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1 **Original research paper**

2

3 **Biology and intracellular pathogenesis of high or low virulent**

4 ***Chlamydophila psittaci* strains in chicken macrophages**

5

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

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17

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

38

39 **Keywords:**

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

41 **1. INTRODUCTION**

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

52 Serovars largely correspond to the currently accepted classification in *ompA* (coding
53 for MOMP) genotypes defined by sequencing (Everett et al., 1999; Geens et al.,
54 2005). Infection with genotypes A and D results in a more elevated pathogenicity for
55 turkeys than genotype B: for genotype B, the incubation period is longer, maximal
56 replication is delayed, the period during which bacteria are observed in the same
57 tissue is shorter and tissue tropism seems to be less extensive (Vanrompay et al.,
58 1995). During the initial replication in epithelial cells from the turkey respiratory
59 system, macrophages are attracted, normally acting as a first line of (innate) defense
60 against infection (Vanrompay et al., 1994a) and *Cp. psittaci* has already been detected
61 in monocytes/macrophages recognized by the specific marker KUL04 (Vanrompay et
62 al., 1995). The present study therefore aims to further shed light on the complex
63 interaction between *Cp. psittaci* and its avian host by studying the biology and
64 intracellular pathogenesis of high and low virulent *Cp. psittaci* strains in the chicken
65 monocyte/macrophage cell line HD11.

66

67 **2. MATERIALS AND METHODS**68 **2.1. Organism and cell culture**

69 The HD11 chicken monocyte/macrophage cell line (Beug et al., 1979) was cultured in
70 Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with
71 5% heat-inactivated fetal calf serum, 1% sodium pyruvate, 1% L-glutamine and 0.5%
72 gentamicin (all products from Invitrogen, Merelbeke, Belgium), further referred to as
73 HD11 culture medium. Cultures were incubated at 5% CO₂ and 37 °C. Four different
74 *Cp. psittaci* strains were used in this study: 84/55 [isolated from the lung of a
75 budgerigar, *ompA* genotype A (Vanrompay et al., 1996)], CP3 [isolated from a
76 pigeon, genotype B (Page and Bankowski, 1959)], 92/1293 [isolated from a pooled
77 homogenate of lungs, cloaca and spleen of diseased turkeys, genotype D (Vanrompay
78 et al., 1993)] and 84/2334, an intermediate strain in the evolution of *Cp. abortus* from
79 *Cp. psittaci*, isolated from a yellow-headed Amazon parrot (Van Loock et al., 2003).
80 All strains were cultured and titrated as described before (Vanrompay et al., 1996).

81

82 **2.2. Transmission electron microscopy**

83 HD11 cells were seeded on Cytodex 3 microcarriers (GE Healthcare, Diegem,
84 Belgium) essentially as described before (Vanrompay et al., 1996). Briefly, 60 mg of
85 microcarriers were hydrated and autoclaved according to the manufacturer's
86 instructions, and subsequently resuspended in HD11 culture medium. Before seeding
87 on the microcarriers, HD11 cells were grown for 72 h with 10% FCS to obtain a large
88 fraction of adherent macrophages. The adherent cells were trypsinized, pelleted and 2
89 x 10⁷ cells were added to the microcarriers in a Petri dish. After incubation for 5 h at
90 37 °C and 5% CO₂, the non-adherent cells were washed away with sterile PBS and

