Legionnaires' disease – A microbial murder mystery

Daddy – a doctor told us that Grandpa is recovering from a nasty infection caused by bacteria living in tap water. Is it really safe for me to take a shower?

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Storyline

We all use and need water on a daily basis. In developed countries, tap water is usually safe for washing purposes and in some regions has even drinking water quality. However, if not regularly flushed or properly disinfected, household water allows the growth of bacteria and can pose a risk for our health. Accordingly, stagnant water in (older) buildings, cooling towers, whirl pools, fountains, garden hoses, water sprinklers or vegetable misting systems can promote the growth of pathogenic bacteria, such as *Legionella pneumophila*. Upon inhalation of *Legionella*-containing tiny water droplets (aerosols), the bacteria reach our lung and can cause a life-threatening pneumonia called "Legionnaires' disease". *Legionella* naturally lives in many waters, including lakes, ponds, rivers and streams, where the bacteria mostly dwell in large biofilm communities. These biofilms are "grazed" by bacteria-consuming amoebae. However, upon ingestion by amoebae, *Legionella* turns the tables and grows within these predators. The peculiar ability to withstand environmental amoebae also enables *Legionella* to overcome and kill immune cells called macrophages in the lung, and the accidental death of these cells triggers a severe pneumonia. An efficient way to reduce the incidence of Legionnaires' disease is to abrogate the growth of *Legionella* in technical water sources using disinfectants and/or high temperatures.

The Microbiology and Societal Context

Legionnaires' disease is a life-threatening pneumonia, which is "accidentally" caused by the widespread environmental bacterium *Legionella pneumophila* growing in free-living amoeba **(Figure 1)**.

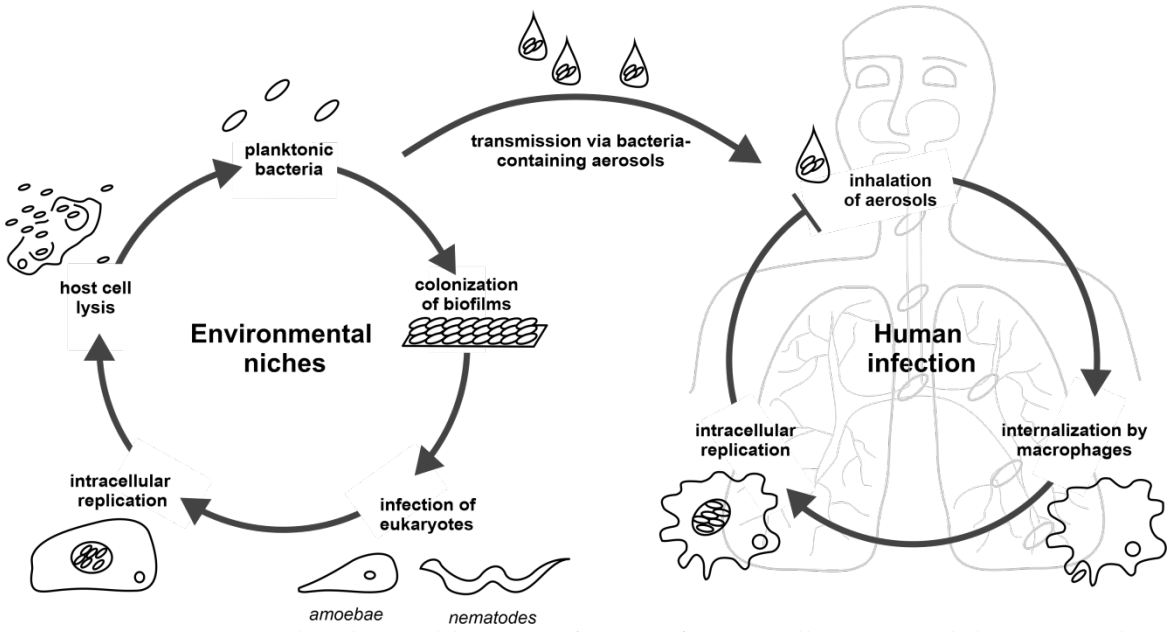

Figure 1: Environmental niches and human infection of *Legionella pneumophila*. *Legionella* bacteria persist in the environment as planktonic cells, colonize biofilms, and infect amoebae as well as nematodes. Highly virulent bacteria are released from their replicative niches in a transmissive (motile) form. Upon inhalation of *Legionella*-containing aerosols, the bacteria replicate within and kill lung immune cells called macrophages, which triggers a severe pneumonia called Legionnaires' disease (Hilbi *et al.*, 2011).

