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Original research paper

Biology and intracellular pathogenesis of high or low virulent *Chlamydophila psittaci* strains in chicken macrophages

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Short Title: Biology of *Cp. psittaci* in chicken macrophages

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Abstract - Within a few days post infection of SPF turkeys, highly pathogenic *Chlamydophila (Cp.) psittaci* genotype A and D strains can be found in blood monocytes/macrophages, while this effect is less pronounced for infection with a milder genotype B strain. To elucidate on the observed difference, we studied the developmental cycle of avian *Cp. psittaci* strains of varying virulence in a matched avian monocyte/macrophage cell line (HD11) by electron microscopy and immunofluorescence and determined the gene transcription of 26 Type III secretion related genes and six control genes upon infection of HD11 cells. The genotype A (84/55) and D (92/1293) strains 1) clearly induced actin recruitment to the site of entry, 2) initiated host cell degeneration at earlier time points, and 3) survived and proliferated better when compared to the milder CP3 strain. Strain 84/2334, genetically intermediate between *Cp. psittaci* and *Cp. abortus*, did not induce actin recruitment. Limited mRNA transcripts for the cell division genes *ftsW* and *ftsK* were in agreement with the observed low replication of *Cp. psittaci* in these host cells. The results also indicated that genes coding for the structural components of the Type III secretion system were transcribed earlier compared to an infection in epithelial cells. Based on the presented results, we postulate that upon infection of blood monocytes/macrophages, *Cp. psittaci* deliberately limits its replication and immediately arms itself to infect other cells elsewhere in the host, whilst using the monocytes/macrophages as a quick transport vehicle.

Keywords:

***Chlamydophila psittaci* / Type III secretion / macrophages / mitochondria / actin**

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1. INTRODUCTION

The obligate intracellular pathogen *Chlamydophila (Cp.) psittaci* can infect both mammalian and avian hosts, with *Psittacidae* and *Columbiformes* among the most frequently infected avian orders (Kaleta and Taday, 2003). Survival and replication within a membrane bound vacuole in the host cell is essential to chlamydial pathogenesis. During their unique biphasic life cycle, *Chlamydiaceae* are able to transfer effector molecules in the host cell by means of a Type III secretion system (T3SS). Genes encoding the T3SS and its effectors seem to be temporally transcribed as early (1.5 to 8 h p.i.), middle (12 to 18 h) or late (by 24 h) in the developmental cycle (Slepenkin et al., 2003), in agreement with the transition from elementary body (EB) to reticulate body (RB), RB multiplication and RB to EB redifferentiation.

Serovars largely correspond to the currently accepted classification in *ompA* (coding for MOMP) genotypes defined by sequencing (Everett et al., 1999; Geens et al., 2005). Infection with genotypes A and D results in a more elevated pathogenicity for turkeys than genotype B: for genotype B, the incubation period is longer, maximal replication is delayed, the period during which bacteria are observed in the same tissue is shorter and tissue tropism seems to be less extensive (Vanrompay et al., 1995). During the initial replication in epithelial cells from the turkey respiratory system, macrophages are attracted, normally acting as a first line of (innate) defense against infection (Vanrompay et al., 1994a) and *Cp. psittaci* has already been detected in monocytes/macrophages recognized by the specific marker KUL04 (Vanrompay et al., 1995). The present study therefore aims to further shed light on the complex interaction between *Cp. psittaci* and its avian host by studying the biology and intracellular pathogenesis of high and low virulent *Cp. psittaci* strains in the chicken monocyte/macrophage cell line HD11.

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2. MATERIALS AND METHODS

2.1. Organism and cell culture

The HD11 chicken monocyte/macrophage cell line (Beug et al., 1979) was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum, 1% sodium pyruvate, 1% L-glutamine and 0.5% gentamicin (all products from Invitrogen, Merelbeke, Belgium), further referred to as HD11 culture medium. Cultures were incubated at 5% CO_2 and 37 °C. Four different *Cp. psittaci* strains were used in this study: 84/55 [isolated from the lung of a budgerigar, *ompA* genotype A (Vanrompay et al., 1996)], CP3 [isolated from a pigeon, genotype B (Page and Bankowski, 1959)], 92/1293 [isolated from a pooled homogenate of lungs, cloaca and spleen of diseased turkeys, genotype D (Vanrompay et al., 1993)] and 84/2334, an intermediate strain in the evolution of *Cp. abortus* from *Cp. psittaci*, isolated from a yellow-headed Amazon parrot (Van Loock et al., 2003). All strains were cultured and titrated as described before (Vanrompay et al., 1996).

2.2. Transmission electron microscopy

HD11 cells were seeded on Cytodex 3 microcarriers (GE Healthcare, Diegem, Belgium) essentially as described before (Vanrompay et al., 1996). Briefly, 60 mg of microcarriers were hydrated and autoclaved according to the manufacturer's instructions, and subsequently resuspended in HD11 culture medium. Before seeding on the microcarriers, HD11 cells were grown for 72 h with 10% FCS to obtain a large fraction of adherent macrophages. The adherent cells were trypisinized, pelleted and 2 x 10^7 cells were added to the microcarriers in a Petri dish. After incubation for 5 h at 37 °C and 5% CO_2, the non-adherent cells were washed away with sterile PBS and

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cells and microcarriers were resuspended in HD11 medium with 10% FCS. The microcarrier cultures were incubated overnight on a rotating platform, distributed into six new Petri dishes with fresh HD11 culture medium to maintain optimal growth conditions and further incubated on the rotating platform for 24 h. Contents of each Petri dish were centrifuged for 4 min at 135 x g and pellets were resuspended in 20 ml HD11 medium supplemented with 5.5 mg/ml glucose (Sigma, Antwerp, Belgium) and each of the four *Cp. psittaci* strains under study at a multiplicity of infection (MOI) of 100. Infected microcarrier cultures were incubated at 37 °C and 5% CO_2 for 3 h whereafter the inoculum was removed by centrifugation and a washing step with sterile PBS. Cultures were then resuspended in HD11 medium supplemented with glucose and further incubated on a rotating platform until sample taking at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 21, 26, 30, 36, 42, 45, 50, 52 and 72 h post inoculation (p.i.). For the negative control, the medium was replaced with HD11 medium with glucose. Infection was checked on dried and acetone fixed spotted microcarriers on a glass slide using a direct immunofluorescence staining at 30 and 50 h (IMAGEN Chlamydia, Oxoid, Drongen, Belgium) as previously described (Vanrompay et al., 1994b). At each time point 100 µl of microcarrier culture was added to 1 ml of Karnovsky fixative (Karnovsky, 1965) and fixed overnight at 4 °C. Afterwards, the fixative was replaced with sodium cacodylate buffer (0.1 M in BiDest, pH 7.4, Sigma) until further processing. Post-fixation was performed using 4% osmiumtetroxide and samples were dehydrated in a step gradient of ethanol at room temperature. The samples were transferred to 100% alcohol/Spurr's resin (1:1) at 4 °C overnight, brought to 100% alcohol/Spurr's resin (1:2) for 8 h (4 °C), and transferred to 100% Spurr's resin and left overnight at 4 °C. Polymerization was performed at 70 °C for 16 h. Sections (70 nm thick) were made using a Reichert

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Ultracut S Ultramicrotome. Formvar-coated single slot copper grids were used. Sections were post-stained with a Leica EM stain for 30 min in uranyl acetate at 40 °C and 10 min in lead citrate stain at 20 °C. The grids were examined with a JEM 1010 Jeol electron microscope equipped with imaging plates which were scanned digitally (Ditabis, Pforzheim, Germany).

