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**The free-living amoeba *Willertia magna*, is particularly resistant to infection by the pathogenic bacteria *Legionella pneumophila***

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Running title:

*L. pneumophila* replication in different free-living amoebas

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## ABSTRACT

*Legionella pneumophila*, the causative agent of Legionnaire's disease, is well characterized as a bacteria surviving and developing, almost exclusively, as intracellular parasite within freshwater protozoa. Several species of protozoa and ciliae have been shown to support the growth of the pathogenic bacteria. In the present study, we report for the first time the behaviour of the protozoan *Willaertia magna* towards *L. pneumophila* and compared it with *Acanthamoeba castellanii* and *Hartmannella vermiformis*, two known *L. pneumophila* permissive protozoa. The results show that *Willaertia* amoebas displayed the ability to internalize the pathogenic bacteria but at a different extent when compared to other species. Surprisingly, and at the opposite of the other two amoebic species, coculture experiments showed that *Willaertia magna* inhibited the growth and the development of *L. pneumophila* and that it was resistant to bacterial induced cytotoxicity. Electron microscopy demonstrated that the formation of replicative phagosomes observed within *Acanthamoeba sp.* and *Hartmannella sp.* is deficient in *Willaertia sp.* These observations describing for the first time the occurrence of a protozoan species that display resistance towards the bacteria *L. pneumophila*, shed new light on a cellular model that may provide useful information on the mechanisms underlying bacterial infection and show that among free-living amoebas, the different species are not equally permissive to *L. pneumophila*.

Key-words: *Willaertia magna*, *Legionella pneumophila*, free-living amoebas, protozoa-bacterial interactions.

## INTRODUCTION

The gram negative bacteria *Legionella pneumophila*, the causative agent of the Legionnaire's disease, is characterized by a facultative intracellular replication inside macrophages, monocytes or epithelial cells (Horwitz, 1983). In the environment, *L. pneumophila* presents a ubiquitous aquatic repartition and replicates inside protozoa as free-living amoebas (Molmeret *et al.*, 2004; Molmeret *et al.*, 2005). In all these phagocytic cells, the genes of the *dot/icm* system of *L. pneumophila* are essential to warrant a successful infection with evasion of the endocytic maturation and inhibition of the phagosome lysosome-fusion inside the diverse cellular hosts (Roy, 2002). Rowbotham was the first to demonstrate an intracellular replication of the bacteria in free-living amoebae (Rowbotham, 1980). Since this previous work, numerous studies have been performed that strengthen the existence of a close relationship between *Legionella* and free-living amoebas. Hence, co-culture experiments in liquid media have shown an important growth promoting effect of amoebas towards *L. pneumophila*. During outbreaks of Legionnaire's disease, water sources are usually found to contain significant amount of free-living amoebas, and conversely, high numbers of *Legionella* species are found when amoebic concentrations are high (Barbaree *et al.*, 1986; Fields *et al.*, 1989; Breiman *et al.*, 1990). In addition, the isolation of *L. pneumophila* from water is strongly improved by addition of amoebas (Sanden *et al.*, 1992). Moreover, Steinert (Steinert *et al.*, 1997) showed that the addition of amoebas can trigger the revival of viable *Legionella* strains that became non cultivable (VBNC state) due to a prolonged stage in poor medium such as distilled water.

Although Fields reported 13 species of amoebas and 2 species of ciliated protozoa that display the ability to sustain *L. pneumophila* replication (Fields, 1996), most of *in vitro* experiments have been performed using two amoebic genera: *Acanthamoeba* (Anand *et al.*, 1983; Holden *et al.*, 1984; Vandenesch *et al.*, 1990; Moffat & Tompkins, 1992; Bozue &

Johnson, 1996; Gao *et al.*, 1997; Neumeister *et al.*, 1997; Harb *et al.*, 1998) and *Hartmannella* (King *et al.*, 1991; Abu Kwaik *et al.*, 1994; Abu Kwaik, 1996.; Abu Kwaik *et al.*, 1998a; Harb *et al.*, 1998) and more recently *Dictyostelium*, which is not really a free-living amoeba (Hagele *et al.*, 2000; Solomon *et al.*, 2000). But, few studies have been carried out with other amoebic genera. For these reasons, cocultures of *L. pneumophila* with *Willaertia magna*, an amoebic species never tested until now, were performed and compared with two bacteria permissive amoebas : *A. castellanii* and *H. vermiformis*.

## MATERIAL AND METHODS

### *Strains*

*L. pneumophila* (Paris serogroup 1 CIP 107 629T), was cultured at 37°C for 4 to 5 days on buffered charcoal yeast extract (BCYE) agar before coculture experiments in order to be in post-exponential phase.

*Willaertia magna* (deposit of this strain was performed at ATCC under the number PTA-7824), *Hartmannella vermiformis* (Ax.5.2e4b) and *Acanthamoeba castellanii* (By 02.2.4) amoebas are environmental strains isolated from a French thermal spa by filtration of water, and grown at 30°C on non nutrient agar (NNA) overlaid with a thin film of *Escherichia coli*. These three strains were established in axenic culture at 37°C in SCGYEM liquid medium containing 10% of foetal calf serum.

