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REVIEW PAPERS

General and Molecular Ecology of *Legionella*

E. L. Golovlev

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142292 Russia

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Abstract—The review is devoted to the general and molecular ecology of bacteria of the genus *Legionella* in natural and anthropogenic environments. Invasion of amoebae and infusoria by legionellae and their replication in these protozoa can be considered to be a preadaptation for invasion of the human immune system. Symbiosis of bacteria and protozoa as a promising model of cellular microbiology and the conception of bacterial ecological niches are discussed in relation to the low fidelity of most bacterial species to their habitats (biotopes). The necessity of elaboration of a similar conception for microbial consortia and associations is emphasized.

Key words: *Legionella*, protozoa, symbiosis, ecology, cell microbiology, evolution, pathogenesis.

LEGIONELLAE AND LEGIONELLOSES

The generic name *Legionella* refers to the etiologic agent of an atypical bacterial pneumonia whose largest outbreak occurred in 1976 in Philadelphia and caused an extraordinarily high lethality of up to 20% [1–4]. The illness was named Legionnaires' disease, since its first victims were Legionnaires at an American Legion convention in Philadelphia. The etiologic agents of the most acute form of legionellosis are *Legionella pneumophila* and *Legionella micdadei* [2–4]. A milder, almost nonlethal form of legionellosis—Pontiac fever—is caused by other *Legionella* species or by *L. pneumophila* and *L. micdadei* serovars. The taxonomy of *Legionella* is still subject of discussion. To date, 36 species of this genus and more than 60 serovars, as well as legionella-like amoebic parasites of as yet unidentified taxonomic position, have been described [1–3]. Because of the low degree of DNA–DNA hybridization with other members of the genus *Legionella*, the species *L. micdadei* was reclassified into the species *T. micdadei* of a new genus *Tatlockia*. Some fluorescent species of legionellae were classified into the third genus, *Fluoribacter*, of the family *Legionellaceae*.

It should be noted that legionellosis-like diseases were first described in the 1950s, although their etiology was unknown. By the late 1980s, legionellosis had become a common, albeit not very frequent, disease in Western Europe. Legionellae mainly infect elderly people and/or those with an impaired immune system. These bacteria are spread aerially from a primary source and not by person-to-person contact. Sources of this disease sometimes remain unknown [1–4]. Epidemiological investigations indicate that legionellae are widespread in most (if not in all) regions of the world and that their sources are present in both natural and anthropogenic environments. As for the legionellosis outbreak in Philadelphia, most specialists suggest that mists from air conditioners in the hotel at which the American Legion convention was accommodated might be the source of infection. On the other hand, the fact that many sick Legionnairs fought in Vietnam gave grounds for the hypothesis that Indochina was the primary source of the wild-type legionella species [1]. This viewpoint is inconsistent with the generally accepted dogma of the ubiquitous distribution of many aquatic and soil bacteria; nevertheless, some indirect data confirm this viewpoint. *Legionella* bacteria are gram-negative aerobic and microaerobic rods and cocci [2]. Uneven rods have a mean size of 0.3–0.9 × 2–20 μm; however, long filaments are always present in legionella cultures. Legionellae are motile due to the presence of a single polar or several subpolar flagella. A characteristic feature of the outer membrane of these bacteria is that it contains large amounts of branched long-chain fatty acids, which make the membrane surface hydrophobic. This largely determines the ecological characteristics of legionellae and their resistance to various unfavorable environmental physicochemical factors, poisons, and antibiotics. Some legionella species synthesize polysaccharide capsules. Their range of temperature tolerance is extremely wide: legionella have been isolated from aquatic habitats with temperatures varying from 6 to 63°C; these bacteria can replicate within a temperature range of 25–37°C with an optimum growth temperature of 32–35°C. The pH tolerance range is between 5.5 and 9.2 (optimum pH for growth is 6.5–6.9). Beyond these limits, *Legionella* bacteria are more sensitive to alkaline than to acidic conditions [1–3]. Legionellae are strict aerobes, which are unable to oxidize sugars but can utilize amino acids as carbon sources. These bacteria require cysteine and

