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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.
Introduction

The Chlamydiales order currently includes the Chlamydiaceae, Parachlamydiaceae, Waddliaceae, Simkaniaeae, Criblamydiaceae and Rhabdochlamydiaceae families (Corsaro and Greub, 2006).

Parachlamydiaceae naturally resist destruction by free-living amoebae. These obligate intracellular bacteria may use the protistan host as a training ground to select virulence traits that help them to resist to the microbicidal effectors of amoebae and macrophages (Greub, 2009; Greub and Raoult, 2004).

Parachlamydia acanthamoebae is a bacterium belonging to the Parachlamydiaceae family. P. acanthamoebae is seriously suspected to be involved in respiratory tract infections (Greub, 2009; Lamoth and Greub, 2009) since serological and molecular evidences are supporting its role as a human respiratory pathogen, mainly in patients with community-acquired (Greub et al., 2003b) and aspiration pneumonia (Greub et al., 2003c). In addition, the disease has been reproduced in a murine model following both intranasal and intratracheal bacterial inoculation (Casson et al., 2008).

The pathogenic potential of Parachlamydia is further supported by the ability of this obligate intracellular bacteria to enter and replicate in human macrophages (Greub et al., 2003a), by preventing the secretion of proinflammatory cytokines (Greub et al., 2005a) and the fusion of its endocytic vacuole with the lysosome (Greub et al., 2005b). Moreover, Parachlamydia is also able to enter and replicate in pneumocytes and lung fibroblasts (Casson et al., 2006).

Members of the Chlamydiales are characterized by a biphasic development cycle comprising the infectious elementary bodies (EBs) and the replicating reticulate bodies (EBs). They have evolved from a common ancestor since more than 700 million years (Greub and Raoult, 2003) that has developed mechanisms for intracellular survival and multiplication in primitive unicellular eukaryotes. Consequently, Chlamydia-related bacteria and classical Chlamydia share a “core genome” of about 700 ORFs that encode for essential bacterial functions that guarantee an efficient intracellular lifestyle including several virulence mechanisms such as the Type III secretion system (T3SS) (Collingro et al., 2011b; Bertelli et al., 2010; Greub et al., 2009; Horn et al., 2004).
T3SS mediates the translocation of bacterial effectors to the cytosol of infected cells to modulate various host cell functions facilitating the survival and the replication of the bacterial pathogens (Coburn et al., 2007). The proteins that constitute the T3SS secretory apparatus are termed structural proteins. An additional set of proteins termed “translocators” function by translocating virulence factors called “effector” proteins into the host cell cytoplasm. T3SS genes are present in all Chlamydia species examined to date and suggest that T3SS is essential for the survival of these strict intracellular bacteria. Proteins that compose the T3SS secretory apparatus and translocators are for the most part well conserved structurally and functionally among Chlamydiales spp. and genes encoding for these T3SS proteins are found in distinct conserved genomic clusters (Betts et al., 2008a; Peters et al., 2007). In contrast, T3SS effectors display little sequence homology and are much harder to identify, except for some members of the family of T3SS secreted Inc proteins which are characterized by a large bi-lobed hydrophobic domain that serves to anchor the proteins in the inclusion membrane (Bannantine et al., 2000). Effectors being the direct modulators of host cell functions and of unique pathogenic properties, every bacterium has adapted its pool of effectors to survive and replicate in their respective environmental niches, which likely explained the poor sequence conservation among these secreted proteins. However, effectors proteins often display common structural traits and have similar enzymatic activities among various bacterial species, including cytoskeletal rearrangement, subversion of signaling pathways, activation or inhibition of apoptosis and modulation of intracellular trafficking (Mota and Cornelis, 2005). In Chlamydiales, effector proteins are secreted in the inclusion lumen, inserted in the inclusion membrane or translocated in the cytosol of the host cell. A few effectors have been identified so far in Chlamydiaceae including Tarp, inclusion membrane proteins IncA to IncG, CADD, Mip and CopN (Beeckman and Vanrompay, 2010; Subtil et al., 2005; Bannantine et al., 2000).

The secretory apparatus is composed of about 20 proteins. The functions of the proteins that compose the injectisome of Chlamydiales bacteria have been mainly attributed based on sequence similarities with proteins composing well described heterologous T3SS apparatus of other bacteria species such as Yersinia and Salmonella (Hueck, 1998). Several studies indicate that the secretin-like SctC protein
form the outer membrane (OM) ring which is connected to the inner membrane (IM) ring through SetD. The inner membrane (IM) ring is formed by SetD together with the lipoprotein SetJ. In the Yersinia T3SS apparatus, the IM ring is associated with a multi-components complex composed of (i) Set R/S/T/U/V (proteins associated with the export apparatus), of (ii) Set N/L (ATPase associated proteins) and of (iii) SetQ which form the cytosolic ring (C-ring) (Diepold et al., 2010; Cornelis, 2002). Several studies focusing on T3SS protein interactions in Chlamydia indicate that the Chlamydiales T3SS apparatus is very similar in structure and function compared to the well studied apparatus of Yersinia and other species (Betts-Hampikian and Fields, 2010; Fields, 2006).

