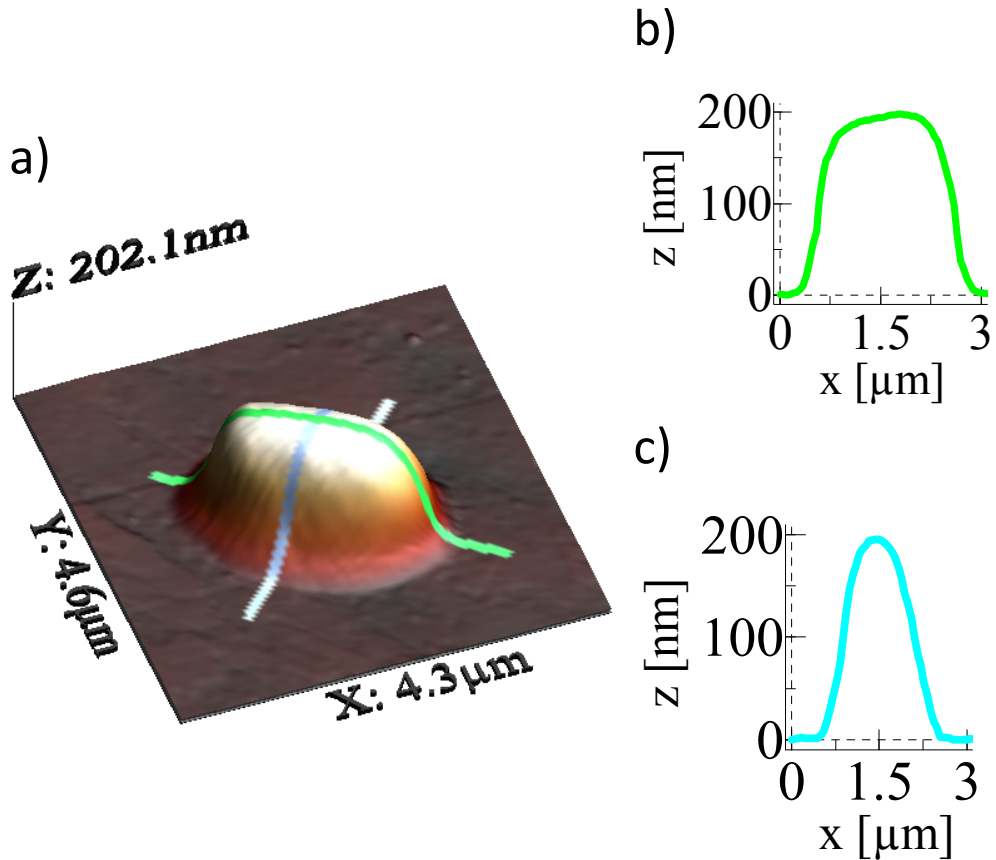


Figure 2.4 a) Atomic force microscope (AFM) topography of a single *S. Typhimurium* bacterium deposited over an HOPG substrate in ambient (air) conditions. b) Longitudinal section of the bacteria with a height of about 200 nm and a length around 2.2 μm . c) Orthogonal section of the bacteria again with 200 nm high and 1.3 μm wide.

2.1.2 *Escherichia coli*

Escherichia coli (*E. coli*) was first described in 1885 by Theodor Escherich, a German bacteriologist. It is a Gram-negative, anaerobic, rod-shaped bacterium that can be commonly found in lower intestines of mammals. They prefer to live at a higher temperature rather than the cooler temperatures. It does not form spores (mechanism of protection). Therefore, it is easy to eradicate by simple boiling or basic sterilization. They are usually benign although some strains, such as O157:H7, which produces Shiga-like toxins, cause severe foodborne infections [13]. These enteric *E. coli* can cause several intestinal and extra-intestinal infections such as urinary tract infection and meningitis. The harmless strains, on the other hand, live in our intestines, where they help our body breakdown the food we eat as well as assist with waste processing, vitamin K production, food absorption and avoiding colonization of other pathogenic bacteria. Predominantly motile enterobacteria, they are about 2.0 micrometers (μm) long and 0.25–1.0 μm in diameter when in physiological media. They usually obtain the energy by aerobic respiration if oxygen is present.