### *Coculture of L. pneumophila with the different amoebas*

Tubes (FALCON<sup>®</sup> 3033) containing 3 ml of SCGYEM medium were seeded with  $5.5 \times 10^4$  trophozoites/ml of the different amoebic strains maintained in exponential growth phase by subcultures every 3-4 days. *L. pneumophila*, grown on BCYE medium, was

suspended in sterile distilled water at  $10^9$ /ml (= 1 OD at 550 nm) and, after appropriate dilutions, was inoculated at a multiplicity of infection (MOI) of 50, i.e around  $2.8 \times 10^6$  bacteria/ml. The tubes were immediately centrifuged at low speed (5 min at 760 g) to initiate interactions between bacteria and amoebas. After 10 min, the pellet was resuspended and the tubes incubated in a slanting position at 37°C.

The cocultures were analyzed for up to 5 days (day 0 to day 4) as follows. At the indicated time, the tubes were placed on ice for 6 min to reduce cell adherence and, after a vigorous agitation by vortex, amoebas were numbered using a hemacytometer. Controls of amoebic growth were also performed in the same conditions in absence of bacteria. The number of total *L. pneumophila* (intra- and extracellular), expressed in CFU/ml, was determined after serial 10 fold dilutions of the coculture medium with sterile H<sub>2</sub>O that were spread in triplicate on BCYE plates and were further incubated at 37°C for at least 6 days. Statistical differences were addressed using Student t-test.

### ***Cytotoxicity of L. pneumophila***

Firstly, the cytotoxicity of *L. pneumophila* towards the different strains of amoebas was studied qualitatively by phase contrast microscopy in 24 well plates containing  $5 \times 10^4$  amoebas/well infected at an MOI of 50 and amoebic monolayer formation was observed after 72 hr infection with *L. pneumophila*.

Bacterial cytotoxicity was also determined on *Acanthamoeba* and *Willaertia* species by trypan blue exclusion test as follows: at 48 and 72 hr of infection (MOI 50), after agitation of the coculture medium, the amoebic suspension was pelleted by low centrifugation and suspended in 200 µl of trypan blue-SCGYEM medium (4:1 v/v) mix. The extent of cell death was determined as the percentage of trypan blue positive cells. This assay could not be performed with *H. vermiformis* due to the punctiform, shrivelled

appearance of the remaining cells resulting from the high cytotoxicity of *L. pneumophila* 72 hours after infection. Trypan blue exclusion tests were also performed on control amoebic cultures grown in the same conditions but without *L. pneumophila*. Statistical differences were addressed using Student t-test.

### ***Transmission electron microscopy***

Axenic cultures of *H. vermiformis*, *A. castellanii* and *W. magna* were infected for 36 hr with *L. pneumophila* at a MOI of 50 and 100. After decantation of the medium, the amoebas were fixed for 20 min at room temperature using 2% glutaraldehyde in serum free SCGYEM medium. After removal of the medium, the fixation of cells was further completed for 30 min with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After scrapping, amoebas were transferred in 1.5 ml Eppendorf microtubes, washed three times (15 min each) by repeated cycles of suspension-centrifugation, and post-fixed for 40 min with 1% OsO<sub>4</sub> in 0.15 M sodium cacodylate buffer. After dehydration of the samples in ethanol solutions of increasing concentration (50°, 70°, 90°, 95°, 2 x 100°), the cells 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 more than 100 cells was observed and the percentage of amoebas displaying a replicative phagosome (i.e vacuoles filled with several bacterias) determined.

## **RESULTS**

We first studied the ability of *L. pneumophila* to infect and growth within the different strains of amoebas. As expected, and in accordance to what was previously published (Anand *et al.*, 1983; Holden *et al.*, 1984; Vandenesch *et al.*, 1990; King *et al.*,

1991; Moffat & Tompkins, 1992; Abu Kwaik *et al.*, 1994; Bozue & Johnson, 1996; Abu Kwaik *et al.*, 1996.; Gao *et al.*, 1997; Neumeister *et al.*, 1997; Abu Kwaik *et al.*, 1998a), the bacteria displayed the ability to grow within *H. vermiformis* and *A. castellanii* with a respective increase of 1.3 and 1.4 log in CFU/ml after 3 days of infection (Fig. 1). Surprisingly, and at the opposite of these two amoebas, *W. magna* did not favour bacterial yield and even reduced it significantly within the coculture medium (Fig. 1). A 2.3 log difference in bacterial yield was measured at day 2 post infection between the *Hartmannella-Acanthamoeba* species and *W. magna*.

