Free-living freshwater amoebae differ in their susceptibility to the pathogenic bacterium *Legionella pneumophila*

Rafik Dey¹,²,³, Jacques Bodennec¹,³, Mouh Oulhadj Mameri¹,² & Pierre Pernin¹,²

¹Université de Lyon, Lyon, France; ²Laboratoire de Biologie Cellulaire, ISPB, Université Lyon, CNRS, UMR5240, Lyon, France; and ³Laboratoire de Physiologie Intégrative Cellulaire et Moléculaire, Université Lyon 1, CNRS, UMR5123, Villeurbanne, France

**Abstract**

*Legionella pneumophila* is known as a facultative intracellular parasite of free-living soil and freshwater amoebae, of which several species have been shown to support the growth of the pathogenic bacteria. We report for the first time the behaviour of two strains (c2c and Z503) of the amoeba *Willaertia magna* towards different strains of *L. pneumophila* serogroup 1 and compared it with *Acanthamoeba castellanii* and *Hartmannella vermiformis*, known to be *L. pneumophila* permissive. In contrast to the results seen with other amoebae, *W. magna* c2c inhibited the growth of one strain of *Legionella* (*L. pneumophila*, Paris), but not of others belonging to the same serogroup (*L. pneumophila*, Philadelphia and *L. pneumo- phila*, Lens). Also, the different *L. pneumophila* inhibited cell growth and induced cell death in *A. castellanii*, *H. vermiformis* and *W. magna* Z503 within 3–4 days while *W. magna* c2c strain remained unaffected even up to 7 days. Electron microscopy demonstrated that the formation of numerous replicative phagosomes observed within *Acanthamoeba* and *Hartmannella* is rarely seen in *W. magna* c2c cocultured with *L. pneumophila*. Moreover, the morphological differences were observed between *L. pneumophila* cultured either with *Willaertia* or other amoebae. These observations show that amoebae are not all equally permissive to *L. pneumophila* and highlight *W. magna* c2c as particularly resistant towards some strains of this bacterium.

**Introduction**

The gram-negative bacterium *Legionella pneumophila*, the causative agent of Legionnaire’s disease, can grow facultatively within macrophages, monocytes or epithelial cells (Horwitz, 1983). *Legionella pneumophila* is found ubiquitously in freshwater environments, where it may replicate within protozoa such as ciliates and free-living amoebae (Molmeret et al., 2004, 2005). Within phagocytic cells, the genes of the *L. pneumophila dot/icm* system are essential for evading endocytic maturation and inhibition of phagosome–lysosome fusion in the cytoplasm of their diverse host cells (Roy, 2002). Intracellular multiplication of *Legionella* in free-living amoebae, discovered by Rowbotham (1980), has since been confirmed by numerous other studies. For instance, coculture experiments in liquid media have shown that amoebae exert an important growth-promoting effect on *L. pneumophila*. During outbreaks of Legionnaire’s disease, the water bodies concerned are usually found to contain significant numbers of free-living amoebae; furthermore, high numbers of *Legionella* species are found when concentrations of amoebae are high (Barbaree et al., 1986). Moreover, the isolation of *L. pneumophila* from water is more successful if amoebae are added (Sanden et al., 1992). Also, the presence of amoebae can trigger the revival of *Legionella* strains that became noncultivable (viable but noncultivable state) following a prolonged period in poor media such as distilled water (Steinert et al., 1997).

Although Fields reported that 13 species of amoebae and two species of ciliated protozoa can support intracellular replication of *L. pneumophila* (Fields, 1996), most *in vitro* experiments have been performed with only two genera of amoebae – *Acanthamoeba* and *Hartmannella*. So far, there have been few attempts to test whether other genera of amoebae can act as hosts for *L. pneumophila* strains. In order to compare the ability of different common freshwater...
amoebae (Acanthamoeba castellanii, Hartmannella vermiformis and two strains of Willaertia magna) to support L. pneumophila growth, we performed coculture experiments using them with different L. pneumophila strains belonging to the same serogroup 1.