2.2 *Gram-positive bacteria*

Gram-positive bacteria as opposed as Gram-negative have a thicker peptidoglycan layer in their cell wall outside the cytoplasmic membrane. During the Gram staining i) a crystal violet dye is applied to the bacteria but ii) the alcoholic solution cannot remove the thick peptidoglycan layer previously stained by the crystal violet and when finally the iii) pink counterstain is applied it may also be absorbed but the darker crystal violet stain predominates visually.

The cytoplasmic membrane is basically composed by a phospholipid-bilayer and as opposed as Gram-negative bacteria it does not have an outer membrane. There is a thick peptidoglycan layer (cell wall) where the chains are cross-linked which confers the rigidity of such bacteria. Teichoic acids and lipids are present, forming lipoteichoic acids, which serve as binding agents, and also for certain types of adherence. They can sometimes have a small periplasmic space

between the wall and the cytoplasmatic (plasma) membrane. Only some species of Gram-positive bacteria have a capsule and/or flagella. A schematic representation of the envelope of a Gram-positive bacterium can be seen in Figure 2.5.

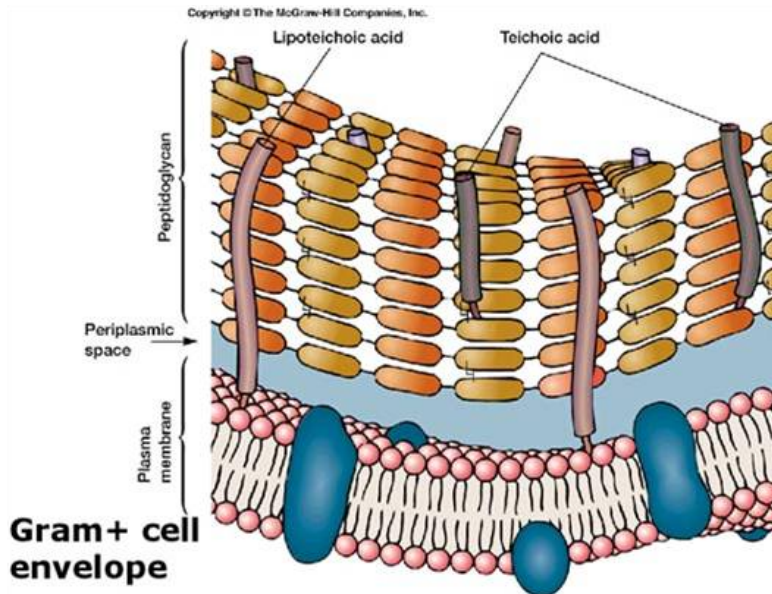


Figure 2.5 Schematic of the envelope of a Gram-positive bacterium with the labels of the main parts (copyright McGraw-Hill).

2.2.1 *Lactobacillus sakei*

Lactobacillus sakei (*L. sakei*) is a rod-shaped, Gram-positive, anaerobic bacterium commonly found living on fresh meat and fish. *L. sakei* took its name from rice alcohol, or sake, which was the product that it was first described in. They are usually used in Europe to ferment and preserve fresh meat and fish thanks to its ability to sustain life even under challenging environmental conditions, its ability to produce bacteriocins able to kill other bacteria like *Listeria*, and its capability to use nutrients in meat for self-growth [14]. They are about 2 micrometers (μm) long and 0.7 to 1.5 μm in diameter when in physiological media as

obtained by electron microscopy (i.e. [15]). They obtain the energy by fermentation.

In figure 2.6a an atomic force microscope (AFM) topographic image in 3D of the *L. sakei* cell is shown. They were deposited over a graphite substrate and left to dry (see dielectric characterization of single bacteria chapter for details) at ambient conditions.

