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Early expression of the Type III secretion system of *Parachlamydia acanthamoebae* during a replicative cycle within its natural host cell *Acanthamoeba castellanii.*

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Summary

The type three secretion system (T3SS) operons of *Chlamydiales* bacteria are distributed in different clusters along their chromosomes and are conserved both at the level of sequence and genetic organization. A complete characterization of the temporal expression of multiple T3SS components at the transcriptional and protein levels has been performed in *Parachlamydia acanthameobae* replicating in its natural host cell *Acanthamoeba castellanii*. The T3SS components were classified in 4 different temporal clusters depending on their pattern of expression during the early, mid and late-phases of the infectious cycle. The putative T3SS transcription units predicted in *Parachlamydia* are similar to those described in *C. trachomatis*, suggesting that T3SS units of transcriptional expression are highly conserved among *Chlamydiales* bacteria. The maximal expression and activation of the T3SS of *Parachlamydia* occurred during the early to mid-phase of the infectious cycle corresponding to a critical phase during which the intracellular bacterium has (i) to evade and/or block the lytic pathway of the amoeba, (ii) to differentiate from elementary bodies (EBs) to reticulate bodies (RBs) and (iii) to modulate the maturation of its vacuole to create a replicative niche able to sustain efficient bacterial growth.

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3   1   Introduction
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6   2   The *Chlamydiales* order currently includes the *Chlamydiaceae*, *Parachlamydiaceae, Waddliaceae,*
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8   3   *Simkaniaceae*, *Criblamydiaceae* and *Rhabdochlamydiaceae* families (Corsaro and Greub, 2006).
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11   4   *Parachlamydiaceae* naturally resist destruction by free-living amoebae. These obligate intracellular
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13   5   bacteria may use the protistan host as a training ground to select virulence traits that help them to resist
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15   6   to the microbicidal effectors of amoebae and macrophages (Greub, 2009; Greub and Raoult, 2004).
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17   7   *Parachlamydia acanthamoebae* is a bacterium belonging to the *Parachlamydiaceae* family. *P.*
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19   8   *acanthamoebae* is seriously suspected to be involved in respiratory tract infections (Greub, 2009;
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21   9   Lamoth and Greub, 2009) since serological and molecular evidences are supporting its role as a human
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23   10   respiratory pathogen, mainly in patients with community-acquired (Greub *et al.*, 2003b) and aspiration
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25   11   pneumonia (Greub *et al.*, 2003c). In addition, the disease has been reproduced in a murine model
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27   12   following both intranasal and intratracheal bacterial inoculation (Casson *et al.*, 2008).
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30   13   The pathogenic potential of *Parachlamydia* is further supported by the ability of this obligate
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32   14   intracellular bacteria to enter and replicate in human macrophages (Greub *et al.*, 2003a), by preventing
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34   15   the secretion of proinflammatory cytokines (Greub *et al.*, 2005a) and the fusion of its endocytic
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36   16   vacuole with the lysosome (Greub *et al.*, 2005b). Moreover, *Parachlamydia* is also able to enter and
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38   17   replicate in pneumocytes and lung fibroblasts (Casson *et al.*, 2006).
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41   18   Members of the *Chlamydiales* are characterized by a biphasic development cycle comprising the
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43   19   infectious elementary bodies (EBs) and the replicating reticulate bodies (EBs). They have evolved
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45   20   from a common ancestor since more than 700 million years (Greub and Raoult, 2003) that has
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47   21   developed mechanisms for intracellular survival and multiplication in primitive unicellular eukaryotes.
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49   22   Consequently, *Chlamydia*-related bacteria and classical *Chlamydia* share a "core genome" of about
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51   23   700 ORFs that encode for essential bacterial functions that guarantee an efficient intracellular lifestyle
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53   24   including several virulence mechanisms such as the Type III secretion system (T3SS) (Collingro *et al.*,
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55   25   2011b; Bertelli *et al.*, 2010; Greub *et al.*, 2009; Horn *et al.*, 2004).
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26   T3SS mediates the translocation of bacterial effectors to the cytosol of infected cells to modulate

27   various host cell functions facilitating the survival and the replication of the bacterial pathogens

28   (Coburn *et al.*, 2007). The proteins that constitute the T3SS secretory apparatus are termed structural

29   proteins. An additional set of proteins termed "translocators" function by translocating virulence

30   factors called "effector" proteins into the host cell cytoplasm. T3SS genes are present in all *Chlamydia*

31   species examined to date and suggest that T3SS is essential for the survival of these strict intracellular

32   bacteria. Proteins that compose the T3SS secretory apparatus and translocators are for the most part

33   well conserved structurally and functionally among *Chlamydiales* spp. and genes encoding for these

34   T3SS proteins are found in distinct conserved genomic clusters (Betts *et al.*, 2008a; Peters *et al.*,

35   2007). In contrast, T3SS effectors display little sequence homology and are much harder to identify,

36   except for some members of the family of T3SS secreted Inc proteins which are characterized by a

37   large bi-lobed hydrophobic domain that serves to anchor the proteins in the inclusion membrane

38   (Bannantine *et al.*, 2000). Effectors being the direct modulators of host cell functions and of unique

39   pathogenic properties, every bacterium has adapted its pool of effectors to survive and replicate in

40   their respective environmental niches, which likely explained the poor sequence conservation among

41   these secreted proteins. However, effectors proteins often display common structural traits and have

42   similar enzymatic activities among various bacterial species, including cytoskeletal rearrangement,

43   subversion of signaling pathways, activation or inhibition of apoptosis and modulation of intracellular

44   trafficking (Mota and Cornelis, 2005). In *Chlamydiales*, effector proteins are secreted in the inclusion

45   lumen, inserted in the inclusion membrane or translocated in the cytosol of the host cell. A few

46   effectors have been identified so far in *Chlamydiaceae* including Tarp, inclusion membrane proteins

47   IncA to IncG, CADD, Mip and CopN (Beeckman and Vanrompay, 2010; Subtil *et al.*, 2005;

48   Bannantine *et al.*, 2000).

49   The secretory apparatus is composed of about 20 proteins. The functions of the proteins that compose

50   the injectisome of *Chlamydiales* bacteria have been mainly attributed based on sequence similarities

51   with proteins composing well described heterologous T3SS apparatus of other bacteria species such as

52   *Yersinia* and *Salmonella* (Hueck, 1998). Several studies indicate that the secretin-like SctC protein

53   form the outer membrane (OM) ring which is connected to the inner membrane (IM) ring through

54   SctD. The inner membrane (IM) ring is formed by SctD together with the lipoprotein SctJ. In the

55   *Yersinia* T3SS apparatus, the IM ring is associated with a multi-components complex composed of (i)

56   Sct R/S/T/U/V (proteins associated with the export apparatus), of (ii) Sct N/L (ATPase associated

57   proteins) and of (iii) SctQ which form the cytosolic ring (C-ring) (Diepold *et al.*, 2010; Cornelis,

58   2002). Several studies focusing on T3SS protein interactions in *Chlamydia* indicate that the

59   *Chlamydiales* T3SS apparatus is very similar in structure and function compared to the well studied

60   apparatus of *Yersinia* and other species (Betts-Hampikian and Fields, 2010; Fields, 2006).

61   Recent experimental evidences obtained in the *Yersinia* model suggest that the apparatus assembly is

62   initiated by the formation of the SctC OM ring and proceed inwards through the assembly of the IM

63   ring SctD and SctJ. Upon completion of the IM ring, additional T3SS components are recruited to the

64   basal apparatus to complete the export apparatus and to form the ATPase and C-ring complexes.

65   Finally, the needle consisted by SctF is assembled to form a mature and functional T3SS. Unlike

66   SctN/L/Q, the exact temporal integration of the IM proteins SctR/S/T/U/V remained unclear (Diepold

67   *et al.*, 2010).

68   Considering the central role of T3SS in *Chlamydiales* biology, a thorough characterization of T3SS

69   expression of conserved components throughout an infectious cycle of *Parachlamydia* within

70   *Acanthamoeba castellanii* amoeba was conducted. We showed that T3SS conservation among

71   *Chlamydiales* is not only restricted to genomic sequence and organization but is also observed at the

72   level of temporal expression during the development cycle.

73   Results

74   **Temporal characterization of an infectious cycle**

75   A thorough characterization of the different stages that compose a complete infectious cycle of

76   *Parachlamydia* within *A. castellanii* was required to accurately define temporal key stages of the

77   internalization, differentiation, replication and extrusion of *Parachlamydia* during a replicative cycle.

78   Amoebae were infected with a MOI of 10 to ensure a nearly 100% rate of infection allowing thus the

79   study of a single replication cycle. As shown by confocal microscopy, between 5 to 10 infectious

80   bacteria were internalized into amoebae 1h post-infection (Figure 1A). The differentiation of EBs into

81   RBs was mostly completed 5h post-infection and inclusions containing multiple replicating bacteria

82   were observed 8h post-infection. Following a lag-phase characterized by the differentiation of EBs

83   into RBs, an exponential growth is observed between 5 and 16h post-infection as shown by

84   quantitative PCR (Figure 1B). The bacterial growth enters stationary-phase between 16h and 24h post-

85   infection which corresponds to the beginning of the re-differentiation of RBs into EBs (Figure 1A and

86   B). As shown in figure 1A, most of the amoebae are lysed 48h post-infection, releasing EBs in the

87   extracellular environment. Based on these data, the infectious cycle was classified as early-, mid-, and

88   late cycles. These times correspond to key developmental stages with (1) early cycle during which

89   bacteria are internalized in the host cell and differentiate from EBs into RBs, (2) mid cycle

90   characterized by exponential growth of dividing RBs and (3), late cycle consisting of the end of

91   bacterial growth, re-differentiation of RBs into EBs and finally release of EBs into the extracellular

92   environment (Figure 1C).

93   **Identification of 16S rRNA as an optimal internal control to measure relative transcriptional**

94   **expression during a replicative cycle**

95   *Chlamydiales* EBs and RBs are two developmental forms characterized by profound differences in

96   membrane composition and structure that play an important role in the efficiency of bacterial lysis

97   during DNA and RNA extraction procedures. Several lysis protocols compatible with the Promega

98   Wizard SV Genomic DNA Purification kit have been evaluated on samples collected at different times

99   corresponding to the different developmental stages that characterize the growth of *Parachlamydia*

100 within *A. castellanii*. These experiments showed that a specific lysis protocol including an incubation

101 of 2 hours with proteinase K at 55°C has to be followed to ensure efficient lysis of both EBs and RBs

102 since an incomplete lysis of EBs was obtained in absence of proteinase K treatment as measured by

103 quantitative real time PCR of the 16S rDNA gene (figure 2 and supplementary figure 1). Thus, a

104 reduced amount of 16S rDNA was observed in absence of proteinase K treatment for samples

105 collected during early (0 to 4h post infection) and late-phase (36 to 48h post infection) of the

106 replicative cycle that are characterized by a significant presence of EBs whereas no difference was

107 measured during the mid-phase (8h to 24h) which is characterized by the sole presence of replicating

108 RBs.

109 The extraction of integral bacterial RNA is incompatible with proteinase K treatment at 55°C for 2

110 hours. Thus, the transcriptional expression measured by quantitative reverse transcription PCR (qRT-

111 PCR) of several putative constitutively expressed housekeeping genes including *16S rRNA, gyrB,*

112 *secY, rpoB* and *hsp60* was compared to the amount of 16S rDNA extracted without proteinase K and

113 measured by qPCR during a replicative cycle of *Parachlamydia* in *A. castellanii* (Figure 2). The fold

114 increase of the different RNAs compared to the 16S rDNA showed that only the *16S rRNA* was

115 constitutively expressed during a replicative cycle (Figure 2A). The ratio of 16S rRNA / 16S rDNA

116 was of about 1 at each time measured during a replicative cycle whereas the ratio observed between

117 other transcripts with the 16S rDNA was greatly varying during the infectious cycle (Figure 2B).