Materials and methods

Strains

The different strains of L. pneumophila serogroup 1 [Lens CIP 108 286, Paris CIP 107 629T and Philadelphia (ATCC 33152)] were cultured at 37 °C for 3–4 days on buffered charcoal yeast extract (BCYE) agar before coculture experiments (Gao & Abu Kwaik, 2000).

The amoebae used in this study were A. castellanii (By 02.2.4) and H. vermiformis (Ax.5.246b), two species known to support L. pneumophila and two strains of Willaertia genus, hitherto untested: W. magna, Z 503 (ATCC 50035) and c2c Maky (ATCC PTA-7824). All amoebae were grown at 30 °C on a lawn of Escherichia coli on non-nutrient agar and were established in axenic culture at 37 °C in serum casein glucose yeast extract medium (SCGYEM) (De Jongkheere, 1977).

Coculture of L. pneumophila with amoebae

Tubes (FALCON® 3033) containing 3 mL of SCGYEM medium were seeded with 5.5 × 10⁴ trophozoites mL⁻¹ of the different amoebae maintained in the exponential growth phase by subculture every 3–4 days. On day 0, the different strains of L. pneumophila, grown on BCYE medium, were suspended in sterile distilled water at 10⁹ mL⁻¹ (1 OD unit at 550 nm), and inoculated into the amoebic cultures at a multiplicity of infection (MOI) of 50. Low-speed centrifugation (5 min at 1000 g) was used to initiate physical interaction between bacteria and amoebae. After 15 min, the pellet was resuspended and the tubes were incubated at 37 °C in a slanting position in order to increase the medium to air exchange surface. The cocultures were analyzed at 24 h intervals from days 0 to 4 with the following objectives:

1. First, the effect of the strains of L. pneumophila on the growth of different amoebae in cocultures. At each time point (see figures), tubes were placed on ice for 6 min to detach cells from the walls (this method was preferred to the use of a cell scraper, because it did not induce mechanical stress) and the cultured microorganisms were resuspended by vortexing. The number of amoebae was determined by counting resuspended cells per unit volume with a haematocytometer. Control values of amoebae grown without bacteria were obtained in the same manner.

2. Simultaneously, the growth of L. pneumophila in coculture with the different amoebic genera was determined from the same tubes by the number of total L. pneumophila (intra- and extracellular), expressed in CFU mL⁻¹, after serial 10-fold dilutions of the coculture medium with sterile H₂O that were spread in triplicate on BCYE plates and incubated at 37 °C for at least 6 days.

Data were analysed by means of Student’s t-test and are means ± SEM of up to eight independent experiments (see the details in the figure legends).

Cytotoxicity of L. pneumophila

Firstly, the cytotoxicity of L. pneumophila strains towards this panel of amoebae was studied qualitatively by phase-contrast microscopy. The effects on amoebal monolayer formation in 24-well plates containing 5 × 10⁴ amoebae per well infected with the bacterium at an MOI of 50 were recorded after 3 and 7 days.

Bacterial cytotoxicity for Acanthamoeba and Willaertia species was also checked by a trypan blue exclusion test. At 72 h of infection (MOI 50), following resuspension by agitation, amoebae were pelleted by low-speed centrifugation and resuspended in 200 μL of a trypan blue–SCGYEM medium (4:1 v/v) mix. Cell death was determined as the percentage of trypan blue-positive cells. Because of the high cytotoxicity of L. pneumophila 72 h after infection, this assay could not be performed with H. vermiformis. Trypan blue exclusion tests were also performed on control cultures of amoebae without bacteria.