The observation that amoebic species display a different behaviour towards *L. pneumophila* was further strengthened by the fact that *Hartmannella* and *Acanthamoeba* growths were particularly affected by the bacteria when compared to *Willaertia* amoebas (Table 1). Firstly, a significant decrease in *Hartmannella* and *Acanthamoeba* number was detected on day 3 of coculture with *L. pneumophila* when compared to day 1, a phenomenon that could not be observed with *Willaertia*. Secondly, a significant drop was observed in *Hartmannella* and *Acanthamoeba* growth cocultured with *Legionella* when compared to their counterparts cultured in absence of the bacteria (control amoebas). Hence, after 3 days of coculture, the number of *Hartmannella* and *Acanthamoeba* was reduced by 82 and 93 % ( $P < 0.001$ ) when compared to the respective amoebas cultured without *L. pneumophila*; a decrease was also observed in the number of *W. magna* cocultured with the bacteria when compared to bacterial-free control, but it was limited to 25 % and less significant ( $P < 0.05$ ).

We then examined the putative cytotoxicity of *L. pneumophila* towards the different species of amoebas. Phase contrast microscopic observations of microplates clearly shown alterations occurring within *Hartmannella* and *Acanthamoeba* populations while *Willaertia magna* was not affected (Fig. 2). Hence, after 72 hr of bacterial infection, few adherent



*Hartmannella* and *Acanthamoeba* amoebas effectively remained when compared to controls, while *Willaertia* still formed a dense monolayer of cells similar to control. Following infection, *Hartmannella* and *Acanthamoeba* trophozoites became progressively rounded, shrivelled, and lost their adherence ability after 2 days in presence of the bacteria. Moreover, further experiments shown that  $28.4 \pm 8\%$  of the very few *Acanthamoeba* amoebas remaining at 72 hr versus only  $3.8 \pm 0.9\%$  of the numerous *W. magna* were trypan blue positive (Fig. 3), showing again a particular resistance of this latter species to bacterial induced cytotoxicity when compared to *A. castellanii*.

We next determined whether the defective growth of *L. pneumophila* in presence of *W. magna* could be due to a defect in intracellular replication. The number of amoebas displaying a replicative phagosome was determined by electron microscopy. In *Acanthamoeba* and *Hartmannella* amoebas, the percentage of cells displaying a replicative phagosome at 36 hr was very high, i.e., 36 % to 51% according to the MOI (Table 2). In contrast, replicative phagosomes were not expressed in *Willaertia* amoebas infected at a MOI of 50, although some rare bacteria were sometimes internalized as demonstrated in figures 5A and 5B. When *L. pneumophila* was cocultured with *W. magna* at a higher ratio (MOI of 100), few amoebas displayed a replicative phagosome but at a very low level (1.5%) when compared to *Acanthamoeba* and *Hartmannella* (Table 2). Hence, electron microscopy showed that in *A. castellanii* and *H. vermiformis*, all the stages of infection could be observed. Shortly after infection, *L. pneumophila* appeared in these two species as isolated bacteria in a vacuole-like structure (Fig. 4A) surrounded by rough endoplasmic reticulum (RER) according to previous descriptions (Abu Kwaik, 1996.). More pronounced invasion leads to the formation of clear distended replicative phagosomes containing numerous bacteria juxtaposed with cytoplasmic amorphous elements (Fig. 4B)

and ended by the necrosis of these heavily infected amoebas. Nevertheless, even in these heavily infected amoebas, the contractile vacuole was still active (Fig. 4C).

In contrast, out of the few *W. magna* displaying intracellular bacteria (only 2/133 *Willaertia* amoebas examined at a MOI of 100), the replicative phagosome showed morphological differences when compared to those observed in *Acanthamoeba* or *Hartmannella* amoebas. These phagosomes were not clearly delimited by a membrane as in *A. castellanii* (compare Fig 5C and 5D with Fig. 4B) and the bacteria inside contain numerous vacuoles usually described as polyhydroxybutyrate granules (Fig. 5D). In *Acanthamoeba* and *Hartmannella* amoebas, 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. 6A), while the *L. pneumophila* observed within the two infested *Willaertia* amoebas appeared as short stubby rods with a dense cytoplasm delimited by a thick laminar outer layer (Fig. 6B). These observations suggest that the replication of *L. pneumophila* cannot occur properly within *W. magna* and may explain why bacterial growth is inhibited in presence of this amoeba (Fig. 1).

## DISCUSSION

The main finding of these experiments is that *W. magna* displays a particular resistance to infection by *L. pneumophila* when compared to the amoebas, *A. castellanii* and *H. vermiformis*. To our knowledge, this is the first study that systematically addresses the ability of *Willaertia* amoebas to resist against *L. pneumophila*. It also confirms previous studies showing that the *Acanthamoeba* and *Hartmannella* species support the proliferation of *L. pneumophila*. Numerous studies effectively shown that multiplication of *L. pneumophila* occurs within various protozoa such as amoebas or ciliates which make that free-living amoebas are now considered as a natural reservoir for the pathogenic