118 Thus, the normalized expression of *gyrB*, *secY*, *rpoB* and *hsp60* relatively to *16S rRNA* demonstrated

119 that the expression of these essential housekeeping genes is rapidly induced following bacterial

120 internalization (Figure 2C). The transcriptional expression of these genes is then decreasing during the

121 late mid-phase of the replicative cycle when bacteria begin to convert from RBs to EBs. As a control,

122 the transcriptional expression of *hc-1*, the homolog of *Chlamydia trachomatis hctB* expressed during

123 RB to EB conversion, was measured during a replicative cycle (Figure 2). Similar to *hctB*, *hc-1*

124 exhibited an increased expression in the late mid-phase corresponding to the beginning of re-

125 differentiation of RBs into EBs. Together, these data indicate that the amount of measured *16S rRNA*

126 transcripts reflects bacterial growth and can be thus considered as an optimal internal control to

127 measure relative transcriptional expression of the T3SS genes of *Parachlamydia* during a replicative

128 cycle within *A. castellanii.*

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130

131   **Temporal transcriptional expression of T3SS components.**

132   Similar to other *Chlamydiae*, genes encoding for T3SS apparatus components are located in distinct

133   genomic clusters distributed along the chromosome (Figure 3A). Four genomic clusters containing

134   conserved genes encoding for the injectisome and the translocon apparatus and exhibiting a very

135   similar genetic organization than other *Chlamydiae* were identified in the genome of *Parachlamydia*

136   (Greub *et al.*, 2009). Unlike *Chlamydiaceae*, no gene showing homology with the flagellar system and

137   having a possible function in chlamydial T3SSs have been identified in the genome of *Parachlamydia*.

138   Thus, the transcriptional expression of the mRNA transcripts of every gene encoding for a protein with

139   a known function was analyzed by qRT-PCR (Figure 3B). The expression of the various T3SS genes

140   during the developmental cycle could be classified in 4 temporal expression clusters (Figure 3B). The

141   first cluster composed of the *copB*, *sycD1* and *sctU* is characterized by a marked repression or lack of

142   activation during the early and mid-phases followed by a significant increased expression in the late-

143   phase (Figure 3B, EL). The second cluster including the *sct Q/W/F/E/G/V/N* genes is characterized by

144   a basal expression during the early and mid-phases and a significant increased expression during the

145   late-phase (Figure 3B, L). The third cluster composed of *sctC/L/J/T* and the multi-cargo secretion

146   chaperone *mcsc* includes genes that exhibit a dramatic increased expression in the early and mid-

147   phases followed by a decreased basal expression during the late-phase (Figure 3B, E). Finally, the

148   fourth temporal cluster is composed of the putative predicted T3SS secreted effector *pkn5* and of a

149   *Parachlamydia* secreted protein localizing to the replicative inclusion membrane (PahN) that are both

150   significantly highly expressed during the mid and beginning of late-phase (Figure 3B, M).

151   Following normalization of expression with the highest temporal expression assigned to 100 and the

152   lowest to 0, a temporal matrix composed of the transcriptional expression of the measured T3SS genes

153   was generated (Figure 4A). These analyses strongly suggest that the expression of the genes that

154   encode for T3SS components can be classified in 4 major defined temporal clusters of gene expression

155   composed of early (E), early-late (EL), mid (M) and late (L) phase-expressed genes.

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156   A bioinformatic analysis of the T3SS genomic clusters loci was performed with the fgenesB software

157   to predict transcriptional units and compared with mRNA expression analysis of T3SS genes. Except

158   for one gene (*pkn5*), the experimental qRT-PCR gene expression data are congruent with the *in silico*

159   prediction (Figure 4B) showing no discrepancies between temporal gene expression and association to

160   a predicted transcriptional unit. Thus *sctU* and *sycD1/copB* (cluster EL), *sctC* and *sctJ/L/T* (cluster E),

161   *sctE/F/G/N/Q* (cluster L), *sctV/sycE* (cluster L) are all located in distinct transcriptional units

162   corresponding to defined temporal clusters (Figure 4). Moreover, the *C. trachomatis* T3SS genes

163   transcriptional expression study performed by Hefty *et al.* (Hefty and Stephens, 2007) strongly

164   indicates that transcriptional expression units of T3SS genes are highly similar between

165   *Parachlamydia* and *C. trachomatis* (Figure 4B). Together these data suggest that T3SSs in

166   *Chlamydiales* are not only conserved at the level of genetic sequence and organization, but also at the

167   level of gene expression.

168   **Temporal protein expression measurement by western blotting: limitations of the technique**.

169   Even though transcription and translation in bacteria are very often coupled, an analysis of protein

170   expression throughout an infectious cycle has to be performed to confirm that a similar temporal

171   expression of T3SS genes and their respective encoded proteins is observed. Proteins encoded by

172   genes belonging to different temporal clusters were selected for protein purifications and mouse

173   immunization programs. Two proteins, Mcsc and SctJ, encoded by genes expressed during early and

174   mid-phase (temporal cluster E), two proteins, CopB and SycD (Scc2), encoded by genes expressed

175   during the early and late-phase (temporal cluster EL) , and CopN (SctW) encoded by a gene expressed

176   during the late-phase (temporal cluster L) were selected. The antisera specificity and the protein

177   expression at different times of the infectious cycle were measure by western blot using specific

178   antibodies (Figure 5). A major drawback of measuring comparative protein amounts during the

179   bacterial growth of obligate intracellular bacteria is the difficulty to normalize the amount of total

180   bacterial proteins collected at different times during the replication cycle. An increased in specific

181   proteins amounts in the late-phase of the replicative cycle may only be due to a significant increased in

182   bacterial numbers and not to a specific up-regulation of protein expression. Thus, specific antiserum

183   raised against whole bacterial cells was used as an internal control to monitor bacterial growth during

184   the development cycle. The expression of SycD and Mcsc was poorly or not detected by western blot

185   before the late-phase of the replicative cycle hindering the possibility to measure their relative

186   expression throughout parachlamydial growth within *A. castellanii*. An estimation of the relative

187   expression of CopB, CopN (SctW) and SctJ during the infectious cycle was calculated by normalizing

188   the measured intensity of the band corresponding to the various proteins to the intensity of the signal

189   corresponding to the whole bacterial cells (Supplementary figure 2). Similar to gene expression

190   analysis of *copB* and *copN (SctW)*, an increasing amount of the protein CopB and CopN (SctW) was

191   observed in the late-phase of the bacterial replicative cycle. Interestingly, a high relative amount of

192   these proteins was also observed at 8hr suggesting that late-phase synthesized proteins of the T3SS are

193   likely functional and still present in high amount during the early-phase of subsequent infection

194   cycles. The protein encoded by the early transcribed gene *sctJ* exhibited the highest relative expression

195   at 8h confirming that de novo SctJ protein synthesis is likely occurring during the early-phase of the

196   infection (Supplementary figure 2). Unfortunately, the amount of bacteria present during the very

197   early-phase of infection is very low and the expression of the analyzed proteins was not detected

198   before 8h post-infection hindering the possibility to accurately monitor protein expression by western

199   blot during the critical first hours of the infectious cycle. In addition, the relative intensity measured at

200   early time of infection by western blot is subjected to imprecision due to very low signal detection.

201   Thus, an alternative method to obtain more accurate protein expression measurement during the entire

202   replicative cycle is required.

203   **Temporal protein expression measurement by quantitative immunofluorescence.**

204   To circumvent western blotting experimental limitations, a quantitative immunofluorescence method

205   proposed by Noursadeghi et al. (Noursadeghi *et al.*, 2008) was adapted to measure bacterial single cell

206   protein expression by quantitative immunofluorescence confocal microscopy (Figure 6). This method

207   relies on the measurement of the summing of protein signals from multiple focus plans covering the

208   entire thickness of the sample. Quantitative fluorescence data are then obtained by generating the

209   histogram of the signal intensity of a Z-projection obtained by summing multiple focal slices. Using

210   this methodology, the protein expression of the late-cycle expressed gene *copB* was analysed during a

211   complete replicative cycle by using a specific anti-CopB mouse anti-sera (Figure 7). Quantitative

212   immunofluorescence histograms were generated for several time points post-infection and showed that

213   the protein CopB is expressed during late-cycle phase, confirming a late-cycle expression of the T3SS

214   component CopB. The same analysis was applied to the Mcsc, SctJ, PahN and SctW (CopN) proteins

215   (Figure 8). Similar to CopB, the temporal expression of these 4 proteins were congruent with the

216   temporal expression of their respective mRNA transcripts (Figure 8 and 3B). The chaperone Mcsc and

217   the inner membrane components SctJ were expressed in higher amount 2h and 8h post-infection,

218   during the early and mid-phase cycles, followed by a marked reduced signal intensity observed from

219   16h post-infection which corresponds to the entry into late-cycle phase. The histogram intensity of the

220   late transcribed gene encoded protein SctW (CopN) showed a gradual reduction of the protein amount

221   from 2h to 16h, indicating that a relative high amount of proteins are present in EBs and in early

222   differentiated RBs during the first phase of the infectious cycle but that no significant induction of

223   protein expression occurred before 24h post-infection which corresponds to a late-phase protein

224   expression. Finally, the protein PahN encoded by a mid-phase transcribed gene showed also an

225   increased expression from 8h post-infection which is maintained during the mid to late-phase of the

226   replicative cycle. These data demonstrate that the temporal pattern of protein expression measured by

227   quantitative immunofluorescence of the 5 selected proteins Mcsc/SctJ/CopN/SctW/PahN is similar to

228   their respective mRNA temporal expression measured by qRT-PCR. These data indicate that protein

229   and mRNA expression of various T3SS components are tightly coupled and that temporal

230   transcriptional expression analysis of multiple T3SS genes can be used to monitor the expression of

231   different T3SS components and thus to characterize its functionality during an infectious cycle.

232   **Discussion**

233   Genome sequencing of multiple *Chlamydiales* bacteria belonging to different distant families have

234   demonstrated that despite marked differences in their genomic size and content, the T3SS loci

235   distributed in different clusters along the chromosomes exhibit a conserved sequence and genetic

236   organization (Collingro *et al.*, 2011a; Bertelli *et al.*, 2010; Greub *et al.*, 2009; Peters *et al.*, 2007).

237   Several studies have demonstrated that specific inhibition of T3SS with chemical compounds totally

238   inhibits bacterial growth in a dose dependent manner strongly indicating that this secretion system is

239   essential for the establishment of *Chlamydiales* replicative conditions (Bertelli *et al.*, 2010; Bailey *et*

240   *al.*, 2007; Muschiol *et al.*, 2006). The temporal expression of T3SS during a replicative cycle has only

241   been studied in *Chlamydiaceae* including *C. trachomatis*, *C. pneumoniae* and *C. psittaci*. In this study,

242   a complete characterization of the temporal expression of multiple T3SS components at the

243   transcriptional and protein level has been performed in *Parachlamydia acanthameobae* replicating in

244   its natural host cell *Acanthamoeba castellanii*. This study is the first description of the T3SS

245   expression during the replicative cycle of a member of the *Chlamydiales* order not belonging to the

246   *Chlamydiaceae* family. The replicative cycle of *Parachlamydia* within *A. castellanii* was separated in

247   3 phases, early, mid and late. The early-phase is characterized by a lag-phase when EBs are

248   internalized in the host cell and differentiate into RBs. The mid-phase is characterized by exponential

249   bacterial replication. The late-phase is starting when bacteria enter stationary phase where RBs

250   redifferentiate into EBs which are then released in the extracellular milieu. This study showed that the

251   temporal transcriptional expression of multiple genes encoding for T3SS components could be

252   classified in 4 different temporal clusters depending on their pattern of expression during the early,

253   mid and late-phases of an infectious cycle. Classical western blotting being of limited value for

254   quantitative temporal assessment of protein expression of obligate intracellular bacteria, a new

255   quantitative immunofluorescence approach allowing accurate measurement of protein expression was

256   used in this study. The expression analysis of selected T3SS proteins encoded by genes belonging to

257   different temporal clusters showed that a similar temporal pattern of transcriptional and protein

258   expression was observed for each of these genes (*copB, mcsc, sctJ, pahN, sctW*).

