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W. chondrophila: from biology to pathogenicity

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Abstract

Waddlia chondrophila is an emerging pathogen causing miscarriages in humans and abortions in ruminants. The full genome of this *Chlamydia*-related bacterium has been recently completed, providing new insights into its biology and evolution. Moreover, new cell biology approaches and the use of novel inhibitors have allowed detailed investigations of its interaction with host cells.

Keywords: intracellular bacteria, *Chlamydia*-related organism, *Waddlia chondrophila*, pathogenesis

Abbreviations: elementary bodies (EBs), reticulate bodies (RBs), aberrant bodies (Abs), endoplasmic reticulum (ER), peptidoglycan (PG), outer membrane protein (OMP), Type III secretion system (T3SS), bacteria containing vacuole (BCV), post infection (p.i.)

1. Introduction

Waddlia chondrophila is an intracellular obligate bacterium that belongs to the *Chlamydiales* order. The *Chlamydiales* order includes six different families, including the *Waddliaceae* family. *Waddlia chondrophila* was first isolated from an aborted bovine fetus in USA in 1990 [1]. Twelve years later, *W. chondrophila* was also isolated from a second bovine case [2]. In 2005, a novel species which shares 91 % of identity with *Waddlia chondrophila,* was identified in Malaysia from a fruit bat and called *Waddlia malaysiensis* [3]. *Waddlia chondrophila* is considered as an abortigenic bacteria in ruminants [1, 2] and is likely responsible of economical losses. The pathogenic role of *W. chondrophila* in humans is supported by a strong association between *W. chondrophila* seropositivity and human miscarriage [4-6]. Moreover, *W. chondrophila* was also detected in respiratory samples from patients with bronchiolitis or pneumonia [7, 8]. Despite the clinical and veterinary importance of this pathogen and its zoonotic potential, little is known regarding the biology and pathogenicity of this bacterium. In this review, we summarize the current knowledge on *Waddlia chondrophila* biology by focussing on its cell biology, genome, metabolism, and membrane proteins.

2. Nascent role of *W. chondrophila* in human miscarriages and respiratory diseases

The potential abortigenic role of *W. chondrophila* in cattle raises the hypothesis that *W. chondrophila* could be involved in human miscarriages, as reported by Baud and colleagues [4]. This prospective study on 438 women comprised 69 women with sporadic miscarriages, 200 with recurrent miscarriages and 169 control women with uneventful pregnancies [4]. By immunofluorescence, 100 women were positive for *Waddlia* with an anti-*Waddlia* IgG titer ≥ 64. The seroprevalence for women with sporadic miscarriages (31.9%) and women with recurrent miscarriage (33%) was higher than for control women (7%, $p<0.05$). The presence of anti-*Waddlia* IgG was confirmed for 97 women by western blot using *W. chondrophila* as the antigen [4]. The specificity of the anti-*Waddlia* antibody was demonstrated by adsorption experiments. In a second

study, a case was documented, which not only exhibited a positive serology for anti-*Waddlia* antibodies, but was also confirmed by *Waddlia* specific real-time PCR performed on cervicovaginal swabs and by immunohistochemistry [5]. Immunohistochemistry demonstrated the presence of *W. chondrophila* in glandular epithelium cells. This is the first evidence of the presence of this bacterium in the human placenta itself.

The transmission mode of *Waddlia* to human remains an important question. Since *C. trachomatis* is a sexually transmitted pathogen whose role in miscarriages is suspected [9], sexual transmission may be possible for *Waddlia*. In the first study, the *C. trachomatis* seropositivity did not differ between the 97 women seropositive for *Waddlia* and the seronegative women [4]. From this absence of correlation between *C. trachomatis* and *Waddlia*, we can conclude that there is neither a significant cross-reactivity nor the same transmission mode. This suggests that *W. chondrophila* is not sexually transmitted. Interestingly, further statistical analysis showed that *Waddlia* seropositivity is associated with animal contact, supporting the zoonotic risk of *W. chondrophila* [4]. This potential is further strengthened when considering that the host range of *W. chondrophila* is known to include cattle [1, 2]. Other transmission modes such as the water network are possible since free-living amoebae present in water are permissive to *W. chondrophila* infection and might represent a huge widespread reservoir for this intracellular bacterium [10]. A recent study on drinking and well water (n=70) confirmed the presence of *W. chondrophila* by qPCR on 10 of the 40 well water samples investigated [11]. Infection by ingestion of contaminated meat or milk is also possible [4].

The implication of *Chlamydia*-related bacteria such as *Simkania negevensis* and *Parachlamydia acanthamoebae* in respiratory disease are well documented [12-14]. The presence of *W. chondrophila* in respiratory samples was also investigated. In one study on 389 patients with community-acquired pneumonia, one case was positive by PCR and sequencing of the 16S rRNA gene fragment revealed 98% and 99.7% sequences similarity to the 16S rRNA gene of *W. chondrophila* isolate WSU 86-1044 and 2032-99, respectively [7]. As part of a second study, a *W.*

chondrophila specific real-time PCR was developed and applied to 32 nasopharyngal samples from children with bronchiolitis not due to respiratory syncytial virus (RSV), the most common etiology of bronchiolitis [8]. Three samples were *Waddlia* positive. Among these three patients, one was also infected by another microbe [8].

