Amoebae as Training Grounds for Intracellular Bacterial Pathogens

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MINIREVIEW

Amoebae as Training Grounds for Intracellular Bacterial Pathogens

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Free-living amoebae are important predators that control microbial communities. They are ubiquitous and have been isolated from various natural sources such as soil, freshwater, salt water, dust, and air. Although their abundance in soil is only limited, they have been implicated in the stimulation of phosphorus and nitrogen turnover and thus play an important role in soil ecosystems (84). Free-living amoebae are also frequently isolated from anthropogenic ecosystems such as tap water, air-conditioning units, and cooling towers, feeding on the microbial biofilm present in those systems. However, several bacteria have developed mechanisms to survive phagocytosis by free-living amoebae and are able to exploit them as hosts.

Transient association with amoebae have been reported for a number of different bacteria including *Legionella pneumophila*, many *Mycobacterium* spp., *Francisella tularensis*, and *Escherichia coli* O157, among others (3, 21, 77, 85). As most of these bacteria are pathogens of humans, amoebae have been suggested to represent their environmental reservoirs, acting as "Trojan horses" of the microbial world (10). To date, only the interaction of *L. pneumophila* with free-living amoebae has been studied in greater detail. *L. pneumophila* is an intracellular pathogen of humans causing Legionnaires' disease. *L. pneumophila* multiplies within its host cell (human macrophages and amoebae) but can also grow outside its host cell and can be cultured by routine methodologies (for reviews, see references 19 and 46). Therefore, it is considered as a facultative intracellular pathogen.

In addition to those facultative intracellular bacteria, various obligate endosymbionts have been observed in free-living amoebae. In this context, it should be noted that the term endosymbiont implies neither a mutualistic nor a parasitic relationship between the bacteria and their host cells. According to the broad concept of symbiosis suggested originally by Anton de Bary (30a) and consistent with the recent awareness of the variability of symbiotic interactions (81), we consider bacteria as endosymbionts when they manage to establish their replicative niche within eukaryotic host cells. Approximately 20% of isolates of ubiquitous *Acanthamoeba* spp. recovered from clinical and environmental sources are found to harbor

such bacterial endosymbionts (37). These endosymbionts have been shown to maintain a stable interaction with their hosts (37), but in contrast to *L. pneumophila*, they cannot be cultured outside their host cells, a primary reason why their identification and analysis have been hampered.

This review examines the impact of the interaction between bacteria and amoebae on the evolution of intracellular bacterial pathogens of humans (i.e., bacteria capable of causing disease) by focusing on recent findings on the stable association of free-living amoebae with their obligate endosymbionts and the transient interaction between *L. pneumophila* and amoebae as a model system.

DIVERSITY OF BACTERIAL SYMBIONTS OF FREE-LIVING AMOEBAE

Bacterial endosymbionts of amoebae were microscopically observed at least three decades ago (83). They have been shown to maintain a stable interaction with their hosts and are able to outlast encystations of the amoebae (37). However, identification and analysis of these endosymbionts have been severely hampered by the fact that they cannot be cultured outside their eukaryotic host cells. Only the advent of molecular biological techniques and the establishment of an rRNA-based phylogenetic system for the classification of prokaryotes enabled a more detailed characterization of these elusive bacteria (8). During the past few years, specific PCR-mediated amplification of bacterial rRNA genes, comparative sequence analysis, and fluorescence in situ hybridization led to the identification of five novel evolutionary lineages of bacterial endosymbionts of free-living amoebae (9, 16, 38, 39, 53–55, 57). These lineages have been found to be affiliated with (i) the *Alphaproteobacteria*, (ii) the *Betaproteobacteria*, (iii) the *Gamaproteobacteria*, (iv) the *Bacteroidetes*, and (v) the *Chlamydiales* (Fig. 1). Interestingly, co-occurrence of phylogenetically different endosymbionts in a single amoebal isolate has never been observed, and significant differences regarding the host range have been shown for the different endosymbionts (55). Apart from one exception (*Neochlamydia hartmannellae* [57]), spontaneous loss of endosymbionts from the original amoeba host or successful eradication of the symbionts from their hosts by antibiotics has never been reported, confirming the stable endosymbiosis. Consistent with this finding, evidence for coevolution between endosymbionts and hosts has been reported for one endosymbiont phylogenetic lineage (14). The phylum-level