2.3. Immunofluorescence assays

We tested whether *Cp. psittaci* strains of different virulence are all able to induce actin polymerization at the site of attachment and entry and whether mitochondria are being attracted to the inclusions. HD11 cells (3 x 10^5 / ml) were seeded on sterile glass coverslips (13 mm) at the bottom of Chlamydia Trac Bottles (Bibby Sterilin Ltd., Stone, UK) for 24 h at 37 °C and 5% CO_2 and subsequently inoculated at an MOI of 100, performing a centrifugation step (1 000 g) at 4 °C for 15 min to synchronize irreversible attachment and entry. One ml of HD11 culture medium with glucose was added and cells were further incubated at 37 °C and 5% CO_2 until PFA fixation and permeabilization with saponin at 15 min, 8 h, 20 h or 28 h p.i. as described before (Beeckman et al., 2007).

Colocalization between *Cp. psittaci* and actin polymerization was assessed using AlexaFluor 488 coupled phalloidin (Molecular Probes, Invitrogen) and an indirect staining for *Cp. psittaci* (Beeckman et al., 2007).

Distribution of the mitochondria was visualized by use of a cross-reacting monoclonal antibody against turkey sperm mitochondria (Korn et al., 2002) (kindly provided by Nancy Korn, Clemson University, Dept. of Animal and Veterinary Sciences, South Carolina). Briefly, cell layers were first incubated with 25 µl of reconstituted hybridoma supernatant for 1 h at 37 °C, rinsed and then incubated with 25 µl of an

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1:25 diluted AlexaFluor 546 goat-anti-mouse secondary antibody (Molecular Probes), again for 1 h at 37 °C. Subsequent rinsing was followed by detection of the chlamydial organisms by a direct immunofluorescence staining (IMAGEN Chlamydia, Oxoid).

All dilutions and washing steps (three) were performed with 1% BSA (Sigma) in PBS. After a final rinsing with PBS, nuclei were visualized in all samples with DAPI (100 µl at a concentration of 100 ng/ml in BiDest for 5 min at room temperature). A final rinsing with BiDest was followed by mounting of the coverslips using Mowiol (Calbiochem, VWR, Haasrode, Belgium) with 0.01% PPD (Sigma) (Valnes and Brandtzaeg, 1985). Images were acquired using a Radiance 2000 confocal laser scanning microscope (Bio-Rad, Nazareth, Belgium, 600 x). Chlamydial replication at 8 h, 20 h and 28 h was assessed using a scoring system from 0 to 5 as previously described (Van Loock et al., 2006).

2.4. Transcription analysis of control genes and T3S genes

As T3S plays an important role in different phases of the chlamydial life cycle, we studied the gene transcription profile of a panel of T3S related genes (26 in total) for different *Cp. psittaci* strains in HD11 cells in order to determine whether virulence could be linked to differences in T3S gene transcription profiles. Total RNA was extracted from 1.5 x 10^6 cells infected at an MOI of 100 (time points 1, 2, 4, 8, 12, 18, 24, 36 and 48 h p.i.) and checked for DNA contamination. All the experiments were performed twice. Instead of 0.5 µg, one microgram of total RNA (spiked with 10 ng of coliphage control RNA) was reverse transcribed using selective hexamers (5'-TTANNN-3' and 5'-CTANNN-3') and qPCR using SYBRGreen technology was performed *in duplo* on each sample. Reverse transcription, primer sequences, cycling

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and normalization were exactly as performed earlier (Beeckman et al., 2008). In addition, primers were developed to determine the transcription of *groEL-1*, *ompA* and *omcB* as markers of the early, middle and late phase of the chlamydial developmental cycle, respectively (Shaw et al., 2000; Slepenkin et al., 2003), the cell division related genes *ftsW* and *ftsK* (Gerard et al., 2001), as well as *dnaK* (*hsp70*) (Cross et al., 1999). An overview of the RT-qPCR primers, designed based on the currently unpublished genome of *Cp. psittaci* 6BC (P. Bavoil & G. Myers, personal communication), can be found in Table 1.

To compare the two independent samples (namely gene transcription ratios of 32 genes from two independent infection experiments per strain, each 16S rRNA normalized) at the same time point, a Paired Student's t-test was used (SPSS Inc., Chicago, Illinois, US). Secondly, an analysis of variance (ANOVA, SPSS Inc.) with post-hoc analysis (both Tukey HSD and Tukey-b) was performed along the time axis to determine significant upregulation time points for each gene for each of the four *Cp. psittaci* strains under study.

2.5.Titration of infectious progeny on BGM cells

To quantify chlamydial replication in HD11 cells, tenfold dilution series from supernatant from cells infected for 48 h with either of the four strains was inoculated onto BGM cells as described before (Vanrompay et al., 1992). Cells were fixed at 48 h p.i. with methanol and stained with IMAGEN Chlamydia (Oxoid). The number of inclusion forming units (IFU/ml) was determined by the method of Spearman & Kaerber (Mayr et al., 1974).

3. RESULTS

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3.1. Transmission electron microscopy

Predominantly monocytes were present in the early infection stages, differentiating into inflammatory macrophages later on in the course of the infection. For all *Cp. psittaci* strains, internalization was often accompanied by an invagination of the HD11 host cell membrane (Figure 1B) or the presence of clathrin-coated pits (except for 84/2334, Table 2). Subsequently, the chlamydial organisms pursued their way to the nucleus and from 6 h p.i. on (8 h for strain 84/55), *Cp. psittaci* EBs or IBs could be observed in the vicinity of the host cell nuclear membrane (Figure 1D). However, not all internalized bacteria arrived at the nucleus, since fusion of the nascent inclusion with lysosomes was detected for all strains (Table 2, Figure 1C), the earliest (2 h p.i.) for the low pathogenic strain CP3. These phagolysosomes then further evolved to the typical morphology given in Figure 1E. For inclusions efficiently escaping the phagolysosomal fusion, the inclusion membrane faded or opened up to the host cell cytoplasm, thus releasing the chlamydiae. As such, a direct interaction between free RBs and the nuclear envelope could sometimes be observed (Figure 1F). However, intact inclusions were also present, containing a limited number of odd shaped RBs (Figure 1G) and at 45 h p.i. phagocytosis of cell fragments could often be observed.

The high virulent strains already induced host cell degeneration from 6 h p.i. on, while for the milder strain CP3 this could be observed six hours later (Table 2). This was characterized by a marked degeneration of the mitochondria (enlarged and cristae more open), dilatation of the endoplasmatic reticulum and nuclear condensation and fragmentation (Figure 1I), which is indicative for apoptosis.