1. ***A brief history of Legionnaires' disease.*** In 1976 the United States of America celebrated 200 years of declaration of independency from Great Britain and, due to its historic past, the city Philadelphia was a center of the festivities. In parallel, the annual convention of the Pennsylvania section of the American Legion took place in Philadelphia from July 21st to 24th. The convention was attended by 4500 veterans, who were accommodated throughout the city. In the aftermath of the events, 221 of the attendants developed a peculiar respiratory ailment and 34 died. Quickly, the term Legionnaires' disease was coined for the mysterious pneumonia and broadcasted nation-wide by the media. Speculations about the causative agent of the disease included viruses, bacteria, fungi or toxic substances, and even terrorist or alien attacks were considered!

The United States Centers for Disease Control and Prevention (CDC) was commissioned to investigate the outbreak. To this end, many environmental and biopsy samples were collected, and the veterans who had returned home in the meantime, had to fill out extensive questionnaires. The investigation excluded known viral and bacterial pathogens, as well as disease transmission in the same room, from person to person, or through food or water. Epidemiological evidence suggested that the causative agent of Legionnaires' disease was air-borne and likely originated from the rooftop cooling towers of the Bellevue Stratford Hotel located in the center of Philadelphia. Yet, what turned out to be the CDC's biggest and most complex medical investigation failed to identify the cause of the deadly epidemic until December 1976.

During the Christmas holidays following the outbreak, Dr. Joseph McDade from the CDC – frustrated and upset by many unsuccessful attempts – went back to the lab again to search for the agent causing Legionnaires' disease. He injected lysates of lung biopsies from pneumonia victims into guinea pigs and subsequently infected egg yolk with tissue from sick animals. This procedure allowed the growth of a novel rod-shaped bacterium staining negative in the Gram reaction. Thus, almost 5 months after the initial outbreak, Dr. McDade finally managed to identify the causative agent, which was later named *Legionella pneumophila*, i.e., the "lung-loving Legionnaires' bacterium".

2. ***Legionnaires' disease – Recent outbreaks and sporadic cases.*** To date, 65 different *Legionella* species have been identified, of which approximately half were linked to clinical cases. However, more than 90% of the Legionnaires' disease cases reported world-wide are caused by *Legionella pneumophila*. Since the initial Legionnaires' disease outbreak in 1976, large epidemics caused by *L. pneumophila* have regularly made headlines. In Holland (Bovenkarspel) in 1999 a contaminated whirlpool infected 242 visitors of a flower show. The hitherto largest outbreak occurred in Spain (Murcia) in 2001 and sickened 449 people. An epidemic in France (Lens) in 2003 with 86 cases showed an exceptionally high case-fatality rate of 21%. After an outbreak in Portugal (Vila Franca de Xira) in 2014 affecting 336 individuals, the first and thus far only incident of a person-to-person transmission was documented. In most of these instances cooling towers were identified as the pathogen source, and contaminated aerosols spread and infected people over several kilometers! Sporadic cases of Legionnaires' disease have also been on the rise world-wide over the last 20 years, and nearly 10,000 cases were reported to the United States CDC in 2018 alone.

Legionnaires' disease affects mostly elderly persons (especially men), smokers and immune-compromised people, but very rarely children. The pneumonia is characterized by influenza-like symptoms as well as by a dry cough and chest pain, 2-10 days after exposure to the bacteria. Fatal outcomes are caused by respiratory or renal failure and/or septic shock. Legionnaires' disease is

generally treated with commonly prescribed antibiotics like azithromycin, ciprofloxacin or tetracycline. Acquired antibiotics resistance has fortunately not been reported for *L. pneumophila*, unlike for other pneumonia agents such as *Streptococcus pneumoniae* or *Mycoplasma pneumonia*. However, since human life expectancy steadily increases, technical appliances associated with transmission of *L. pneumophila* are in widespread use, and global warming might promote the growth of environmental bacteria, Legionnaires' disease is likely to remain a health problem and even gain increased importance in the near future.