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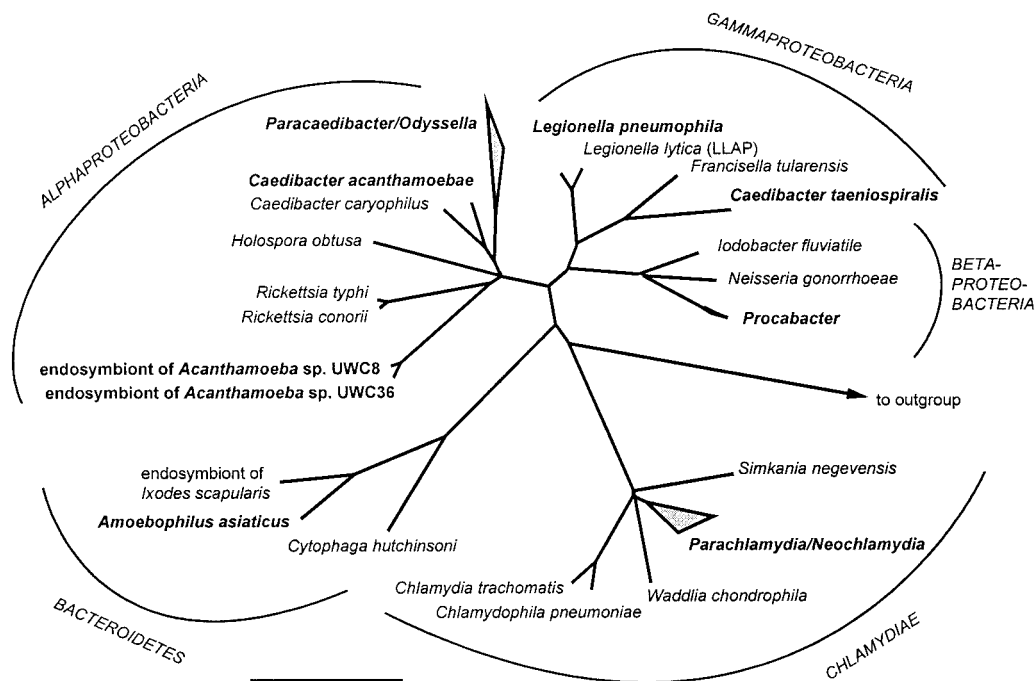
FIG. 1. 16S rRNA-based phylogenetic tree showing the affiliation of *L. pneumophila* and obligate endosymbionts of free-living protozoa. Bar, 10% estimated evolutionary distance.

diversity of the endosymbionts suggests that the capability to exploit free-living amoebae by endosymbionts has evolved several times, independently, in different lineages within the domain *Bacteria*. But there is also unity in variety. For many of these lineages, endosymbionts with very similar or almost identical 16S rRNA gene sequences were found in geographically dispersed regions and different ecosystems, suggesting a ubiquitous distribution of these organisms and their hosts.

A common feature of most of the recently identified endosymbionts of free-living amoebae is their close phylogenetic relationship to other obligate intracellular bacteria. For example, the *Bacteroidetes*-related symbiont "*Candidatus* Amoebophilus asiaticus" is monophyletic with two different insect symbionts (55). Furthermore, several of the closest relatives of the different amoebal endosymbionts are well-known intracellular pathogens of humans and animals (Fig. 1). For instance, the two alphaproteobacterial lineages (16, 38, 53) form a common branch with the rickettsiae comprising many important intracellular pathogens like *Rickettsia typhi*, the causative agent of typhus fever. In addition, the endosymbionts of amoebae belonging to the *Chlamydiales* are the closest relatives of previously known chlamydiae (9, 39, 57), which are a deeply branching evolutionary lineage exclusively containing important intracellular pathogens of animals and humans, including *Chlamydia trachomatis* and *Chlamydophila pneumoniae*. The emerging theme is that many obligate intracellular bacteria thriving in free-living protozoa share a common ancestor with intracellular bacteria pathogenic for humans. This theme is further supported by the identification of a gammaproteobacterial endoparasite of the ciliate *Paramecium tetraurelia* as being related to the *Francisella* group containing the highly infectious pathogen *Francisella tularensis*, the causative agent of