91 cells and microcarriers were resuspended in HD11 medium with 10% FCS. The
92 microcarrier cultures were incubated overnight on a rotating platform, distributed into
93 six new Petri dishes with fresh HD11 culture medium to maintain optimal growth
94 conditions and further incubated on the rotating platform for 24 h. Contents of each
95 Petri dish were centrifuged for 4 min at 135 x g and pellets were resuspended in 20 ml
96 HD11 medium supplemented with 5.5 mg/ml glucose (Sigma, Antwerp, Belgium) and
97 each of the four *Cp. psittaci* strains under study at a multiplicity of infection (MOI) of
98 100. Infected microcarrier cultures were incubated at 37 °C and 5% CO₂ for 3 h
99 whereafter the inoculum was removed by centrifugation and a washing step with
100 sterile PBS. Cultures were then resuspended in HD11 medium supplemented with
101 glucose and further incubated on a rotating platform until sample taking at 0, 1, 2, 3,
102 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 21, 26, 30, 36, 42, 45, 50, 52 and 72 h post
103 inoculation (p.i.). For the negative control, the medium was replaced with HD11
104 medium with glucose. Infection was checked on dried and acetone fixed spotted
105 microcarriers on a glass slide using a direct immunofluorescence staining at 30 and 50
106 h (IMAGEN Chlamydia, Oxoid, Drogen, Belgium) as previously described
107 (Vanrompay et al., 1994b). At each time point 100 µl of microcarrier culture was
108 added to 1 ml of Karnovsky fixative (Karnovsky, 1965) and fixed overnight at 4 °C.
109 Afterwards, the fixative was replaced with sodium cacodylate buffer (0.1 M in
110 BiDest, pH 7.4, Sigma) until further processing. Post-fixation was performed using
111 4% osmiumtetroxide and samples were dehydrated in a step gradient of ethanol at
112 room temperature. The samples were transferred to 100% alcohol/Spurr's resin (1:1)
113 at 4 °C overnight, brought to 100% alcohol/Spurr's resin (1:2) for 8 h (4 °C), and
114 transferred to 100% Spurr's resin and left overnight at 4 °C. Polymerization was
115 performed at 70 °C for 16 h. Sections (70 nm thick) were made using a Reichert

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

121

122 **2.3. Immunofluorescence assays**

123 We tested whether *Cp. psittaci* strains of different virulence are all able to induce
124 actin polymerization at the site of attachment and entry and whether mitochondria are
125 being attracted to the inclusions. HD11 cells (3×10^5 / ml) were seeded on sterile
126 glass coverslips (13 mm) at the bottom of Chlamydia Trac Bottles (Bibby Sterilin
127 Ltd., Stone, UK) for 24 h at 37 °C and 5% CO₂ and subsequently inoculated at an
128 MOI of 100, performing a centrifugation step (1 000 g) at 4 °C for 15 min to
129 synchronize irreversible attachment and entry. One ml of HD11 culture medium with
130 glucose was added and cells were further incubated at 37 °C and 5% CO₂ until PFA
131 fixation and permeabilization with saponin at 15 min, 8 h, 20 h or 28 h p.i. as
132 described before (Beeckman et al., 2007).

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

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

141 1:25 diluted AlexaFluor 546 goat-anti-mouse secondary antibody (Molecular Probes),
142 again for 1 h at 37 °C. Subsequent rinsing was followed by detection of the
143 chlamydial organisms by a direct immunofluorescence staining (IMAGEN
144 Chlamydia, Oxoid).

145 All dilutions and washing steps (three) were performed with 1% BSA (Sigma) in
146 PBS. After a final rinsing with PBS, nuclei were visualized in all samples with DAPI
147 (100 µl at a concentration of 100 ng/ml in BiDest for 5 min at room temperature). A
148 final rinsing with BiDest was followed by mounting of the coverslips using Mowiol
149 (Calbiochem, VWR, Haasrode, Belgium) with 0.01% PPD (Sigma) (Valnes and
150 Brandtzaeg, 1985). Images were acquired using a Radiance 2000 confocal laser
151 scanning microscope (Bio-Rad, Nazareth, Belgium, 600 x). Chlamydial replication at
152 8 h, 20 h and 28 h was assessed using a scoring system from 0 to 5 as previously
153 described (Van Loock et al., 2006).

154

155 **2.4. Transcription analysis of control genes and T3S genes**

156 As T3S plays an important role in different phases of the chlamydial life cycle, we
157 studied the gene transcription profile of a panel of T3S related genes (26 in total) for
158 different *Cp. psittaci* strains in HD11 cells in order to determine whether virulence
159 could be linked to differences in T3S gene transcription profiles. Total RNA was
160 extracted from 1.5×10^6 cells infected at an MOI of 100 (time points 1, 2, 4, 8, 12, 18,
161 24, 36 and 48 h p.i.) and checked for DNA contamination. All the experiments were
162 performed twice. Instead of 0.5 µg, one microgram of total RNA (spiked with 10 ng
163 of coliphage control RNA) was reverse transcribed using selective hexamers (5'-
164 TTANNN-3' and 5'-CTANNN-3') and qPCR using SYBRGreen technology was
165 performed *in duplo* on each sample. Reverse transcription, primer sequences, cycling