259   Based on our experimental data, a model of temporal expression of T3SS during an infectious cycle

260   can be proposed (Figure 9). The T3SS is a multi-component complex machinery containing protein

261   with diverse functions and in varying quantities. A varying stoechiometric composition of the T3SS

262   could likely define the functionality of the apparatus. The levels of proteins in bacterial cells are

263   controlled by multiple mechanisms and are defined by both the rate of protein synthesis and protein

264   degradation, which are varying with the physiological status of the bacteria during a growth cycle. The

265   relative protein content of the T3SS in EBs is likely similar to the protein content observed during the

266   late-phase of the replicative cycle when RBs redifferentiate into EBs before cell lysis and bacterial

267   release in the extracellular milieu. During that time, the T3SS is likely not required for secretion of

268   bacterial effectors and maintained in a dormant state ready to be activated. Upon cell contact and

269   bacterial internalization of EBs, the T3SS is likely quickly activated to allow rapid secretion of

270   effector proteins to modulate host cell functions. Varying de novo rapid synthesis of T3SS

271   components depending of protein stability, quantity and functionality likely occurs during the critical

272   early-phase of bacterial infection to promote bacterial survival, differentiation, maturation and

273   initiation of replication. The subversion of host cell defense mechanisms has likely to be operated

274   during the early-phase of the infection to ensure bacterial evasion from the lytic pathway. Similarly, a

275   rapid modulation of host cell functions ensuring a fast maturation of the replicative vacuole is a pre-

276   request to trigger bacterial replication. Thus, the importance of a highly functional and efficient T3SS

277   during the early-phase of infection would likely confer a significant advantage for the intracellular

278   bacteria against the defense mechanisms of the host cell. In mid-phase, once the bacteria have

279   established conditions allowing efficient bacterial replication such as replicative vacuole maturation,

280   subversion of host cell defenses and establishment of systems allowing efficient scavenging of host

281   cell nutrients, the importance and the number of T3SS per bacteria are reduced or only maintained in

282   bacteria being in direct contact with the inclusion membrane. Finally, increased de novo synthesis of

283   some T3SS components in the late-phase trigger the shutdown of the apparatus (SctW) and the

284   formation of a silent T3SS in EBs ready to be rapidly activated when requested.

285   A significant similarity between the transcriptional expression units of *Parachlamydia* and *C.

286   trachomatis* was observed, suggesting that T3SSs sequences, genetic organization and transcriptional

287   expression units are highly conserved among *Chlamydiales* bacteria (Hefty and Stephens, 2007).

288   However, several studies indicate that the temporal expression of these transcriptional units may vary

289   among *Chlamydiales* bacteria (Beeckman *et al.*, 2008; Hefty and Stephens, 2007; Lugert *et al.*, 2004;

290   Slepenkin *et al.*, 2003). Some discrepancies may originate from several issues including chlamydial

291   species specificities, growth kinetics differences, host cell lines and variation of the limit of detection

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292 of the molecular techniques used in the different experimental assets. The regulation of the T3SS

293 expression in *Chlamydiales* is poorly understood. T3SS genes and operons are preceded by predicted

294 *E. coli* σ70-like promoter elements but no transcriptional regulators have yet been identified (Hefty

295 and Stephens, 2007). Similarly, the explanation of the separation of the *Chlamydiales* T3SS elements

296 in distinct genomic clusters and transcriptional units expressed in different temporal classes remained

297 to be determined. Transcriptional regulation of T3SS genes in other bacterial species such as *Yersinia*

298 and *Salmonella* is mainly controlled by AraC-like transcriptional activators whose activities are

299 regulated by several regulatory pathways responding to multiple environmental signals sensed during

300 the infection process (Francis *et al.*, 2002). No gene encoding for an AraC-like transcriptional

301 activators has been identified in *Chlamydiales* genomes, suggesting that C*hlamydiales* T3SSs are

302 regulated by a different mechanism. The absolute requirement of T3SS for chlamydial survival and

303 replication in host cells may suggest that T3SSs are controlled by the global regulatory network

304 system of *Chlamydiales* bacteria via various transcriptional regulators and/or alternative regulatory

305 systems. Recent data suggest that transcriptional expression of chlamydial T3SS operons is controlled

306 by the degree of DNA supercoiling that varies temporally during a developmental cycle (Case *et al.*,

307 2010). Thus, the differences of sensitivity of the promoters to alteration of DNA supercoiling may

308 define temporal transcriptional expression differences of T3SS operons and of other unrelated

309 chlamydial genes.

310 The *Chlamydiales* T3SS is rapidly activated upon contact with the host cell and remains probably

311 functional until bacterial detachment from the inclusion membrane and RBs conversion into EBs

312 (Fields, 2006; Clifton *et al.*, 2004; Fields *et al.*, 2003). Even though cell contact-dependent activation

313 of T3SS is conserved among bacteria (Hueck, 1998), the exact mechanism governing the activation in

314 *Chlamydiales* remains to be identified and marked differences compared to other bacterial species

315 T3SS could be observed considering the peculiar developmental cycle of *Chlamydiae*. For instance,

316 the *Chlamydiales* needle protein SctF contains cystein residues that are, like the proteins forming the

317 envelope, linked by disulfide bridges in EBs (Betts *et al.*, 2008b) indicating that a reduction of these

318 bonds could be required for a complete activation of the secretion. This hypothesis is supported by the

319   observation that the classical activation of T3SSs secretion obtained by Ca2+ depletion did only

320   poorly stimulated the secretion of the Tarp effector from purified cell-free *C. trachomatis* EBs

321   (Jamison and Hackstadt, 2008).

322   Based on T3SS studies in other bacteria, some components of the chlamydial T3SS likely exhibit

323   secretion regulatory functions such as SctW (CopN), SctU and SctL. SctW contains domains similar to

324   *Yersinia* YopN and TyeA which are negative regulators of T3SS secretion (Joseph and Plano, 2007;

325   Goss *et al.*, 2004). The secretion of effector proteins by the T3SS is partially controlled by the

326   complex YopN/TyeA which interacts with the secretory apparatus to prevent secretion before

327   activation of the system upon detection of the right stimuli by the needle-tip complex (Joseph and

328   Plano, 2007). The breaking of the YopN/TyeA interaction triggered by the stimuli that activate the

329   T3SS induce the secretion of YopN, which render the apparatus operational for effectors secretion.

330   The homologous SctW protein of *Chlamydiales* could have a similar regulatory function in addition of

331   its secreted effector activity that trigger cell cycle arrest by altering the microtubule cytoskeleton

332   (Huang *et al.*, 2008; Fields and Hackstadt, 2000). The expression of SctW observed in late-phase of

333   the replicative cycle of *Parachlamydia* is compatible with the dual function of this protein. The

334   expression and the recruitment of SctW to the secretory apparatus in late-phase coincide with

335   deactivation of the T3SS and thus inhibition of secretion. During the early-phase of infection, SctW is

336   rapidly secreted which renders again the T3SS secretion competent.

337   The cleavage of the SctU *Yersinia* homolog YscU is required to permit secretion of effector proteins

338   likely by allowing the recruitment of the ATPase complex SctN/L to the apparatus (Riordan and

339   Schneewind, 2008). The transcriptional expression of the *sctU* gene is highly reduced in the mid-phase

340   of the replicative cycle of *Parachlamydi*a, which suggest that once the T3SS apparatus is assembled

341   and activated, the requirement and the expression of the this protein is possibly decreased as observed

342   in this study, or that single SctU proteins might be shared by several T3SS apparatus when its

343   requirement decreases.

344   Regulation of the T3SS during the replicative cycle might be operated through the controlled temporal

345   expression of some components required to initiate protein-protein interactions or functionality. For

346   instance, studies on the *Yersinia* T3SS indicate that the assembly of the cytosolic complex consisting

347   of the ATPase SctN, the interacting protein SctL and the C ring component SctQ require the presence

348   of all of its components (Diepold *et al.*, 2010; Jackson and Plano, 2000). Thus, in *Parachlamydia*, the

349   late-phase cycle expressed proteins SctN and SctQ would not be active until the early-phase

350   expression of maximal amounts of SctL initiates or increases the early formation of the

351   SctN/SctL/SctQ complex, triggering its docking onto the IM ring and the subsequent full activation of

352   the T3SS secretion system. Similarly, the early expression of the multiple cargo secretion chaperone

353   Mcsc would initiate or increase a protein-protein interaction hub consisting of Mcsc-effector-SctQ

354   complexes and activation of T3SS-dependent effectors secretion.

355   The CopB protein identified in *Parachlamydia* is poorly homologous to the translocator proteins

356   CopB and CopB2 of *Chlamydiaceae* bacteria (Chellas-Gery *et al.*, 2011) . Similar to *sctU*, the

357   expression of CopB is reduced during the mid-phase. Even though no sequence homologies are

358   observed, CopB of *Parachlamydia* exhibit more similarities with the predicted secondary structures of

359   the *C. trachomatis* CopB2 than with CopB (data not shown). In addition, no co-localisation of CopB

360   with the inclusion membrane of *Parachlamydia* has been observed in this study suggesting that this

361   protein is possibly secreted in the host cell cytoplasm similarly to CopB2 of *C. trachomatis* (Chellas-

362   Gery *et al.*, 2011).

363   In conclusion, the differential temporal expression of T3SS components during the replicative cycle

364   of *Parachlamydia* suggests that the maximal expression and activation of T3SS injectisomes occurs

365   during the early to mid-phase of the infectious cycle, which corresponds to a critical phase during

366   which the intracellular bacterium has to evade and/or block lytic effectors of the amoebal host cell, to

367   differentiate from EBs to RBs and to modulate the maturation of its replicative vacuole to create

368   replicative condition able to sustain efficient bacterial replication. Based on several studies mainly

369   performed in other bacteria than *Chlamydiales*, the functions of several T3SS components are well

370   described whereas for other components, no functionality has yet been attributed. In addition of a

371　described enzymatic, regulatory or structural function, a temporal activity remains to be characterized

372　for many proteins that compose the chlamydial T3SS machinery. The T3SS machinery is likely a

373　dynamic structure which can fluctuate during a replicative cycle to modulate its functionality. The

374　expression of each component of the T3SS during the replicative cycle depends of several molecular

375　criteria including function, activity, stoechiometric ratio, stability and turnover of each protein. In

376　addition, some proteins might be shared between different apparatus of the cell. Finally, recent data

377　strongly suggest that unlike other described bacterial species, the *Chlamydiales* T3SS is likely deeply

378　involved in additional roles than solely protein secretion and translocation (Peters *et al.*, 2007). For

379　instance, the attachment to inclusion membranes and the differentiation of RBs into EBs may be

380　controlled by the T3SS. Thus, differential temporal expression of various components of the

381　chlamydial T3SS may be required to modulate these multiple regulatory activities.

382　## Experimental procedures

383　**Bacterial strain.** *P. acanthamoebae* strain Hall's coccus were grown at 32 °C within *Acanthamoeba*

384　*castellanii* in 75-cm^2 cell culture flasks (Corning, New-York, USA) with 30 ml of peptone-yeast

385　extract-glucose (PYG) broth (Greub and Raoult, 2002). After 5 days of incubation, cultures were

386　harvested and the broth was filtered through a 5μm pore to eliminate both amoebal trophozoites and

387　cysts and to collect bacteria in the flow through. *A. castellanii* ATCC 30010 was cultured in 75-cm^2

388　cell culture flasks at 25°C.

389　**Infection procedure.** Amoebae harvested from 24h fresh culture were centrifuged at $200 \times g$ for 5

390　min, resuspended in RPMI-HEPES supplemented with 200 mM L-glutamine (Gibco-BRL, Life

391　Technologies, Paisley, Scotland) and 10% fetal calf serum (Gibco-BRL). Then, 10^5 amoeba cells/ml

392　were incubated for 2h at 25 °C in 24-well cell culture plates (Corning). Non-adherent cells were

393　washed and the remaining adherent cells were incubated for 16h at 25°C.

394　The amount of bacteria after 5 days of amoeabal co-culture was equal to about 10^8 cells per ml as

395　measured by quantitative PCR of the 16S rDNA. Host cell lysis assay following infection of *A.*

396 *castellanii* with limiting serial dilution of a bacterial filtrate confirmed that most collected bacteria are

397 living cells.

398 Wells containing 10^5 amoebae were infected with a 10^6 bacteria/ml suspension corresponding to a

399 multiplicity of infection (MOI) of about 10. Plates were centrifuged at $1790 \times g$ for 10 min at room

400 temperature. After 30 min of incubation at 32 °C, amoebal cells were washed with RPMI-HEPES and

401 further incubated for different periods at 32 °C in RPMI-HEPES supplemented with 200 mM L-

402 glutamine and 10% fetal calf serum.