In conclusion, *W. chondrophila* is likely involved in human miscarriage and possibly involved in respiratory disease. Additional studies are needed to precisely evaluate the incidence and importance of this bacterium in these clinical settings.

3. *W. chondrophila*, an emerging veterinary pathogen

W. chondrophila was isolated twice from aborted bovine fetuses in USA and in Germany [1, 2]. The German fetus was coinfected with *Neospora caninum* which precluded a definitive diagnosis on the cause of abortion. A serological test was developed and applied to bovine sera, showing an association between anti-*Waddlia* antibodies and bovine abortion [15]. Moreover, 2 bovine fetuses were experimentally infected with *W. chondrophila* and one fetus died within 2 weeks [15]. These results support an abortigenic potential of *W. chondrophila* in cattle.

Previous studies reported in this review, showed the potential role of *W. chondrophila* in abortion in ruminants and humans, raising the question of whether this bacterium has the same role in other mammals. In this prospect, the potential role of *W. chondrophila* in porcine abortion was assessed by Koschwanez and colleagues [16]. None of the aborted fetuses (n=286) were positive for the *Waddlia* specific real time PCR suggesting that *Waddlia* is not an abortigenic agent in Swiss sows [16].

Recently, it was reported that *W. chondrophila* is able to enter and proliferate in two different fish cell lines suggesting a potential pathogenicity toward fishes that could constitute a potential reservoir of *W. chondrophila* [17]. This hypothesis is supported by the identified role of other *Chlamydia*-related bacteria, such as *Candidatus* Piscichlamydia salmonis [18] and *Candidatus* Clavochlamydia salmonicola [19], in epitheliocystis disease in fish.

4. The biphasic developmental cycle of *W. chondrophila*

W. chondrophila was first cultivated in bovine turbinate cells (BT). A cytopathic effect was observed about 2-3 days post infection (p.i.). Light microscopy revealed bacteria within cytoplasmic inclusions ranging in size from 0.2-0.4 μm [1]. Further studies in BT cells and in P388D1 mouse macrophages demonstrated a biphasic developmental cycle similar to these of other members of the *Chlamydiales* order. This cycle begins with infectious elementary bodies (EBs), which enter in the host cells. Once inside the cells, EBs convert to metabolically active reticulate bodies (RBs), which divide by binary fission (**Fig. 1**). Finally, RBs redifferentiate into EBs, which are released after cell lysis and can initiate a new infectious cycle [20]. EBs of *W. chondrophila* are characterized by a nuclear condensation (**Fig. 1A**). The mechanism regulating nuclear condensation is still poorly understood.

The bacteria of the *Chlamydiales* order also exhibit an alternative developmental stage characterized by an abnormal size, enlarged RB-like structures called aberrant bodies (ABs) [21]. Aberrant bodies are considered as persistent forms. In endometrial cells (Ishikawa), *W. chondrophila* develop into aberrant bodies as early as 72h p.i. and their number and their size increase over time [21]. When the culture medium is renewed every day, aberrant bodies are observed in only 10 % of bacteria containing vacuole compared to 100 % without medium change suggesting that aberrant bodies developed in response to starvation [21]. Interestingly, these persistent forms can revert to proliferating bacterial stages when fresh medium is added after 6 days p.i. [21]. We think that persistent forms, present in endometrial cells of the glandular decidual epithelium, will revert to proliferating bacteria in early days of pregnancy due to local metabolic changes, then leading to inflammation and miscarriage. This explains the occurrence of recurrent miscarriages [4, 6].

5. An overview of the *Waddlia* Genome

The complete annotated genome sequence is required for the thorough characterization of a genetically intractable obligate intracellular bacterium such as *W. chondrophila* since it allows the identification of virulence factors by homology and the understanding of its biology.

Waddlia chondrophila WSU 86-1044 genome consist of a circular chromosome of 2.1Mb and a circular plasmid of 15.6 Kb, with a G+C content of 43.8% and 37.6% respectively [22]. This corresponds to a 2 fold larger genome size compared to the genomes of *Chlamydiaceae* (**Table 1**). *W. chondrophila* possesses two copies of the gene *dnaA* whose positions are not linked to the minimum of the cumulative GC skew corresponding to the origin of replication [23-25]. The genome encompasses 2 rRNA operons, 27 tRNA genes and 1934 protein coding genes [22]. Among these proteins, 65% possess a putative function or a family membership, 13% are conserved hypothetical proteins and 23% show no similarity to known proteins [22]. A core set of *Chlamydiales* genes was determined and comprises, among others, all essential genes coding for proteins involved in DNA replication, transcription and RNA translation (493 genes). A large proportion of these genes encode for family specific proteins and proteins poorly conserved at the amino acid level.