bacteria (Abu Kwaik *et al.*, 1998b; Molmeret *et al.*, 2004; Molmeret *et al.*, 2005). Fields reported that five amoebic genera are able to support the intracellular growth of *L. pneumophila* (Fields, 1996). However, out of these 5 genera, only two (*Acanthamoeba* and *Hartmannella*) have been repeatedly tested in coculture with *L. pneumophila* (Anand *et al.*, 1983; Holden *et al.*, 1984; Vandenesch *et al.*, 1990; King *et al.*, 1991; Moffat & Tompkins, 1992; Abu Kwaik *et al.*, 1994; Abu Kwaik, 1996; Bozue & Johnson, 1996; Gao *et al.*, 1997; Neumeister *et al.*, 1997; Abu Kwaik *et al.*, 1998a; Harb *et al.*, 1998). Recently, it was shown that *Dictyostelium discoideum* is also able to support the bacterial growth and proliferation (Hagele *et al.*, 2000; Solomon *et al.*, 2000). Our results with *A. castellanii* and *H. vermiformis* are in agreement with these previous studies although the bacteria grow to a lesser extent in our experiments when compared to available data in the literature. This may be explained by the fact that bacterial growth determination was performed upon spontaneous lysis of amoebas instead of the artificial lysis that was often used in other studies. However, *Acanthamoeba* and *Hartmannella* species displayed a 2.3 log higher ability to support bacterial growth when compared to *W. magna* tested exactly in the same conditions. Concomitantly to the lack of *L. pneumophila* replication, the bacteria did not display any obvious cytotoxicity towards *W. magna*, demonstrating again the particular resistance of this latter species when compared to the other two permissive genera.

Different possibilities, related to either the bacteria or the amoeba itself, may explain the particular resistance of *W. magna*. Until now, in all studies that reported a defect of *L. pneumophila* growth in coculture with macrophages and/or amoebas, the underlying mechanism results from the use of mutant strains of the bacteria that split in two great categories. Several bacterial mutants of the *dot/icm* system are defective in evasion of phagolysosomal fusion and consequently, have lost their capacity of intracellular replication (Cianciotto & Fields, 1992; 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 egress from the phagocytic cell despite an active intracellular replication (Alli *et al.*, 2000). However, these two possibilities can be excluded in our study since the bacterial strain of *L. pneumophila* used in coculture experiments with *W. magna* was the same that displayed an obvious multiplication within *A. castellanii* and *H. vermiformis* associated to a pronounced cytolytic effect in these two species. Consequently, the mechanism of the defective replication and the absence of cytopathogenicity in *W. magna* lie in the amoeba itself rather than in the bacterial strain. *Willaertia* may reduce *L. pneumophila* growth in coculture either by inhibiting its intracellular replication and/or by blocking bacterial induced lysis. Another explanation may be the occurrence of differences in the membrane composition of amoebas that may result in a lower efficiency of bacterial internalization within *W. magna*. Some studies have shown heterogeneity in the attachment and uptake mechanisms of *L. pneumophila* by *H. vermiformis* and *A. polyphaga* (Harb *et al.*, 1998). Although none of the hypothesis may be excluded, our observations support the possibility that *L. pneumophila* replication is inhibited by *W. magna*.

Hence, a defective replication of the bacteria in *W. magna* amoebas clearly occurs as shown by the very low occurrence of replicative phagosomes in this species when compared to *Acanthamoeba* and *Hartmannella* amoebas. The microscopic observations suggest that the fusion of phagosome with lysosomes is not inhibited by *L. pneumophila* within *Willaertia* amoebas. Moreover, in the very few *W. magna* that displayed a replicative phagosome, this latter subcellular organelle appeared not as clearly delimited as the one in other amoebic species. To test the hypothesis of an efficient phago-lysosomal formation within *Willaertia* amoebas, colocalisation studies of *L. pneumophila* with late endosomal and lysosomal markers such as acid phosphatase or LAMP-1 glycoprotein could be performed.

The expression of replicative phagosomes in *W. magna* only occurred for the highest MOI (i.e 100), suggesting that the cell defence mechanisms of *Willaertia* amoebas may be partially overwhelmed at this bacterial concentration. Concomitantly, this observation demonstrates that ingestion of *L. pneumophila* occurs in *W. magna*. Furthermore, even when *L. pneumophila* exceptionally succeeded in this limited intracellular replication within *W. magna*, it displayed a particular aspect recalling the “cyst-like” or mature intracellular form described by Garduno in HELA cells (Garduno *et al.*, 2002). The authors suggested that these “cyst-like” bacteria are resilient and metabolically dormant bacteria that showed to be more resistant to diverse chemical and physical stresses. It is thus tempting to speculate that the bacteria observed within *Willaertia* are individuals that display a particular ability to resist to the intracellular environment of this amoeba. Concomitantly, the observations showing that the *L. pneumophila* internalized within *Willaertia* displays a dormant like morphology similar to the one described by Garduno (Garduno *et al.*, 2002) strengthen our assumption that *W. magna* is an amoebic species having a particular resistance towards the bacteria, at least in our experimental conditions.