tularemia (14). *F. tularensis* has also been shown to survive and replicate within intact trophozoites of *Acanthamoeba castellanii* and has been found in excreted vesicles and cysts (1). Taken together, these findings implicate the interaction with amoebae during the evolution of intracellular bacterial pathogens. Through interaction with unicellular eukaryotes, the common ancestor of protozoan symbionts and human pathogens might have evolved mechanisms for intracellular survival in eukaryotic cells. Evidence for these "training grounds" for pathogens is supported by numerous studies on the interaction between legionellae and amoebae (10, 46), which are discussed below. Additional support comes from a more detailed analysis of the interaction between the recently identified chlamydia-related symbionts and endosymbionts and their amoeba hosts.

NEOCHLAMYDIA AND *PARACHLAMYDIA*: ENVIRONMENTAL COUNTERPARTS OF PATHOGENIC CHLAMYDIAE?

The majority of the identified endosymbionts of free-living amoebae belongs to the *Chlamydiales*, thrives within a host vacuole, and possesses the unique chlamydial biphasic developmental cycle. During this cycle, metabolically active intracellular reticulate bodies undergo binary fission and subsequently differentiate to metabolically inactive elementary bodies which are adapted to extracellular survival and infection of eukaryotic host cells (Fig. 2) (48). Consequently, the chlamydia-related endosymbionts of amoebae, also referred to as environmental chlamydiae, are classified within the new genera *Neochlamydia* or *Parachlamydia* of the order *Chlamydiales* (9, 32, 57). These studies and the finding of other chlamydia-like bacteria (*Simkania negevensis*, *Waddlia chondrophila*, and
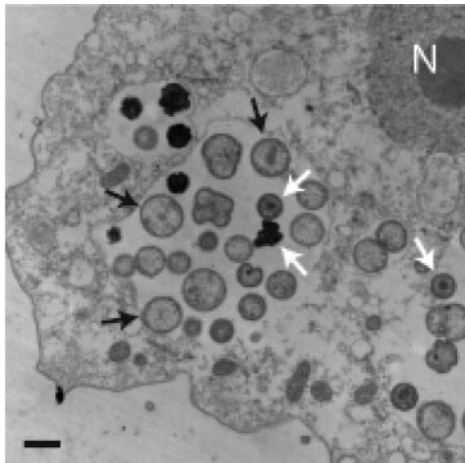
FIG. 2. Electron micrograph of environmental chlamydiae within an *Acanthamoeba* host cell. The two different stages of the developmental cycle are indicated by black (reticulate bodies) and white (elementary bodies) arrows. N, nucleus; bar, 1 μm.

Fritschea bemisiae) as contaminants of a tissue culture (63), within an aborted bovine fetus (49, 89), or as symbionts of insects (98) have dramatically changed our view of chlamydial diversity and ecology. Further evidence for a wide distribution of novel chlamydiae in the environment is provided by PCR-based culture-independent monitoring of environmental and clinical samples (28, 29, 56, 79). Today, several broad-range PCR assays and a comprehensive set of oligonucleotide probes for application in fluorescence in situ hybridization are available for the assessment of chlamydial diversity and occurrence of chlamydia-related bacteria in the environment (70, 82).

The presence of a developmental cycle similar to the developmental cycle of pathogenic chlamydiae suggests that amoebal symbionts and human pathogens share a common life style. Pathogenic chlamydiae possess a specialized transport protein which catalyzes the import of host cell ATP in exchange for ADP (100). Such transport proteins are very rare and have to date been identified only in chlamydiae, rickettsiae, mitochondria, and plastids (102). Although chlamydiae still contain the functional capacity to produce their own ATP, they use the ATP/ADP translocase in certain stages of the developmental cycle, thereby thriving as energy parasites within their host cells (61). By using a set of degenerate primers and a combination of arbitrary PCR approaches, a gene coding for an ATP/ADP transport protein in the *Parachlamydia*-related symbiont UWE25 has been identified (91). Detection of transcripts and biochemical characterization of the heterologously expressed protein clearly indicate that these environmental chlamydiae, similar to the pathogenic chlamydiae, are able to utilize the ATP pool of their amoeba host cells. Interestingly, phylogenetic analysis suggested that nonmitochondrial ATP/ADP transport proteins have evolved within the common ancestor of present-day chlamydiae and that they have been subsequently acquired by rickettsiae and plastids or cyanobacteria via lateral gene transfer (44, 91). Additional insights into the lifestyle of symbiotic chlamydiae were recently gained by analysis of the complete genome sequence of the *Parachlamydia*-related symbiont UWE25 of *Acanthamoeba* sp. These data reveal striking

similarities between the biology of symbiotic and pathogenic chlamydiae and show that the symbiont UWE25 is highly adapted to intracellular life, being dependent on a variety of host metabolites (52).

