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Permissivity of insect cells to *Waddlia chondrophila*, *Estrella lausannensis* and *Parachlamydia acanthamoebae*

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Abstract

Recent large scale studies questioning the presence of intracellular bacteria of the *Chlamydiales* order in ticks and fleas revealed that arthropods, similarly to mammals, reptiles, birds or fishes, can be colonized by *Chlamydia*-related bacteria with a predominant representation of the *Rhabdochlamydiaceae* and *Parachlamydiaceae* families. We thus investigated the permissivity of two insect cell lines towards *Waddlia chondrophila*, *Estrella lausannensis* and *Parachlamydia acanthamoebae*, three bacteria representative of three distinct families within the *Chlamydiales* order, all documented in ticks and/or in other arthropods. We demonstrated that *W. chondrophila* and *E. lausannensis* are able to very efficiently multiply in these insect cell lines. *E. lausannensis* however induced a rapid cytopathic effect, which somehow restricted its replication. *P. acanthamoebae* was not able to grow in these cell lines even if inclusions containing a few replicating bacteria could occasionally be observed.

Keywords: Intracellular bacteria; *Chlamydia*-related bacteria; Cell permissivity; Host range; Reservoir; Vector

1. Introduction

All bacteria belonging to the *Chlamydiales* order are strict intracellular organisms sharing a biphasic life cycle that involves two distinct bacterial forms, an infectious Elementary Body (EB) capable of attaching to and entering into its host cell, but that cannot replicate before it differentiates into a metabolically active form, called Reticulate Body (RB). At the end of the multiplication phase, RBs differentiate back into infectious particles and lyse their host cells to start a new cycle [32]. *Chlamydiales* bacteria have been isolated from samples of multiple origins such as mammals, birds, fishes, reptiles or protozoa revealing the large and probably still largely underestimated diversity of their ecological niches (reviewed in [22, 36]). Besides the well-studied human and animal pathogens belonging to the *Chlamydiaceae* family (such as *Chlamydia pneumoniae, C. trachomatis* or *C. abortus*), this clade also comprises 8 other family-level lineages whose members are very diverse being either emerging pathogens able to grow in cells of various origins (*Waddlia chondrophila, Simkania negevensis)* or harmless environmental species only replicating in amoebae (*Protochlamydia amoebophila, Neochlamydia hartmanellae*). Recent large scale studies questioning the presence of *Chlamydiales* bacteria in ticks and fleas [13], [Pilloux et al. submitted 2015] revealed that arthropods may be colonized by these bacteria and thus could serve as reservoir and vectors for potential novel pathogens. In this context, we investigated the permissivity of two insect cell lines, *Aedes albopictus* larva cells and *Spodoptera frugiperda* ovary cells (Sf9), towards *Waddlia chondrophila, Estrella lausannensis* and *Parachlamydia acanthamoebae*, three *Chlamydia*-related bacteria representative of three different families, all belonging to the

Chlamydiales order, documented in fleas and/or ticks and whose pathogenic potential for humans and cattle is confirmed or highly suspected [2-6, 9, 15, 16, 23, 34, 37, 14].

2. Materials and methods

2.1. Cell culture and bacterial strains

Aedes albopictus clone C6/36 larva cells (ATCC® CRL-1660™) and *Spodoptera frugiperda* ovarian epithelial cells (Sf9) (ATCC® CRL-1711™) were routinely maintained respectively at 28°C and 5%CO_2 in Dulbecco's modified essential medium (DMEM; Gibco Invitrogen, Basel, Switzerland) supplemented with 10% foetal calf serum (Biochrom, Berlin, Germany) or at 27°C in Grace insect medium (GIM; Gibco Invitrogen, Basel, Switzerland) supplemented with 10% foetal calf serum.

W. chondrophila strain WSU 86-1044 (ATCC VR-1470), *E. lausannensis* strain CRIB 30 and *P. acanthamoebae* strain Hall's coccus were grown at 32°C within *Acanthamoeba castellanii* strain ATCC 30010, as described in [19].