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

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

181

182 **2.5. Titration of infectious progeny on BGM cells**

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

189

190 **3. RESULTS**

191 **3.1. Transmission electron microscopy**

192 Predominantly monocytes were present in the early infection stages, differentiating
193 into inflammatory macrophages later on in the course of the infection. For all *Cp.*
194 *psittaci* strains, internalization was often accompanied by an invagination of the
195 HD11 host cell membrane (Figure 1B) or the presence of clathrin-coated pits (except
196 for 84/2334, Table 2). Subsequently, the chlamydial organisms pursued their way to
197 the nucleus and from 6 h p.i. on (8 h for strain 84/55), *Cp. psittaci* EBs or IBs could
198 be observed in the vicinity of the host cell nuclear membrane (Figure 1D). However,
199 not all internalized bacteria arrived at the nucleus, since fusion of the nascent
200 inclusion with lysosomes was detected for all strains (Table 2, Figure 1C), the earliest
201 (2 h p.i.) for the low pathogenic strain CP3. These phagolysosomes then further
202 evolved to the typical morphology given in Figure 1E. For inclusions efficiently
203 escaping the phagolysosomal fusion, the inclusion membrane faded or opened up to
204 the host cell cytoplasm, thus releasing the chlamydiae. As such, a direct interaction
205 between free RBs and the nuclear envelope could sometimes be observed (Figure 1F).
206 However, intact inclusions were also present, containing a limited number of odd
207 shaped RBs (Figure 1G) and at 45 h p.i. phagocytosis of cell fragments could often be
208 observed.

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

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

216

217 **3.2. Immunofluorescence assays**

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

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

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

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

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

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

270

271 **3.3. Transcription analysis of control genes and T3S genes**

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

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

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

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

292 For *Cp. psittaci* **84/55** (Table 4, column 3-5), no transcription could be observed for
293 *sctU* (cluster 1), *copB1* (cluster 2.2), *sctN* (cluster 3) or *capI* (non-clustered) and both
294 *incA* and *incC* (non-clustered) were shown to be transcribed from 1 h p.i. on.
295 Transcription results of the both translocon-coding gene clusters revealed that cluster
296 4 (*scc3*, *copB2*, *copD2*) was generally spoken transcribed later than the *scc2-copB1-*
297 *copD1* cluster.

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

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

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

315

316 **3.4. Titration of infectious progeny on BGM cells**

317 A titration was performed on the supernatant of infected HD11 cells at 48 h p.i.
318 through inoculation of dilution series of this supernatant on BGM cells. In
319 concordance with immunofluorescence stainings, TCID₅₀/ml for 92/1293 progeny was
320 the highest (2.5×10^8 / ml), closely followed by 84/55 (2×10^8 / ml). CP3 had
321 proliferated the least in the HD11 cells (TCID₅₀ 5×10^7 /ml) while an 84/2334
322 infection generated an intermediate value of 1×10^8 / ml.

323

324 **4. DISCUSSION**

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

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

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

351

352 Within 15 minutes upon attachment to the host cell, *Cp. psittaci* 92/1293 induces
353 recruitment of actin to the site of invasion, as earlier demonstrated by Beeckman *et al.*
354 (2007). To determine whether this phenomenon contributes to virulence, we assessed
355 the induction of actin recruitment by the four strains under study. Both high and low
356 virulent strains recruited actin filaments to the site of infection and this effect was
357 more pronounced for the higher virulent strains (Figure 2). This could be linked to a
358 higher transcription of the *tarp* gene in the more virulent strains. Remarkably, no
359 colocalization of *Cp. psittaci* and actin polymerization could be observed for an
360 infection of HD11 cells with strain 84/2334, although the Tarp encoding gene can
361 definitely be transcribed by this strain (Table 5). Similarly, at all time points p.i., no
362 association with clathrin-coated pits could be observed for an 84/2334 infection
363 (Table 2). It could be that *Cp. psittaci* 84/2334 induces actin recruitment later on,
364 and/or that other, not yet identified, entry mechanisms are deployed. The observed