Noteworthy is the bridging between extracellular *Cp. psittaci* 84/2334 and the host cell membrane at 8 h p.i. (Figure 1H).

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3.2. Immunofluorescence assays

In order to investigate the host cell reaction to infection with *Cp. psittaci* strains of different virulence, HD11 cells were stained for host cell actin (15 min p.i.) or mitochondria (8, 20 and 28 h p.i.), bacteria and the nucleus (all time points).

At 15 min p.i., strains 92/1293 and 84/55 had attached greatly to the cells, thereby inducing strong host cell actin polymerization at the site of attachment (Figure 2: A and C). Both the level of attachment and the accompanying actin polymerization were less pronounced for inoculation with the less virulent strain CP3 (Figure 2B). Attachment of *Cp. psittaci* 84/2334 was higher than observed for CP3, but lower compared to the two highly virulent strains 92/1293 and 84/55. However, no colocalization of actin polymerization and the bacteria could be observed for this strain (Figure 2D).

No significant differences could be observed in the staining pattern for the mitochondria or the nuclei at **8 h p.i.** with the four *Cp. psittaci* strains (Figure 3, first column). As could be expected from the lower attachment of CP3 at 15 min p.i., fewer bacteria had entered the host cells compared to the other strains. The highest amount of bacteria at this time point could be observed for 92/1293, while comparable amounts of 84/55 and 84/2334 had entered the cells. As confirmed by the electron microscopy experiments, bacteria could already be observed in the vicinity of the nucleus for all strains. Twelve hours later (**20 h p.i.**), bacteria had further developed, but no dense uniformly stained inclusions could be observed yet, except for the mini-inclusions observed for CP3 (Figure 3, second column). For 84/55, an association between the inclusions and host mitochondria could be observed, although not for every inclusion, and heavily infected host cells showed clear signs of degeneration.

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Similar effects could be observed for the genotype D strain (92/1293), and more scattered green fluorescent staining in the host cells was observed. For 84/2334, more bacteria could be visualized compared to an infection with CP3. Host cells had degenerated less when compared to an inoculation with 92/1293 or 84/55, while for CP3 no degeneration of the HD11 cells could be observed. No clear association between CP3 or 84/2334 and mitochondria could be noticed at this time point (Table 2). At **28 h p.i.**, bacterial development had proceeded for all strains (Figure 3, third column), but no large, dense, uniformly stained inclusions could be visualized. In concordance with earlier time points, strains 92/1293 and 84/55 had proliferated the most, while growth of CP3 was still very limited. Typically, plenty, clearly separate, mini-inclusions could be observed for 84/2334. Mitochondria were no longer stained in all host cells, especially for 92/1293 or 84/55 inoculated cells, possible as a result of the more pronounced host cell degeneration (Figure 1, Table 2). Additionally, inoculation with *Cp. psittaci* strains of different virulence had an effect on the size of the host cell nuclei, as visualized by DAPI. Condensed nuclei could be observed in heavily infected cells, whereas the surrounding not or only moderately infected cells had enlarged nuclei when compared to the negative controls. For strain 84/2334, both infected and uninfected cells in the same sample had enlarged nuclei, comparable in size to those of the uninfected host cells in 92/1293 or 84/55 inoculated samples. These effects could already be observed at 20 h p.i., but were more pronounced at 28 h p.i. and were not present in CP3 inoculated samples.

Assessing bacterial proliferation at **48 h p.i.**, immunofluorescence staining of *Cp. psittaci* 84/55 or 92/1293 infected monocytes/macrophages revealed a significant bacterial load (most for 92/1293). Multiple inclusions could be observed per cell and host cells were degenerating. For inoculation with CP3, results were in concordance

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with previous time points: CP3 proliferation was limited, giving rise to multiple mini-inclusions per cell. As observed at earlier time points, multiple 84/2334 mini-inclusions could be observed per cell, as well as a few larger inclusions (data not shown).

3.3. Transcription analysis of control genes and T3S genes

Firstly, the transcription pattern of a number of control genes (*groEL-1*, *ompA*, *omcB*, *ftsW*, *ftsK* and *dnaK*) was determined. All newly developed primer couples yielded an amplicon of the expected size when tested on *Cp. psittaci* 92/1293 genomic DNA. However, for *groEL-1*, no amplicon could be obtained when tested on copy DNA, despite several PCR optimization steps. An overview of the upregulation time points is given in Table 3.

The bacterial cell division genes *ftsW* and *ftsK* were only poorly transcribed and transcription dropped below the detection limit after 12 h p.i, except for 92/1293 (no transcription after 24 h). For *ompA*, a typical mid-cycle gene, transcription was predominantly observed from 8 to 18 h p.i., except for 84/55, where limited transcription was already observed from 1 h p.i. on. The typical late-cycle gene *omcB* was transcribed from 1 h p.i. on for all four strains, although very limited, and reached its peak transcription at 8 h p.i., followed by a secondary upregulation of transcription late in the infection cycle.

As for the transcription of T3S related genes, *incB* and *copB2* were excluded from further analysis, because no working primers could be developed. None of the strains under study transcribed the *sctS* or *ca530* genes to a detectable level. An overview of significant gene transcription upregulation time points along the time axis, as

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determined by ANOVA, is given in Table 4 and Table 5 and the most remarkable results will be summarized hereafter.

For *Cp. psittaci* **84/55** (Table 4, column 3-5), no transcription could be observed for *sctU* (cluster 1), *copB1* (cluster 2.2), *sctN* (cluster 3) or *cap1* (non-clustered) and both *incA* and *incC* (non-clustered) were shown to be transcribed from 1 h p.i. on. Transcription results of the both translocon-coding gene clusters revealed that cluster 4 (*scc3*, *copB2*, *copD2*) was generally spoken transcribed later than the *scc2-copB1-copD1* cluster.

Striking gene transcription results for **CP3** infection in chicken macrophages (Table 4, column 6-8) include the late transcription of the non-clustered genes *incA* (24 h) and *cap1* (12 h). Transcription of *copB1* was strongly delayed when compared to the other genes of cluster 2.2 as was also the case for *sctC* compared to the other genes in cluster 3.

Transcription results for a *Cp. psittaci* **92/1293** infection of HD11 cells (Table 5, column 3-5) include upregulation of *sctT* (cluster 2.1) transcription at the 8 h p.i. time point only, but not further on in the developmental cycle. In addition, transcription of *incC* (cluster 3) occurred early in the infection while the non-clustered *incA* gene did not seem to be transcribed at all. Transcription of the non-clustered *tarp* was upregulated already at 4 h p.i., whereas *cadd* transcription could be detected from 2 h p.i. on. The *cap1* gene was upregulated from 8 h pi on, with the highest transcription observed at 36 h p.i.

Noteworthy for **84/2334** (Table 5, column 6-8) is that this bacterium transcribes the *pkn5* gene (cluster 3), while none of the other strains do so, and that *pkn5* and *ca037* share the same transcription upregulation profile. No transcription could be observed for the genes of cluster 2.2 (*scc2*, *copB1*, *copD1)*.