2.2. Infection procedure

Insect cells were seeded at $1x10^5$ cells per well in 24-wells microplates (Corning) the day before infection. Infection was performed at 28°C or 27°C, as described in [24], with a 5 days-old culture of bacteria in A *castellanii* diluted, if not otherwise described, 1:1000 for *W. chondrophila* (MOI: 1-20) and *P. acanthamoebae* (MOI: 0.1-1) and 1:2000 for *E. lausannensis* (MOI: 1-10)

2.3. Immunofluorescence and confocal microscopy

At different time points after infection, immunofluorescence was performed on cells cultivated on glass coverslips following precisely the protocol described in [27] except that rabbit anti-*P. acanthamoebae* and mouse anti-*E. lausannensis* were used respectively at a 1:1000 and 1:500 dilution. Secondary antibody was diluted 1:500 and mixed with a 1/50 dilution of Concanavalin A (Molecular Probe) and 150 ng/ml DAPI (Molecular Probe, Eugene, Oregon, USA). Cells were observed under an epifluorescent microscope (Axioplan 2, Zeiss, Feldbach, Switzerland) or a confocal microscope (AxioPlan 2 LSM 710, Zeiss).

2.4. Electron microscopy

One T25 flask of *Aedes albopictus* or Sf9 cells was infected as described above with *W. chondrophila* and *E. lausannensis* diluted 1/200. 24 hours post-infection, cells were washed once with PBS and harvested. After one centrifugation step of 10 min at 1'000 rpm, cells were resuspended in phosphate buffer (19ml of 0.2 M NaH_2PO_4 + 81ml of 0.2M Na_2HPO_4, pH 7.4) containing 0.2% glutaraldehyde (Fluka Biochemika, Buchs, Switzerland) and 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, USA)

and incubated overnight at 4°C. After two more washing steps with phosphate buffer, cells were prepared as described previously [10]. Thin sections on grids were examined with a transmission electron microscope Philips CM 100 (Philips, Eindhoven, The Netherlands).

2.5. Quantitative PCR

Cells were harvested at different time points after infection, genomic DNA was extracted following manufacturer's instructions (Wizard SV Genomic DNA purification kit, Promega, Madison, WI) and qPCR was performed as described in [24]. To account for variation in the inocula used for infection, bacterial growth is expressed as a fold increase in the number of bacteria after normalization using time point "0h" as reference.

2.6. Cell viability

Cell viability was determined with a propidium iodide assay as described in [14] except that propidium iodide was added immediately after infection. Positive control was obtained by incubating non-infected cells 5 minutes with MetOH at -20°C. Results were normalized considering the positive control as 100% mortality.

3. Results

3.1. Growth kinetic in insect cells

Two insect cell lines, *Aedes albopictus* larva cells and Sf9 (*S. frugiperda* ovarian epithelial cells) were infected with either *W. chondrophila, E. lausannensis* or *P. acanthamoebae* and bacterial growth was monitored using specific quantitative PCRs (qPCRs) developed in our laboratory [11, 16, 30]. In parallel, bacterial multiplication was

also assessed by immunofluorescence and confocal microscopy using in-house polyclonal antibodies and by electron microscopy.

3.1.1. W. chondrophila and E. lausannensis

Results shown in Fig. 1 indicated that *W. chondrophila* and *E. lausannensis* are both able to efficiently replicate in insect cells, leading to an increase of bacterial genomic DNA copies of about 3 logs in 48 hours for *W. chondrophila* (Fig. 1 panels a and b) and of about 2 logs in 48 hours for *E. lausannensis* (Fig. 1 panels c and d). During the first 8 hours following infection, EBs enter their insect cell host, differentiate into RBs and create a replicative niche able to support their exponential multiplication. Inclusions containing dividing bacteria can already be observed by immunofluorescence and confocal microscopy 24 hours post infection (Fig. 2A, panels a,b,c,and d). Similarly to what has been described in other cell lines [24, 25], *W. chondrophila* then exponentially replicate until 48 hours p.i. and ultimately lyse their host to release infectious particles ready to infect new insect cells (data not shown). Replication of *W. chondrophila* and *E. lausannensis* was also documented in Sf9 cells by electron microscopy 24 hours post infection (Fig. 2B). The *W. chondrophila*-containing vacuoles were surrounded by tightly associated mitochondria, a feature that was also described in other cell lines [12, 25].