3. *Ecology and evolution of* Legionella pneumophila. *Legionella pneumophila* has been recovered from natural water sources like rivers, streams, lakes, ponds and hot springs, as well as from technical water systems including air-conditioners, cooling towers, fountains, garden hoses, water sprinklers, vegetable misting systems and thermal spas. In the environment, *L. pneumophila* colonizes complex microbial communities termed biofilms, which house prokaryotic and eukaryotic (micro)organisms (**Figure 1**). These biofilms comprise dynamic networks of architectural and metabolic interactions and represent the prevailing environmental niche of microbes. Free-living protozoa such as amoebae and ciliates feed on bacteria ("graze") in biofilms. Remarkably, upon ingestion by amoebae, e.g., *Acanthamoeba castellanii* or *Dictyostelium discoideum*, *L. pneumophila* turns the tables and grows within these phagocytic predators. Intracellularly, *L. pneumophila* is protected to some extent from antibiotics, biocides as well as from osmotic and thermal stresses. Accordingly, the pathogen "hides" in the amoebae, which – counterintuitively – are a "safe haven" for the bacteria.

Accidental infection of humans by *L. pneumophila* occurs upon inhalation of bacteria-containing tiny water droplets (aerosols), generated by showers, air-conditioning systems, cooling towers, whirlpools, etc. In the lung, the pathogen infects alveolar macrophages, which are specialized phagocytes of the mammalian innate immune system. The bacterial mechanism of intracellular replication and host cell killing is very similar in amoebae and macrophages (Figure **1**), and macrophage death is a prerequisite for triggering Legionnaires' disease. To reach the alveolar macrophages, the infection route via contaminated aerosols is crucial. In contrast, drinking an *L. pneumophila* suspension (without aspiration), and thus administering the bacteria solely through the gastrointestinal route, likely does not cause disease (do not try this at home!). Taken together, *L. pneumophila* is an accidental pathogen, which adopts its virulence toward free-living amoebae to overwhelm human immune cells. In other words, amoebae are an "evolutionary crib" or an "evolutionary training ground" for *Legionella* virulence.

The human lung is a dead end for *L. pneumophila*, and therefore, likely does not allow co-evolutionary processes to happen. Rather, the pathogen acquired and evolved its virulence traits in co-existence with environmental amoebae. In fact, more than 10% of the proteins produced by *L. pneumophila* are devoted to define the interactions with protozoa. In agreement with the notion of horizontal gene transfer from amoebae to *L. pneumophila* (**Figure 2**), the bacterial genome contains a number of genes, which encode eukaryotic-like proteins or fragments (domains) thereof. These proteins are potential or proven virulence factors, which exert similar functions as their eukaryotic counterparts and target host cell processes for the benefit of the pathogen.

4. *Physiology and bi-phasic life style of* Legionella pneumophila. *Legionella pneumophila* is a fastidious bacterium, which for extracellular growth primarily uses proteins, peptides and amino acids as carbon and energy sources. The opportunistic pathogen is an obligate aerobe bacterium (i.e., a non-fermenter) and grows optimally between 25°C and 45°C. Recent studies indicated that *L. pneumophila* has broader metabolic capacities and likely also utilizes polysaccharides

(cellulose, chitin, starch and glycogen) and – in particular intracellularly – metabolizes carbohydrates (sugars) such as glucose and inositol, or glycerol and glycerolipids.

Growth and virulence of *L. pneumophila* are reciprocally linked in a bi-phasic cycle: growing bacteria are non-virulent, while non-growing bacteria are transmissive (virulent, motile and stress resistant). The bi-phasic cycle is controlled by extrinsic cues (nutrients, environmental conditions), as well as by an intrinsic, hydrophobic small signalling molecule termed LAI-1 (*Legionella* autoinducer-1, 3-hydroxypentadecane-4-one) (**Figure 2**). LAI-1 is produced and detected by components of the cell density-dependent Lqs (*Legionella* quorum sensing) regulatory system. In addition to controlling the growth phase switch, the Lqs system is also a major regulator of *L. pneumophila* virulence, motility, natural competence for DNA uptake and bacterial filament production.