The *W. chondrophila* plasmid is present in about 11 copies per cell and encodes 22 proteins that show no homology to other chlamydial plasmid proteins, except an integrase that exhibits 52% of identity to the plasmid integrase pCpA1_003 of *C. psittaci* [22]. The plasmid encodes two transposases and 7 chromosomal regions, ranging from 57 bp to 889 bp, that show 99% to 100% of identity with these two transposases [22]. Two adjacent genes present on the plasmid were also found integrated in the *W. chondrophila* chromosome sharing 88% of identity with their plasmid counterparts. One of these two genes encodes a protein homologous to MazF, an endoribonuclease of the toxin-antitoxin system, MazEF [22, 26]. This system could be involved in the plasmid stability during cell division. Surprisingly, the adjacent gene of *mazF* does not show sequence

similarities with *mazE* gene which encodes the labile antitoxin that prevents the lethal effect of the stable toxin, MazF [22]. The biological function of the plasmid is still unknown. *W. chondrophila* and almost all bacteria of the *Chlamydiales* order are considered as untractable organisms since targeted mutagenesis is not possible. Nevertheless, the presence of the *W. chondrophila* plasmid implies that plasmid-based genetic could be possible for this bacterium as recently reported for *C. trachomatis* [27], leaving an opened-window to genetic approaches.

Recently, the second isolate of *W. chondrophila* (strain 2032/99) identified in Germany was sequenced [28]. The general features of the chromosome are similar to *W. chondrophila* strain WSU 86-1044 (Table 1). Interestingly, *W. chondrophila* 2032/99 does not harbor a plasmid. However, 9 plasmid proteins have homologs in *W. chondrophila* 2032/99 genome distributed over 5 contigs, suggesting a chromosomal integration in this strain [28]. A common ancestor is shared by plasmids of *Chlamydiaceae* [29]. The presence of one protein encoded on the *W. chondrophila* plasmid homologue to a plasmid-encoded protein of the *Chlamydiaceae*, support the hypothesis of a common evolutionary origin of all chlamydial plasmids.

6. Metabolic secrets uncovered by the *Waddlia* genome

W. chondrophila genome analysis revealed that this strict intracellular bacteria is able to produce energy independently from its host through oxidative phosphorylation. *W. chondrophila* harbours a complete TCA cycle and glycolysis allowing production of reduced cofactors that are funnelled along the electron transport chain to generate ATP [22]. In addition to a V_1V_0 ATPase complex conserved in the *Chlamydiaceae*, *W. chondrophila* possesses a F_1F_0 ATP synthase complex increasing ATP production. This feature could improve the adaptation of *W. chondrophila* to energy-depleted environment. Moreover, a glyoxylate shunt is present allowing utilization of fatty acids or acetate as carbon source [22].

Interestingly, *W. chondrophila* possesses genes to produce at least ten of the twenty classical amino acids compared to *C. trachomatis* that is only able to produce three amino acids (**Table 2**). Unlike

197 *Chlamydia* spp., *W. chondrophila* genome completely lacks the genes involved in the tryptophan
198 biosynthesis. This bacterium seems also unable to synthesize tyrosine and phenylalanine [22].
199 Nevertheless, five transporters dedicated to general or specific aromatic amino acids such as
200 tyrosine and phenylalanine are encoded in the *W. chondrophila* genome. Several oligopeptide and
201 amino acid transporters or permeases are also identified allowing importing these compounds from
202 the environment [22].
203 Concerning lipid metabolism of *W. chondrophila*, additional enzymes for glycerophospholipid,
204 glycerolipid and sphingolipid metabolism are found compared to other members of the
205 *Chlamydiales* order. *W. chondrophila* also possesses a complete operon encoding the mevalonate
206 pathway in the biosynthesis of isoprenoid precursors that is not present in the *P. amoebophila* and
207 *C. trachomatis* genomes [22].
208 Unlike other *Chlamydiales*, *W. chondrophila* possesses all enzymes to convert L-glutamine in UMP
209 and all derivatives of pyrimidine. In contrast, this bacterium does not possess a complete purine
210 biosynthesis pathway but an active purine conversion, specific to *W. chondrophila,* is present. In the
211 *W. chondrophila* genome, no homolog to the *P. amoebophila* NAD+/ADP transporter were
212 identified but it seems that *W. chondrophila* is able to synthesize NAD from an intermediary
213 metabolite such as quinolinate or nicotinamide imported through another system. Interestingly, *W.*
214 *chondrophila* genome encodes five nucleotide transporters similar to *ntt_1,2* and *3* of *P.*
215 *amoebophila* potentially involved in the import of all nucleotides including ATP [22]. These genes,
216 likely originated by serial duplications, suggest that the *Chlamydiales* ancestors were already
217 intracellular and imported nucleotides from host cells more than 1 billion years ago [30].
218 Taking together, this genome analysis showed that *W. chondrophila* harbours many enzymes for the
219 synthesis of co-factors, nucleic acids and amino acids, and a complete central metabolism providing
220 energy necessary for biosynthesis. This analysis also suggests that *W. chondrophila* is more
221 independent of the host cell compared to other *Chlamydiales* and represent the best chance to date
222 for cultivating a member of the *Chlamydiales* order on an axenic medium.