Globally, our results show that at least one amoebic species may not be a good vector for the pathogenic bacteria. Our observations are in agreement with a recent publication of Declerck (Declerck *et al.*, 2005) in which the authors shown that *Naegleria lovaniensis* is less permissive than *A. castellanii* to invasion and replication of *L. pneumophila*. This observation, reported for the first time for a *Naegleria* species, seems perfectly in line with our results since *Naegleria* and *Willaertia* are two related genera, which belong to the same family of *Vahlkampfiidae* (De Jonckheere *et al.*, 1984; Page, 1987; De Jonckheere, 1997). Indeed, these two genera share some characteristics that clearly differentiate them from the group of *Hartmannella spp.* and *Acanthamoeba spp.* An important difference between

these amoebas is their mode of locomotion: *Hartmannella* and *Acanthamoeba* amoebas are moving by slow and progressive pseudopodial deformations while *Willaertia* and *Naegleria* amoebas do it by fast deformations with emission of eruptive lobopods. *Hartmannella* and *Acanthamoeba* mitosis is similar to that of higher eucaryotic cells, while *Willaertia* and *Naegleria* division occurs through promitosis characterized by the persistence of the nuclear membrane and of the nucleolus.

The results reported herein demonstrate marked differences between the three tested amoebic species in their ability to serve as host cells for the replication of *L. pneumophila*. Consequently, the distribution and the density of the pathogenic bacteria 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* may be a useful model to study the mechanisms of host resistance to *L. pneumophila*.

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Amoeba species	Time (day of co-culture)			
	0	1	2	3
<i>Acanthamoeba</i>	$5.38 \times 10^4 \pm$	$1.35 \times 10^5 \pm$	$1.13 \times 10^5 \pm$	$3.64 \times 10^4 \pm$
<i>castellanii</i>	$3.86 \times 10^3$	$5.91 \times 10^4$	$5.67 \times 10^4 **$	$3.05 \times 10^4 **$
<i>Hartmannella</i>	$5,50 \times 10^4 \pm$	$1.97 \times 10^5 \pm$	$2.32 \times 10^5 \pm$	$1.26 \times 10^5 \pm$
<i>vermiformis</i>	$4,60 \times 10^3$	$4.63 \times 10^4$	$3.76 \times 10^4 *$	$2.96 \times 10^4 **$
<i>Willaertia</i>	$5.41 \times 10^4 \pm$	$1.77 \times 10^5 \pm$	$2.92 \times 10^5 \pm$	$2.42 \times 10^5 \pm$
<i>magna</i>	$2.52 \times 10^3$	$3.93 \times 10^4$	$5.26 \times 10^4$	$4.71 \times 10^4$

**Table. 1. Effect of bacterial infection on the growth of amoebas**

The different amoebic species were cocultured with *L. pneumophila* at an MOI of 50 and cells number determined as described in methods. The results are expressed as the number of amoebic cells/ml of medium and are the mean  $\pm$  standard deviation of 6 (*Hartmannella*) to 8 (*Acanthamoeba* and *Willaertia*) independent experiments. Statistical differences between the growth of *Willaertia* and other amoebic species are indicated (\*:  $P < 0.05$ ; \*\*:  $P < 0.001$ ). The number of *Acanthamoeba* and *Hartmanella* determined on day 3 of co-culture represents a drop of  $\sim 93$  and  $82$  % in the number of respective control amoebas grown in the same conditions for 3 days without *L. pneumophila*. This drop was limited to  $\sim 25$  % in the case of coculture experiments using *W. magna*.

**Table. 2 : Occurrence of replicative phagosomes within amoebas**

	<i>Acanthamoeba castellanii</i>	<i>Hartmannella vermiformis</i>	<i>Willaertia magna</i>
MOI*= 50	36 %	42%	0 %
MOI = 100	51 %	ND	1,5 %

\* MOI : Multiplicity of infection

Amoebas were cocultured for 36 hours with *L. pneumophila* at a MOI of 50 or 100, fixed and processed for electron microscopy. The results are expressed as the percentage of cells displaying a replicative phagosome and were obtained out of the observation of more than 100 cells. The determination of phagosome occurrence at a MOI of 100 could not be performed in *H. vermiformis* on account of too many bacterial-induced lysed cells (ND: not determined).

**Fig. 1. Growth of *Legionella pneumophila* in coculture with the three amoebic genera.**

Amoebas were axenised in SCGYEM medium and co-cultured with the bacteria added on day 0 as described in methods. The growth of bacteria was determined up to 4 days in presence of the different species of amoebas. The data are the average  $\pm$  standard deviation of 6 independent experiments for *Hartmannella vermiformis* (open squares) to 8 for *Willaertia magna* (closed squares) and *Acanthamoeba castellanii* (closed circles). Statistical differences in the bacterial growth in co-culture with *Willaertia magna* or *Acanthamoeba castellanii* at day 1 (\*:  $P < 0.05$ ) and between *Willaertia magna* and other two amoebic species between day 2 to day 4 (\*\*:  $P < 0.001$ ) are indicated.

**Fig. 2. Effect of *L. pneumophila* on amoebic monolayer formation.**

Representative phase contrast microscopic images of the different amoebic species cocultured, either with or without *L. pneumophila*, at a MOI of 50 for 72 hr as described in methods. Note the destruction of amoebic monolayer in *Hartmannella* and *Acanthamoeba* species cultured in presence of the bacteria when compared to controls and the relative resistance of *Willaertia magna*.