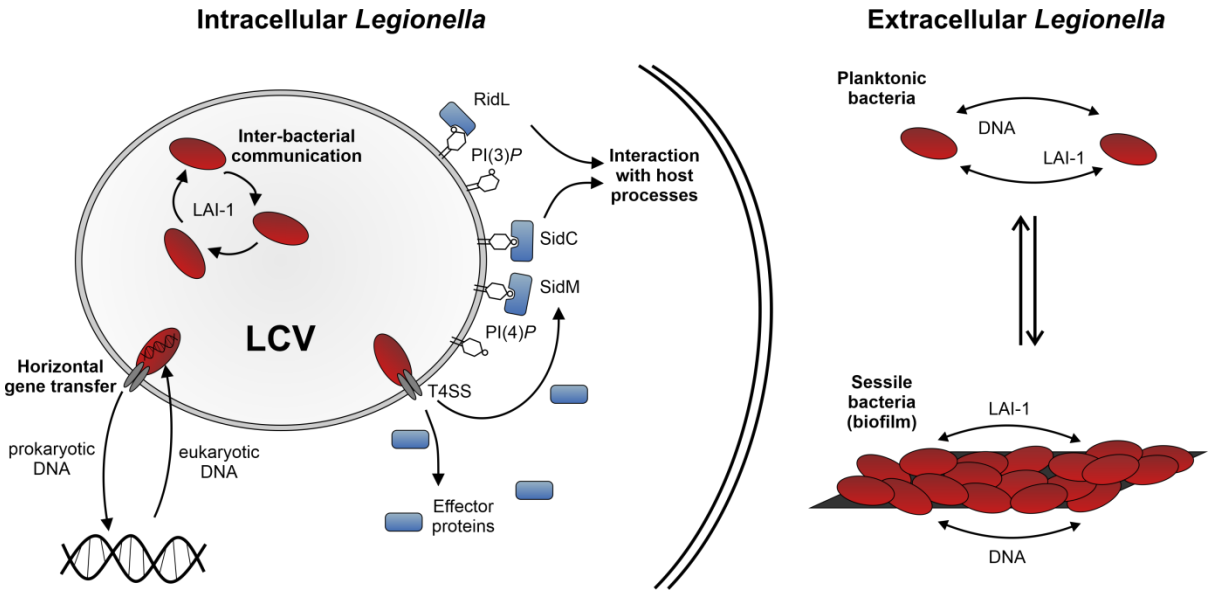

Fig. 2. Virulence and communication of *Legionella pneumophila*. The virulence of *L. pneumophila* depends on a type IV secretion system (T4SS), which secretes more than 300 different „effector" proteins into host cells to establish a replication-permissive "*Legionella*-containing vacuole" (LCV). For cell-cell communication, *L. pneumophila* uses the α-hydroxyketone signaling molecule LAI-1 (*Legionella* autoinducer-1), which is produced and sensed by the Lqs quorum sensing system. The *L. pneumophila* genome harbors genes of eukaryotic origin, indicating an extensive horizontal transfer of DNA between prokaryotes and eukaryotes. In the extracellular environment, planktonic and sessile (biofilm) *Legionella* communicate through LAI-1 and might exchange DNA (Hilbi *et al.*, 2011).

5. *Virulence and intracellular growth of* Legionella pneumophila. Similar to many other pathogenic bacteria, the outer membrane lipopolysaccharide (LPS) of *L. pneumophila* is a major virulence factor. Strikingly, *L. pneumophila* strains producing serogroup 1 LPS are clinically most relevant, while not prevalent in the environment. Accordingly, serogroup 1 LPS is critically involved in *L. pneumophila* pathogenesis. Another key virulence determinant is the flagellum, in particular its major structural component called flagellin. The flagellum is essential for bacterial motility and chemotaxis. Both, *L. pneumophila* LPS and flagellin activate the innate immune system and cytokine production of metazoan hosts through distinct multi-subunit machineries termed „inflammasomes".

L. pneumophila is a facultative intracellular bacterium, which is able to replicate in eukaryotic cells as different as amoebae, macrophages or epithelial cells. Within these cells, *L. pneumophila* resists degradation and establishes a distinct, replication-permissive compartment termed the

"*Legionella*-containing vacuole" (LCV) (**Figure 2**). This compartment does not acidify, but extensively communicates with different host cell vesicle trafficking pathways. Pivotal processes of LCV formation and maturation are a phosphoinositide (PI) lipid conversion, subversion of small GTPase enzymes, and acquisition and modulation of a host cell organelle called the endoplasmic reticulum (ER).

Intracellular replication of *L. pneumophila* requires a complex bacterial machinery termed the Icm/Dot (Intracellular multiplication / Defective organelle transport) type IV secretion system (T4SS) (Figure **2**). The T4SS is an essential virulence factor, and its deletion abrogates intracellular replication and bacterial virulence. The T4SS translocates more than 300 different "effector" proteins into host cells, where they subvert pivotal processes, such as signal transduction, membrane trafficking and cytoskeletal dynamics. Importantly, the effector proteins govern the conversion of the initial (bactericidal) phagosome into the protective LCV. While the T4SS is conserved in the genus *Legionella*, the effector proteins are not and in fact, are astonishingly diverse among different species. Many of these proteins adopt unique and novel biological activities targeting and modifying host cell components. Based on the manifold and sophisticated molecular interactions between *L. pneumophila* and host cells, the pathogen has been dubbed the „world's best cell biologist".