Transmission electron microscopy

Axenic cultures of H. vermiformis, A. castellanii and W. magna c2c Maky were infected for 36 h with L. pneumophila Paris at an MOI of 50 and 100. After decantation of the medium, amoebae were fixed (at room temperature) with 2% glutaraldehyde, successively in serum-free SCGYEM medium (20 min) and then in 0.1 M sodium cacodylate buffer (pH 7.4; 30 min). The cells were washed and postfixed for 40 min with 1% OsO₄ in 0.15 M sodium cacodylate buffer. After dehydration in ethanol solutions (50°, 70°, 90°, 95° and 2 × 100°), amoebae were embedded in EPON resin. Ultrathin sections were stained with uranyl acetate, followed by lead citrate, and examined using a JEOL 1200 CX electron microscope at 80 kV. A minimum of > 100 cells was observed for each of the three species, and the percentage of amoebae displaying a replicative phagosome (i.e. vacuoles filled with several bacteria) was determined. Electron microscopy was also performed on the two strains of W. magna infected with L. pneumophila Lens and Philadelphia in order to compare their morphology with W. magna c2c Maky infected with L. pneumophila Paris.
Results

We first screened the impact on the growth of the different free-living amoebae that the strain *L. pneumophila* Paris had when cocultured at an MOI of 50 for up to 4 days (Fig. 1a). Cocultures with *L. pneumophila* resulted in a strong reduction in the growth of *H. vermiformis* (c. 82% and c. 88% decrease at day 3 and day 4, respectively) and of *A. castellanii* (c. 93% and c. 95% at day 3 and day 4, respectively) when compared with controls. On the other hand, the growth of both strains of *W. magna* was unaffected by *L. pneumophila* Paris because no significant difference was observed in the number of amoebae grown with or without this bacterium.

With regard to the cytotoxicity of *L. pneumophila* for different amoebae, phase-contrast microscopy revealed that a disruption of monolayer formation was induced in *A. castellanii* and *H. vermiformis* after 72 h of bacterial infection, while *W. magna* c2c was unaffected (Fig. 1b)

![Graph](https://example.com/graph.png)

**Fig. 1.** Effect of *Legionella pneumophila* on the growth of different amoebae in coculture. (a) Effect of the bacterial infection on the growth of amoebae. The different amoebae were cultured at 37 °C for up to 4 days (d3 and d4 represent, respectively, day 3 and day 4 in culture) either with (grey bars) or without (black bars) *L. pneumophila* Paris at an MOI of 50. White bars represent the number of amoebae on day 0 of experiments. The data are expressed as the number of amoebae mL⁻¹ of medium and are the mean ± SEM of four to eight independent experiments. Statistical differences (Student’s t-test) between the growth of amoebae cultured either with or without bacteria are indicated (*P < 0.01; **P < 0.001). (b and c) Effect of *L. pneumophila* on monolayer formation by amoebae. Representative phase-contrast images of the different amoebae cultured in 24-well plates either with or without the strains of *L. pneumophila* at an MOI of 50 for 3 days (b) or 7 days (c). Note the destruction of monolayers of *Hartmannella* and *Acanthamoeba* cultured in the presence of the bacteria when compared with controls and also the relative resistance of *Willaertia magna* (strain c2c). Also, note the destruction of monolayers of *W. magna* Z503 cultured in the presence of the bacteria for 7 days when compared with controls and the c2c strain.
irrespective of the strain of \textit{L. pneumophila} used in these experiments. Moreover, 28.4 ± 3.6%, 11.01 ± 1.1% and 10.9 ± 2.6% of the few remaining \textit{A. castellanii} on day 3 of coculture with, respectively, \textit{L. pneumophila} Paris, Lens and Philadelphia were trypan blue positive; for \textit{W. magna} c2c, it was only 3.8 ± 0.4%, 2.9 ± 0.9% and 3.2 ± 0.7%, respectively.