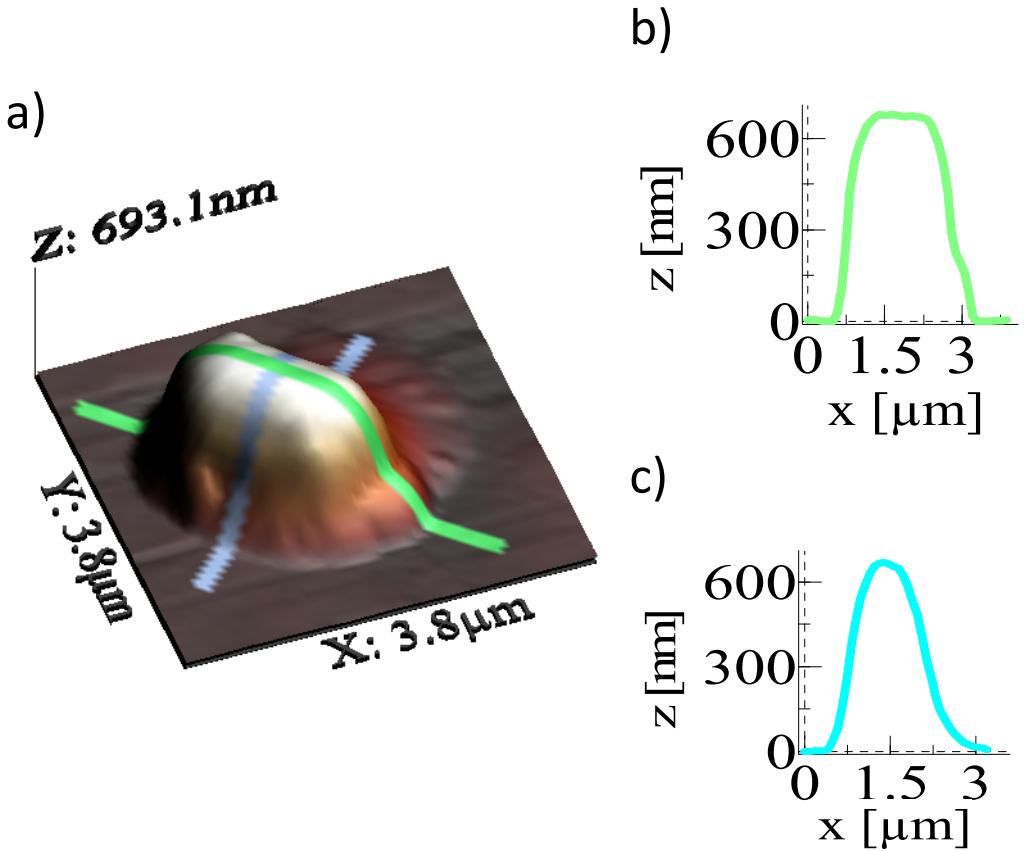


Figure 2.6

a) Atomic force microscope (AFM) topography of a single *L. sakei* bacterium deposited over an HOPG substrate in ambient (air) conditions. b) Longitudinal section of the bacteria with a height of about 690 nm and a length around 2.2 μm. c) Orthogonal section of the bacteria again with 690 nm high and 1.8 μm wide.

They were deposited over a graphite substrate and left to dry (see dielectric characterization of single bacteria chapter for details) at ambient conditions which made them to slightly collapse although the cytoplasm volume is conserved.

The height is around 690 nm (Figure 2.6b and Figure 2.6c) which is lower than the diameter when in suspension although its width (1.8 μm Figure 2.6c) is larger. The length is about 2.2 μm within the range of the bacteria in liquid medium (Figure 2.6b).

2.2.2 *Listeria innocua*

Listeria innocua (*L. innocua*) is one of the six species belonging to the genus *Listeria*. It is widely found in the environment (such as soil) and food sources. It can survive in extreme pH and temperature, and high salt concentration [16]. It is a rod-shaped, Gram-positive and bacterium. It is a non-spore forming bacterium. It may live individually or organize into chains with other *L. innocua* bacteria. *L. innocua* very much resembles its other family members, the pathogenic *Listeria monocytogenes* (*L. monocytogenes*) [16]. *L. innocua* is important because it is very similar to the food-borne pathogen *L. monocytogenes* but non-pathogenic in character. Thus its genome was sequenced in order to compare it to the genome of *L. monocytogenes* to learn what makes the latter pathogenic. They are about 2 micrometers (μm) long and 0.7 to 1 μm in diameter when in physiological media as obtained by electron microscopy (i.e [17]). It obtains the energy under the aerobic metabolism of glucose, where *L. innocua* forms lactic acid and acetic acid. However, under anaerobic conditions, the metabolism of glucose yields only lactic acid.