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

367

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

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

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

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

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

429

430 To address the contribution of the T3SS during the chlamydial development in avian
431 monocytes/macrophages, the mRNA levels of a set of T3S related genes were
432 determined from 1 to 48 h p.i. of chicken HD11 cells. Transcription results for strain
433 92/1293 strongly differed for several genes with former results obtained in BGM cells
434 (Beeckman et al., 2008). For example, for a *Cp. psittaci* 92/1293 infection of HD11
435 cells, *sctT* was now shown to be transcribed. Overall, genes encoding structural
436 components of the T3SS seem to be transcribed earlier for a *Cp. psittaci* infection in
437 monocytes/macrophages compared to a similar infection in epithelial cells as
438 described in Beeckman et al. (2008). Taking into account the limited proliferation of
439 *Cp. psittaci* within monocytes/macrophages, we propose that the bacteria use their

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

446 When turning to the transcription of T3S effector encoding genes, we found that the
447 *Cp. psittaci* CP3 *cap1* gene was transcribed remarkably late (12 h p.i.) compared to
448 the transcription pattern for 92/1293 in BGM-cells [1 h p.i. (Beeckman et al., 2008)].
449 The same was observed for 92/1293 (8 h) and 84/2334 (12 h) and no transcription of
450 *cap1* could be observed in 84/55. For an infection of *Chlamydia (C.) trachomatis* in
451 McCoy or HeLa cells, early transcription of this gene was observed (Belland et al.,
452 2003; Balsara et al., 2006), as also for *Cp. psittaci* 92/1293 in BGM cells [1 h
453 (Beeckman et al., 2008)]. The corresponding Cap1 protein is known to stimulate
454 protective CD8⁺ T-cells (peptides are presented in association with MHC class I) to
455 transform to CTLs, thus reinforcing the cellular immune response. The fact that the
456 expression of Cap1 by *Cp. psittaci* in HD11 cells is delayed may therefore indicate
457 that *Cp. psittaci* is trying to avoid recognition of the infected macrophages by CTLs in
458 order to use the macrophage as a transport vehicle throughout the body for as long as
459 required. Transcription of the non-clustered *tarp* and *cadd* genes was noticed much
460 earlier (1-4 h and 1-2 h p.i., respectively) when compared to an infection in BGM cells
461 [12 h and 10 h p.i., respectively (Beeckman et al., 2008)]. The gene encoding the
462 serine-threonine kinase Pkn5 is not transcribed for an infection of HD11 cells with
463 *Cp. psittaci* 84/55, CP3 or 92/1293, although the immediately adjacent *sctC* gene is.
464 *Cp. psittaci* 84/2334 is the only strain transcribing *pkn5*. In addition, *sctC* and *sctQ*

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

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

477

478 5. CONCLUSION

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

490

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614 **FIGURE LEGENDS**

615 **Figure 1** Electron microscopic photographs of *Cp. psittaci* infections in HD11 cells.
616 The cells were seeded on microcarriers for 24 h, followed by infection with *Cp.*
617 *psittaci* (MOI=100). After three hours, unbound inoculum was washed away and
618 cultured were resuspended in HD11 medium with glucose until sample taking and
619 processing for TEM. A. Negative control at 12 h. B. Invagination induced by *Cp.*
620 *psittaci* 84/55 at 1h p.i. C. Fusion of *Cp. psittaci* 84/55 phagosome with a primary
621 lysosome (darker organelle) at 8 h p.i. D. Association of a *Cp. psittaci* 92/1293 EB
622 with the host cell nucleus. E. Typical morphology of phagolysosomes, image from a
623 *Cp. psittaci* 84/55 infection at 26 h p.i. F. Close interaction between a *Cp. psittaci*
624 92/1293 RB and the host cell nuclear membrane. G. Typical small inclusion of *Cp.*
625 *psittaci* 92/1293 at 36 h p.i. H. *Cp. psittaci* 84/2334 in between two host cells at 8 h
626 p.i. The arrow points at a kind of bridge formation between the IB and the host cell
627 membrane. I. Cell degeneration induced by *Cp. psittaci* 82/2334 (12 h p.i.). Scale bars
628 represent 500 nm, except for panels A (5 μ m) and I (2 μ m).