MEDICAL SIGNIFICANCE OF ENVIRONMENTAL CHLAMYDIAE

Classical chlamydiae are important pathogens of humans and animals. *Chlamydophila* (formerly classified as *Chlamydia*) *pneumoniae* is responsible for about 10% of community-acquired pneumonia (73) and has recently been associated with intrinsic asthma and cardiovascular disease (reviewed in references 23 and 68). *Chlamydia trachomatis* causes trachoma and is considered to be the world's leading cause of blindness, with about 6 million people affected as a result of this disease. In addition, *C. trachomatis* is the most frequent sexually transmitted bacterial pathogen, with an estimated 90 million new cases occurring each year worldwide (90). *Chlamydophila* (formerly classified as *Chlamydia*) *psittaci* can cause systemic fatal disease in birds and is also able infect mammals and humans, causing a wide variety of diseases (90). The diverse assembly of recently identified, ubiquitously distributed environmental isolates of chlamydiae represents a large environmental reservoir of bacteria that are closely related to human pathogens, with an as-yet-undefined relevance for public health. Thus, the expanding diversity of the *Chlamydiales* also deserves attention from a medical point of view. Interestingly, recent data suggest a possible clinical importance for environmental chlamydiae. Kahane and coworkers have reported high antibody prevalence against *Simkania negevensis* in infants suffering from bronchiolitis (62), and it has been suggested that the same organism may also be associated with community-acquired pneumonia in adults (69). In addition, specific antibodies against "Hall's coccus," an amoebal endosymbiont belonging to the genus *Parachlamydia*, have been detected in patients with pneumonia of unknown etiology (18, 74). The thoughts that novel chlamydia-related bacteria are capable of thriving within humans is also supported by the PCR-mediated retrieval of several of their 16S rRNA gene sequences from clinical specimens of respiratory disease patients (28, 29, 79). Although these findings do not yet prove that environmental chlamydiae do actually cause disease, they clearly indicate the need for a more thorough analysis of these newly discovered bacteria and their role in infectious disease.

THE ROLE OF FREE-LIVING AMOEBAE IN THE EVOLUTION OF CHLAMYDIAE

The natural reservoir of pathogenic chlamydiae is still unknown. In this context, the finding that (besides environmental chlamydiae) *Chlamydophila pneumoniae* is also able to multiply in free-living amoebae (31) indicates that protozoa might serve as an environmental reservoir for chlamydiae. Although the persistence of this interaction during encystment of the amoeba host has not been demonstrated, this observation shows that a single chlamydial organism can thrive in both protozoa and humans. Consistent with this finding, it has recently been reported that the amoeba symbiont *Parachlamydia acanthamoebae* is able to enter and multiply in human macro-
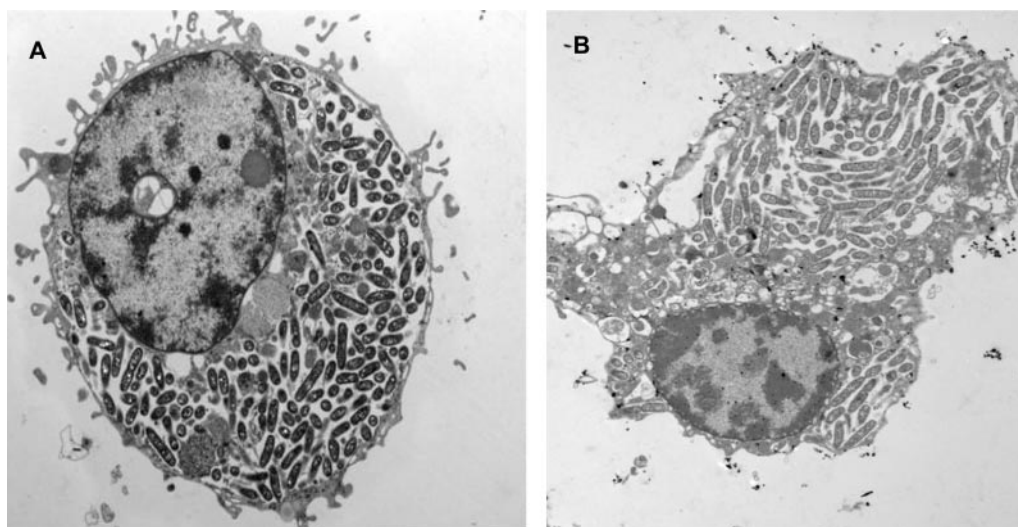
FIG. 3. Electron micrographs of U937 macrophages (A) and *A. polyphaga* (B) infected by *L. pneumophila* (strain AA100) at 24 h. The infection is very similar in both host cells. *Legionella* is cytoplasmic at 24 h in the two panels.