403 **Confocal microscopy.** Infected amoeabe were washed with PBS, fixed with ice-cold methanol for 4

404 min. Cells were then washed three times with PBS and were then blocked and permeabilised for 1h in

405 a blocking solution (PBS/0.1% Saponin/10% fetal calf serum). Coverslips were incubated with

406 primary mouse antisera directed against different specific proteins (CopB, SycD1, Mcsc, SctW, SctJ,

407 PahN) or against total whole cells of *Parachlamydia* for 1h at room temperature in blocking solution.

408 After washing three times with PBS/0.1% saponin, coverslips were incubated for 1h with secondary

409 anti-mouse antibodies in blocking solution containing concanavalin A (Invitrogen, Basel, Switzerland)

410 and DAPI (dilactate, D3571, Molecular Probes, OR, USA) to counterstain amoebae and label DNA,

411 respectively. After two washing in PBS/0.1% saponin, two in PBS and one with deionised water, the

412 coverslips were mounted onto glass slides using Mowiol (Sigma-Aldrich, MO, USA). Cells were

413 observed on a confocal fluorescence microscope (Zeiss LSM710 Meta, Jena, Germany). Files were

414 analysed using Image J for microscopy (www.macbiophotonics.com) softwares. All the assays were

415 performed at least in triplicate.

416 **Quantitative reverse transcription polymerase chain reaction (qRT-PCR).** Amoeabe were

417 infected in 24-wells plates as described before. Samples were harvested at different times

418 post-infection by scraping amoeba cells from the well surface and by resuspending the

419 samples in RNAprotect bacteria reagent (Qiagen GmbH, Germany). Total RNA was extracted

420 using the RNeasy Plus Mini Kit (Qiagen GmbH, Germany) as described by the manufacturer.

421 The RNA samples were treated with the DNA-free kit (Ambion, Applied Biosystems,

422 California, USA) to remove contaminating DNA from the RNA preparation.

423   The analysis of mRNA quantification was performed by a one step reverse transcription polymerase

424   chain reaction using the One step MESA GREEN qRT-PCR MasterMix Plus for SYBR® assay

425   (Eurogentec, Seraing, Belgium). The design of the specific primers listed in table 1 was done with the

426   Primer3plus software (http://primer3.sourceforge.net/webif.php). The qRT-PCR assay was performed

427   in a total volume of 20 μl with 10 μl of 2x reaction buffer, 100nM forward primer, 100nM reverse

428   primer, 0.25U/μl Euroscript reverse transcriptase, 0.1U/μl RNase inhibitor and RNAse-free water. The

429   thermocycling conditions were set up as follow: (i) reverse transcription, 30min, 48°C, (ii) Meteor *Taq*

430   activation and Euroscript inactivation, 5min, 95°C, (iii) 40 cycles, 15sec, 95°C, 1min, 60°C, (iv) melt

431   curve analysis, temperature increment of 0.3% from 60 to 95°C. The analysis of the 16S rRNA was

432   used as an internal control to normalize the quantification of every specific mRNA collected at

433   different times during an infectious cycle. The results are presented as a relative quantification taking

434   samples at time 0 (EBs) as a reference of 1. Water was used as a negative control and the qRT-PCR

435   reaction without reverse transcriptase was used as a control to measure DNA contamination in the

436   extracted RNA samples. All measurements were performed at least in triplicates.

437   **Protein purification and generation of antisera**. *P. acanthamoebae sctJ*, *sctW*, *copB*, *mcsc*, *pah-N*

438   and *sycD (scc2)* ORFs were amplified from purified *P. acanthamoebae* genomic DNA using primers

439   listed in table 2 using the high fidelity Phusion polymerase (Finnzymes, Espoo, Finland). Using the

440   Champion pET directional TOPO Expression Kits (Invitrogen), *sctJ* and *mcsc* were cloned in frame

441   with a N-terminal and C-terminal peptide containing a polyhistidine (6xHis) tag to generate the

442   plasmids pET200/D-TOPO-*sctJ* and pET101/D-TOPO-*mcsc*, respectively (Table 2). The four bases

443   CACC were added to the forward primers to allow directional in-frame cloning of the PCR fragments

444   into plasmids pET200/D-TOPO and pET101/D-TOPO. Following digestion of the PCR amplicons and

445   the plasmids with NcoI/HindIII, *copB* (nt 1-960) and *pahN* were ligated into the plasmid

446   pBADmycHis (Invitrogen) to generate the plasmids pBADmycHis-*copB* and pBADmycHis-*pahN*

447   encoding for C-terminal polyhistidine tag (6xHis) recombinant proteins. Similarly, *copN* and *scc2*

448   amplicons and the plasmid pET28a were digested with NdeI/BamHI to generate by ligation the

449   plasmids pET28a-*copN* and pET28a-*scc2* encoding for N-terminal polyhistidine tag recombinant

450   proteins. The proteins were expressed in BL21 star (DE3) *E. coli* strains following 0.5mM IPTG

19

451   (pET200/D-TOPO-*sctJ* / pET101/D-TOPO-*mcsc* / pET28a-*copN* / pET28a-*scc2*) induction at 37°C or

452   in TOP10 *E. coli* strains following 0.02% arabinose (pBADmycHis-*copB* / pBADmycHis-*pahN*)

453   induction at 37°C. The BL21 and TOP10 *E.coli* strains expressing the protein of interest were lysed

454   with the FastBreak cell lysis solution (Promega) and recombinant His-tagged proteins were purified in

455   native or denaturing conditions using the MagneHis protein purification system (Promega) according

456   to the manufacturer's recommendation. Purified recombinant His-tagged proteins were used to

457   conduct mouse immunization programs at the Eurogentec animal facility (Eurogentec, Seraing,

458   Belgium) to elicit the production of specific mouse antisera.

459   **Western blot**. For Immunoblot analysis, protein samples were separated by SDS-PAGE, transferred

460   to 0.45 μm nitrocellulose membranes and blocked with 5% non-fat powder milk in TBST (50mM

461   Tris-Base, 150mM NaCl, 0.2% Tween pH7.4). The nitrocellulose membranes were incubated with

462   mouse primary antibodies (anti-SctJ, SctW, CopB, Mcsc, SctF) diluted in 0.5% milk-TBST, washed 3

463   times, incubated with secondary antibody conjugated to horseradish peroxidase diluted in 0.5% milk-

464   TBST and detected by chemiluminescence. The density of the bands on western blots was measured

465   with the gel analysis method of the ImageJ software as described by Luke Miller

466   (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/).   Western

467   blot have been done at least in triplicates for each analyzed proteins.

468   **Quantitative immunofluorescence.** Proteins were labeled by immunofluorescence staining with

469   specific mouse polyclonal antibodies as described in confocal microscopy. A series of images of

470   multiple focal planes covering the entire thickness of the sample were collected by z-stack confocal

471   microscopy. Using the ImageJ software, a Z-projection composed of the summing of the slices was

472   generated. The region of interest (ROI) including only the signal of intensity emitted by the bacteria

473   was defined by applying a median filter (2x2.0 pixels) and by adjusting the Isodata threshold setup. A

474   histogram of the ROI composed of the signal intensity on the x-axis and of the frequency on the y-axis

475   was generated. A mean value and a standard deviation of the histogram of the frequency distribution

476   of fluorescence intensity were also generated. The results were then exported and analyzed using

477   GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). The

478   signal intensity for each frequency was normalized by using a value of 100 for the highest intensity.

479   Several images from one experiment performed at least in triplicates have been analyzed for each

480   protein.

481

482   **References**

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601

602   **Tables**

603   **Table 1.** List of primers used in qRT-PCR

Name	Sequence	Tm	Product Size
qRTPCR-16S-F	TGACGATGCGACGCCGTGTG	60.38	
qRTPCR-16S-R	GCAGCTGCTGGCACGGAGTT	60.25	142
qRTPCR-sycD#1-F	AGGGCTTGCTCCGAAAGACCTCA	59.87	
qRTPCR-sycD#1-R	ACAGGCCGCCAATCCCATCG	59.48	175
qRTPCR-copB-F	TAGGCGCGGGTGCAGGTTTG	59.97	
qRTPCR-copB-R	CAATGCTGGCGGCCCTGGAA	59.97	167
qRTPCR-sycD#2-F	GCAAGCCATGGGCCTTAGCGA	59.78	
qRTPCR-sycD#2-R	TCGACGCTGCAAGTCCGAGTG	59.47	161
qRTPCR-sctJ-F	GCTCGGCAAGACCCGCTTGA	59.7	
qRTPCR-sctJ-R	AGGCGACGCAAGCCATGTCG	60.38	144
qRTPCR-sctL-F	GCTTCAAAGCCTTCTTTTTGAGCGGC	60.01	
qRTPCR-sctL-R	GCGCCTAGCTCTAAGGTCGTTTCTG	59.46	161
qRTPCR-sctT-F	GTGCGGCAAAACACGCGCTC	60.64	
qRTPCR-sctT-R	ACAGTGCGCTTGCCACTGGAG	59.92	119
qRTPCR-sctC-F	TGCTCCTGCTGGTGGAGTCGT	59.84	
qRTPCR-sctC-R	TGCAGTAGGCTGCGGCACAA	59.27	139
qRTPCR-pkn5-F	AAAGCGGGGATCGCCTGAGC	59.49	
qRTPCR-pkn5-R	CCCTCCTCCCGGGACGTCAA	59.61	170
qRTPCR-sctQ-F	TTGGGAAGCGTTTGCGCAGC	59.37	
qRTPCR-sctQ-R	CCCACCAAACGGGACCTTCAATCG	60	170
qRTPCR-sctF-F	GCGCGAAAGCCAAACTGCTTGA	59.44	
qRTPCR-sctF-R	AGTTCGCTGCACTGATAACCGCTG	59.83	138
qRTPCR-sctD-F	CCGGAAGAAGAGGACGCCGC	59.84	
qRTPCR-sctD-R	GGTGCGCCATCTTCAGCGACA	60.05	143
qRTPCR-sctW-F	ACCGCGGAGCAAGCGGAAAG	60.32	
qRTPCR-sctW-R	GCGCTGCGCAGAAAGTGGC	60.11	111
qRTPCR-sctU-F	ACGTGCTCAGGCAGTTGTCACG	59.93	
qRTPCR-sctU-R	CGCTCAGCTAGGGCGTCTTCG	59.67	117
qRTPCR_Mcsc-F	TGGCACGTTTGCTTCACCTCATCA	59.35	
qRTPCR_Mcsc-R	TGGCGGGGATGAGAAGGCGG	60.95	102
qRTPCR_sctE-F	AGCGGATCCTGTGAAAATGCGGG	59.81	
qRTPCR_sctE-R	TTCGCGAGATCACTTTTAGCATGGATGT	59.01	158
qRTPCR_sctG-F	ACTTCCCTTTGCTAATCGAGGCTGGT	59.95	
qRTPCR_sctG-R	TGCCCGGGACTCATCGCTTCT	59.98	103
qRTPCR_sctN-F	AGCGCGTCGGCATTTTTGCG	60.11	
qRTPCR_sctN-R	GTCACGAAGCTCACGCCCCC	60.04	128
qRTPCR_sctV-F	GATCACCAAGGGTGCGGAACGG	60.05	
qRTPCR_sctV-R	AGCGCGCATATCGGCGTCAA	59.91	100
qRTPCR-pahN-F	GCCCCGTGTGGAATCTGCACC	60.31	
qRTPCR-pahN-R	AGCAAGCAAACAACTCACAGGCCA	59.76	124
qRT-PCR_gyrB-F	CCTGCGTATGCGCCCTTTCCA	59.8	
qRT-PCR_gyrB-R	TGGTGCCGCGTTTGGTGGTT	60	135
qRTPCR_hsp60-1-F	CAAGCACGAAAACATGGGCGCT	59.2	