3.1.2. P. acanthamoebae

No growth of *P. acanthamoebae* could be detected by qPCR or immunofluorescence in the two insect cell lines tested (Fig.1 and 2A, panels e and f) even after 7 days. However, when cells were infected with a 10 fold higher bacterial load (MOI of 1-10), rare inclusions containing replicating bacteria could be observed by confocal microscopy

in *Aedes albopictus* cells (Fig. 2A, panel g). This bacterial replication is very limited and restricted to about 3% of all infected cells.

3.2. Cytophatic effect

Direct examination by confocal microscopy of *E. lausannensis* infection revealed that these bacteria, like *W. chondophila*, efficiently multiplied in insect cells but these cells were more rapidly lysed than those infected with W. *chondrophila*. This observed cytopathic effect was confirmed in a host cell viability assay based on propidium iodide incorporation that showed, 48 hours post infection, 100% mortality of *Aedes albopictus* cells infected with *E. lausannensis* versus 50% in cells infected with *W. chondrophila* and 23% in non-infected cells (Fig. 3). Results were normalized using methanol-treated cells at time of infection as a positive control of 100% mortality. Mortality rate over 100% are thus explained by an increase, at later time points, of the total number of cells that ultimately died. We could observe a weak cytopathic effect of *P. acanthamoebae* infection that probably resulted more from the lower fitness of infected cells than from the few replicating bacteria.

In Sf9 cells, a similar cytopathic effect of *E. lausannensis* was observed by confocal microscopy (data not shown), however, due to interferences of the culture medium with the propidium iodide assay, this increased cell mortality could not be precisely quantified.

4. Discussion

In the present study, we demonstrated that *W. chondrophila* and *E. lausannensis* are able to enter and efficiently multiply in two different insect cell lines. *E. lausannensis* growth is however slightly less efficient than *W. chondrophila* growth, a difference that can be explained by the rapid cytopathic effect of *E. lausannensis,* which restricts the number of replication rounds possible.

W. chondrophila is an emerging pathogen for humans and animals and it is able to readily grow at temperatures ranging from 25°C to 37°C in mammalian cell lines of diverse origins [12, 21, 25, 28], in fish cells [24] as well as in protozoa [17, 31]. *E. lausannensis,* whose pathogenic potential is currently under investigation, was first isolated from an environmental water sample and is also able to grow at different temperatures in protozoa, fishes and mammalian cells [14, 24, 30]. Strikingly, these two organisms that display such a broad host range, both encode an extended family of OmpA proteins with beta-barrel structure that were recently shown to be dominant in the outer membrane of *W. chondrophila* and to play a role in adhesion of this bacteria to its host [1, 7, 8, 26, 29]. In addition, *Simkania negevensis*, another *Chlamydia*-related bacteria that also possess a large family of 37 MOMP-like proteins in its outer membrane similarly displays a wide host range [1, 27]. Based on these observations, we recently hypothesized that the large diversity of the OmpA protein family is linked to the wide host range of these bacteria [26]. In addition, ability to grow in multiple and diverse eukaryotic cells correlates in most cases with virulence towards mammals [27].