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do not require vitamins for growth [2–4]. The high sensitivity of *Legionella* to hydrogen peroxide and oxygen superoxide, which is atypical of pathogenic bacteria, leads to the necessity to add activated charcoal to yeast extract–containing media [5]. The requirement of *Legionella* for iron is rather high (the optimum concentration of iron in growth media ranges from 5 to 50 mg/l), which is explained by the inability of legionellae to synthesize siderophores [6]. These bacteria are unable to grow at high concentrations of NaCl in the medium. Aluminum, lead, cadmium, copper, and manganese inhibit their growth only at concentrations exceeding 10 mg/l [1]. Among the biological properties of *Legionella* bacteria that are essential for understanding their ecology, noteworthy are the ability of these bacteria to parasitize in protozoa, to form associations with other bacteria and algae, to produce biofilms, and to transit to a viable but nonculturable state under hostile conditions [2–4, 7–12]. *Legionella* bacteria are of interest for researchers in the fields of general microbiology, theoretical ecology, and evolution, since they are a suitable object for devising a concept of the ecological niche for bacteria and studying the coevolution of humans and microbes. The interest of researchers in *Legionella* is also due to their being occasional human parasites [13], for which their division into pathogenic and nonpathogenic species makes no biological sense.

GENERAL ECOLOGY OF LEGIONELLAE

Occurrence of *Legionella* in natural habitats. Epidemiological investigations performed in Indochina showed that humans are usually infected by legionellae through inhalation of legionella-containing mists near waterfalls, whirlpools, and rapidly flowing rivers and streams [1]. Due to hydrophobicity of their surface, legionella cells exhibit an affinity for the water–air interface and hence concentrate in surface film and microscopic water droplets 0.8–10 μm in diameter [1]. *Legionella* bacteria have also been found in tropical sea water and in warm water bodies with a temperature of about 35–37°C, irrespective of their geographical position, for instance, in thermal water springs or ponds with an inflow of hot process water [3]. In northern countries, legionellae were also found in water bodies with moderate temperatures. Thus, Fliermans *et al.* detected legionellae in 67 lakes, rivers, and streams of the northern United States (up to Illinois) and concluded that these bacteria are natural for aquatic biotopes in this geographical zone [14]. *Legionella* bacteria are often found in cyanobacterial mats and in other associations with the cyanobacteria of the genera *Fisherella, Formidium*, and *Oscillatoria*, as well as in associations with algae and some aquatic plants. In such associations, legionellae obviously grow at the expense of compounds (e.g., amino acids) that are excreted by cyanobacteria or algae [3, 8, 14]. Thus, in a complete laboratory medium, *Legionella* grew two times faster in the presence of the cyanobacterium *Fisherella* sp. than without it [3]. It should be noted that potentially infectious *Legionella* strains, which were isolated through passages in guinea pigs, have also been found in epidemiologically safe water bodies of the moderate climatic zone [15]. This indicates that such water bodies are a permanent source of this bacterium, which, however, occurs primarily in a physiologically inactive state.

Due to their specific nutritional requirements, *Legionella* usually come into trophic relations with other organisms, such as cyanobacteria, algae, and some bacteria, which presumably supply *Legionella* with cysteine [16]. For the same reasons, *Legionella* is often found in the biofilms formed on various surfaces present in water bodies and water-containing processing systems [9, 10].

Already the first attempts to isolate *Legionella* bacteria in pure cultures showed that they are associated with amoeba, primarily those belonging to the genera *Hartmanella* and *Acantamoeba* [1–3]. Legionellae turned out to be natural parasites of protozoa, in which they replicate and survive unfavorable conditions in a viable but nonculturable state [3, 11, 12]. The most frequently infected amoebic species belong to the genera *Acanthamoeba* (the most susceptible species is *A. castellanii*), *Hartmanella* (the most susceptible species is *H. vermiformis*), *Naegleria*, and *Echinamoeba* [3]. There is evidence indicating that *Legionella* can also infect the infusorium *Tetrahymena pyriformis* [18].

Thus, legionellae inhabit primarily natural aquatic environments, although they are also found in wet soils, various sediments and even in subsurface waters at depths of up to 1170 m [3]. It is likely that the main factors supporting *Legionella* populations in nature are sufficiently high humidity and temperature, as well as the presence of protozoan hosts [3].