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3.4. Titration of infectious progeny on BGM cells

A titration was performed on the supernatant of infected HD11 cells at 48 h p.i. through inoculation of dilution series of this supernatant on BGM cells. In concordance with immunofluorescence stainings, $TCID_{50}$/ml for 92/1293 progeny was the highest (2.5 x 10^8 / ml), closely followed by 84/55 (2 x 10^8 / ml). CP3 had proliferated the least in the HD11 cells ($TCID_{50}$ 5 x 10^7 /ml) while an 84/2334 infection generated an intermediate value of 1 x 10^8 / ml.

4. DISCUSSION

Considerable evidence exists indicating that *Chlamydophila psittaci* is nearly endemic in the European turkey industry. Previous research has shown that the virulence of *Cp. psittaci* strains for turkeys depends on the *ompA* ('outer membrane protein A') genotype of the infecting strain: genotypes A and D result in a severe systemic infection of the animals, while genotype B is less invasive (Vanrompay et al., 1995). As a consequence, the poultry industry suffers predominantly from genotype A and D infections. In experimentally infected turkeys, the infection starts with chlamydial replication in epithelial cells and macrophages of the respiratory tract, thereby inducing severe inflammation. Subsequently, chlamydiae are found in blood monocytes/macrophages recognized by the specific marker KUL04 (Vanrompay et al., 1995) and the infection spreads to various organs.

We hypothesize that the described differences in virulence are correlated with the ability of the infecting strain to invade blood monocytes/macrophages, thereby using them as a transport vehicle to reach other tissues in the host. To elucidate on the complex interaction between *Cp. psittaci* and its avian host, we characterized the

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chlamydial development and its interaction with the host cell in the chicken monocyte/macrophage cell line HD11. Studies investigating the interaction between *Cp. psittaci* 6BC and human monocytes or macrophages have been performed in the past, revealing that both cell types support intracellular replication of *Cp. psittaci* (Rothermel et al., 1986; Carlin and Weller, 1995). However, Roshick *et al*. (2006) recently demonstrated the importance of performing chlamydial research in the matched host cell system, hence our choice for the avian monocyte/macrophage cell line HD11. Two high virulent (84/55 and 92/1293) and one low virulent (CP3) *Cp. psittaci* strains were included in the study, in addition to 84/2334, formerly classified as serovar A but genetically an intermediate between avian *Cp. psittaci* and mammalian *Cp. abortus* (Van Loock et al., 2003).

Within 15 minutes upon attachment to the host cell, *Cp. psittaci* 92/1293 induces recruitment of actin to the site of invasion, as earlier demonstrated by Beeckman *et al.* (2007). To determine whether this phenomenon contributes to virulence, we assessed the induction of actin recruitment by the four strains under study. Both high and low virulent strains recruited actin filaments to the site of infection and this effect was more pronounced for the higher virulent strains (Figure 2). This could be linked to a higher transcription of the *tarp* gene in the more virulent strains. Remarkably, no colocalization of *Cp. psittaci* and actin polymerization could be observed for an infection of HD11 cells with strain 84/2334, although the Tarp encoding gene can definitely be transcribed by this strain (Table 5). Similarly, at all time points p.i., no association with clathrin-coated pits could be observed for an 84/2334 infection (Table 2). It could be that *Cp. psittaci* 84/2334 induces actin recruitment later on, and/or that other, not yet identified, entry mechanisms are deployed. The observed

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bridging between extracellular *Cp. psittaci* 84/2334 and the host cell membrane at 8 h p.i. (Figure 1H) could represent such a mechanism and requires further investigation.

Next, we characterized the intracellular development and host cell interaction of the four *Cp. psittaci* strains. While fusion of internalized CP3 organisms with lysosomes was already observed at 2 h p.i., this was only the case at 8 h the earliest for the other three strains. As a result, fewer and smaller phagolysosomes were present in CP3 infected cells. Combined with the lower attachment and recruitment of actin, this could account for the smaller inclusion size observed at 20 h and 28 h p.i. (Figure 3) and lower infectious progeny at 48 h p.i. (see section 3.4). Contrary to an infection in epithelial cells (Vanrompay et al., 1996), none of the strains completely occupied the host cell cytoplasm at the later time points of the experiment. The limited survival and replication of *Cp. psittaci* in avian monocytes/macrophages was also reflected by the low gene transcription levels of *ftsW* and *ftsK*, encoding proteins required for cell division (Gerard et al., 2001). Possible explanations for the limited proliferation of *Cp. psittaci* in monocytes/macrophages compared to an infection in epithelial cells could thus include the fact that not all bacteria in the inoculum effectively attach to and enter the host cells (especially for the low virulent strain CP3) and a less efficient escape of phagolysosomal fusion. On the other hand, the chlamydial organisms can only benefit from this limited proliferation, as chlamydial replication would otherwise further activate these essentially antigen presenting cells (APCs). Indeed, higher replication within the inclusion would involve more secretion of chlamydial proteins, which, just as proteins from bacteria replicating in the cytoplasm, could be processed by the proteasome into peptide fragments. These peptides would then be presented in combination with MHC I at the cell surface to $CD8^+$ T-cells, initiating their

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differentiation into cytotoxic T-lymphocytes (CTLs). In addition, activated macrophages would more frequently/efficiently phagocytose extracellular organisms or engulf other infected cells, followed by a presentation of chlamydial antigens in association with MHC II to $CD4^+$ T-cells. The subsequent IFN-γ production by $CD4^+$ Th1 cells would then result in further activation of CTLs.

We have demonstrated that only a limited proliferation, or barely survival, of *Cp. psittaci* is possible within avian monocytes/macrophages. One could therefore wonder why chlamydiae infect these cells in the first place. It could of course be that the monocytes/macrophages simply phagocytose the chlamydial bodies. However, the induction of actin recruitment to the entry site actively mediating internalization would, as least partially, contradict this. The answer could come from former studies in *Cp. pneumoniae* suggesting that blood monocytes/macrophages act as vectors that systemically disseminate *Cp. pneumoniae* by hematogenous and lymphatic routes (Moazed et al., 1998; Gieffers et al., 2004) and *Cp. psittaci*, revealing presence of the bacteria in monocytes/macrophages recognized by the specific marker KUL04 (Vanrompay et al., 1995). Moreover, heterophils have very recently been shown to function as a transport vehicle for *Cp. psittaci* in infected birds (Johns et al., 2009). The question which then remains is: how do these infected macrophages halt to cross the blood/tissue barrier? Referring to research performed on *Cp. pneumoniae*, May *et al.* report that *Cp. pneumoniae* induces rolling and adhesion of infected macrophages to non-inflamed vessel walls. More specific, *Cp. pneumoniae* infected monocytic cells attach to the endothelium through upregulation or activation of VLA-4 (very late antigen-4), LFA-1 (lymphocyte function-associated antigen-1) and Mac-1 (macrophage antigen-1) (May et al., 2003). At least one of these integrins, LFA-1, can bind ICAM-2 (Intercellular adhesion molecule 2) which is expressed on resting

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endothelium (de Fougerolles et al., 1991). In a similar way *Cp. psittaci* infected macrophages could adhere to endothelial cells at locations where macrophages can easily escape from the blood vessel (extravasation), e.g. spleen and high endothelial venules, to invade these tissues. This hypothesis is strengthened by the fact that in diseased birds, *Cp. psittaci* is often found in macrophages and in the spleen (Vanrompay et al., 1995).