P. acanthamoebae only encodes one homolog of MOMP and its efficient growth seems to be restricted to amoebal hosts [18, 20]. Several studies have reported a limited replication of this bacteria in host cells such as bone-marrow derived macrophages, fish or insect cell lines [24, 33, 35]. This present work also demonstrated that growth of *P.*

acanthamoebae in insect cells is limited and restricted to a few cells. Sixt *et al.* also reported a limited *P. acanthamoebae* replication in *Aedes albopictus*, Sf9 and *Drosophila* S2 cells and they observed a bacteria-induced programmed cell death in the last two cell lines [35]. We did not observe nuclear fragmentation in *P. acanthamoebae*-infected cells and cell viability was stable during the first 72 hours post infection, which is not in agreement with apoptosis. Furthermore, preliminary experiments performed in *Aedes albopictus* cells in presence of a pan-caspase inhibitor did not demonstrate an enhanced multiplication of *P. acanthamoebae* (A. Croxatto, unpublished).

In conclusion, we demonstrated the permissivity of insect cells to *Waddla chondrophila* and *Estrella lausannensis* and further highlighted the very broad host range of these possible pathogens and the potential role of insects as reservoir or vectors for these strict intracellular bacteria.

5. Acknowledgments

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6. Conflict of interest

The authors have no conflict of interest.

6. References

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7. Figure legends

Fig. 1 Bacterial growth within insect cells

W. chondrophila (panels a and b), *E. lausannensis* (panels c and d) and *P. acanthamoebae* (panels e and f) replication measured by qPCR in *Aedes albopictus* cells (panels a, c and e) and in Sf9 cells (panels b, d and f). Values are normalized to the number of bacteria at 0h post infection (p.i). Results are the mean +/-SD of at least four independent experiments performed in duplicates.

Fig.2 Bacterial replication assessed by confocal and electron microscopy

A. Immunofluorescence and confocal microscopy of *W. chondrophila* (panels a and b), *E. lausannensis* (panels c and d) and *P. acanthamoebae* (panels e and f) in *Aedes albopictus* cells (panels a, c and e) and in Sf9 cells (panels b, d and f) 24 hours post-infection. Panel g displays one rare inclusion of *P. acanthamoebae* in *Aedes albopictus* cells 4 days post infection. Bacteria (green) are stained with species-specific polyclonal antibodies and insect cells (red) are stained with Texas Red-conjugated Concanavalin A. Scale bar 10 μm.
B. Electron micrographs of *W. chondrophila* (panel a) and *E. lausannensis* (panel b) in Sf9 cells 24 hours post infection. Scale bar: 1 μm.

Fig. 3 Cell viability

Aedes albopictus cells viability was determined with a propidium iodide assay at different time points after infection. Results are the mean +/- SD of 2 independent experiments performed in triplicates.