**Fig. 3. Cytotoxicity of *L. pneumophila* towards *Acanthamoeba* and *Willaertia* amoebas.**

Amoebas were cocultured with bacteria for 2 and 3 days as described in methods. The extent of cell death was determined by trypan blue exclusion test and the percentage of trypan blue positive cells calculated. The data are the average  $\pm$  standard deviation of 5 independent experiments. Statistical differences between *Acanthamoeba* (closed bars) and *Willaertia* (open bars) are indicated (\*:  $P < 0.001$ ). The percentage of trypan blue positive cells in control amoebic cultures, without *L. pneumophila*, was always below 1%.

**Fig. 4. Transmission electron micrographs of *Acanthamoeba* infected by *L. pneumophila*.**

Cocultures of *L. pneumophila* with *Acanthamoeba* amoebas for 36 hours, were fixed and treated for electron microscopy as described in methods. **Panel A** : first stage of *Acanthamoeba* infection by *L. pneumophila* : the bacteria are contained in phagosomes that are surrounded by rough endoplasmic reticulum (**rer**). **Panel B** : typical replicative phagosome observed at a more pronounced infection stage. **Panel C** : massively infected pre-lytic amoeba displaying a contractile vacuole (**CV**). **N**: nucleus.

**Fig. 5. Transmission electron micrographs of the rare *Willaertia* infected by *L. pneumophila*.**

Cocultures of *L. pneumophila* with *Willaertia* amoebas for 36 hours were fixed and treated for electron microscopy as described in methods. The different micrographs show the few cells of *W. magna* that were found to be infected by the bacteria. **Panel A** : single *L. pneumophila* within a vacuole not surrounded by rer. **Panel B**: *W. magna* showing an isolated intracytoplasmic *L. pneumophila* strongly altered. **Panels C and D** show, at MOI 100, the only two *W. magna* (2/133 cells observed) displaying a replicative phagosome. Note that in contrast to *Acanthamoeba*, *Willaertia* phagosomes are not clearly delineated and that bacteria are strongly vacuolized (compare these figures with Fig. 4 panels B and C). Bacteria are indicated by arrows on panels A and B. **N**: nucleus; **nu**: nucleolus.

**Fig. 6. Sections of *L. pneumophila* in *Acanthamoeba* and *Willaertia* phagosomes.**

Cocultures of *L. pneumophila* with *Acanthamoeba* (Panels A) and *Willaertia* amoebas (Panels B) for 36 hr were fixed and treated for electron microscopy as described in

methods. Note in panel A, the typical membrane ultrastructure of gram-negative bacteria internalized within *Acanthamoeba* (i.e., a wall with a wavy outer membrane surrounding a clear periplasmic space). In contrast, in panel B the bacteria contained within *Willaertia* display a thick laminar outer layer (indicated by arrows) and a darkened cytoplasm containing large vacuoles usually described as poly-hydroxybutyrate inclusions (see discussion for details).



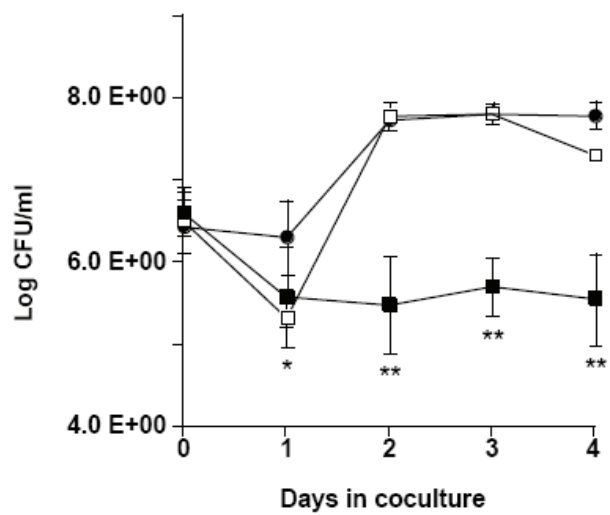


Fig. 1.