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qRTPCR_hsp60-1-R	GCTCCAGCTGCGACATTGCG	59.3	141
qRTPCR_secY-F	TGGGGAAGCGGCAAAGCGAA	59.6	
qRTPCR_secY-R	TCACCAGCGACGATACCTGGCTT	60	135
qRTPCR_rpoB-F	ACGGCGCTGAAAGGGTTGTTGT	59.8	
qRTPCR_rpoB-R	TCCGCGCATGTGGCGTTCTT	60	80
qRTPCR_hc1-F	GGCACAAAAGGCCGCGCAAA	59.9	
qRTPCR_hc1-R	AGCAGCATGTTTAGGCGCAGCA	59.9	87

604

605   Tm calculation: thermodynamic parameters and salt correction formula: SantaLucia JR, 1998

606   **Table 2.** Cloning primers and plasmids

primers	sequences	plasmids	His-tag position	Comments
sctJ-For	**CACC**GAATCGCGGAAAACGATTG			
sctJ-Rev	TTAAGATTCTTTAGCCACAATGATTGAC	pET200/D-TOPO	N-terminal	direct PCR product TOPO directional cloning
mcsc-For	**CACC**ATGGTAAAATTAAAGCTAGATAGAATCATCG			
mcsc-Rev	AAGGGTATGTTTTTTTTCTTCTTGTG	pET101/D-TOPO	C-terminal	direct PCR product TOPO directional cloning
copB-For (NcoI)	GGTGGT**CCATGG**TGAATGAATTTAATATTTCATCACAGGGACA			
copB-Rev (HindIII)	GGTGGT**AAGCTT**TAAATTTCCTGTTTGGCTCGCTGTTC	pBADmycHis	C-terminal	subcloning nt 1-960
copN-For (NdeI)	GGTGGT**CATATG**CCCGATTGGAATATCCAACAAG			
copN-Rev (BamHI)	GGTGGT**GGATCC**TTATTCTTTATCTTCCTCTTCTTCCTGTT	pET28a	N-terminal	
scc2-For (NdeI)	GGTGGT**CATATG**AAAGGTGAACAAAGTCAAATCAAAAAGG			
scc2-Rev (BamHI)	GGTGGT**GGATCC**CTATGAAATCGGTTGATTTTCTTCAGC	pET28a	N-terminal	
pahN-For (NcoI)	GGTGGT**CCATGG**CTATAAGTTTATATTCAAATCAACAACC			
pahN-Rev (EcoRI)	GGTGGT**GAATTC**GTATTAGACGTTGCAGGTTTTTTCTTC	pBADmycHis	C-terminal	

607

608   Primers: The restriction enzymes used to digest and clone the PCR products in the plasmids pBADmycHis and pET28a are indicated in brackets. Sequences: the restriction

609   enzymes cleavage sequences are indicated in bold and underlined. The four bases CACC added to the forward primers to allow directional in-frame cloning of the PCR

610   fragments into plasmids pET101/D-TOPO and pET200/D-TOPO are indicated in bold.

26

611   **Figure legends**

612   **Fig 1.** Temporal characterization of the replicative cycle. (A) *Acanthamoeba castellanii* were infected

613   with *Parachalmydia acanthamoebae* at a MOI of 10 to obtain an amoebal infectious rate of nearly

614   100% ensuring that only a single infectious cycle occurred in every experiment. Samples were

615   collected at the indicated times post-infection to cover the complete replicative cycle. Bacteria (green)

616   were labeled by immunofluorescence using specific mouse anti-*P. acanthamoebae* polyclonal

617   antibodies. *A. castellanii* (red) were stained with concanavalin A. An average of 2-5 infectious

618   particles per amoebal cell were observed 1h post-infection. Internalization and differentiation of EBs

619   into RBs occurred during the 5 first hours of the infection. Replicating RBs located in bacterial

620   inclusions were observed from 8 to 24h post-infection. Redifferentiation of RBs into EBs occurred

621   between 24-48h post-infection. Finally cell lysis and extrusion of infectious EBs from host cells were

622   observed 48h post-infection. (B) Bacterial growth measured by qPCR of the 16S rDNA. A lag-phase

623   corresponding to bacterial differentiation of EBs to RBs is observed during the first 5 hours of

624   infection followed by an exponential bacterial growth. An entry into stationary-phase was measured

625   between 16 and 24h post-infection. (C) The replicative cycle was classified in 3 phases: Early, mid

626   and late. The early cycle is characterized by the differentiation of EBs into RBs. Bacterial replication

627   and exponential growth occurs during the mid-phase. The late-phase is characterized by an entry into

628   stationary-phase during which RBs redifferentiate into EBs and by the final cell lysis and release of

629   EBs into the extracellular milieu.

630   **Fig 2.** Expression analysis of several housekeeping genes during an infectious cycle to identify the

631   optimal internal control for quantitative reverse transcription analyses (qRT-PCR). (A) Fold increase

632   of 16S rRNA, *gyrB/ hsp60-1/secY/rpoB/ hc-1* mRNA transcripts and of 16S DNA during a replicative

633   cycle of *Parachlamydia* within *A. castellanii*. DNA extraction was performed with (16S DNA + Lyz +

634   protK) and without proteinase K (16S DNA + Lyz) to obtain DNA extraction method similar to RNA

635   extraction which cannot be performed with proteinase K treatment. Proteinase K treatment is essential

636   to obtain complete lysis of EBs. The fold increase was normalized to 1 at 16h since no difference

637   between samples treated with and without porteinase K is observe at 16h post-infection when the

638   bacterial population is only composed of RBs as shown in supplementary figure 1. (B) The ratio

639   between 16S rRNA, *gyrB/ hsp60-1/secY/rpoB/ hc-1* RNA and 16S DNA was calculated at each time

640   and showed that only 16rRNA is expressed constitutively by exhibiting a ratio 16rRNA/16S DNA of

641   about 1 at each time of the replicative cycle. (C) Temporal transcriptional expression of *gyrB/ hsp60-*

642   *1/secY/rpoB/ hc-1* normalized to the expression of the internal control 16S rRNA. A significant

643   increase of expression of the essential housekeeping genes *gyrB/ hsp60-1/secY/rpoB* is observed

644   during the early to mid-phase of the replicative cycle when EBs differentiate into RBs and during the

645   first-phase of exponential bacterial growth. As previously shown in C. *trachomatis*, the gene encoding

646   for the histone-like protein Hc-1 exhibits an increased expression in the late mid-phase when RBs re-

647   differentiate into EBs.

648   **Fig 3.** Temporal transcriptional expression of T3SS genes. (A) Genetic organization of identified

649   T3SS genes (adapted from (Greub *et al.*, 2009)). The identified genes encoding for *Parachlamydia*

650   T3SS proteins are localized in 4 main defined genomic clusters. Clusters 1 to 3 are much conserved

651   among *Chlamydiales* bacteria. A difference is observed for the cluster 4 between *Chlamydiaceae* and

652   other sequenced *Chlamydia*-related bacteria. The conserved genes are represented by different colors

653   according to their respective functions. Hypothetical proteins are represented in light gray and genes

654   encoding for proteins with identified functions likely not involved in T3SS are represented in dark

655   gray. Capital letters refer to sct gene names according to the unified nomenclature suggested by Hueck

656   in 1998 (Hueck, 1998). *sycE* and *sycD*: genes encoding for SycE-like and SycD/LcrH-like T3SS

657   chaperones. All SycD/LcrH predicted T3SS chaperones contain conserved tetratricopeptide repeats

658   domains (TPRs). Unlike *Chlamydiaceae*, no genes showing homology with the flagellar system that

659   could have a possible function in chlamydial T3SS have been identified. (B) Transcriptional

660   expression of conserved T3SS genes measured by quantitative reverse transcription PCR (qRT-PCR).

661   The mRNA expression of the various T3SS genes was classified in 4 temporal clusters: EL (early-late

662   phase), L (late-phase), E (early-phase), M (mid-phase) according to the temporal pattern of expression

663   during the replicative cycle. The cluster EL exhibits a marked reduction of mRNA amount during the

664   mid-phase followed by an increased expression in late-phase. Cluster L is characterized by a basal

665   expression during early and mid-phase and a significant increased expression during the late-phase.

666   Cluster E is composed of genes that are transcribed during the early and beginning of mid-phase.

667   Finally, the cluster M includes genes that are expressed in the mid-phase.

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669   **Fig 4.** Transcriptional expression cluster analysis of T3SS genes. (A) The highest degree of expression

670   of each gene measured at different time of the infectious cycle was normalized to 100 and the lowest

671   to 0 to generate a matrix of transcriptional expression. The 4 cluster of temporal expression (EL, L, E,

672   M) are indicated on the left part of the matrix. (B) Transcriptional units prediction. A bioonformatic

673   analysis of the T3SS genomic clusters was conducted with the fgenesB software to predict

674   transcriptional units (dotted arrows). Transcriptional units deduced from the experimental

675   transcriptional expression analysis by qRT-PCR (solid arrows). Each gene associated with a predicted

676   transcriptional unit belongs to the same temporal expression cluster. Operon prediction of *C.*

677   *trachomatis* genomic loci containing T3SS genes as described by Hefty and al. (Hefty and Stephens,

678   2007) (dashed arrows). The transcriptional units predicted by the temporal transcriptional clustering of

679   *Parachlamydia* are in congruence with the transcriptional unit prediction of the fgenesB software

680   analysis and/ are highly similar to the operon prediction described in *C. trachomatis*.

681

682   **Fig. 5.** Protein expression analysis by western blot. The amount of the T3SS proteins Mcsc, SctJ,

683   SctW (CopN), CopB and SycD were analyzed at different times post-infection by western blot using

684   specific mouse antisera. A polyclonal mouse antisera directed against whole bacterial cells was used as

685   an internal control to estimate bacterial number at each time collected during the infectious cycle. The

686   expression of SycD and Mcsc was not or poorly detected at 8h and 16h hindering the possibility to

687   measure their relative protein expression during the infectious cycle by western blot. The relative

688   expression of each protein throughout the replicative cycle is roughly estimated at each time by

689   calculating the ratio between the intensity of a given band and the intensity corresponding to whole

690   bacterial number as shown in supplementary Figure 2. Thus, the expression of the protein CopB is

691   induced in the late-phase of the replicative cycle. SctW (CopN) exhibits a significant increased level

692   36h post-infection corresponding to a late-phase expression but the protein is also present in high

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693   concentrations at 8h post-infection. A high level of SctJ is detected at 8h post-infection corresponding

694   to the early/mid-phase of the replicative cycle.

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696   **Fig. 6**. Quantitative immunofluorescence microscopy. Proteins are labeled by immunofluorescence

697   staining with specific mouse polyclonal antibodies. A series of images of multiple focal planes

698   covering the entire thickness of the sample were collected by z-stack confocal microscopy. Using the

699   ImageJ software, a Z-projection composed of the summing of the slices is generated. The region of

700   interest (ROI) including only the signal of intensity emitted by the bacteria is defined. A histogram of

701   the ROI composed of the signal intensity on the x-axis and of the frequency on the y-axis is generated.

702   A mean value and a standard deviation of the histogram of the frequency distribution of fluorescence

703   intensity are also generated. This method allows the accurate measurement of protein expression at the

704   single cell level.

705

706   **Fig. 7**. Protein expression analysis of the late-phase transcribed gene *copB*. CopB was detected with a

707   specific mouse antisera by immunofluorescence staining. (A) Z-projection images generated at

708   different time during infection. Each image contains magnification of various level of CopB

709   expression observed in different bacteria. At 2h post-infection, a heterogenous CopB expression is

710   observed among EBs. Most bacteria exhibit a low CopB expression with only a minor subset showing

711   moderate to high protein expression. CopB expression remains low at 8h post-infection with some

712   RBs expressing CopB moderately. At 16h post-infection, the majority of bacteria poorly expressed

713   CopB but patches of bacteria likely located in the same inclusion exhibit significant increased level of

714   expression. At 24h, 36h and 48h post-infection, most bacteria highly expressed CopB. (B) Histograms

715   of the frequency distribution of fluorescence intensity were generated with each Z-projection showed

716   in panel A. The mean frequency for each histogram was also calculated. These data demonstrate that

717   bacteria induced CopB expression at about 24h post-infection, which corresponds to the

718   transcriptional temporal expression of the *copB* gene (figure 3B).