Remarkably, none of the four strains in this study transcribed the *groEL-1* gene upon infection of HD11 cells. Primer incompatibility can be ruled out because the primers were tested on genomic DNA, generating an amplicon of the expected size. Although transcription of the late-cycle *omcB* gene was generally spoken delayed compared to the mid-cycle *ompA* gene transcription for all strains (Table 3), transcripts could be detected from 1 h p.i. on, indicating that, contrary to other chlamydial species, *Cp. psittaci omcB* gene transcription in monocytes/macrophages is not restricted to the later stages of the infectious cycle.

To address the contribution of the T3SS during the chlamydial development in avian monocytes/macrophages, the mRNA levels of a set of T3S related genes were determined from 1 to 48 h p.i. of chicken HD11 cells. Transcription results for strain 92/1293 strongly differed for several genes with former results obtained in BGM cells (Beeckman et al., 2008). For example, for a *Cp. psittaci* 92/1293 infection of HD11 cells, *sctT* was now shown to be transcribed. Overall, genes encoding structural components of the T3SS seem to be transcribed earlier for a *Cp. psittaci* infection in monocytes/macrophages compared to a similar infection in epithelial cells as described in Beeckman et al. (2008). Taking into account the limited proliferation of *Cp. psittaci* within monocytes/macrophages, we propose that the bacteria use their

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T3SS to enter the cells, allow for a limited proliferation and directly arm themselves again to infect new (epithelial) host cells elsewhere in the body, after attachment of the infected mononuclear cell to the blood vessel wall (see above). The observation that the inclusion membrane often disappears could be important as contact with the inclusion membrane through the T3SS is a prerequisite for RB replication (Wilson et al., 2006).

When turning to the transcription of T3S effector encoding genes, we found that the *Cp. psittaci* CP3 *cap1* gene was transcribed remarkably late (12 h p.i.) compared to the transcription pattern for 92/1293 in BGM-cells [1 h p.i. (Beeckman et al., 2008)]. The same was observed for 92/1293 (8 h) and 84/2334 (12 h) and no transcription of *cap1* could be observed in 84/55. For an infection of *Chlamydia (C.) trachomatis* in McCoy or HeLa cells, early transcription of this gene was observed (Belland et al., 2003; Balsara et al., 2006), as also for *Cp. psittaci* 92/1293 in BGM cells [1 h (Beeckman et al., 2008)]. The corresponding Cap1 protein is known to stimulate protective $CD8^+$ T-cells (peptides are presented in association with MHC class I) to transform to CTLs, thus reinforcing the cellular immune response. The fact that the expression of Cap1 by *Cp. psittaci* in HD11 cells is delayed may therefore indicate that *Cp. psittaci* is trying to avoid recognition of the infected macrophages by CTLs in order to use the macrophage as a transport vehicle throughout the body for as long as required. Transcription of the non-clustered *tarp* and *cadd* genes was noticed much earlier (1-4 h and 1-2 h p.i., repectively) when compared to an infection in BGM cells [12 h and 10 h p.i., repectively (Beeckman et al., 2008)]. The gene encoding the serine-threonine kinase Pkn5 is not transcribed for an infection of HD11 cells with *Cp. psittaci* 84/55, CP3 or 92/1293, although the immediately adjacent *sctC* gene is. *Cp. psittaci* 84/2334 is the only strain transcribing *pkn5*. In addition, s*ctC* and *sctQ*

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have the same upregulation pattern, just as is the case for *pkn5* and *ca037* (Table 5). These results are in agreement with Hefty & Stephens (2007) stating that *sctC* and *pkn5* do not belong to the same operon. Taking into account the transcription profile of *sctD*, this would seem to suggest that in the 84/2334 genome sequence the gene order has switched from *ca037-sctQ-pkn5-sctC* to *ca037-pkn5-sctQ-sctC*. Genome sequencing could provide a definitive answer.

No transcription could be observed for the translocon components CopB1 and CopD1, neither for their chaperone Scc2 after an 84/2334 infection. Primer incompatibility could be the cause, but it could also be that gene cluster 2.2 is not present in the *Cp. psittaci* 84/2334 genome. However, as these genes are present in all other *Chlamydiaceae*, this option is less likely. Again, genome sequencing could shed light on this issue.

5. CONCLUSION

The present study revealed a limited proliferation of high and low virulent avian *Cp. psittaci* strains in the matched avian monocyte/macrophage cell line HD11. In concordance with its observed lower virulence for turkeys, *Cp. psittaci* CP3 attached to and invaded the host cells less efficiently, the infection was less productive and induced less cell degeneration compared to the other strains. Infection with the abortus-like 84/2334 strain was characterized by the absence of actin recruitment at the site of entry and a remarkable T3S gene transcription profile. Further experiments, including genome sequencing, will provide more information on the specific characteristics of this strain. We also proposed that *Cp. psittaci* uses blood monocytes/macrophages as a vehicle to spread to other organs and provided possible mechanisms deployed by the bacteria to reach this goal.

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FIGURE LEGENDS

Figure 1 Electron microscopic photographs of *Cp. psittaci* infections in HD11 cells. The cells were seeded on microcarriers for 24 h, followed by infection with *Cp. psittaci* (MOI=100). After three hours, unbound inoculum was washed away and cultured were resuspended in HD11 medium with glucose until sample taking and processing for TEM. A. Negative control at 12 h. B. Invagination induced by *Cp. psittaci* 84/55 at 1h p.i. C. Fusion of *Cp. psittaci* 84/55 phagosome with a primary lysosome (darker organelle) at 8 h p.i. D. Association of a *Cp. psittaci* 92/1293 EB with the host cell nucleus. E. Typical morphology of phagolysosomes, image from a *Cp. psittaci* 84/55 infection at 26 h p.i. F. Close interaction between a *Cp. psittaci* 92/1293 RB and the host cell nuclear membrane. G. Typical small inclusion of *Cp. psittaci* 92/1293 at 36 h p.i. H. *Cp. psittaci* 84/2334 in between two host cells at 8 h p.i. The arrow points at a kind of bridge formation between the IB and the host cell membrane. I. Cell degeneration induced by *Cp. psittaci* 82/2334 (12 h p.i.). Scale bars represent 500 nm, except for panels A (5 µm) and I (2 µm).