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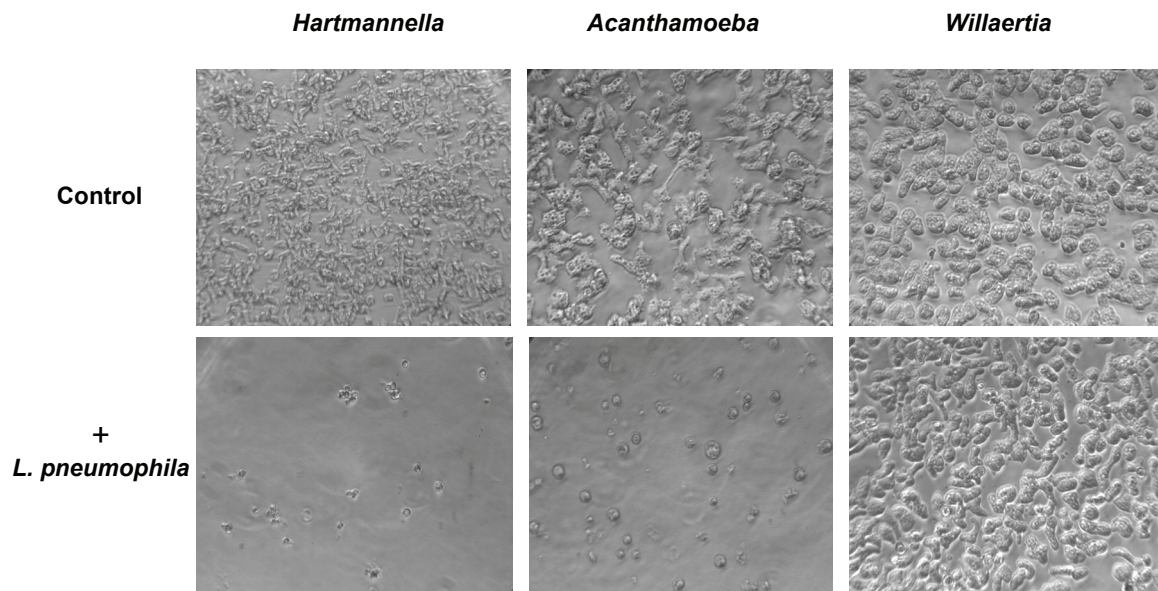


Fig. 2.

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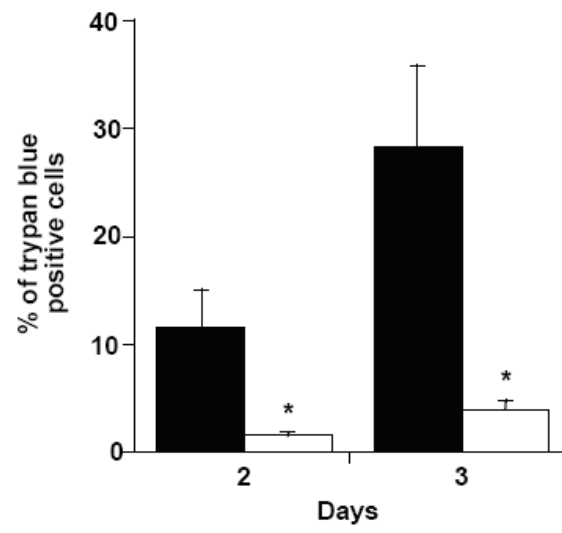


Fig. 3.

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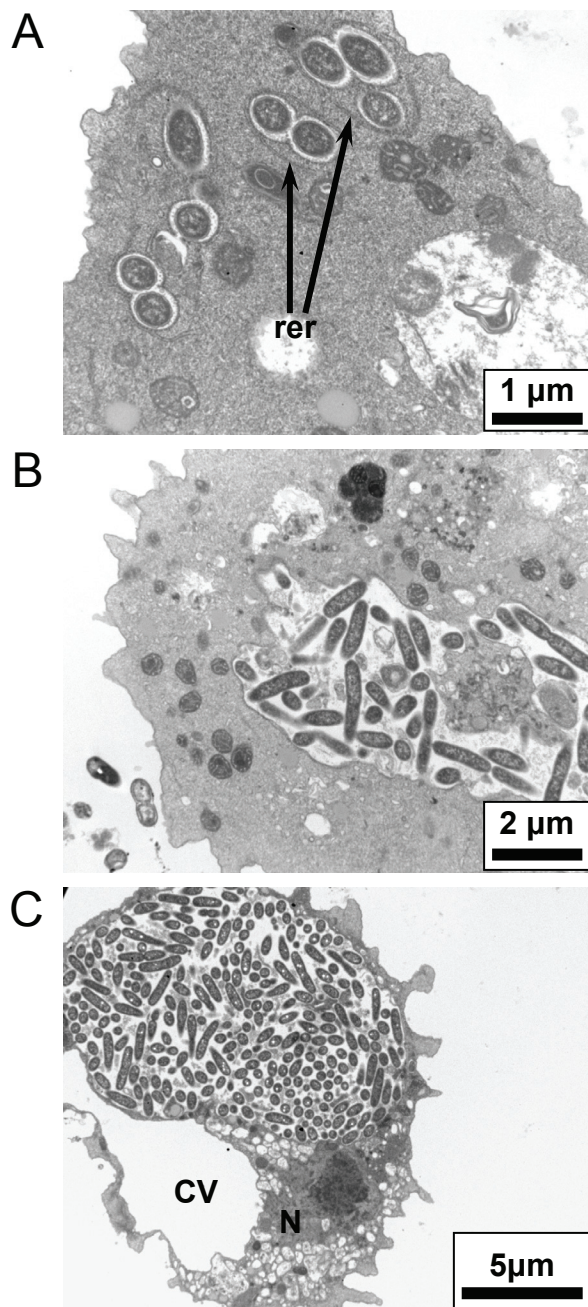


Fig. 4.