In control cultures of amoebae, devoid of bacteria, the percentage of trypan blue-positive cells was < 1%. Similar to what was observed with \textit{W. magna} c2c, the monolayer formation of Z503 was not affected at day three by \textit{L. pneumophila} Paris. With \textit{L. pneumophila} Lens and Philadelphia, Z503 monolayers started to shrink in the presence of numerous floating rounded cells. The percentage of Z503 trypan blue-positive cells was approximately three times higher than what was observed with the c2c strain. These observations indicated that the \textit{W. magna} c2c strain is less sensitive to bacteria than the Z503 strain. This difference between the two \textit{W. magna} is even more obvious when amoebae are cultured up to 7 days with the \textit{L. pneumophila} strains. Effectively, Fig. 1c demonstrates a disruption of the \textit{W. magna} Z503 monolayer in contrast to the c2c, which remains unaffected irrespective of the \textit{L. pneumophila} strain used.

From these preliminary results, we focused our attention on the combination of \textit{L. pneumophila} Paris and \textit{W. magna}. We compared the growth kinetics of this bacterium in coculture with \textit{A. castellanii} and \textit{H. vermiformis}. As expected, the bacterium grew in \textit{H. vermiformis} and \textit{A. castellanii} with a respective increase of 1.3 and 1.5 log in CFU mL \(^{-1}\) after 3 days of infection (Fig. 2a). Unlike these two permissive amoebae, bacterial growth, being reduced markedly during the same period, was not favoured in coculture with \textit{W. magna} (c2c Maky and Z503). A significant (c. 2.3−2.6 log) difference in bacterial yield was recorded at days 2−3 postinfection between the \textit{Hartmannella–Acanthamoeba} species and the \textit{W. magna} c2c (Fig. 2a). In contrast, when we examined the ability of the \textit{L. pneumophila} strains Lens and Philadelphia to grow on days 3 and 4 in the presence of \textit{H. vermiformis} and \textit{W. magna}, the bacteria grew up to 4 days in all cases (Fig. 2b), although to different extents [a difference of c. 2 log CFU mL \(^{-1}\) was observed after 3 days of \textit{L. pneumophila} Philadelphia cocultured with \textit{W. magna} c2c and other amoebae (This difference in bacterial growth when cocultured with \textit{W. magna} c2c or other amoebae is also reinforced by visual observations of the 24-well plates that were used to study the effect of \textit{L. pneumophila} strains on monolayer formation (Fig. 1b and c). In the presence of \textit{L. pneumophila} Lens and Philadelphia, an intense bacterial proliferation was observed with \textit{W. magna} Z503 (the culture medium was ‘cloudy’ due to the high bacterial density). This phenomenon was never observed when the different bacteria were cocultured with \textit{W. magna} c2c.].

We next determined whether the defective growth of \textit{L. pneumophila} Paris in the presence of \textit{W. magna} c2c could be due to a fault in intracellular replication. Ultrastructural examination showed that the number of \textit{Acanthamoeba} and \textit{Hartmannella} displaying replicative phagosomes at 36 h postinfection was very high, i.e. 36−51% according to the MOI (Table 1). In contrast, replicative phagosomes were not detected in \textit{Willaertia} c2c amoebae infected at an MOI of 50. When cocultures of \textit{W. magna} c2c were infected at a higher
ratio (MOI of 100), few amoebae displayed a replicative phagosome (1.5% i.e. 2/133 amoebae) a very low level compared with Acanthamoeba and Hartmannella (Table 1), although some rare bacteria were sometimes observed as demonstrated in Fig. 3b. We also found that W. magna Z503 strain displayed more replicative phagosomes than the c2c strain: 7% of W. magna Z503 cells displayed a replicative phagosome when cocultured with L. pneumophila Lens and 13% with L. pneumophila Philadelphia. All the stages of infection could be observed ultrastructurally in A. castellanii and H. vermiformis. Shortly after infection, L. pneumophila Paris appeared in these two species as isolated bacteria in a vacuole-like structure (Fig. 3a) surrounded by a rough endoplasmic reticulum (RER) as described previously (Abu Kwaik, 1996). More pronounced invasion led to the formation of distended replicative phagosomes containing numerous bacteria and finally to necrosis of these heavily infected amoebae. Nevertheless, even in such amoebae, the contractile vacuole was still evident (Fig. 3d).