Figure 2 Actin skeleton distribution at 15 min post inoculation with different *Cp. psittaci* strains. HD11 cells were infected at an MOI of 100 by centrifugation at 4 °C, where after the temperature was shifted to 37 °C for 15 min to allow chlamydial induction of host cell actin polymerization. Chlamydial organisms are shown in red while the actin skeleton is visualized in green. Colocalization of actin polymerization and chlamydial EBs is depicted in yellow (overlap of green and red fluorescence). Nuclei of the host cells were stained with DAPI. Representative confocal images are shown. A. *Cp. psittaci* 84/55. B. *Cp. psittaci* CP3. C. *Cp. psittaci* 92/1293. D. *Cp. psittaci* 84/2334. The scale bar represents 10 µm.

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Figure 3 Distribution of mitochondria in HD11 cells after inoculation with different *Cp. psittaci* strains. *Chlamydophila psittaci* was inoculated onto HD11 cells by centrifugation, followed by incubation at 37 °C for 8 h (first column), 20 h (second column) or 28 h p.i. (last column). Mitochondria were stained indirectly by AlexaFluor 546 (red), while *Cp. psittaci* was directly fluorescently green stained. Nuclei of the host cells were stained with DAPI (blue). Representative confocal images are shown. A. *Cp. psittaci* 84/55. B. *Cp. psittaci* CP3. C. *Cp. psittaci* 92/1293. D. *Cp. psittaci* 84/2334. The scale bar represents 10 µm.

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TABLE LEGENDS

Table 1 Oligonucleotide primers used for RT-qPCR analysis. Legend: Ta, annealing temperature.

Table 2 Overview of specific characteristics observed at different time points p.i. with the four *Cp. psittaci* strains under study.

Table 3 Gene transcription patterns of control genes for different *Cp. psittaci* strains in HD11 cells. Total RNA from 1.5 x 10^6 HD11 cells inoculated with *Cp. psittaci* 92/1293 at an MOI of 13 was isolated at 1, 2, 4, 8, 12, 18, 24, 36 and 48 h p.i. The experiments were performed twice and all samples were tested in duplicate. For each gene the time point at which a specific PCR product was first detected is provided (time point at which the absolute copy number without 16S rRNA normalization was higher than 100). The time point where maximal significant upregulation of the genes was observed is given in the third column, as determined by an ANOVA with post-hoc analysis along the time axis (both Tukey HSD and Tukey-b test, $P < 0.01$). The fourth column describes further significant upregulation time points after 16S rRNA normalization, in order of their importance.

Table 4 T3S gene transcription patterns for *Cp. psittaci* 84/55 and CP3 in HD11 cells. In order to describe the gene transcription profile for each of the genes under study, the time point where maximal significant upregulation of the genes was observed is given in a second column per strain. The third column describes other significant upregulation time points after 16S rRNA normalization, in order of their importance.

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Table 5 T3S gene transcription patterns for *Cp. psittaci* 92/1293 and 84/2334 in HD11 cells. In order to describe the gene transcription profile for each of the genes under study, the time point where maximal significant upregulation of the genes was observed is given in a second column per strain. The third column describes other significant upregulation time points after 16S rRNA normalization, in order of their importance.

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Figure 1

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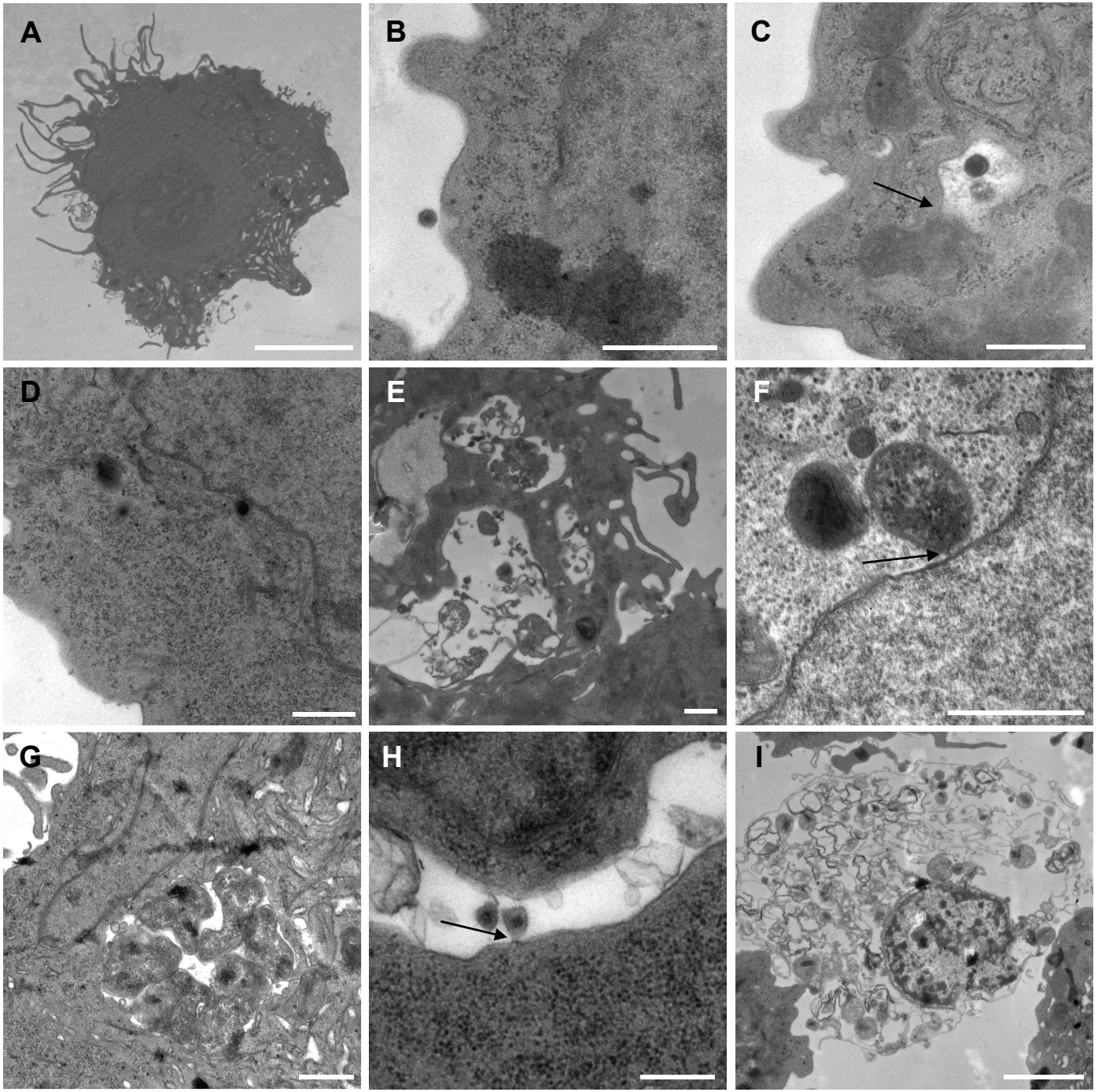