Recent experimental evidences obtained in the Yersinia model suggest that the apparatus assembly is initiated by the formation of the SetC OM ring and proceed inwards through the assembly of the IM ring SetD and SetJ. Upon completion of the IM ring, additional T3SS components are recruited to the basal apparatus to complete the export apparatus and to form the ATPase and C-ring complexes. Finally, the needle consisted by SetF is assembled to form a mature and functional T3SS. Unlike SetN/L/Q, the exact temporal integration of the IM proteins SetR/S/T/U/V remained unclear (Diepold et al., 2010).

Considering the central role of T3SS in Chlamydiales biology, a thorough characterization of T3SS expression of conserved components throughout an infectious cycle of Parachlamydia within Acanthamoeba castellanii amoeba was conducted. We showed that T3SS conservation among Chlamydiales is not only restricted to genomic sequence and organization but is also observed at the level of temporal expression during the development cycle.

Results

Temporal characterization of an infectious cycle

A thorough characterization of the different stages that compose a complete infectious cycle of Parachlamydia within A. castellanii was required to accurately define temporal key stages of the internalization, differentiation, replication and extrusion of Parachlamydia during a replicative cycle.
Amoebae were infected with a MOI of 10 to ensure a nearly 100% rate of infection allowing the study of a single replication cycle. As shown by confocal microscopy, between 5 to 10 infectious bacteria were internalized into amoebae 1h post-infection (Figure 1A). The differentiation of EBs into RBs was mostly completed 5h post-infection and inclusions containing multiple replicating bacteria were observed 8h post-infection. Following a lag-phase characterized by the differentiation of EBs into RBs, an exponential growth is observed between 5 and 16h post-infection as shown by quantitative PCR (Figure 1B). The bacterial growth enters stationary-phase between 16h and 24h post-infection which corresponds to the beginning of the re-differentiation of RBs into EBs (Figure 1A and B). As shown in figure 1A, most of the amoebae are lysed 48h post-infection, releasing EBs in the extracellular environment. Based on these data, the infectious cycle was classified as early-, mid-, and late cycles. These times correspond to key developmental stages with (1) early cycle during which bacteria are internalized in the host cell and differentiate from EBs into RBs, (2) mid cycle characterized by exponential growth of dividing RBs and (3), late cycle consisting of the end of bacterial growth, re-differentiation of RBs into EBs and finally release of EBs into the extracellular environment (Figure 1C).

**Identification of 16S rRNA as an optimal internal control to measure relative transcriptional expression during a replicative cycle**

*Chlamydiales* EBs and RBs are two developmental forms characterized by profound differences in membrane composition and structure that play an important role in the efficiency of bacterial lysis during DNA and RNA extraction procedures. Several lysis protocols compatible with the Promega Wizard SV Genomic DNA Purification kit have been evaluated on samples collected at different times corresponding to the different developmental stages that characterize the growth of *Parachlamydia* within *A. castellanii*. These experiments showed that a specific lysis protocol including an incubation of 2 hours with proteinase K at 55°C has to be followed to ensure efficient lysis of both EBs and RBs since an incomplete lysis of EBs was obtained in absence of proteinase K treatment as measured by quantitative real time PCR of the 16S rDNA gene (figure 2 and supplementary figure 1). Thus, a reduced amount of 16S rDNA was observed in absence of proteinase K treatment for samples...
collected during early (0 to 4h post infection) and late-phase (36 to 48h post infection) of the
replicative cycle that are characterized by a significant presence of EBs whereas no difference was
measured during the mid-phase (8h to 24h) which is characterized by the sole presence of replicating
RBs.