719

720   **Fig 8.** Protein expression analysis of Mcsc, SctJ, PahN and SctW (CopN). Two protein (Mcsc and

721   SctJ) encoded by genes that are transcribed during the early and mid-phase, one protein (PahN)

722   encoded by a mid-phase transcribed gene and one protein (SctW) encoded by a late-phase transcribed

723   gene were analyzed by quantitative immunofluorescence. Similar to transcriptional expression, the

724   Mcsc and SctJ protein exhibit an early to mid-phase increased protein expression at 2h and 8h post-

725   infection followed by a reduced expression during the mid to late-phase of the infection cycle. The

726   expression of the PahN protein was induced during mid-phase and remained highly expressed

727   throughout the rest of the infectious cycle. Relative high amount of SctW were measured 2h post-

728   infection followed by a gradual decrease in protein levels until 16h post-infection. A significant

729   increase in protein expression is then observed in the late-phase cycle at about 24h post-infection,

730   which corresponds to transcriptional expression of the *sctW* gene.

731

732   **Fig 9.** Model of expression of T3SS components during an infectious cycle of *Parachlamydia* within

733   *A. castellanii*. (A) Schematic overview of the transcriptional expression of the T3SS genes during the

734   3 phases of the replicative cycle. Early-phase: An increased transcriptional expression of

735   *sctC/D/J/T/S/R/L* genes is observed during the early-phase of the cycle. A basal transcriptional

736   expression is observed for the genes encoding other components of the T3SS. Mid-phase: A reduced

737   and moderate expression of *sctC/D/J/T/S/R/L* genes is detected while no change in basal expression of

738   *sctV/N/Q/F/W* genes is observed. The level of *sctU/copB* transcripts is almost undetected suggesting

739   than the transcriptional expression of these genes is highly reduced during the mid-phase of the cycle.

740   In the contrary, the transcriptional expression of the gene encoding for the putative effector protein

741   Pkn5 is significantly induced. Late-phase: A basal expression of the genes *sctC/D/J/T/S/R/L* that were

742   highly expressed during the early-phase is observed whereas an increased transcriptional expression of

743   the genes *sctV/N/Q/F/W/U* and *copB* (or *CopB2*) is measured during the late-phase of replication. (B)

744   Relative T3SS proteins content during the 3 different phases of the cycle. The T3SS is a multi-

745   component complex machinery containing protein with diverse functions and in varying quantities. A

746   varying stoechiometric composition of the T3SS could likely define the functionality of the apparatus.

747   The levels of proteins in bacterial cells are controlled by multiple mechanisms and are defined by both

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748 the rate of protein synthesis and protein degradation, which are varying with the physiological status

749 of the bacteria during a growth cycle. The relative protein content of the T3SS in EBs is likely similar

750 to the protein content observed during the late-phase of the replicative cycle when RBs redifferentiate

751 into EBs before cell lysis and bacterial release in the extracellular milieu. During that time, the T3SS

752 is likely not required for secretion of bacterial effectors and is maintained in a dormant state ready to

753 be activated. Upon cell contact and bacterial internalization of EBs, the T3SS is likely quickly

754 activated to allow rapid secretion of effector proteins to modulate host cell functions. Rapid de novo

755 synthesis of some T3SS components likely occurs during the critical early-phase of bacterial infection

756 to promote bacterial survival, differentiation, maturation and initiation of replication. The subversion

757 of host cell defense mechanisms has likely to be operated during the early-phase of the infection to

758 ensure bacterial evasion from the lytic pathway. In mid-phase, once the bacteria have established

759 conditions allowing efficient bacterial replication such as replicative vacuole maturation, subversion of

760 host cell defenses and establishment of systems allowing efficient scavenging of host cell nutrients,

761 the importance and the number of T3SS per bacteria are reduced. Finally, increased de novo synthesis

762 of some T3SS components in the late-phase trigger the shutdown of the apparatus (SctW) and the

763 formation of a silent T3SS in EBs ready to be rapidly activated when requested. IM: Inner membrane.

764 OM: Outer membrane. RVM: Replication vacuole membrane

765 **Supplementary figure 1**. Proteinase K treatment is required for complete EBs lysis during DNA

766 extraction. Bacterial growth quantified by DNA copy number in a logarithmic (A) and linear (B) scale

767 measured by qPCR during a replicative cycle of *Parachlamydia* within *A. castellanii* from DNA

768 extracted with and without proteinase K treatment. A significant difference is observed when extracted

769 samples contain a bacterial population composed of a majority of EBs demonstrating the absolute

770 requirement of proteinase K treatment for complete bacterial lysis of both EBs and RBs.

771 **Supplementary figure 2.** Estimation of protein expression during a replicative cycle by western blot.

772 Total bacterial number was detected with an antisera directed against whole bacterial cells (α-Para).

773 The relative expression of CopB, SctW (CopN) and SctJ at different times of bacterial growth was

774 estimated by calculating the ratio between the intensity of the bands corresponding to the different

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3   775   proteins and the intensity of the signal obtained with total anti-*Parachlamydia* antisera. The results
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5   776   were normalized to 1 at 8h post-infection.
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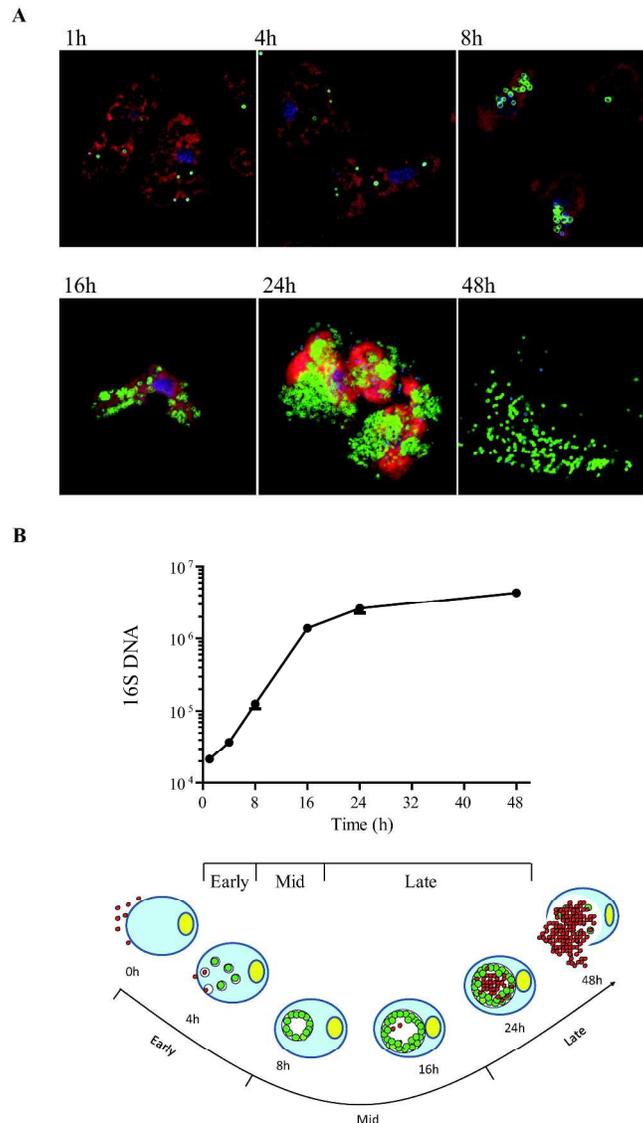41
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778 **Acknowledgments**

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Fig. 1

Fig 1. Temporal characterization of the replicative cycle. (A) Acanthamoeba castellanii were infected with Parachalmydia acanthamoebae at a MOI of 10 to obtain an amoebal infectious rate of nearly 100% ensuring that only a single infectious cycle occurred in every experiment. Samples were collected at the indicated times post-infection to cover the complete replicative cycle. Bacteria (green) were labeled by immunofluorescence using specific mouse anti-P. acanthamoebae polyclonal antibodies. A. castellanii (red) were stained with concanavalin A. An average of 2-5 infectious particles per amoebal cell were observed 1h post-infection. Internalization and differentiation of EBs into RBs occurred during the 5 first hours of the infection. Replicating RBs located in bacterial inclusions were observed from 8 to 24h post-infection. Redifferentiation of RBs into EBs occurred between 24-48h post-infection. Finally cell lysis and extrusion of infectious EBs from host cells were observed 48h post-infection. (B) Bacterial growth measured by qPCR of the 16S rDNA. A lag-phase corresponding to bacterial differentiation of EBs to RBs is observed during the first 5 hours of infection followed by an exponential bacterial growth. An entry into stationary-phase was measured between 16 and 24h post-infection. (C) The replicative cycle was classified in 3 phases: Early, mid

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and late. The early cycle is characterized by the differentiation of EBs into RBs. Bacterial replication and exponential growth occurs during the mid-phase. The late-phase is characterized by an entry into stationary-phase during which RBs redifferentiate into EBs and by the final cell lysis and release of EBs into the extracellular milieu.
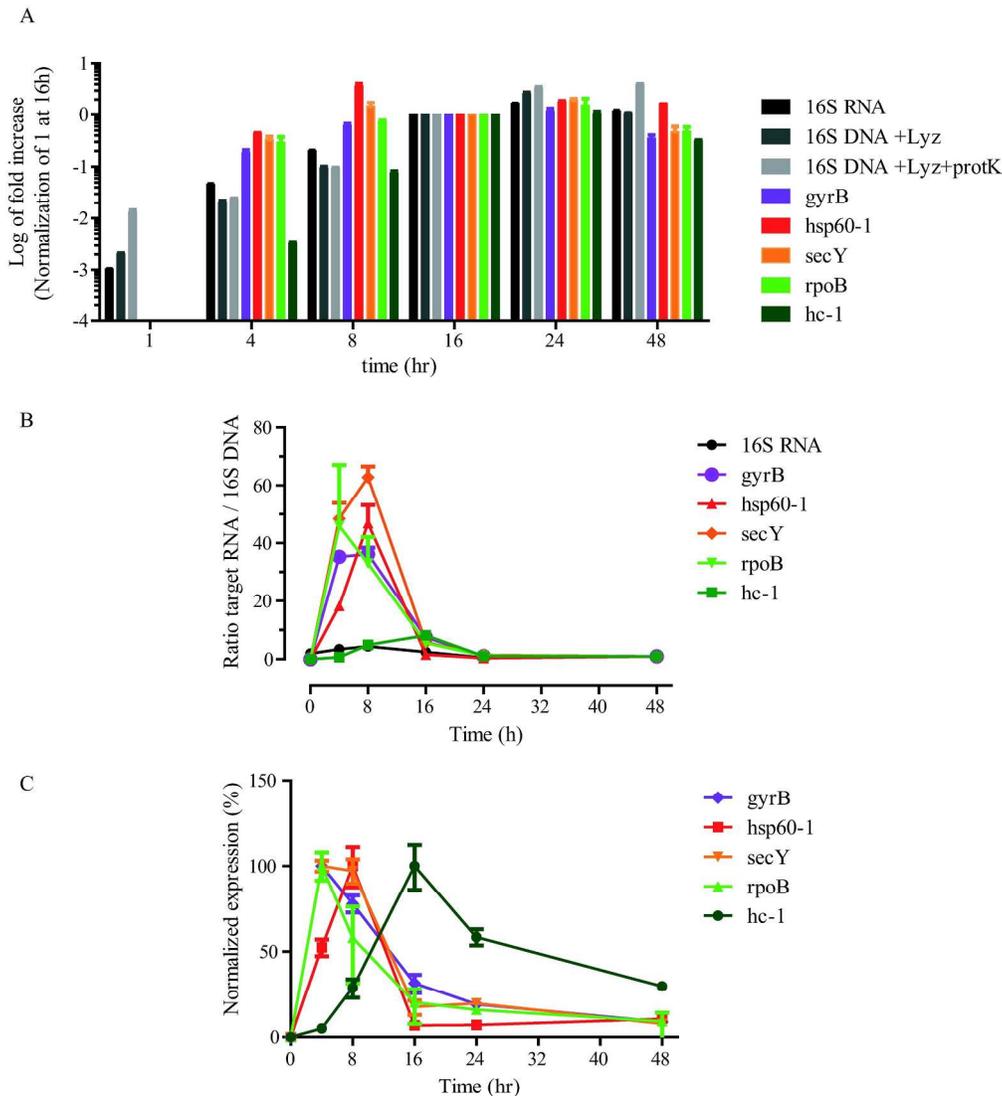
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Fig. 2

Fig 2. Expression analysis of several housekeeping genes during an infectious cycle to identify the optimal internal control for quantitative reverse transcription analyses (qRT-PCR). (A) Fold increase of 16S rRNA, gyrB/ hsp60-1/secY/rpoB/ hc-1 mRNA transcripts and of 16S DNA during a replicative cycle of Parachlamydia within A. castellanii. DNA extraction was performed with (16S DNA + Lyz + protK) and without proteinase K (16S DNA + Lyz) to obtain DNA extraction method similar to RNA extraction which cannot be performed with proteinase K treatment. Proteinase K treatment is essential to obtain complete lysis of EBs. The fold increase was normalized to 1 at 16h since no difference between samples treated with and without porteinase K is observe at 16h post-infection when the bacterial population is only composed of RBs as shown in supplementary figure 1. (B) The ratio between 16S rRNA, gyrB/ hsp60-1/secY/rpoB/ hc-1 RNA and 16S DNA was calculated at each time and showed that only 16rRNA is expressed constitutively by exhibiting a ratio 16rRNA/16S DNA of about 1 at each time of the replicative cycle. (C) Temporal transcriptional expression of gyrB/ hsp60-1/secY/rpoB/ hc-1 normalized to the expression of the internal control 16S rRNA. A significant increase of expression of the essential housekeeping genes gyrB/ hsp60-1/secY/rpoB is observed during the early to mid-phase of the replicative cycle when EBs differentiate into

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RBs and during the first-phase of exponential bacterial growth. As previously shown in C. trachomatis, the gene encoding for the histone-like protein Hc-1 exhibits an increased expression in the late mid-phase when RBs re-differentiate into EBs.