Occurrence of *Legionella* in anthropogenic environments. In anthropogenic environments, legionellae colonize habitats ecologically similar to their natural habitats, that is, warm water reservoirs, evaporators, and process systems with running and sufficiently aerated water (air conditioners, heating systems with moderate temperatures, plumbing systems, cooling towers, showers, swimming pools, etc.). Generally, legionellae reside in anthropogenic habitats containing sufficient amounts of water with a temperature of 35–45°C and an oxygen concentration of at least 2.2 mg/l. In rooms with air conditioners, the formation of aerosols considerably increases the risk of legionellosis among humans [1, 3, 4].

The adherence of *Legionella* cells to hydrophobic surfaces is one of the major factors responsible for the formation of biofilms in various process systems and water reservoirs [1, 9, 10]. The most developed legionella-containing biofilms have been found on the inner surfaces of plastic tubes and tanks, rubber and plastic gaskets in metal joints and valves, etc. Although legionellae can produce biofilms on smooth metal sur-

faces, they predominantly colonize plastic surfaces, which are rough (this facilitates colonization) and serve as sources of nutrients due to plastic leaching [1, 4, 9, 19]. It should be noted that some authors consider biofilms to be the main source of legionellae in both natural and anthropogenic environments [9].

Due to the high tolerance of *Legionella* to metal ions, their increased concentrations in potable water systems made of metals do not prevent the formation of legionella-containing biofilms there [19]. And even though they are not detected in new copper pipelines, viable *Legionella* cells can easily be detected there five years later [1].

Studies of the microflora of tap and process waters have also demonstrated the association of *Legionella* with bacterial and protozoan partners. It is the presence of these partners and the formation of mixed bacterial biofilms that may be responsible for the efficient reproduction of legionellae in anthropogenic aquatic environments [9–16]. Another observation important for understanding the ecology of *Legionella* in anthropogenic habitats is its high resistance to chlorine and other water disinfectants [20].

The key factors responsible for the infection of humans in anthropogenic environments by legionellae are most likely environmental temperature and the probability of aerosol formation (the latter facilitates infection through inhalation of legionella-contaminated mists). For this reason, air conditioners, cooling systems, and showers are believed to be the most dangerous sources of legionella infection.

MOLECULAR ECOLOGY OF LEGIONELLAE

Molecular mechanisms of *Legionella* interaction with protozoa. Some authors believe that *Legionella* cells can replicate only inside infected protozoan cells [21]. This seems questionable, since warm-water cyanobacterial mats can also provide all necessary nutrients to legionellae. There is no doubt that *Legionella* is unable to grow alone in natural or anthropogenic aquatic environments [1–4] and that protozoa play a significant role in the maintenance of the population of this bacterium.

Investigation of the interaction of *Legionella* with the amoebae *H. vermiformis* and *A. castellanii* showed that the mechanisms of bacterial invasion of amoebae are species-specific and depend on the host organism [21–23]. Thus, the initial stage of invasion of *H. vermiformis* by *L. micdadei* involves the recognition of the amoebic cell wall lectin containing a typical galactose–*N*-acetylgalactosamine (Gal/GalNAc) carbohydrate motif [22–24]. At the same time, *L pneumophila* interacts with another galactose-containing lectin of this amoeba [24]. The bacterial ligands that bind to this lectin are probably type IV pili, which are usually involved in DNA transfer during genetic transformation and bacterial adherence to various surfaces and which are responsible for the twitching motility of some bacteria. *Legionella* mutants defective in the expression of proteins of these pili are unable to attach to protozoan cells [25]. The pili may also be responsible for the adhesion of *Legionella* cells to biofilms. Thus, we deal with a receptor-mediated interaction. Conversely, the lectin receptor of *Acanthamoeba polyphaga* plays an insignificant part, if any, in legionella attachment [23].

The regulatory mechanisms that control bacterial invasion are as yet poorly understood. It has been shown that the surface chaperonin Hsp60 plays a role in the invasion of HeLa cells [26]. This may indicate that the bacterial invasion of protozoa is a more active process than phagocytosis, which is responsible for amoebic nutrition and the bactericidal activity of phagocytes. Hickey and Chianciotto [27] identified an iron- and Fur-repressed *L. pneumophila* gene that promoted intracellular infection and encoded a protein homologous to the synthetase of aerobactin, one of the siderophores of *E. coli*. These findings contradict the data of Reeves *et al.* showing the absence of siderophores in legionellae [6]. It is likely that iron deficiency induces some virulence factors in legionellae that are responsible for their invasion of eukaryotic cells, as it has been shown for other pathogenic bacteria [28]. Siderophore activity is considered to be one of the six postulated virulence factors of *L. pneumophila* [21]. It remains unknown how *Legionella* obtains iron inside protozoan cells.