phages (43) and thus might have the potential to be a human pathogen. This is also supported by the identification of several genes, like, e.g., a complete type III secretion system, that have been associated with pathogenicity of chlamydiae in the genome of the *Parachlamydia*-related symbiont UWE25 (52). Moreover, phylogenetic analyses of those genes have indicated that they are already present in the last common ancestor of chlamydia-related symbionts of amoebae and pathogenic chlamydiae (52).

Taken together, there is cumulative evidence to suggest an important role of protozoa in the evolution of chlamydiae. Data from comparative genome analysis and the fact that both the environmental chlamydiae and the recognized pathogenic chlamydiae possess orthologous genes for ATP/ADP translocases, which are exclusively found in intracellular bacteria, can be used to infer that the last common ancestor of both lineages was also characterized by an intracellular lifestyle (52, 91). This chlamydial ancestor evolved around 700 million years ago, long before higher multicellular eukaryotes had evolved (52). Thus, a conceivable scenario for the contribution of protozoa to the evolution of chlamydiae is that a common ancestor of all chlamydiae has developed mechanisms for intracellular survival and multiplication in primitive unicellular eukaryotes. One might speculate that these ancient eukaryotes grazed on microorganisms and digested them in a phagocytic vacuole in a manner similar to that of today's free-living amoebae. The chlamydial ancestor must have evolved to alter its fate within the phagosomes by exporting proteins into the phagosome membrane and possibly also the host cytosol to evade degradation and to exploit the eukaryote as a host. Long-term coexistence of both organisms eventually led to the stable symbiotic interaction observed today between free-living amoebae and environmental chlamydiae. Sometime during this evolutionary process, a chlamydial clone evolved to infect as a parasite of multicellular eukaryotes, making a different ecological niche accessible, thus leading to diversification of chlamydiae. Considering the broad host range of the members of the *Chlamydiales*, such host changes might have occurred more

than once. Therefore, significant diversity exists within the formerly uniform order *Chlamydiales*. A better understanding of the evolution, ecology, and virulence of *Chlamydiales* will require a much more encompassing analysis of its recently discovered members. This knowledge not only is important for basic science but might also eventually assist in the selection of suitable antichlamydial drug targets and vaccine strategies.

LEGIONELLAE AS FACULTATIVE SYMBIONTS OF FREE-LIVING AMOEBAE

L. pneumophila is a gram-negative facultative intracellular bacillus responsible for Legionnaires' disease. It replicates within evolutionarily distant eukaryotic host cells such as protozoa and mammalian macrophages (Fig. 3). In aquatic environments, *L. pneumophila* is ubiquitous and grows within protozoan hosts. At least 13 species of amoebae and 2 species of ciliated protozoa support intracellular replication of *L. pneumophila* (33). Among the most predominant amoebae in water sources are hartmannellae and acanthamoebae, which have been also isolated from water sources associated with Legionnaires' disease outbreaks (33). Interaction between *L. pneumophila* and protozoa is considered to be central to the pathogenesis and ecology of *L. pneumophila* (46, 85). In humans, *L. pneumophila* reaches the lungs after inhalation of contaminated aerosol droplets (33, 36). The main sources of contaminated water droplets are hot water and air-conditioning systems, but the bacteria have been isolated from fountains, spas, pools, dental and hospital units, and other man-made water systems (36). No person-to-person transmission has ever been described. Once in the lungs, *L. pneumophila* is ingested by alveolar macrophages, which are thought to be the major site of bacterial replication. This ingestion results in an acute and severe pneumonia. Approximately one-half of the 46 species of *Legionella* have been associated with human disease. *L. pneumophila* is responsible for 90% of cases of Legionnaires' disease. However, all the *Legionella* species may be capable of intracellular growth and infliction of human disease under

appropriate conditions. Infections due to less common species of legionellae are not frequently diagnosed and reported and are less studied than *L. pneumophila* (34).