Relevance for Sustainable Development Goals and Grand Challenges

Goal 3. Good health and well-being. The reduction or eradication of *Legionella* bacteria in technical water systems would obviously significantly reduce the number of Legionnaires' disease cases and thus significantly improve human health.

Goal 6: Clean water and sanitation. The reduction or eradication of *Legionella* bacteria in technical water systems should preferentially not occur through compounds compromising the water quality. Rather, physical approaches such as (temporarily) flushing the systems and/or elevating the temperature should be favored.

Goal 7: Affordable and clean energy. Heating up technical water sources is an energetically costly process. There is an ongoing discussion about to what extent energy-consuming hygiene considerations (high water temperatures) should be prioritized over potentially live-risking energy saving considerations (lower water temperatures).

Goal 8: Decent work and economic growth. New measures and tests to diagnose and eradicate *Legionella* bacteria in technical water systems can be marketed through existing enterprises or newly founded spin-off companies.

Goal 9: Industry, innovation and infrastructure. Innovative new treatment strategies to eradicate *Legionella* species are urgently needed. Basic research is required to better understand the physiology, virulence and pathogenesis, biofilm colonization and formation, as well as motility and spread of *Legionella*. Novel approaches for a more rapid detection of *Legionella* are desirable. The concept of biocontrol has not been explored to reduce pathogen numbers.

Goal 11: Sustainable cities and communities. The flux and treatment of drinking water and waste water needs to be considered to reach or maintain more sustainable cities and communities. *Legionella*-safe or -reduced construction should be considered for new as well as for existing buildings.

Potential Implications for Decisions

1. Individual

a) Consider the potential microbial contamination of stagnant technical water systems (buildings, cooling towers, whirl pools, fountains, garden hoses, water sprinklers or vegetable misting systems). Protect yourself by, e.g., flushing the systems prior to use if possible.

2. Community and national policies

a) The regulations for household and drinking water (food!) have to be regularly updated and adapted by the health, nutrition and energy authorities according to the latest scientific findings and technological applications.

Pupil Participation

1. Class discussion about the topic and the underlying microbiology:

a) Regarding water-borne pathogens: is it generally safe (for children) to shower?

b) Which measures could help to reduce the incidence of *Legionella* in buildings?

c) How can we grow and identify novel, fastidious bacteria with an unknown physiology?

d) What are the requirements for suitable animal models to study infectious diseases?

e) How does a microbial prey possibly evolve to become a predator?

f) Why is antibiotics resistance (to date) not an issue for treating Legionnaires' disease?

The Evidence Base, Further Reading and Teaching Aids

1. Episode on Legionnaires' disease on the American documentary-style television program "Forensic Files" (http://www.forensicfiles.com). Accessible through YouTube (https://www.youtube.com/watch?v=7X2FLyLSC9E).
2. Wikipedia website entry on Legionnaires' disease (https://en.wikipedia.org/wiki/1976_Philadelphia_Legionnaires%27_disease_outbreak).
3. CDC website entry on *Legionella* (https://www.cdc.gov/legionella/).
4. Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar, R.G., *et al.* (1977). Legionnaires' disease: description of an epidemic of pneumonia. *The New England Journal of Medicine* **297,** 1189-1197.
5. McDade, J.E., Shepard, C.C., Fraser, D.W., Tsai, T.R., Redus, M.A. and Dowdle, W.R. (1977). Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *The New England Journal of Medicine* **297,** 1197-1203.
6. Newton, H.J., Ang, D.K., van Driel, I.R. and Hartland, E.L. (2010). Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clinical Microbiology Reviews* **23,** 274-298.
7. Hilbi, H., Hoffmann, C. and Harrison, C.F. (2011). *Legionella* spp. outdoors: colonization, communication and persistence. *Environmental Microbiology Reports* **3,** 286–296.
8. Gomez-Valero, L. and Buchrieser, C. (2013). Genome dynamics in *Legionella*: the basis of versatility and adaptation to intracellular replication. *Cold Spring Harbor Perspectives in Medicine* **3,** a009993.
9. Swart, A.L., Harrison, C.F., Eichinger, L., Steinert, M. and Hilbi, H. (2018). *Acanthamoeba* and *Dictyostelium* as cellular models for *Legionella* infection. *Frontiers in Cellular and Infection Microbiology* **8,** 61.
10. Steiner, B., Weber, S. and Hilbi, H. (2018). Formation of the *Legionella*-containing vacuole: phosphoinositide conversion, GTPase modulation and ER dynamics. *International Journal of Medical Microbiology* **308,** 49-57.
11. Personnic, N., Striednig, B. and Hilbi, H. (2018). *Legionella* quorum sensing and its role in pathogen-host interactions. *Current Opinion in Microbiology* **41,** 29-35
12. Hochstrasser, R. and Hilbi, H. (2020). *Legionella* quorum sensing meets cyclic-di-GMP signaling. *Current Opinion in Microbiology* **55,** 9-16.