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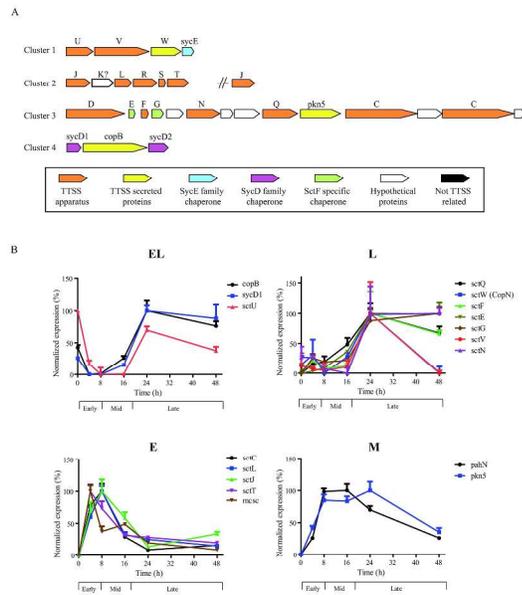51
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Fig. 3

Fig 3. Temporal transcriptional expression of T3SS genes. (A) Genetic organization of identified T3SS genes (adapted from (Greub et al., 2009)). The identified genes encoding for Parachlamydia T3SS proteins are localized in 4 main defined genomic clusters. Clusters 1 to 3 are much conserved among Chlamydiales bacteria. A difference is observed for the cluster 4 between Chlamydiaceae and other sequenced Chlamydia-related bacteria. The conserved genes are represented by different colors according to their respective functions. Hypothetical proteins are represented in light gray and genes encoding for proteins with identified functions likely not involved in T3SS are represented in dark gray. Capital letters refer to sct gene names according to the unified nomenclature suggested by Hueck in 1998 (Hueck, 1998). sycE and sycD: genes encoding for SycE-like and SycD/LcrH-like T3SS chaperones. All SycD/LcrH predicted T3SS chaperones contain conserved tetratricopeptide repeats domains (TPRs). Unlike Chlamydiaceae, no genes showing homology with the flagellar system that could have a possible function in chlamydial T3SS have been identified. (B) Transcriptional expression of conserved T3SS genes measured by quantitative reverse transcription PCR (qRT-PCR). The mRNA expression of the various T3SS genes was classified in 4 temporal clusters: EL (early-late phase), L (late-phase), E (early-phase), M (mid-phase) according to the temporal pattern of expression during the replicative cycle. The cluster EL exhibits a marked reduction of mRNA amount during the mid-phase followed by an increased expression in late-phase. Cluster L is characterized by a basal expression during early and mid-phase and a significant increased expression during the late-phase. Cluster E is composed of genes that are transcribed during the early and beginning of mid-phase. Finally, the cluster M includes genes that are expressed in the mid-phase.
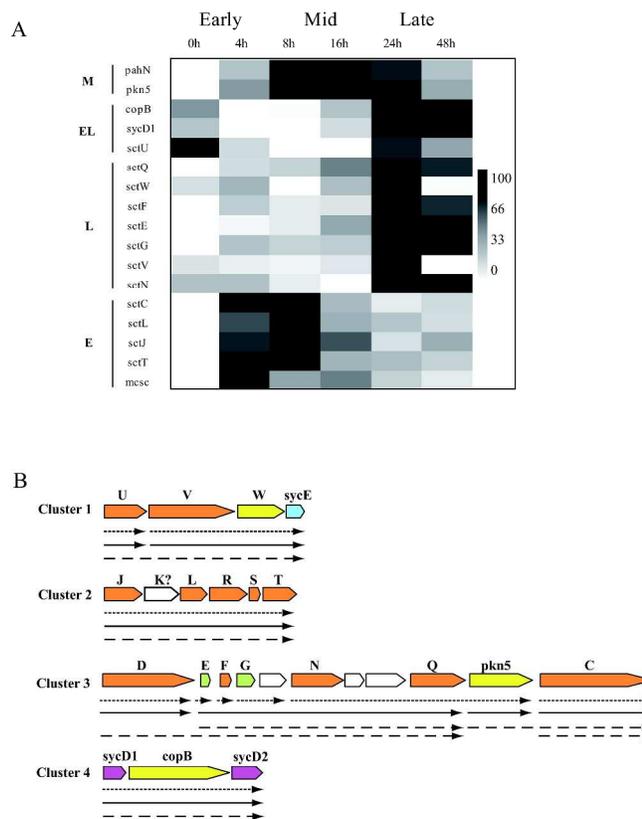263x174mm (300 x 300 DPI)

Fig. 4

Fig 4. Transcriptional expression cluster analysis of T3SS genes. (A) The highest degree of expression of each gene measured at different time of the infectious cycle was normalized to 100 and the lowest to 0 to generate a matrix of transcriptional expression. The 4 cluster of temporal expression (EL, L, E, M) are indicated on the left part of the matrix. (B) Transcriptional units prediction. A bioonformatic analysis of the T3SS genomic clusters was conducted with the fgenesB software to predict transcriptional units (dotted arrows). Transcriptional units deduced from the experimental transcriptional expression analysis by qRT-PCR (solid arrows). Each gene associated with a predicted transcriptional unit belongs to the same temporal expression cluster. Operon prediction of C. trachomatis genomic loci containing T3SS genes as described by Hefty and al. (Hefty and Stephens, 2007) (dashed arrows). The transcriptional units predicted by the temporal transcriptional clustering of Parachlamydia are in congruence with the transcriptional unit prediction of the fgenesB software analysis and/ are highly similar to the operon prediction described in C. trachomatis.
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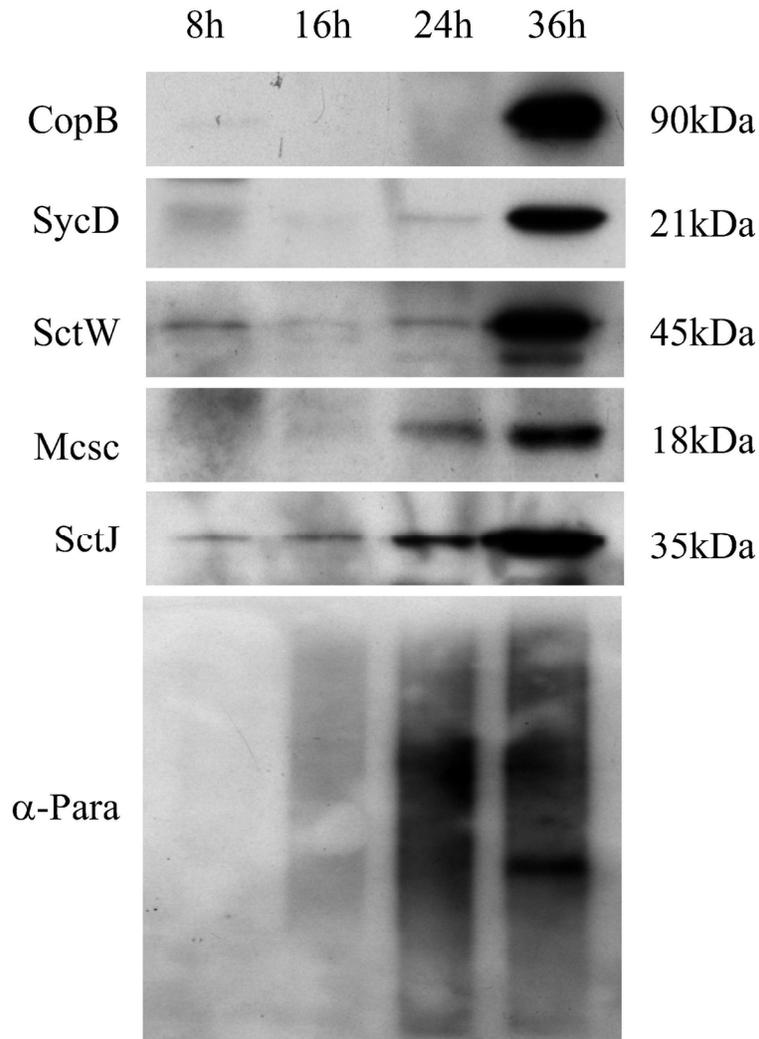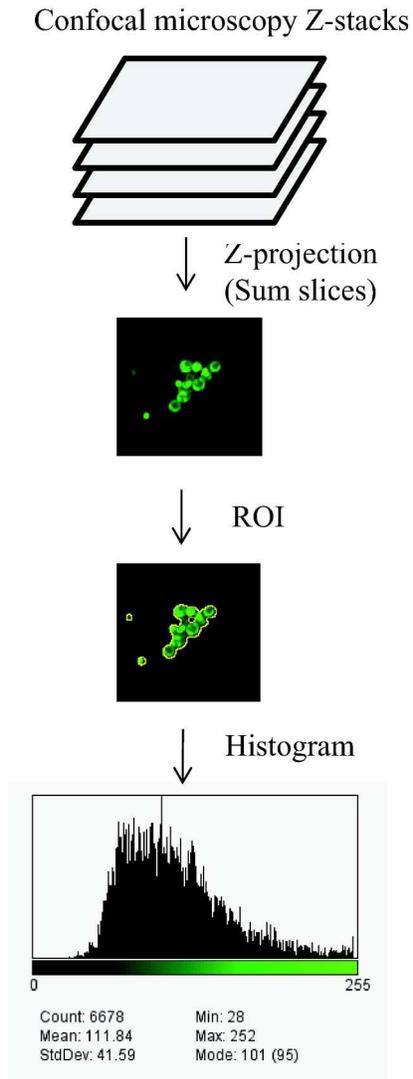51
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Fig.5

Fig. 5. Protein expression analysis by western blot. The amount of the T3SS proteins Mcsc, SctJ, SctW (CopN), CopB and SycD were analyzed at different times post-infection by western blot using specific mouse antisera. A polyclonal mouse antisera directed against whole bacterial cells was used as an internal control to estimate bacterial number at each time collected during the infectious cycle. The expression of SycD and Mcsc was not or poorly detected at 8h and 16h hindering the possibility to measure their relative protein expression during the infectious cycle by western blot. The relative expression of each protein throughout the replicative cycle is roughly estimated at each time by calculating the ratio between the intensity of a given band and the intensity corresponding to whole bacterial number as shown in supplementary Figure 2. Thus, the expression of the protein CopB is induced in the late-phase of the replicative cycle. SctW (CopN) exhibits a significant increased level 36h post-infection corresponding to a late-phase expression but the protein is also present in high concentrations at 8h post-infection. A high level of SctJ is detected at 8h post-infection corresponding to the early/mid-phase of the replicative cycle.
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Confocal microscopy Z-stacks