7. Insights into the evolution of the chlamydial cell wall from *Waddlia* genome

The presence of peptidoglycan (PG) in the chlamydial cell wall has been debated for a long time. Recent studies demonstrated that *Chlamydiaceae* exhibit almost a complete pathway for peptidoglycan biosynthesis. Moreover, some PG synthesis enzymes are functional. Interestingly, PG synthesis genes are expressed primarily during reticulate body development and division suggesting a potential role of PG in cell division. *Chlamydiales*, including *W. chondrophila*, do not possess the *ftsZ* gene. FtsZ is a highly conserved tubulin-like protein involved in cell division of most bacteria. FtsZ localizes at midcell, polymerize to a ring structure called Z-ring and recruits proteins involved in cell division such as FtsI, FtsW and AmiC. The absence of *ftsZ* gene, in *W. chondrophila* and in other *Chlamydiales*, supports a FtsZ-independent cell division mechanism where PG could be involved. MreB, an actin homolog which localized at midcell, might represent a functional homolog of FtsZ in *W. chondrophila* (Jacquier *et al.*, submitted).

The inability to detect PG could be explained by the fact that all attempts were made on EBs [31]. In EBs, a highly disulphide-linked proteinaceous layer serves as a functional equivalent to PG [32]. In contrast, RBs contain less cross-linked membrane proteins. Several proteins form this cross-linked proteins complex via disulfide bond, including, OmcA and OmcB which belong to the polymorphic outer membrane protein (OMP) family, and the outer membrane protein beta-barrel porins OmpA and PorB.

OmpA was shown to be involved in different mechanisms such as attachment, infection, surface exposure and has antigenic properties. Previous studies, performed on *Chlamydia* spp., demonstrated that OmpA is an adhesin promoting non-specific interaction with host cells [33]. OmpA may also act as porin during chlamydial proliferation [34]. Surprisingly, a novel OMP family comprising 11 putative β-barrel proteins or porins with C-rich signature was identified in *W. chondrophila* genome [22]. Characterization of these putative adhesins is in progress.

Homologs to *omcA* and *omcB* genes were detected in the *W. chondrophila* genome. Furthermore, the five adjacent genes shares similarities with the N-terminal region of OmcA/B and possess conserved cystein residues, supporting an extended *omc* family in *W. chondrophila*. OmcB protein of *C. trachomatis* was shown to be a surface-exposed protein that functions as an adhesin suggesting the potential role of these Omc family proteins in adhesion [35].

A putative autotransporter protein sharing similarities with *P. amoebophila* gene was also identified. This protein likely belongs to the chlamydial polymorphic membrane protein (PMP) family. The pmp proteins are classical autotransporters with a passenger domain surface-localized or secreted, responsible of their function such as adhesion [36]. The *C. trachomatis* pmp family has 9 members and *C. pneumoniae* 21 members [37, 38]. This autotransporter could function as an adhesin necessary for *W. chondrophila* infection.

Type III secretion system (T3SS), also called injectisome, translocates bacterial proteins into host cell cytoplasm [39]. This machinery is composed of about 25 proteins, localized in the bacterial cell envelope, i.e. the inner and the outer membrane, and is able to span eukaryotic plasma membrane to inject bacterial proteins [40]. Many pathogens, such as *Salmonella* and *Yersinia*, possess a T3SS in order to hijack host cell processes [41, 42]. The T3SS components are highly conserved compared to its effectors which are mostly species-specific. All *Chlamydiales* genomes encode an almost complete T3SS distributed in 4 clusters of genes. This unusual spread of T3SS-encoding genes along the chlamydial chromosome indicates that the T3SS was already present in the common ancestor of *Chlamydia* and *Waddlia* which diverged about 1 billion years ago. Like other members of the *Chlamydiales* order, *W. chondrophila* possesses almost all genes coding the T3SS. The main differences reside in the effector proteins of *W. chondrophila*. The only waddlial effector identified by homology to known *Chlamydiaceae* effectors, is Pkn5, suggesting that filling the gap in this area will probably improve our knowledge of *W. chondrophila* biology.

The functionality and the role of the T3SS in *Waddlia* survival and proliferation in human macrophages were demonstrated by using T3SS specific inhibitors, which inhibited bacterial

growth in human macrophages. It could be interesting to assess the role of the T3SS in specific events such as adhesion, internalization and recruitment of mitochondria or endoplasmic reticulum (ER).

8. Growth of *Waddlia* in different cell lines

W. chondrophila was first reported to be able to grow in BT cells and in mouse macrophages. *W. chondrophila* can also be cultivated in buffalo green monkey cells, Mc Coy cells, human fibroblasts [2], peripheral blood mononuclear cells (PBMC) [43], Vero cells, A549 pneumocytes and in Ishikawa endometrial cells [21].