Although fragmentary, these data suggest that bacterial reception produces a number of pleiotropic regulatory responses, including a physiological stress response. This has been shown for at least one *L. pneumophila* gene, *gspA* (encoding global stress protein), which is activated in response to phagocyte invasion and to various stress factors (heat, oxidative, and osmotic stresses) [29]. The product of the *gspA* gene exhibits a relatively high homology (41.3 and 36.5%) with two heat shock proteins of *E. coli*. One of the two promoters of this gene can be recognized in *E. coli* cells by the σ^{32} subunit, which controls the expression of one of the heat shock response regulons [30].

Bacterial reception induces a number of processes in the host cell. Thus, the activation of *H. vermiformis* phosphoprotein phosphatases results in the drastic dephosphorylation of tyrosyl residues in lectin and some other host proteins, including those of the actin cytoskeleton [21, 22, 31]. Conversely, the invasion of human cells by *Salmonella typhimurium* and *Shigella flexneri* is accompanied by the phosphorylation of protein tyrosyl residues, which disturbs the signal transduction system of the host cell [24]. Therefore, tyrosine phosphatases destroy the cytoskeleton of *Hartmanella*, which is believed to facilitate microfilament- independent receptor-mediated phagocytosis and bacterial motility. Furthermore, dephosphorylation of the Gal/GalNAc receptor and cytoskeleton proteins may block receptors and thus impair signal transduction in

the host cell. Probably, it is this process that blocks, after cell invasion, the fusion of the phagosome with the lysosome [24]. All these events disorganize cells [24, 31]. In contrast to the interaction with *Hartmanella*, the interaction of *Legionella* with *A. polyphaga* is associated with only a slight dephosphorylation of phosphoproteins [23].

The next stage of invasion is the phagocytosis of bacterial cells. Pagocytosis in amoebae is of two types, conventional (as in *H. vermiformis*) and coiling (as in *A. castellanii*) [21, 34]. The latter is also typical of human immune cells. During coiling phagocytosis, an invaded bacterium is enclosed within a multilayer sac [22]. Invasion of amoebae by other representatives of the family *Legionellaceae*, e.g., by *T. micdadei*, has been shown to occur by the mechanism of microfilament-independent conventional phagocytosis. Whether or not the coiling phagocytosis in *A. castellani* depends on microfilaments remains unknown [21, 22].

Impairment of the signal system of host cells is an efficient mechanism of the action of pathogenic bacteria on eukaryotic cells. ADP-ribosylation of eukaryotic proteins is considered to be part of this mechanism. It was shown that the ADP-ribosylating activity of *L. pneumophila* is associated with a protein that is probably incorporated into the outer membrane and becomes activated when contacting the membranes or the cytoplasmic components of phagocytes [36]. The main targets for bacterial ADP-ribosyl transferases are the regulatory proteins of eukaryotic cells. It cannot be excluded that the invasion of humans and animals by protozoa is accompanied by the ADP-ribosylation of so-called G-proteins, which are specific GTP–binding heterotrimeric membrane proteins acting as the primary components of the signal transduction pathway and leading to the activation of adenylate cyclase. *Legionella* may also block the G-protein–dependent signal transduction pathway at the level of the synthesis of phosphoinositides [37]. All these processes have been studied in detail with respect to human immune cells [36]; however, relevant publications devoted to protozoa are scarce.

The binding of bacteria to the receptors of *H. vermiformis* cells induces the synthesis of new proteins. In *A. polyphaga* cells and human phagocytes, this does not take place, probably because these proteins are synthesized constitutively (at least in phagocytes) [21, 32]. The physiological role of these proteins and the respective signal transduction pathways in *Hartmanella* have not been studied; however, they may be involved in the process of invasion, since blocking their synthesis by antibiotics inhibits the penetration of bacteria into amoebic cells [32]. The proteins are synthesized only if protozoa come into contact with viable virulent bacterial cells, but not with avirulent or killed *Legionella* or *E. coli* cells [21].