The extraction of integral bacterial RNA is incompatible with proteinase K treatment at 55°C for 2
hours. Thus, the transcriptional expression measured by quantitative reverse transcription PCR (qRT-
PCR) of several putative constitutively expressed housekeeping genes including 16S rRNA, gyrB, 
secY, rpoB and hsp60 was compared to the amount of 16S rDNA extracted without proteinase K and
measured by qPCR during a replicative cycle of Parachlamydia in A. castellanii (Figure 2). The fold
increase of the different RNAs compared to the 16S rDNA showed that only the 16S rRNA was
constitutively expressed during a replicative cycle (Figure 2A). The ratio of 16S rRNA / 16S rDNA
was of about 1 at each time measured during a replicative cycle whereas the ratio observed between
other transcripts with the 16S rDNA was greatly varying during the infectious cycle (Figure 2B).
Thus, the normalized expression of gyrB, secY, rpoB and hsp60 relatively to 16S rRNA demonstrated
that the expression of these essential housekeeping genes is rapidly induced following bacterial
internalization (Figure 2C). The transcriptional expression of these genes is then decreasing during the
late mid-phase of the replicative cycle when bacteria begin to convert from RBs to EBs. As a control,
the transcriptional expression of hc-1, the homolog of Chlamydia trachomatis hctB expressed during
RB to EB conversion, was measured during a replicative cycle (Figure 2). Similar to hctB, hc-1
exhibited an increased expression in the late mid-phase corresponding to the beginning of re-
differentiation of RBs into EBs. Together, these data indicate that the amount of measured 16S rRNA
transcripts reflects bacterial growth and can be thus considered as an optimal internal control to
measure relative transcriptional expression of the T3SS genes of Parachlamydia during a replicative
cycle within A. castellanii.
Temporal transcriptional expression of T3SS components.

Similar to other Chlamydiae, genes encoding for T3SS apparatus components are located in distinct genomic clusters distributed along the chromosome (Figure 3A). Four genomic clusters containing conserved genes encoding for the injectisome and the translocon apparatus and exhibiting a very similar genetic organization than other Chlamydiae were identified in the genome of Parachlamydia (Greub et al., 2009). Unlike Chlamydiaceae, no gene showing homology with the flagellar system and having a possible function in chlamydial T3SSs have been identified in the genome of Parachlamydia. Thus, the transcriptional expression of the mRNA transcripts of every gene encoding for a protein with a known function was analyzed by qRT-PCR (Figure 3B). The expression of the various T3SS genes during the developmental cycle could be classified in 4 temporal expression clusters (Figure 3B). The first cluster composed of the copB, sycD1 and sctU is characterized by a marked repression or lack of activation during the early and mid-phases followed by a significant increased expression in the late-phase (Figure 3B, EL). The second cluster including the sct Q/W/F/E/G/V/N genes is characterized by a basal expression during the early and mid-phases and a significant increased expression during the late-phase (Figure 3B, L). The third cluster composed of sctC/L/J/T and the multi-cargo secretion chaperone mcsc includes genes that exhibit a dramatic increased expression in the early and mid-phases followed by a decreased basal expression during the late-phase (Figure 3B, E). Finally, the fourth temporal cluster is composed of the putative predicted T3SS secreted effector pkn5 and of a Parachlamydia secreted protein localizing to the replicative inclusion membrane (PahN) that are both significantly highly expressed during the mid and beginning of late-phase (Figure 3B, M).

Following normalization of expression with the highest temporal expression assigned to 100 and the lowest to 0, a temporal matrix composed of the transcriptional expression of the measured T3SS genes was generated (Figure 4A). These analyses strongly suggest that the expression of the genes that encode for T3SS components can be classified in 4 major defined temporal clusters of gene expression composed of early (E), early-late (EL), mid (M) and late (L) phase-expressed genes.
A bioinformatic analysis of the T3SS genomic clusters loci was performed with the fgenesB software to predict transcriptional units and compared with mRNA expression analysis of T3SS genes. Except for one gene (pkn5), the experimental qRT-PCR gene expression data are congruent with the \textit{in silico} prediction (Figure 4B) showing no discrepancies between temporal gene expression and association to a predicted transcriptional unit. Thus $sctU$ and $sycD1/copB$ (cluster EL), $sctC$ and $sctJ/L/T$ (cluster E), $sctE/F/G/N/Q$ (cluster L), $sctV/sycE$ (cluster L) are all located in distinct transcriptional units corresponding to defined temporal clusters (Figure 4). Moreover, the \textit{C. trachomatis} T3SS genes transcriptional expression study performed by Hefty \textit{et al.} (Hefty and Stephens, 2007) strongly indicates that transcriptional expression units of T3SS genes are highly similar between \textit{Parachlamydia} and \textit{C. trachomatis} (Figure 4B). Together these data suggest that T3SSs in \textit{Chlamydiales} are not only conserved at the level of genetic sequence and organization, but also at the level of gene expression.

\textbf{Temporal protein expression measurement by western blotting: limitations of the technique.}