In addition to recognized *Legionella* species, a number of *Legionella*-related bacteria designated *Legionella*-like amoebal pathogens (LLAPs) (85, 86) have been described (6). Interestingly, many LLAPs have been associated with Legionnaires' disease (17, 74). In contrast to other *Legionella* species, however, most LLAPs cannot be cultured in vitro on artificial media but are isolated by coculture with protozoa (33). The recent developments in the use of PCR for bacterial identification in environmental samples will facilitate better identification of legionellae and LLAPs. Further cellular and molecular biology studies are needed to better understand the intracellular life of these endosymbionts.

THE ROLE OF FREE-LIVING AMOEBAE IN PERSISTENCE OF LEGIONELLAE IN THE ENVIRONMENT

It is most likely that the association of legionellae with protozoa is a major factor in the continuous presence of the bacteria in the environment. Many strategies have been used to eradicate legionellae from sources of infection in water and plumbing systems that have been associated with disease outbreaks. These strategies include chemical biocides such as chlorine, overheating of the water, and UV irradiation (20, 66, 76). Such interventions have been successful for short periods of time, after which the bacteria reappear in these sources (20, 103). Thus, eradication of *L. pneumophila* from the environmental sources of infection requires continuous treatment of the water with agents such as monochloramine or copper-silver ions in addition to maintenance of the water temperature above ~55°C (30, 51, 66, 67). Compared to in vitro-grown *L. pneumophila*, amoeba-grown bacteria have been shown to be highly resistant to chemical disinfectants and to treatment with biocides (11). Amoeba-grown *L. pneumophila* has been shown to manifest a dramatic increase in its resistance to harsh environmental conditions such as fluctuation in temperature, osmolarity, pH, and exposure to oxidizing agents (4). Protozoa have been shown to release vesicles containing *L. pneumophila* that are highly resistant to biocides (15). The ability of *L. pneumophila* to survive within amoebic cysts further contributes to the resistance of *L. pneumophila* to physical and biochemical agents used in bacterial eradication (11, 13). It is likely that eradication of the bacteria from the environment should start by preventing protozoan infection, an integral part of the infectious cycle of *L. pneumophila*. Extracellular *L. pneumophila* is more susceptible to environmental conditions and is not protected from biocides and disinfectants.

THE ROLE OF FREE-LIVING AMOEBAE IN PATHOGENESIS OF LEGIONELLAE

There are many lines of evidence to suggest that protozoa play major roles in the transmission of *L. pneumophila* as an infectious particle for Legionnaires' disease (87). First, many protozoan hosts that allow intracellular bacterial replication, the only documented means of bacterial amplification in the environment, have been identified (5, 33, 46). Second, in out-

breaks of Legionnaires' disease, amoebae and bacteria have been isolated from the same source of infection, and the isolated amoebae supported intracellular replication of the bacteria (35). Third, following intracellular replication within protozoa, *L. pneumophila* exhibits a dramatic increase in resistance to harsh conditions including high temperature, acidity, and high osmolarity, which may facilitate bacterial survival in the environment (4). Fourth, intracellular *L. pneumophila* within protozoa are more resistant to chemical disinfection and biocides compared to in vitro-grown bacteria (11–13). Fifth, protozoa have been shown to release vesicles of respirable size that contain numerous *L. pneumophila* isolates. The vesicles are resistant to freeze-thawing and sonication, and the bacteria within the vesicles are highly resistant to biocides (15). Sixth, following their release from the protozoan host, the bacteria exhibit a dramatic increase in their infectivity for mammalian cells in vitro (27). In addition, it has been demonstrated that intracellular bacteria within *Hartmanella vermiformis* are dramatically more infectious and are highly lethal in mice (22). Seventh, the number of bacteria isolated from the source of infection of Legionnaires' disease is usually very low or undetectable, and thus, enhanced infectivity of intracellular bacteria within protozoa may compensate for the low infectious dose (78). Eighth, viable but nonculturable *L. pneumophila* can be resuscitated by coculture with protozoa (95). This observation may suggest that the failure to isolate the bacteria from environmental sources of infection may be due to this "dormant" phase of the bacteria that cannot be recovered on artificial media. Ninth, there has been no documented case of bacterial transmission between individuals. The only source of transmission is environmental droplets generated from man-made devices such as shower heads, water fountains, whirlpools, and cooling towers of air-conditioning systems (33).