Within one hour after penetration into a protozoan cell, the bacterial cell appears to be enclosed in a phagosome surrounded by host mitochondria and vesicles [33]. However, bacterial invasion is not associated with the fusion of the phagosome and lysosome, as it takes place during the nutrition of the protozoa [34]. By the 4th h of invasion, the phagosome becomes surrounded by a multilayer structure composed of the rough endoplasmic reticulum with attached ribosomes [33] (the phagosome of *L. micdadei* is enclosed in the smooth endoplasmic reticulum [24]). It is suggested that the phagosome enclosed in the multilayer endoplasmic reticulum envelope is recognized by the host cell as its native organelle [33].

It is believed that the aforementioned 4-h period of invasion is required to provide for bacterial adaptation to the new environment and to recruit the host organelles for bacterial reproduction [22]. Bacterial reproduction in host cells occurs at a rate close to that observed in a complete medium. Preliminarily starved bacteria reproduce still faster [35]. At the early stage of replication, bacterial cells are nonmotile, since they lack flagella. At the second stage of replication, when host cells begin to lyse, bacteria look like short rods and are again motile, probably, to be released from lysed host cells [21].

The persistence and multiplication of *Legionella* bacteria in protozoan cells are important for understanding their ecology and the epidemiology of legionelloses. First, *Legionella*-infected protozoa are highly virulent organisms [38]. Second, bacteria occurring in protozoan cells are much more tolerant to environmental factors, such as temperature, pH, and the presence of disinfectants [3, 30]. Moreover, when released from host amoebae, *Legionella* bacteria become more resistant to antibiotics than when cultivated in laboratory media [39]. Intracellular replication enhances the virulence of legionellae and facilitates their resuscitation from the viable but nonculturable state [40, 41]. Amoebae of the genus *Acanthamoeba* have recently attracted the attention of researchers owing to their ability to form extracellular vesicles containing viable virulent bacteria [41]. The amoebae isolated from a cooling system produced vesicles 2.1–6.4 μm in size (vesicle formation was most active at temperatures between 30 and 48°C). The vesicles were resistant to environmental factors and presumably infectious [41].

The key point in the biology of *Legionella* bacteria is the relation between the mechanisms of their invasion of protozoan and human immune cells. Some authors believe that, despite some minor differences, these mechanisms are similar and that the intracellular parasitism of legionellae in amoebae may be a mechanism of their preadaptation to the invasion of human macrophages [33]. The processes of invasion of amoebae and macrophages differ only at the stage of bacterial penetration into them [21]. Of the three differences postulated for the interaction of bacteria with host cells, the first (specific induction of protein synthesis in host cells) has been demonstrated for only *H. vermiformis;*

the second (the absence of coiling phagocytosis in amoebae) has been disproved by detecting such phagocytosis in *A. polyphaga;* and the third (the absence of a specific lectin receptor in human phagocytes) cannot be considered essential, since the role of such a receptor may be played by some other proteins (it should be noted in this connection that the β-integrin of human phagocytes is immunologically similar to Gal/GalNAc lectin [24]). It should be emphasized that the differences between the mechanisms of invasion of protozoan and human immune cells may be due to the differences between the amoebic species investigated. At the same time, the fundamental similarity of the structural and functional organization of eukaryotic cells makes the abstract concept of occasional parasitism [13] more real at the level of cellular biology.

It should be noted that the recently emerged science of cellular microbiology, whose subject has been declared to be primarily the model processes of pathogenesis in eukaryotic cells [42, 43], is in fact closely related to general microbiology, since the investigation of the invasion of eukaryotes by nonpathogenic or avirulent bacteria should give answers to very important medical problems, e.g., the mechanisms of the appearance of new diseases, therapeutic strategies, the invention of new drugs, and to fundamental problems related to the coevolution of bacteria, eukaryotes, and humans.

Investigation of *Legionella* bacteria will essentially contribute to the further development of the concept of an ecological niche for bacteria. The perfect preadaptation of these bacteria to anthropogenic environments and human immune cells indicates that habitat (or biotope) is not an actual element of an ecological niche and that the traditional division of microorganisms into soil, aquatic, phytopathogenic, and so on makes no biological sense. High-fidelity microbial forms, such as the neustonic organism *Nevskia,* seem to be the exception rather than the rule. The close association of *Legionella* with cyanobacteria and protozoa poses the problem of the elaboration of a concept of an ecological niche not for individual organisms, which naturally follows from the Gauze principle and the Hutchinson model, but for microbial consortia, communities, or, at least, community species. Interestingly, researchers in the field of the ecology of higher organisms seem to be arriving at similar conclusions [44].

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