In contrast, out of the few W. magna c2c displaying intracellular bacteria (only 1.5% at an MOI of 100), the replicative phagosome was different from those observed in Acanthamoeba or Hartmannella in lacking a clearly delimiting membrane. Additionally, the bacteria inside contained numerous vacuoles usually described as polyhydroxybutyrate granules (Fig. 3e and h). In Acanthamoeba and Hartmannella, the bacteria displayed the characteristic morphology of the typical gram-negative cell envelope composed of a wavy outer membrane around a clear periplasmic space (Fig. 3g), while the L. pneumophila observed within the two infected Willaertia c2c appeared as short stubby rods with a dense cytoplasm delimited by a thick laminar outer layer (Fig. 3h). Figure 3c shows a Willaertia Z503 with a replicative phagosome filled with L. pneumophila Lens. The bacteria are contained within a vacuole-like structure with a clear delimiting membrane and display an external periplasm (see Fig. 3c and i) in contrast to what was observed with the Willaertia c2c strain. In W. magna Z503, bacterial morphology resembled one observed within the c2c strain, with vacuoles usually described as polyhydroxybutyrate granules (compare Fig. 3f and i with e and h). Similar observations were made when W. magna Z503 were infected with L. pneumophila Philadelphia (not shown).

### Discussion

Numerous studies have reported the multiplication of L. pneumophila within various protozoa such as amoeba, ciliates (Fields, 1996) or cellular slime mould Dictyostelium discoideum (Hagele et al., 2000; Solomon et al., 2000), which has led to free-living amoeba being considered as natural reservoirs for this pathogen. But out of the five amoebic genera reported by Fields (1996) as able to support the intracellular growth of L. pneumophila, only two (Acanthamoeba and Hartmannella) have been tested repeatedly in coculture with L. pneumophila (Abu Kwaik et al., 1998; Molmeret et al., 2004, 2005). As far as we are aware, this study is the first to consider the interactions between W. magna amoeba and L. pneumophila systematically.

The main and unexpected finding is that, among the different amoeba tested, W. magna is an inefficient vector for the strain L. pneumophila Paris when compared with A. castellanii and H. vermiformis. Indeed, these last two permissive amoeba displayed a 2.3 log greater ability to support bacterial growth than W. magna tested under exactly the same conditions. Furthermore, the different L. pneumophila strains did not display any obvious cytotoxicity towards the W. magna strain c2c. Taken together, these results demonstrate that W. magna c2c displays a particular resistance against L. pneumophila Paris growth and the different L. pneumophila strains-induced cytotoxicity. Interestingly, the results also demonstrate that W. magna c2c strain is particularly resistant towards L. pneumophila strains-induced cytotoxicity when compared with Z503 belonging to the same genus.

The particular resistance of W. magna c2c may be accounted for by factors relating to the bacterium or the amoeba. Until now, in all studies that reported a defect of L. pneumophila growth in coculture with macrophages and/ or amoeba, the underlying mechanism results from the use of two main categories of bacterial mutant strains. First, several mutants of the dot/icm system are defective in evasion of phagolysosomal fusion and, consequently, have lost their capacity of intracellular replication (Swanson & Isberg, 1996; Gao et al., 1997; Coers et al., 2000). In contrast, the rib mutants are defective in their cytotoxic effect and are unable to escape from the phagocytic cell despite an active intracellular replication (Alli et al., 2000). Willaeria c2c may reduce L. pneumophila Paris growth in coculture either by inhibiting intracellular replication and/or by blocking bacterial-induced lysis. Another explanation for the observed defective replication of L. pneumophila Paris in W. magna