In PBMC-derived macrophages, the entry of bacteria is taking place during the first 8h after inoculation. In about 24-36 h, the number of bacteria increase by 2-3 logs and the generation time is estimated to 2.8 h. At 48 h p.i., a cytopathic effect is observed with a host cell survival rate decreasing to 50%. This cytopathic effect is associated to the increase in bacterial number at 24 h p.i. [43].

In Vero cells and A549 pneumocytes, the growth kinetic of *W. chondrophila* is very similar to that observed in PBMC-derived macrophages, with a latent phase lasting 8 h during which bacteria enter in the cells and differentiate into RBs [21]. This phase is followed by a proliferation phase where the number of bacteria per cell increase by 2.5 log between 8 to 24 h p.i.. The infected cells are then lysed and released bacteria can initiate a new infection cycle. At 72 h p.i., the number of bacteria reaches a plateau because all the cells are infected. In Ishikawa endometrial cells, the growth kinetic of *W. chondrophila* is similar until 24 h p.i. [21]. The bacterial number then slightly decreases between 24 h and 96 h, and no second round of infection was observed. A cytopathic effect of *W. chondrophila* is shown in Vero and A549 cells by measuring cell viability. In contrast, in Ishikawa cells, the bacteria do not exert a significant cytopathic effect [21]. This suggests that the bacteria remain in the cell without proliferation after 24 h p.i. and without inducing cell lysis. Interestingly, aberrant bodies appeared in Ishikawa cells at 72 h p.i. (See section 4.).

As *Candidatus* Piscichlamydia salmonis, a new family member belonging to the *Chlamydiales* order, is responsible of a common infection in many fish species, called epitheliocystis, the permissivity of two fish cell lines, to *W. chondrophila* was investigated [17]. By quantitative PCR and immunofluorescence, it was shown that *W. chondrophila* is able to proliferate in these two fish cell lines, EPC-175 (Fathead Minnow) and RTG-2 (rainbow trout). This rapid proliferation was associated with a cytopathic effect [17]. Thus, *W. chondrophila* can be used to develop an *in vivo* model of epitheliocystis in fish species.

9. Cell biology studies provide insight into the relationship between *Waddlia* and its host

As previously reported in this review, *W. chondrophila* extensively proliferate within human macrophages (PBMC) and induce cell lysis. Different strategies can be adopted by bacterial pathogens to survive within macrophages. The bacterial pathogen can avoid delivery in a degradative compartment such as lysosomes or develop strategies to survive in this compartment. A common tactic is to hijack the endocytic pathway to finally create a proliferation niche that has unique properties. In order to understand how *W. chondrophila* survive in human macrophages, intracellular trafficking was studied [44]. The bacterial intracellular trafficking is followed by the successive acquisition of organelle-specific marker on the bacteria-containing vacuoles (BCV). It was shown that *W. chondrophila* transiently acquired the early endosome marker, EEA1, with a peak at 15 min [44]. Interestingly, *W. chondrophila* never co-localize with LAMP1 and v-ATPase, two late endosome-lysosome markers. Moreover, by using the Lysotracker probe, a fluorescent acidotrophic probe, to monitor phagosome acidification, it was shown that vacuoles containing *W. chondrophila* never co-localize with this probe suggesting that the vacuoles are not acidic [44]. These results demonstrate that *W. chondrophila* BCVs interact with early but not late endosomal compartment and that *W. chondrophila* rapidly evade the endocytic pathway [44].

Host cell mitochondria are rapidly and gradually recruited to *W. chondrophila* BCVs, with 50% positive at 2 h p.i. and more than 80% at 8 h p.i. (**Fig. 2A**). Electron microscopy shows an intimate

326 association between mitochondria and *W. chondrophila* BCVs (**Fig. 2B**). Some vacuoles containing
327 *W. chondrophila* are already surrounded by mitochondria as early as 30 min p.i. suggesting that
328 recruitment start just after phagocytosis [44]. A specific association between *W. chondrophila* and
329 host cell mitochondria was already shown in 1990 using BT cells and P388D1 mouse macrophages
330 [1, 45]. A clear association between host cell mitochondria and the bacteria-containing vacuoles
331 was also observed in Vero and Ishikawa cells [21]. One hypothesis could be that mitochondria are
332 required to support bacterial proliferation. After treatment of PBMC-derived macrophages,
333 simultaneously, with Nocodazole and Cytochalasin D, which are respectively, a microtubule-
334 depolymerizing agent and an actin microfilament-depolymerizing agent, mitochondrial recruitment
335 on the BCVs and bacterial growth are inhibited without affecting bacterial entry [44]. However,
336 when used alone, these inhibitors did not impair the mitochondrial recruitment suggesting that two
337 independent mechanisms, i.e. actin-dependent and microtubule-dependent, are involved in this
338 recruitment.
339 To further characterize *W. chondrophila* trafficking, acquisition of ER (Calnexin) and Golgi (GM
340 130) marker were assessed. At 8 h p.i., when bacterial proliferation starts, *W. chondrophila* BCVs
341 are clearly positives for the ER marker but not for the Golgi marker. The *W. chondrophila* BCVs
342 positives for Calnexin or PDI (another ER marker), increase from 4 to 8 h. No close association is
343 observed between BCVs and ER membrane. The *W. chondrophila* inclusions are surrounded by two
344 layers, an inner layer composed of mitochondria and an outer layer composed of ER (**Fig 2A** and
345 **2B**). This results from a sequential process where vacuoles containing *W. chondrophila* first
346 associate with mitochondria and secondly recruit ER.
347 To evaluate the potential role of the COPI-dependent ER-Golgi vesicular transport on the
348 generation of proliferation niche sustaining *W. chondrophila* proliferation, infected cells were
349 treated with BFA which inhibits ARF1, the small GTPase regulating COPI vesicle formation. It was
350 shown that the intracellular growth is inhibited when cells are treated with BFA from 2.5 to 5.5h
351 p.i., supporting the role of the COPI-dependent ER-Golgi vesicular transport during a short period