Z-projection
(Sum slices)

ROI

Histogram

Count: 6678	Min: 28
Mean: 111.84	Max: 252
StdDev: 41.59	Mode: 101 (95)

Fig. 6

Fig. 6. Quantitative immunofluorescence microscopy. Proteins are labeled by immunofluorescence staining with specific mouse polyclonal antibodies. A series of images of multiple focal planes covering the entire thickness of the sample were collected by z-stack confocal microscopy. Using the ImageJ software, a Z-projection composed of the summing of the slices is generated. The region of interest (ROI) including only the signal of intensity emitted by the bacteria is defined. A histogram of the ROI composed of the signal intensity on the x-axis and of the frequency on the y-axis is generated. A mean value and a standard deviation of the histogram of the frequency distribution of fluorescence intensity are also generated. This method allows the accurate measurement of protein expression at the single cell level.
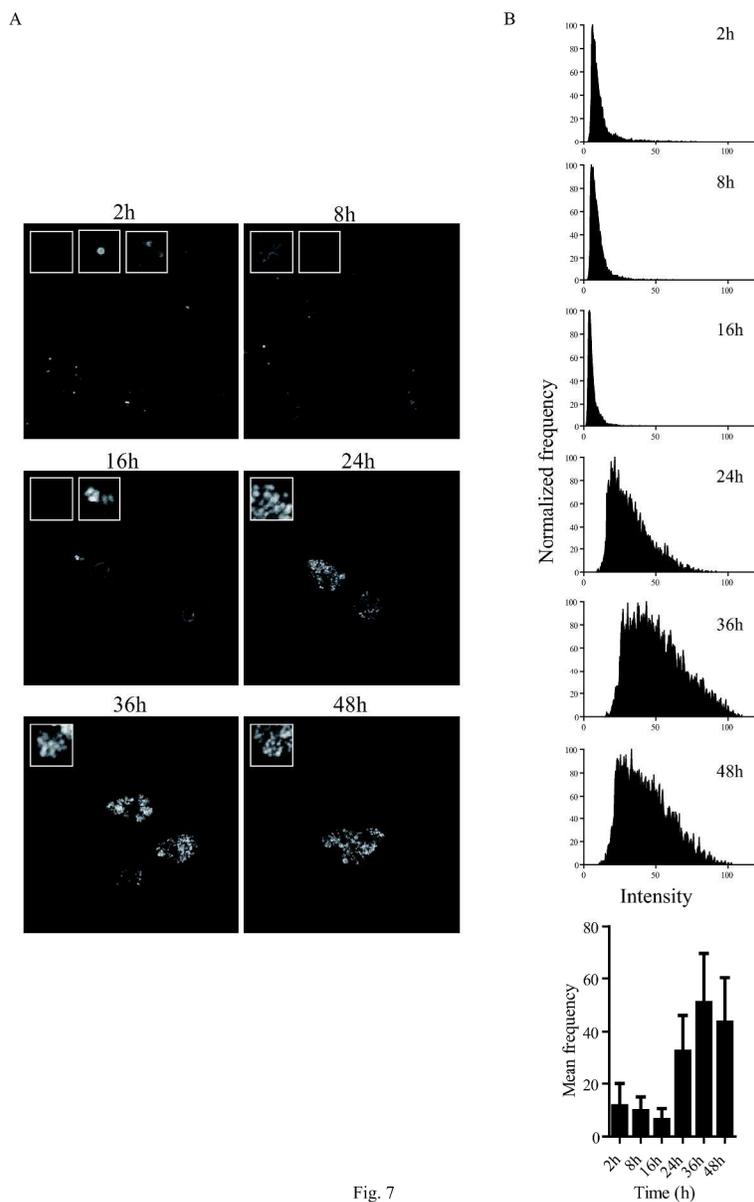164x491mm (300 x 300 DPI)

Fig. 7

Fig. 7. Protein expression analysis of the late-phase transcribed gene copB. CopB was detected with a specific mouse antisera by immunofluorescence staining. (A) Z-projection images generated at different time during infection. Each image contains magnification of various level of CopB expression observed in different bacteria. At 2h post-infection, a heterogenous CopB expression is observed among EBs. Most bacteria exhibit a low CopB expression with only a minor subset showing moderate to high protein expression. CopB expression remains low at 8h post-infection with some RBs expressing CopB moderately. At 16h post-infection, the majority of bacteria poorly expressed CopB but patches of bacteria likely located in the same inclusion exhibit significant increased level of expression. At 24h, 36h and 48h post-infection, most bacteria highly expressed CopB. (B) Histograms of the frequency distribution of fluorescence intensity were generated with each Z-projection showed in panel A. The mean frequency for each histogram was also calculated. These data demonstrate that bacteria induced CopB expression at about 24h post-infection, which corresponds to the transcriptional temporal expression of the copB gene (figure 3B).
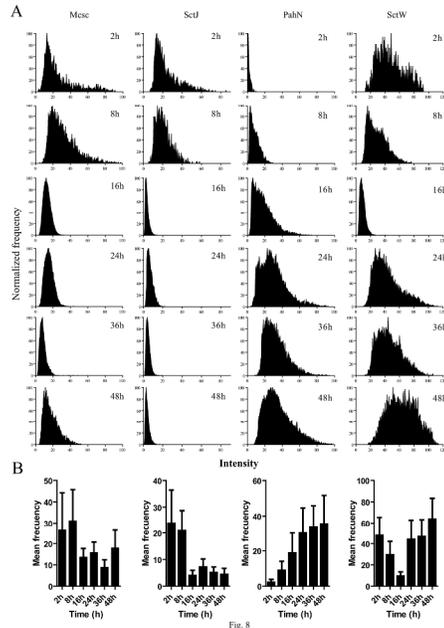
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Fig 8. Protein expression analysis of Mcsc, SctJ, PahN and SctW (CopN). Two protein (Mcsc and SctJ) encoded by genes that are transcribed during the early and mid-phase, one protein (PahN) encoded by a mid-phase transcribed gene and one protein (SctW) encoded by a late-phase transcribed gene were analyzed by quantitative immunofluorescence. Similar to transcriptional expression, the Mcsc and SctJ protein exhibit an early to mid-phase increased protein expression at 2h and 8h post-infection followed by a reduced expression during the mid to late-phase of the infection cycle. The expression of the PahN protein was induced during mid-phase and remained highly expressed throughout the rest of the infectious cycle. Relative high amount of SctW were measured 2h post-infection followed by a gradual decrease in protein levels until 16h post-infection. A significant increase in protein expression is then observed in the late-phase cycle at about 24h post-infection, which corresponds to transcriptional expression of the sctW gene.
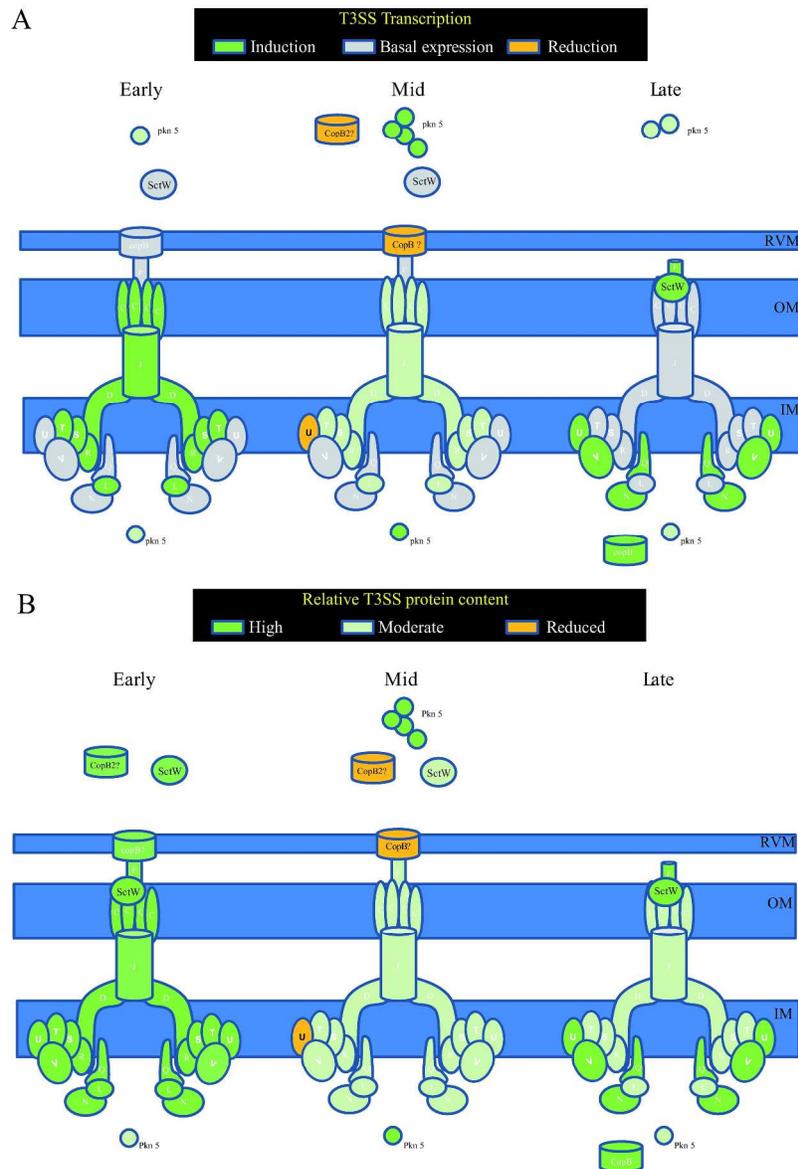297x187mm (300 x 300 DPI)

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Fig. 9

Fig 9. Model of expression of T3SS components during an infectious cycle of Parachlamydia within A. castellanii. (A) Schematic overview of the transcriptional expression of the T3SS genes during the 3 phases of the replicative cycle. Early-phase: An increased transcriptional expression of sctC/D/J/T/S/R/L genes is observed during the early-phase of the cycle. A basal transcriptional expression is observed for the genes encoding other components of the T3SS. Mid-phase: A reduced and moderate expression of sctC/D/J/T/S/R/L genes is detected while no change in basal expression of sctV/N/Q/F/W genes is observed. The level of sctU/copB transcripts is almost undetected suggesting than the transcriptional expression of these genes is highly reduced during the mid-phase of the cycle. In the contrary, the transcriptional expression of the gene encoding for the putative effector protein Pkn5 is significantly induced. Late-phase: A basal expression of the genes sctC/D/J/T/S/R/L that were highly expressed during the early-phase is observed whereas an increased transcriptional expression of the genes sctV/N/Q/F/W/U and copB (or CopB2) is measured during the late-phase of replication. (B) Relative T3SS proteins content during the 3 different phases of the cycle. The T3SS is a multi-component complex machinery containing protein with

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diverse functions and in varying quantities. A varying stoechiometric composition of the T3SS could likely define the functionality of the apparatus. The levels of proteins in bacterial cells are controlled by multiple mechanisms and are defined by both the rate of protein synthesis and protein degradation, which are varying with the physiological status of the bacteria during a growth cycle. The relative protein content of the T3SS in EBs is likely similar to the protein content observed during the late-phase of the replicative cycle when RBs redifferentiate into EBs before cell lysis and bacterial release in the extracellular milieu. During that time, the T3SS is likely not required for secretion of bacterial effectors and is maintained in a dormant state ready to be activated. Upon cell contact and bacterial internalization of EBs, the T3SS is likely quickly activated to allow rapid secretion of effector proteins to modulate host cell functions. Rapid de novo synthesis of some T3SS components likely occurs during the critical early-phase of bacterial infection to promote bacterial survival, differentiation, maturation and initiation of replication. The subversion of host cell defense mechanisms has likely to be operated during the early-phase of the infection to ensure bacterial evasion from the lytic pathway. In mid-phase, once the bacteria have established conditions allowing efficient bacterial replication such as replicative vacuole maturation, subversion of host cell defenses and establishment of systems allowing efficient scavenging of host cell nutrients, the importance and the number of T3SS per bacteria are reduced.

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