Figure 2

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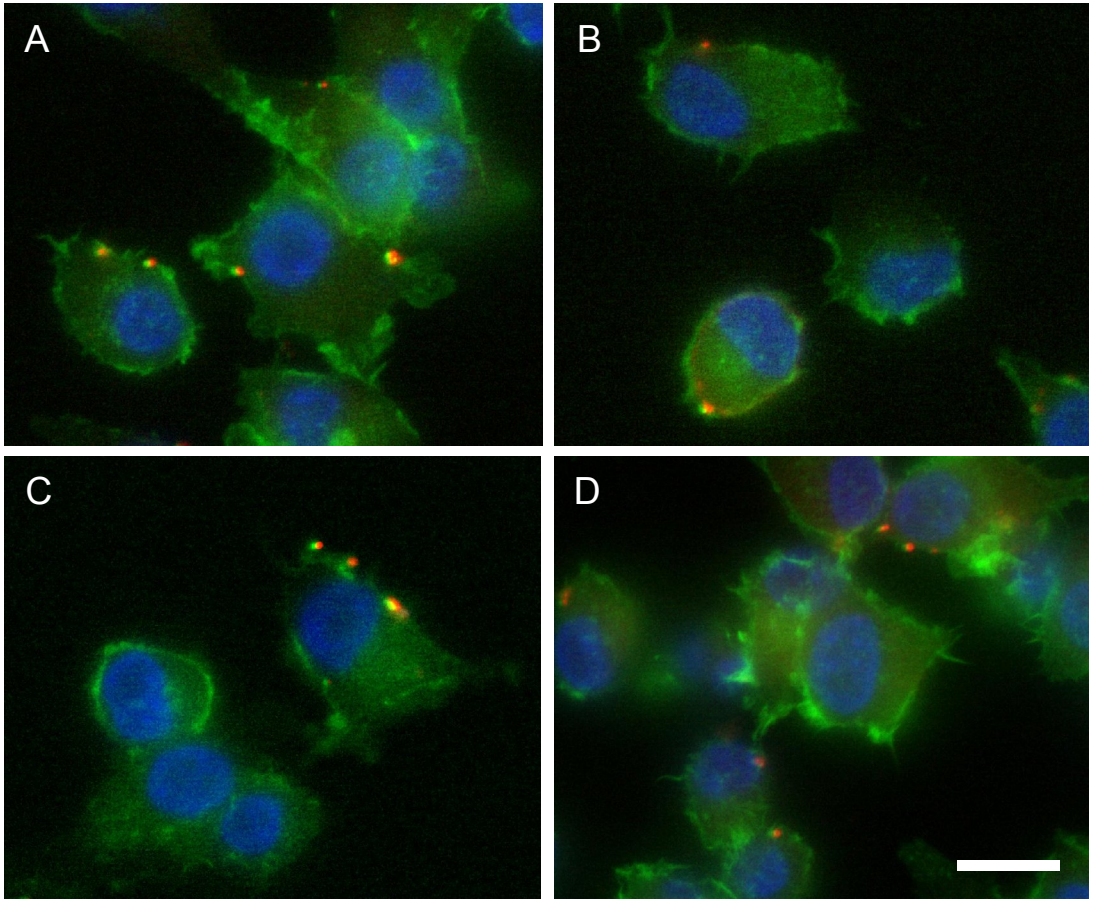

Figure 3

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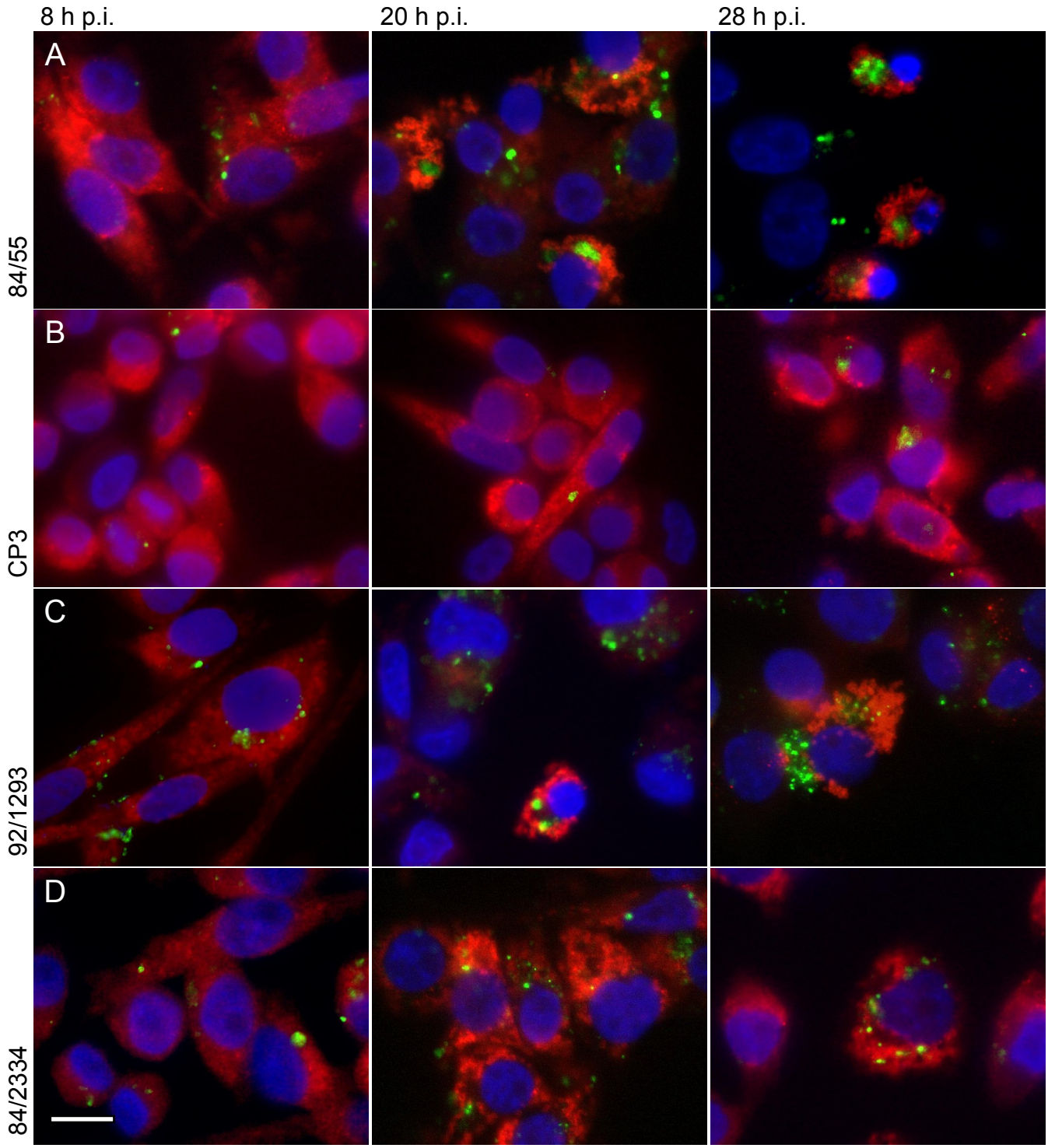