for the biogenesis of the *Waddlia* proliferative organelle [44].

In summary, after phagocytosis, the *W. chondrophila* BCVs transiently interact with early endosomes and rapidly evade from the endocytic pathway. Host cell mitochondria are then gradually recruited and form an intimate association with the BCVs. Finally, ER co-localized with vacuoles containing *W. chondrophila*. At 8h p.i., mature *W. chondrophila* proliferative vacuoles are formed with intimate association with mitochondria and ER co-localization (**Fig. 3**). Further experiments are required to shed light on molecular mechanisms involved in the subversion of the endocytic pathway and in the establishment of proliferative vacuoles.

Recently, it was reported that *W. chondrophila* possesses a functional catalase (KatA) [46]. Catalases are involved in the degradation of reactive oxygen species (ROS) produced in macrophages, by the NADPH oxidase, as microbicidal effectors. KatA is likely a second factor involved in the resistance to macrophage killing in addition to the avoidance of the lysosomal degradation.

10. Conclusions

In conclusion, *W. chondrophila* is an emerging pathogen likely involved in human miscarriage and respiratory disease, as well as in bovine abortion. This bacterium is also considered as a potential zoonotic agent. Altogether, the study of the biology of this zoonotic agent is essential to prevent *Waddlia*-associated morbidity.

Two aspects of the *W. chondrophila* biology, shared with other *Chlamydiales*, make difficult the study of this bacterium: 1) *W. chondrophila* is an obligate intracellular bacteria; 2) *W. chondrophila* is a genetically untractable organism. Interestingly, *W. chondrophila* is more independent of the host compared to other *Chlamydiales* and is probably a good candidate for growth on cell free medium.

W. chondrophila possesses a plasmid which suggests that plasmid-based genetic could be possible. In this respect, development of axenic medium (cell free) and transformation techniques would

considerably facilitate the experiments and give us the opportunity to better explore the *W. chondrophila* biology, a still poorly known landscape. This would significantly improve our knowledge on *W. chondrophila* biology that could be extended to other members of the *Chlamydiales* order.

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512 **Table 1. Genome comparison between *Chlamydia trachomatis* D/UW-3/CX, *Waddlia***
513 ***chondrophila* WSU 86-1044 and *Waddlia chondrophila* 2032/99 (adapted from [28])**

514

	Chlamydia trachomatis D/UW-3/CX [47]	*Waddlia chondrophila* WSU 86-1044 [22]	*Waddlia chondrophila* 2032/99* [28]
Genome size	1'042'519	2'116'324	2'141'577
GC content	41%	44%	43%
% coding	89%	92%	93%
Predicted CDSs	895	1934	2'028
Nb of tRNAs	37	37	34
Nb of rRNA operons	2	2	2
Plasmid size	7'493	15'593	-

515

516 *Unfinished genome

517

518

519
520 **Table 2. Presence of genes, in *Chlamydia trachomatis* D/UV-3/CX and *Waddlia chondrophila***
521 **WSU 86-1044 genome, allowing production of amino acids (adapted from [22]). Genes present**
522 **are highlighted in green and these absent in red.**
523

Amino acids	*Chlamydia trachomatis* D/UW-3/CX	*Waddlia chondrophila* WSU 86-1044
Alanine		
Arginine		
Asparagine		
Aspartate		
Cysteine		
Glutamate		
Glutamine		
Glycine		
Histidine		
Isoleucine		
Leucine		
Lysine		
Methionine		
Phenylalanine		
Proline		
Serine		
Threonine		
Tryptophan		
Tyrosine		
Valine		

524

Figure legends

Figure 1. Electron microscopy of macrophages infected with *W. chondrophila* at 16h p.i..

A. EBs (arrows) and RBs (arrow heads) present in one inclusion. Scale bar represents 2µm.

B. Binary fission of RBs. Scale bars represent 1µm on the top panel and 0.5µm on the bottom panel.

Figure 2. Mitochondrial recruitment to *W. chondrophila*-containing vacuoles in Ishikawa cells (A). *W. chondrophila* inclusion, in macrophages, surrounded by a bilayer of mitochondria and ER (B).