### Table 1. Occurrence of replicative phagosomes within amoebae

<table>
<thead>
<tr>
<th>Acanthamoeba castellanii</th>
<th>Hartmannella vermiformis</th>
<th>Willaertia magna c2c Maky</th>
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<tbody>
<tr>
<td>MOI = 50</td>
<td>36%</td>
<td>42%</td>
</tr>
<tr>
<td>MOI = 100</td>
<td>51%</td>
<td>ND</td>
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<tr>
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<td></td>
<td>1.5%</td>
</tr>
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Amoebae were cocultured for 36 h with Legionella pneumophila Paris at a MOI of 50 or 100, fixed and processed for electron microscopy. Results are expressed as the percentage of cells displaying a replicative phagosome sampled from > 100 cells. In Hartmannella vermiformis phagosome occurrence at a MOI of 100 could not be determined due to the presence of too many bacterial-induced lysed cells (ND, not determined).
c2c amoebae is that the bacterial strain is specifically lacking in factors that are needed for its growth in this particular host. Differences in the membrane composition of amoebae may also result in a lower efficiency of bacterial internalization within *W. magna* c2c. Although none of these hypotheses may be presently excluded, our ultrastructural observations, especially the scarcity of replicative phagosomes in *W. magna* c2c compared with the situation with *Acanthamoeba* and *Hartmannella*, favour defective replication of *L. pneumophila* Paris in *W. magna*. To test whether the fusion of phagosome with lysosomes is not inhibited by *L. pneumophila* within *Willaertia* c2c amoebae, colocalization studies of *L. pneumophila*, with late endosomal and lysosomal markers such as acid phosphatase or lysosome-associated-membrane-protein (LAMP-1) glycoprotein, could be performed. Moreover, the expression of replicative phagosomes occurring only for the highest MOI (i.e. 100), in very few *W. magna* c2c (2/133), suggests that the cell defence mechanisms of *Willaertia* amoebae may be partially overwhelmed at this bacterial concentration. Interestingly, this replicative phagosome appeared not as clearly delimited as those in other permissive amoebic species (*Hartmannella*)
and Acanthamoeba), and the bacteria observed within resemble the 'cyst-like' or mature intracellular form described in HELA cells by Garduno et al. (2002), who suggested that it could be metabolically dormant L. pneumophila. This bacterial morphology was also observed within the W. magna Z503 strain when infected with L. pneumophila. It is thus possible that this particular bacterial morphology may be an adaptation phenotype to several cellular environments such as amoebas of the Willaertia genus. All these ultrastructural observations suggest that the replication of L. pneumophila Paris cannot occur properly within W. magna and may explain why bacterial growth is inhibited in the presence of this amoeba.

Overall, our results demonstrate the marked resistance of one strain of W. magna towards L. pneumophila. They are in agreement with a recent publication (Declerck et al., 2005) showing that the amoeba Naegleria lovanisiensis is clearly less permissive than A. castellani to invasion and replication of L. pneumophila. This observation seems perfectly coherent with our own results because Naegleria and Willaertia are related genera of the family Vahlkampfiidae (De Jonckheere et al., 1984, 1997; Page, 1987). Indeed, these genera share several characteristics (eruptive pseudopods, division by promitosis) that clearly differentiate them from Hartmannella and Acanthamoeba.

In conclusion, our observations show that growth of L. pneumophila depends on both the bacterial strain itself and on the species of host amoebae. More precisely, our results demonstrate that all free-living amoebae are not equally permissive to L. pneumophila, because at least one strain of the bacterium (L. pneumophila Paris) was inhibited when cocultured with W. magna c2c. Consequently, the distribution and the density of the pathogenic microorganism in the environment may be subject to variations according to the composition and the evolution of the amoebic population in the biotope, with potential implications on the occurrence of outbreaks of Legionnaire's disease. Our results also suggest that W. magna c2c may be a useful model to study the mechanisms of host resistance to L. pneumophila.

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References