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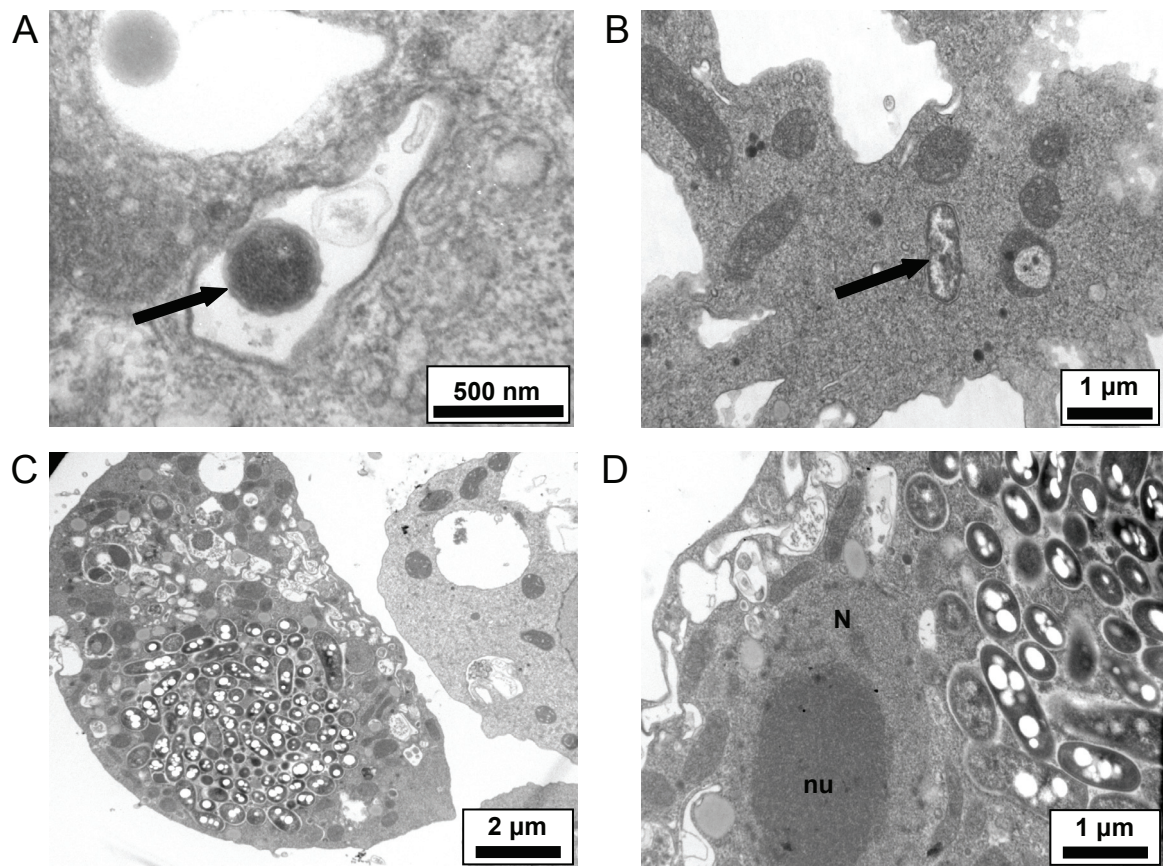


Fig. 5.

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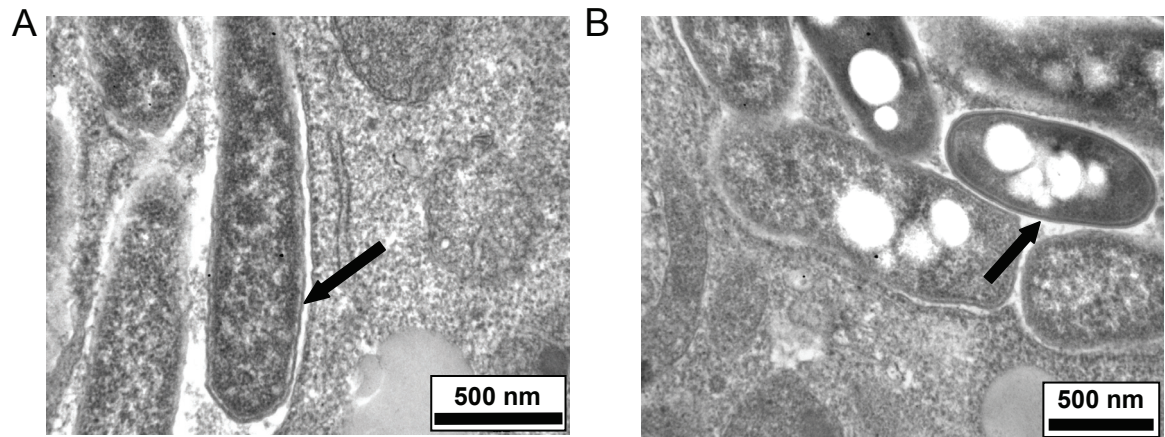


Fig. 6.

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