Figure 1

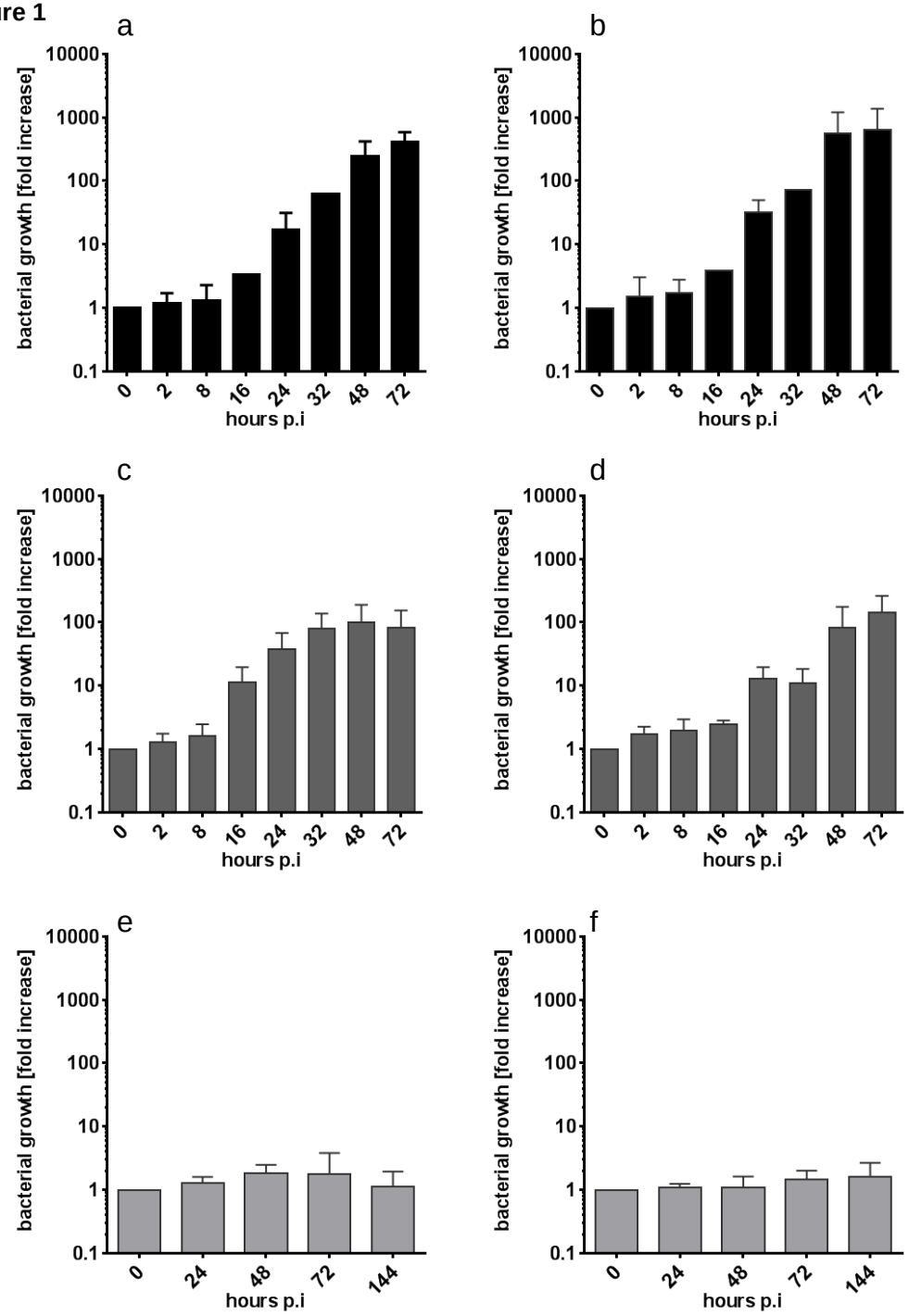
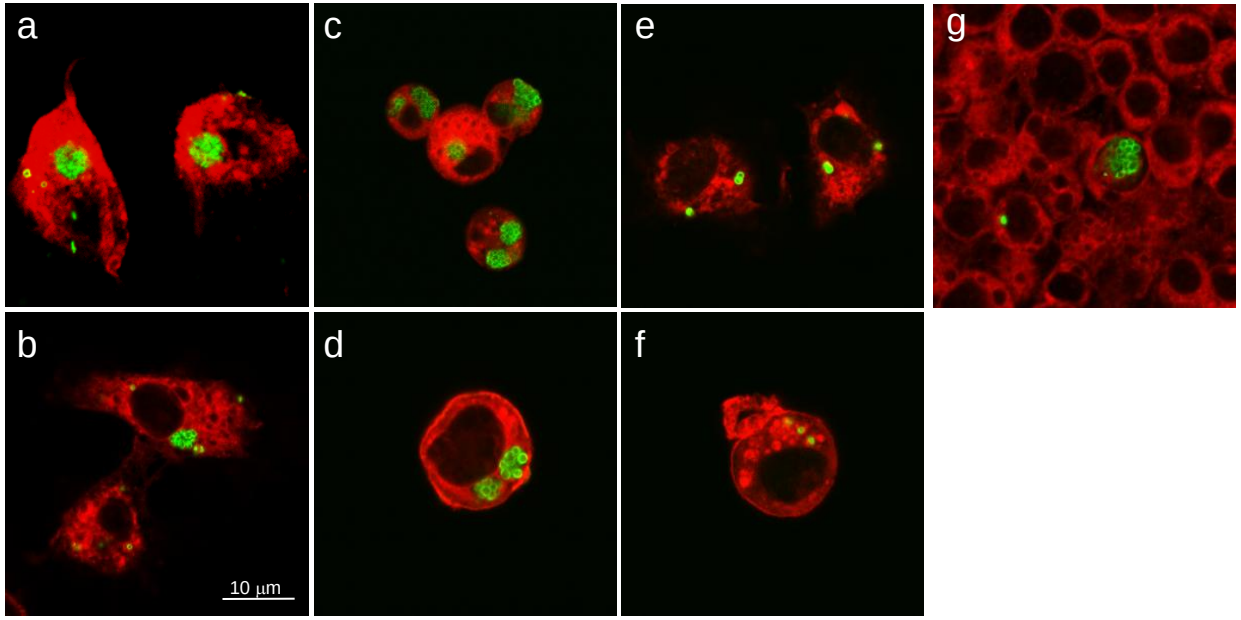