629

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

639

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

648

649 **TABLE LEGENDS**

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

652

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

655

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

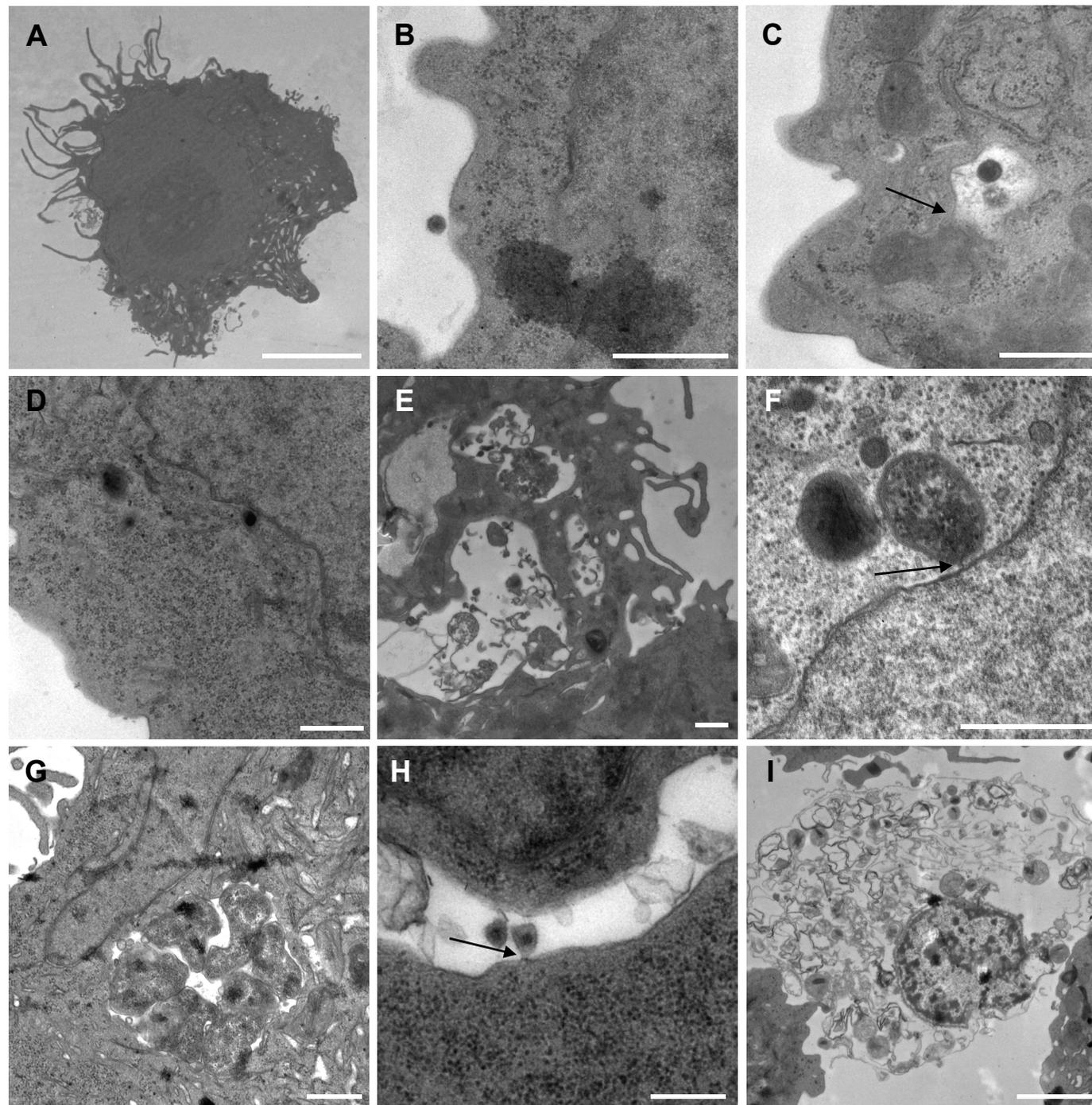
667