FIG. 4. Electron micrograph of U937 cells infected by *L. pneumophila* at 8 h postinfection. The LCP is intact and is surrounded by the endoplasmic reticulum.

These findings indicate a rather sophisticated host-parasite interaction and a tremendous adaptation of legionellae to parasitize protozoa. This host-parasite interaction is central to the pathogenesis and ecology of these bacteria.

ENTRY, INTRACELLULAR TRAFFICKING, SURVIVAL, AND REPLICATION OF *L. PNEUMOPHILA* WITHIN MACROPHAGES AND AMOEBAE

The attachment and entry mechanisms of *L. pneumophila* entry into its protozoan hosts and variations in these mechanisms have been reported for both *H. vermiformis* and *Acanthamoeba* spp. Attachment of *L. pneumophila* to *H. vermiformis* is mediated by adherence to a protozoan receptor characterized as a putative galactose/*N*-acetylgalactosamine (Gal/GalNAc) lectin (47, 71, 101). Host protein synthesis by *A. polyphaga* is not required for invasion by *L. pneumophila*, whereas it is required for invasion of *H. vermiformis* (47). Following this initial host-parasite interaction, uptake of *L. pneumophila* by *A. castellanii* occurs by coiling phagocytosis (3, 21). However, the uptake of *L. pneumophila* by *H. vermiformis* occurs mainly through cup-shaped invaginations (or zipper phagocytosis) on the surface of the amoeba, in addition to occasional coiling phagocytosis (3, 21). Human macrophages are able to phagocytose heat- or formalin-killed *L. pneumophila* by coiling phagocytosis (59), which indicates that coiling phagocytosis does not play a role in the intracellular fate of *L. pneumophila*. However, the infectivity of *A. castellanii* and macrophages by *L. pneumophila* has been shown to be similar (50). In addition, the adherence receptors of macrophages used by *L. pneumophila* do not seem to affect its intracellular survival profoundly as the bacterium multiplies within phagocytes after entry under different opsonizing or nonopsonizing conditions (26, 60, 72, 80)

Similar to its intracellular fate within macrophages, *L. pneumophila* is enclosed, after entry into amoebae, in a phagosome surrounded by host cell organelles such as mitochondria, vesicles, and a multilayer membrane derived from the rough endoplasmic reticulum (RER) of amoeba (3, 58, 97, 99) (Fig. 4). The bacterial phagosome is blocked from fusion to the lysosomes (21). The role of the RER in the intracellular infection is not known, but the RER is not required as a source of proteins for the bacteria (2). Interestingly, examination of the intracellular infection of macrophages, alveolar epithelial cells, and protozoa by another *Legionella* species, *Legionella micdadei*, showed that within all of these host cells, the bacteria were localized to RER-free phagosomes (42). Whether other *Legionella* species replicate within RER-free phagosomes is still to be determined. Following formation of this phagosome within protozoan cells, bacterial replication is initiated. The 4-h period prior to initiation of intracellular replication may be the time required for *L. pneumophila* to recruit host cell organelles that may be required for replication. Alternatively, the 4-h period may be a lag phase of metabolic and environmental adjustment of the bacteria to a new niche.

ROLE OF THE *dot/icm* GENES IN EVASION OF THE ENDOCYTIC PATHWAY

The main virulence system of *L. pneumophila* is the Dot/Icm type IV secretion system. Because the Dot/Icm secretion sys-

tem is ancestrally related to type IV secretion systems that mediate conjugal DNA transfer between bacteria (24), *L. pneumophila* may utilize this transporter to transfer macromolecules into the host cell to evade endocytic fusion (88). The Dot/Icm-mediated transfer is thought to occur through the insertion of a pore in the host cellular membrane through which the effector proteins are transported (64, 65). With few exceptions, the function of individual Dot/Icm proteins is unknown. However, the *dot/icm* genes were present in all tested *Legionella* species (7).