a
b
c
d
e
f
bacterial growth [fold increase]
10000
1000
100
10
1
0.1
0
2
8
16
24
32
48
72
144
hours p.i

Figure 2

A



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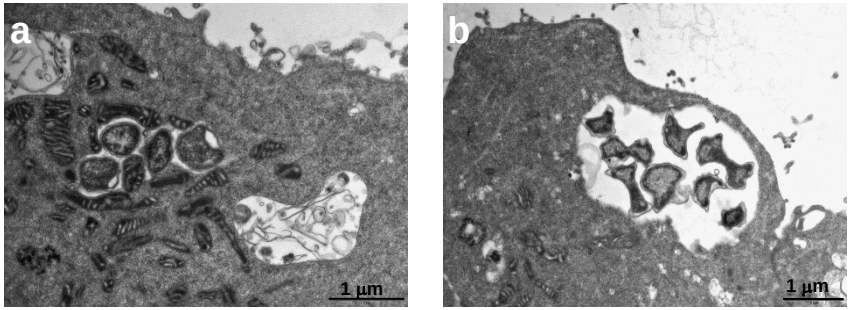
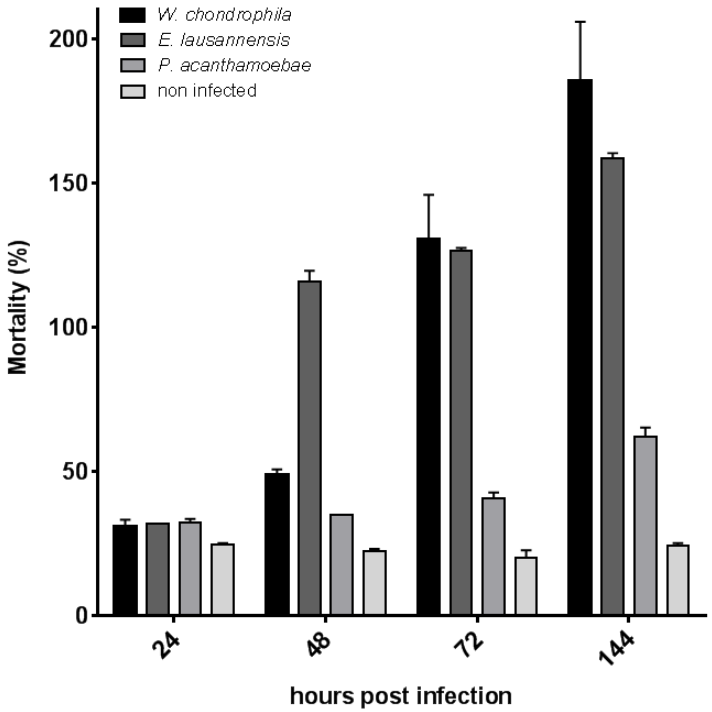


Figure 3


W. chondrophila
E. lausannensis
P. acanthamoebae
non infected
200
150
100
50
0
Mortality (%)
24
48
72
144
hours post infection