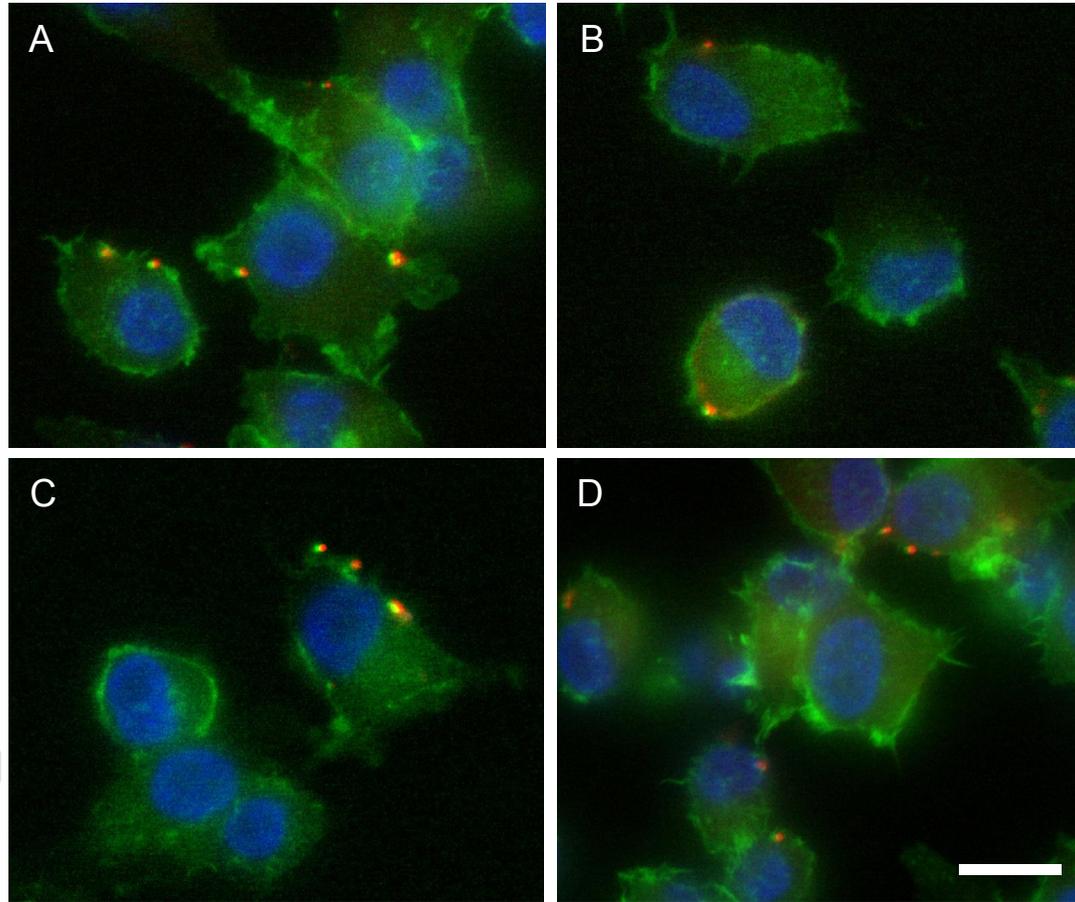
668 **Table 4** T3S gene transcription patterns for *Cp. psittaci* 84/55 and CP3 in HD11 cells. In
669 order to describe the gene transcription profile for each of the genes under study, the time
670 point where maximal significant upregulation of the genes was observed is given in a
671 second column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