A. Confocal microscopy analysis at 12h p.i.. Mitochondria are stained with mitotracker (in red) and *W. chondrophila* are detected with antibody (in green).

B. Electron microscopy analysis at 8h p.i.. Arrows indicate ER. M: mitochondria. Scale bars represent 2µm on the right panel and 0.5µm on the left panel.

Figure 3. Adhesion, internalization and trafficking of *W. chondrophila* during infection of macrophages.

1. Adhesion is a critical step for bacterial infection. Several protein families could be involved in this step such as the T3SS, the Omc family, the OmpA family and the pmp family.

2. Internalization could be induced by the T3SS effectors translocated directly to the host cell cytoplasm or to the BCV membrane. Once inside the cell, *W. chondrophila* BCVs transiently interact with the early endosome. The catalase KatA degrades ROS produced by the NADPH oxidase in the phagosome lumen.

3. After evasion of the endocytic pathway, BCVs quickly recruit mitochondria. This recruitment could be induced by T3SS effectors. Mitochondria recruitment and proliferation are inhibited by nocodazole and cytochalasin B, microtubules and actin filaments inhibitors, respectively.

4. The COPI vesicular transport is required to reach the ER, as BFA treatment decrease bacterial proliferation. T3SS effectors could be involved in this step. Finally, *W. chondrophila* proliferate in this particular compartment surrounded by a bi-layer of organelles, an inner layer composed of mitochondria and an outer layer composed of ER.

Little is known on the molecular mechanisms involved in adhesion, internalization and trafficking steps during *W. chondrophila* infection. Further investigations on the potential role of the T3SS effectors, the Omc family, the OmpA family and the pmp family in these steps are required to improve our knowledge on *W. chondrophila* pathogenicity.

Figure 1

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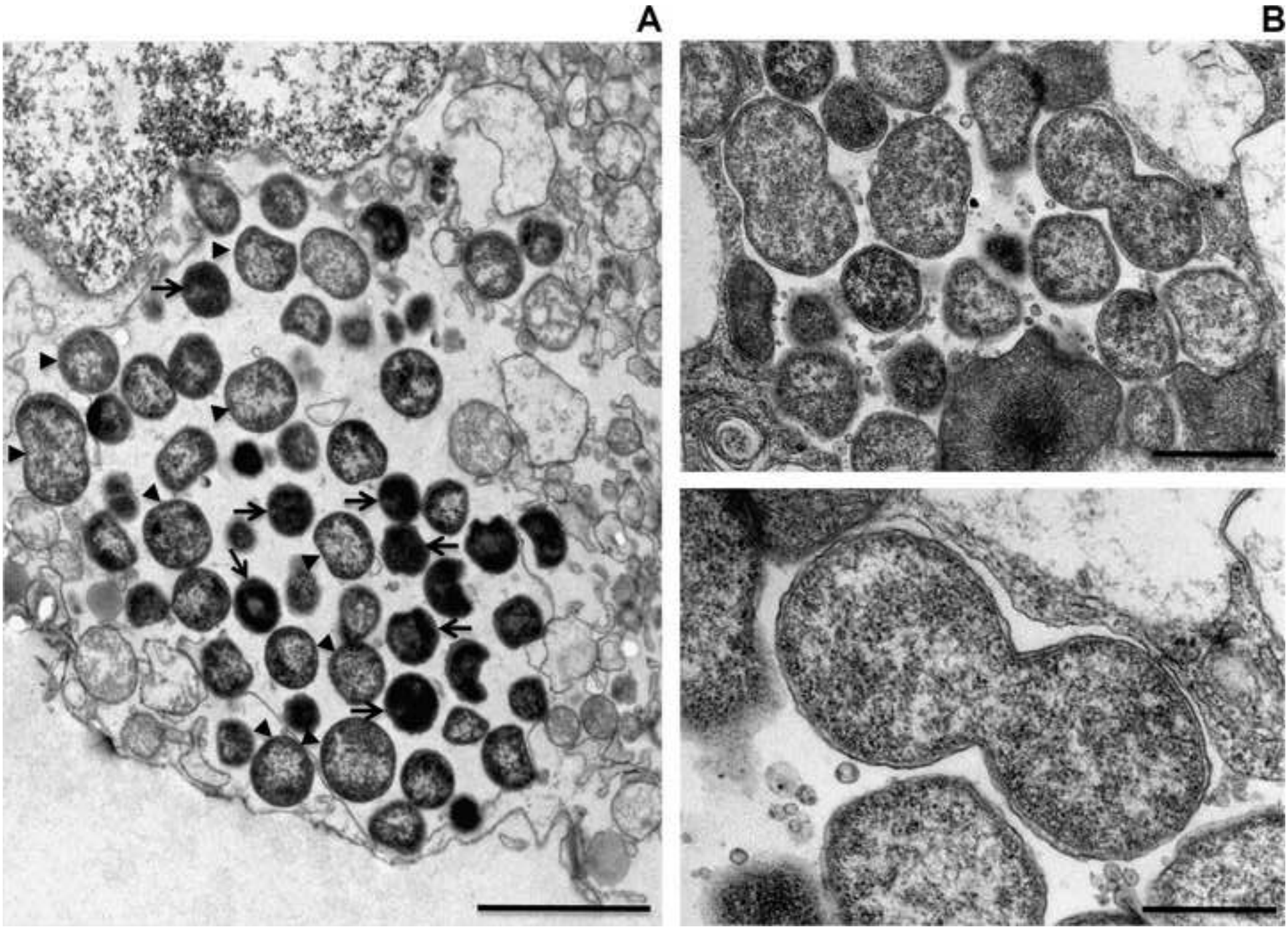