Glossary

Acanthamoeba castellanii – water-borne amoeba; model organism for *Legionella* growth
aerosol – tiny water droplets, which can carry and spread pathogens
alveolar macrophage – innate immune cell in the lung taking up and killing bacteria
amoeba – environmental, unicellular (protozoan), motile organism feeding on bacteria
antibiotic – drug preferentially inhibiting/killing bacteria
biocontrol – control of a microbial pathogen by (products of) other bacteria
biofilm – architecturally and metabolically connected, sessile community of microorganisms
carbohydrate – organic compound ("sugar"), building block of polysaccharides
case-fatality rate – proportion of deaths due to a disease compared to the total number of people diagnosed with a disease
Centers for Disease Control and Prevention (CDC) – United States national public health institute with the mandate to protect public health and safety.
chemotaxis – oriented cellular motility along a gradient of a chemical attractant
ciliate – environmental, unicellular (protozoan), motile organism with hair-like protrusions
cytokine – immunological signaling molecule produced by different eukaryotic cells
Dictyostelium discoideum – soil-borne "social" amoeba undergoing a developmental cycle upon starvation; model organism for intracellular *Legionella* growth
endoplasmic reticulum (ER) – cellular organelle that synthesizes lipids and modifies proteins
epidemic – disease affecting a large number of people in a community, population, or region
epithelial cell – eukaryotic cell lining the outer surfaces of organs (e.g. lung, intestine, skin)
flagellum – rotating bacterial protrusion allowing directional motility
global warming – global climate change due to rising concentrations of anthropogenic CO_2
Gram staining – fundamental classification procedure based on bacterial cell wall differences
Horizontal gene transfer – exchange of genetic material between cells in the same generation
Hydrophobic – water-repellant (fat-soluble)
Inflammasome – multi-subunit protein complex activating specific cytokines
innate immune system –unspecific, non-adaptive branch of the immune system
LAI-1 (*Legionella* autoinducer-1) – small, hydrophobic *L. pneumophila* signalling molecule (3-hydroxypentadecane-4-one)
Legionella pneumophila – environmental, amoeba-resistant, motile, aerobe, Gram-negative bacterium
Legionnaires' disease – severe pneumonia caused by *Legionella* species
lipopolysaccharide (LPS) – complex outer membrane component of Gram-negative bacteria
– cell density-dependent *L. pneumophila* regulatory system based on the production and detection of the small signalling molecule LAI-1
McDade, Joseph – CDC employee who first isolated and described *L. pneumophila*
metabolism – general term for the live-sustaining chemical reactions in an organism
metazoan – multicellular organisms (opposite: protozoa)
nematode – roundworm, e.g., *Caenorhabditis elegans*
niche – ecological match of a species with distinct environmental conditions
non-fermenter – bacterium gaining energy through respiration rather than fermentation
obligate aerobe – bacterium performing respiration only with O_2 a terminal electron acceptor
opportunistic pathogen – infectious microorganism that can cause disease when resistance of the host is low
pathogenicity – ability/capacity of an organism to causes disease
phagocyte – eukaryotic cell, which actively takes up particles and microorganisms
phosphoinositide (PI) lipid – signaling lipid of eukaryotic cells

pneumonia – lung inflammation
polysaccharide – macromolecule comprising (different) carbohydrates
prokaryote – (bacterial) cell with no separated nucleus and organelle
protozoa – unicellular organisms (opposite: metazoan)
septic shock – life-threatening condition characterized by low blood pressure due to sepsis ("blood poisoning"), a systemic inflammatory immune reaction in response to infections.
s**mall GTPase** – regulatory enzyme ("molecular switch": GTP-bound [on], GDP-bound [off]
type IV secretion system (T4SS) – complex protein secretion machinery
vesicle trafficking pathway – cellular transport route of cargo-loaded vesicles (lipid spheres)
virulence factor – microbial product that promotes virulence (i.e., the pathogenicity degree)