673

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

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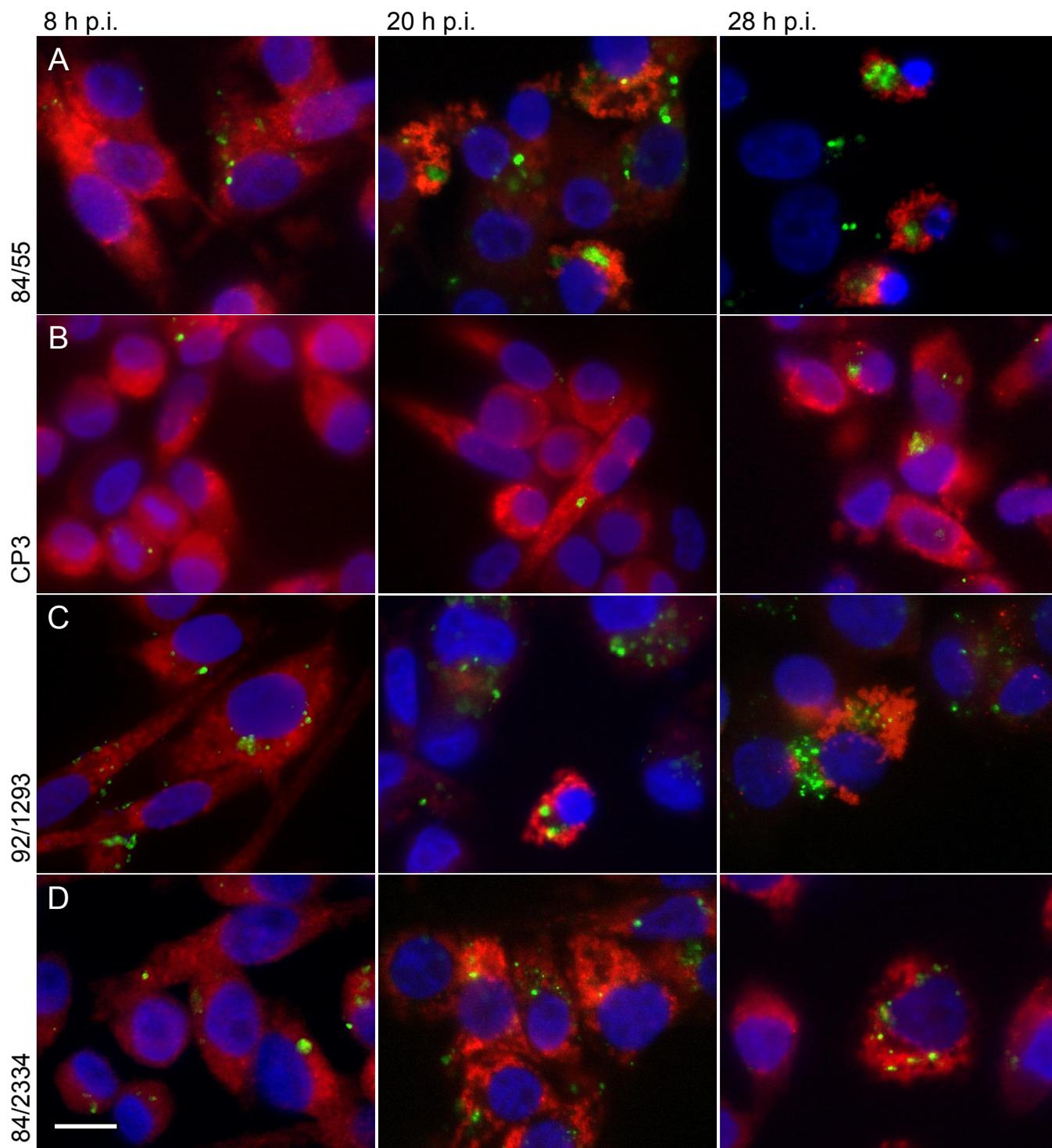


Table 1

Gene		Primer sequence	T _a	Size (bp)
<i>ftsW</i>	F ^a	5'- ATGGAGCAAAGCGATGGTTAG -3'	60 °C	300
	R ^b	5'- CGCCCACAACAAGAATACATAA -3'		
<i>ftsK</i>	F	5'- ACCACCAACTCCCATTGTCTC -3'	60 °C	323
	R	5'- CACGCAAGGATTCAGGTTTAG -3'		
<i>dnaK</i>	F	5'- GCATTCAAAGGAAACGAAACC -3'	55 °C	308
	R	5'- AAGTAGGCAGGAACCGTAATCA -3'		
<i>groEL-1</i>	F	5'- CGAAGACAAACACGAGAACAT -3'	55 °C	305
	R	5'- CGGCGATGAGATTACCAA -3'		
<i>ompA</i>	F	5'- TATGGGAATGTGGTTGTGC -3'	60 °C	290
	R	5'- TGCTCTTGACCAGTTTACGC -3'		
<i>omcB</i>	F	5'- CTGCGACAACACCAACCTC -3'	60 °C	287
	R	5'- GACGACAACATTACGGGCTAT -3'		

^a Forward primers (F).

^b Reverse primers (R).

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

Table 3

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

^aNo signal observed

^bAn amplicon could be obtained when using genomic DNA but not cDNA

^cFaint signal

Table 4

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

^aNo signal observed^bNo statistically significant upregulation (P > 0.10)

NT: not tested

Table 5

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

^aNo signal observed^bNo statistically significant upregulation (P > 0.10)

NT: not tested