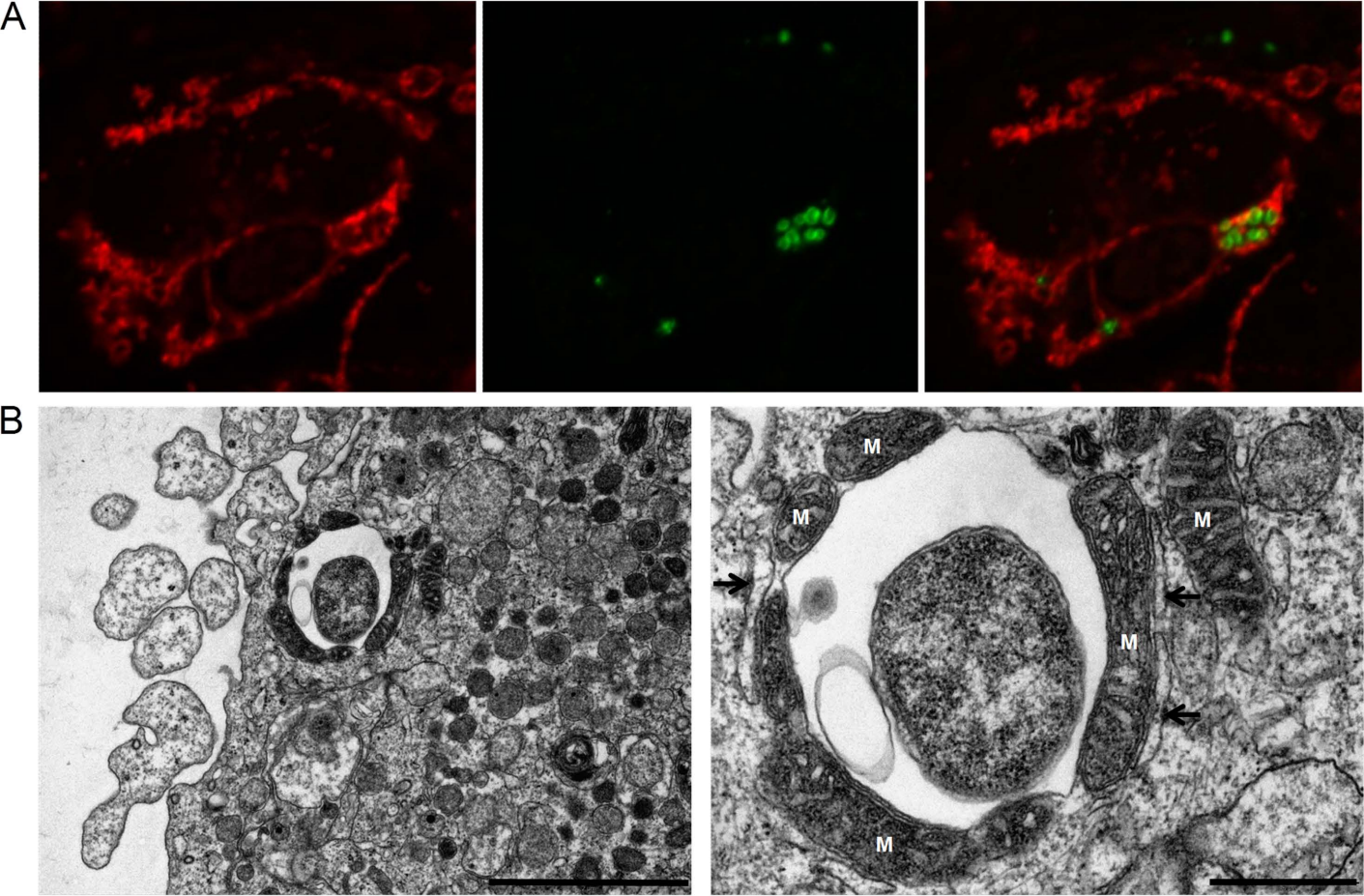
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Figure 3

T3SS

Omc family

OmpA family

Pmp

Effectors

1. Adhesion

2. Internalization

?

?

ROS

KatA

Early endosome

Late endosome

Lysosome

?

?

COPI vesicular transport

BFA

3. Mitochondria recruitment

Nocodazole + Cytochalasin B

4. ER co-localization