Most of the *dot/icm* genes required for intracellular growth within human cells are also required for intracellular growth in the protozoan host *A. castellanii* (92). In addition, enhanced phagocytosis of wild-type *L. pneumophila* by *A. castellanii* has also been demonstrated to be dependent on *dot/icm* genes (50). Although some loci have been shown to be essential only for the intracellular growth of *L. pneumophila* in macrophages (40), numerous loci have been identified as essential for survival and intracellular replication of *L. pneumophila* in *A. polyphaga* or *H. vermiformis* and in macrophages (25, 41, 96). The slime mold *Dictyostelium discoideum*, a unicellular soil organism, has been shown to support intracellular multiplication of *L. pneumophila* (45, 93, 94). In the amoebal form, the cells are highly motile and are very active in phagocytosis. This model is interesting because genetic tools are available for analysis of host-pathogen interactions (93, 94). The intracellular fate of *L. pneumophila* in infected *D. discoideum* cells is very similar to that in macrophages, including the recruitment of RER and evasion of lysosomal fusion (94) and the dependence of intracellular growth on *dot/icm* gene functions (94). The similarity between the infections of different protozoa by *L. pneumophila* supports the idea that the ability of *L. pneumophila* to parasitize macrophages and hence to cause human disease is a consequence of its prior adaptation to intracellular growth within protozoa.

EGRESS OF *L. PNEUMOPHILA* FROM AMOEBAE

A detailed ultrastructural analysis of late stages of intracellular replication has been performed to examine egress of *L. pneumophila* from both macrophages and amoebae by electron microscopy (75). The membrane of the *L. pneumophila*-containing phagosome (LCP) within both macrophages and *Acanthamoeba polyphaga* cells is intact up to 8 h postinfection (75). However, at 12 h, the majority of the LCPs are disrupted within both hosts, while the plasma membrane remains intact (75). At 18 and 24 h postinfection, cytoplasmic elements such as mitochondria, lysosomes, vesicles, and amorphous material are dispersed among the bacteria, and these bacteria are considered cytoplasmic (75). Thus, by 18 to 24 h postinfection, the majority of the remaining host cells harbor cytoplasmic bacteria, and this transient cytoplasmic presence of *L. pneumophila* precedes lysis of the plasma membrane (75). Interestingly, within both macrophages and amoebae, bacterial replication proceeds in the cytoplasm (75).

Therefore, rather than simultaneous lysis of both the phagosomal and the plasma membranes, the phagosomal membrane is disrupted first. These disruptions of the LCP may be the result of multifactorial events linked to apoptosis, the pore-

forming activity of the type IV secretion system, and mechanical pressure due to the increase of the phagosomal size (65).

CONCLUSION

The interaction between legionellae and free-living amoebae shows strong similarity with processes that occur during infection of mammalian cells by legionellae. In addition, free-living amoebae seem to play a crucial role for persistence and dispersal of legionellae in the environment, and there is convincing evidence that intracellular multiplication of *L. pneumophila* in free-living amoebae is a prerequisite for the infection of humans. It thus seems conceivable that these intracellular bacterial pathogens have developed and evolved mechanisms for survival in eukaryotic host cells during the interaction with free-living amoebae. This hypothesis of amoebae acting as a (evolutionary) training ground for intracellular bacterial pathogens is further supported by the analysis of chlamydia-related symbionts of free-living amoebae, which still use strategies for host cell interaction that were developed 700 million years ago in interplay with early unicellular eukaryotes (and in the absence of higher, multicellular organisms). Clearly, although other protozoa might have acted in the same manner, the interaction between intracellular bacteria and prokaryotes other than amoebae has rarely been addressed. Future studies should examine obligate and facultative symbionts of other protozoa in order to elucidate the role of unicellular eukaryotes in the transition of free-living bacteria to intracellular bacteria that eventually become able to infect animals and humans. A better understanding of such processes will help to develop novel strategies and targets for vaccines and antibiotics against intracellular bacterial pathogens.

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