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Table 1

Gene		Primer sequence	Ta	Size (bp)
ftsW	F[a]	5'- ATGGAGCAAAGCGATGGTTAG -3'	60 °C	300
	R[b]	5'- CGCCCACAACAAGAATACATAA -3'		
ftsK	F	5'- ACCACCAACTCCCATTGTCTC -3'	60 °C	323
	R	5'- CACGCAAGGATTCAGGTTTAG -3'		
dnaK	F	5'- GCATTCAAAGGAAACGAAACC -3'	55 °C	308
	R	5'- AAGTAGGCAGGAACCGTAATCA -3'		
groEL-1	F	5'- CGAAGACAAACACGAGAACAT -3'	55 °C	305
	R	5'- CGGCGATGAGATTACCAA -3'		
ompA	F	5'- TATGGGAATGTGGTTGTGC -3'	60 °C	290
	R	5'- TGCTCTTGACCAGTTTACGC -3'		
omcB	F	5'- CTGCGACAACACCAACCTC -3'	60 °C	287
	R	5'- GACGACAACATTACGGGCTAT -3'		

[a] Forward primers (F).
[b] Reverse primers (R).

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Table 2

	84/55	CP3	92/1293	84/2334
Invaginations	1 - 6 h	2 - 4 h	1 - 4 h	2 h
Clathrin-coated pits	1 h	1 h	1 h	/
Fusion with lysosomes	8 h	2 h	8 h	8 h
Arrival at the nucleus	8 h	6 h	6 h	6 h
Disappearance of inclusion	8 h	2 h	6 h	/
Association with mitochondria	12 h	/	12 h	20 - 28 h
Host cell degeneration	6 h	12 h	6 h	6 h

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Table 3

Cluster	Gene	First detection	Major upregulation timepoint	Secondary timepoints of upregulation
84/55	*ftsW*	2h	4h[c]	2-12h[c]
	ftsK	/[a]	8h[c]	
	dnaK	4h	4h	8h
	groEL-1[b]			
	ompA	1h	8h	12h
	omcB	1h	8-12h	36h
CP3	*ftsW*	1h	12h	2-8h[c]
	ftsK	4h	12h[c]	4h[c]
	dnaK	4h	8h	4-18h
	groEL-1			
	ompA	8h	12h	8h
	omcB	1h	8-12h	36h
92/1293	*ftsW*	/	8-12h[c]	
	ftsK	/	24h[c]	4-18h[c]
	dnaK	8h	8h	12h & 48h
	groEL-1			
	ompA	8h	8h	12-18h
	omcB	1h	48h	8h & 18-24h
84/2334	*ftsW*	/	/	/
	ftsK	/	4h[c]	
	dnaK	/	/	/
	groEL-1			
	ompA	8h	8h	
	omcB	1h	8h	24-48h

[a] No signal observed
[b] An amplicon could be obtained when using genomic DNA but not cDNA
[c] Faint signal

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Table 4

Cluster	Gene	84/55 First detection	84/55 Major upregulation time point	84/55 Secondary time points of upregulation	CP3 First detection	CP3 Major upregulation time point	CP3 Secondary time points of upregulation
Cluster 1	*scc1*	2h	12h	4h	2h	2h	36h & 18h
	sctW	2h	2h		1h	2h	12h
	sctV	1h	8h	2h	1h	2h	8h
	sctU	/[a]	/	/	1h	4h	1h
Cluster 2.1	*sctJ*	1h	4h	12h & 1h	1h	2h	8-12h
	sctL	2h	12h	4h	1h	8h	36h
	sctR	2h	4-12h		1h	12h	4h
	sctS	/	/	/	/	/	/
	sctT	8h	8h		/	/	/
Cluster 2.2	*scc2*	1h	8h	48h & 1h	1h	12h	36h
	copB1	/	/	/	4h	4h	36-48h
	copD1	1h	8h	1h	1h	12h	2h
Cluster 3	*sctD*	2h	4h	12h	1h	8h	1h
	sctN	/	/	/	/	/	/
	ca037	1h	8h	1h	1h	8h	2h & 24h
	sctQ	4h	24h	4h	2h	8h	36-48h
	pkn5	/	/	/	/	/	/
	sctC	1h	8h	1h	1h	12h	2h
Cluster 4	*scc3*	1h	12h	48h & 2h	1h	4h	12h
	copB2	NT	NT	NT	NT	NT	NT
	copD2	4h	4h	18h	/	/	/
Non-clustered	*incC*	1h	4h	1h	1h	2h (1-4h)	18h
	incB	NT	NT	NT	NT	NT	NT
	incA	1h	18h	1h	24h	24h	
	cap1	/	/	/	12h	12h	
	tarp	1h	8h	36h	1h	36h	4h
	ca530	/	/	/	/	/	/
	cadd	1h	8h		1h	8h	18h
Normalizing	*16S rRNA*	1h	-	-	1h	-	-

[a] No signal observed
[b] No statistically significant upregulation (P > 0.10)
NT: not tested

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Table 5

Cluster	Gene	92/1293 First detection	92/1293 Major upregulation time point	92/1293 Secondary time points of upregulation	84/2334 First detection	84/2334 Major upregulation time point	84/2334 Secondary time points of upregulation
Cluster 1	*scc1*	1h	12h (8-18h)	1h	/	/	/
	sctW	1h	12-18h	4h	2h	2h	48h & 24h
	sctV	1h	8-12h	24h	1h	4-8h	48h
	sctU	/[a]	/	/	2h	4h	48h & 24h
Cluster 2.1	*sctJ*	1h	24h	1h (& 4h[b])	/	/	/
	sctL	2h	8h	48h	/	/	/
	sctR	1h	24h	8h	1h	4h	1h,48h&24h
	sctS	/	/	/	/	/	/
	sctT	8h	8h		2h	2h	48h,12h&24h
Cluster 2.2	*scc2*	2h	18h	4h	/	/	/
	copB1	/	/	/	/	/	/
	copD1	1h	18h	8h & 36h	/	/	/
Cluster 3	*sctD*	2h	18h	8h	1h	8h	48h
	sctN	4h	18h	8h	/	/	/
	ca037	1h	8-12h	36h	1h	8h	24h & 48h
	sctQ	1h	24h	8h	18h	48h	24h
	pkn5	/	/	/	1h	8h	1h,24h&48h
	sctC	1h	18h	8h & 1h	18h	48h	18-24h
Cluster 4	*scc3*	1h	4h	36-48h	1h	8h	
	copB2	NT	NT	NT	NT	NT	NT
	copD2	/	/	/	2h	4h	
Non-clustered	*incC*	1h	8h	1h	1h	8h	2h
	incB	NT	NT	NT	NT	NT	NT
	incA	/	/	/	/	/	/
	cap1	8h	36h	8h	12h	12h	36h
	tarp	4h	18h	4h	1h	2h (1-4h)	24h
	ca530	/	/	/	/	/	/
	cadd	2h	12-18h	48h	/	/	/
Normalizing	*16S rRNA*	1h	-	-	1h	-	-

[a] No signal observed
[b] No statistically significant upregulation (P > 0.10)
NT: not tested

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