Even though transcription and translation in bacteria are very often coupled, an analysis of protein expression throughout an infectious cycle has to be performed to confirm that a similar temporal expression of T3SS genes and their respective encoded proteins is observed. Proteins encoded by genes belonging to different temporal clusters were selected for protein purifications and mouse immunization programs. Two proteins, Mcsc and SctJ, encoded by genes expressed during early and mid-phase (temporal cluster E), two proteins, CopB and SycD (Scc2), encoded by genes expressed during the early and late-phase (temporal cluster EL), and CopN (SctW) encoded by a gene expressed during the late-phase (temporal cluster L) were selected. The antisera specificity and the protein expression at different times of the infectious cycle were measure by western blot using specific antibodies (Figure 5). A major drawback of measuring comparative protein amounts during the bacterial growth of obligate intracellular bacteria is the difficulty to normalize the amount of total bacterial proteins collected at different times during the replication cycle. An increased in specific proteins amounts in the late-phase of the replicative cycle may only be due to a significant increased in bacterial numbers and not to a specific up-regulation of protein expression. Thus, specific antiserum
raised against whole bacterial cells was used as an internal control to monitor bacterial growth during the development cycle. The expression of SycD and Msce was poorly or not detected by western blot before the late-phase of the replicative cycle hindering the possibility to measure their relative expression throughout parachlamydial growth within *A. castellanii*. An estimation of the relative expression of CopB, CopN (SctW) and SctJ during the infectious cycle was calculated by normalizing the measured intensity of the band corresponding to the various proteins to the intensity of the signal corresponding to the whole bacterial cells (Supplementary figure 2). Similar to gene expression analysis of *copB* and *copN (SctW)*, an increasing amount of the protein CopB and CopN (SctW) was observed in the late-phase of the bacterial replicative cycle. Interestingly, a high relative amount of these proteins was also observed at 8hr suggesting that late-phase synthesized proteins of the T3SS are likely functional and still present in high amount during the early-phase of subsequent infection cycles. The protein encoded by the early transcribed gene *sctJ* exhibited the highest relative expression at 8h confirming that de novo SctJ protein synthesis is likely occurring during the early-phase of the infection (Supplementary figure 2). Unfortunately, the amount of bacteria present during the very early-phase of infection is very low and the expression of the analyzed proteins was not detected before 8h post-infection hindering the possibility to accurately monitor protein expression by western blot during the critical first hours of the infectious cycle. In addition, the relative intensity measured at early time of infection by western blot is subjected to imprecision due to very low signal detection. Thus, an alternative method to obtain more accurate protein expression measurement during the entire replicative cycle is required.

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

To circumvent western blotting experimental limitations, a quantitative immunofluorescence method proposed by Noursadeghi et al. (*Noursadeghi et al.*, 2008) was adapted to measure bacterial single cell protein expression by quantitative immunofluorescence confocal microscopy (Figure 6). This method relies on the measurement of the summing of protein signals from multiple focus plans covering the entire thickness of the sample. Quantitative fluorescence data are then obtained by generating the histogram of the signal intensity of a Z-projection obtained by summing multiple focal slices. Using
this methodology, the protein expression of the late-cycle expressed gene copB was analysed during a complete replicative cycle by using a specific anti-CopB mouse anti-sera (Figure 7). Quantitative immunofluorescence histograms were generated for several time points post-infection and showed that the protein CopB is expressed during late-cycle phase, confirming a late-cycle expression of the T3SS component CopB. The same analysis was applied to the Mcsc, SctJ, PahN and SetW (CopN) proteins (Figure 8). Similar to CopB, the temporal expression of these 4 proteins were congruent with the temporal expression of their respective mRNA transcripts (Figure 8 and 3B). The chaperone Mcsc and the inner membrane components SctJ were expressed in higher amount 2h and 8h post-infection, during the early and mid-phase cycles, followed by a marked reduced signal intensity observed from 16h post-infection which corresponds to the entry into late-cycle phase. The histogram intensity of the late transcribed gene encoded protein SetW (CopN) showed a gradual reduction of the protein amount from 2h to 16h, indicating that a relative high amount of proteins are present in EBs and in early differentiated RBs during the first phase of the infectious cycle but that no significant induction of protein expression occurred before 24h post-infection which corresponds to a late-phase protein expression. Finally, the protein PahN encoded by a mid-phase transcribed gene showed also an increased expression from 8h post-infection which is maintained during the mid to late-phase of the replicative cycle. These data demonstrate that the temporal pattern of protein expression measured by quantitative immunofluorescence of the 5 selected proteins Mcsc/SctJ/CopN/SetW/PahN is similar to their respective mRNA temporal expression measured by qRT-PCR. These data indicate that protein and mRNA expression of various T3SS components are tightly coupled and that temporal transcriptional expression analysis of multiple T3SS genes can be used to monitor the expression of different T3SS components and thus to characterize its functionality during an infectious cycle.

Discussion

Genome sequencing of multiple Chlamydiales bacteria belonging to different distant families have demonstrated that despite marked differences in their genomic size and content, the T3SS loci distributed in different clusters along the chromosomes exhibit a conserved sequence and genetic organization (Collingro et al., 2011a; Bertelli et al., 2010; Greub et al., 2009; Peters et al., 2007).
Several studies have demonstrated that specific inhibition of T3SS with chemical compounds totally inhibits bacterial growth in a dose dependent manner strongly indicating that this secretion system is essential for the establishment of Chlamydiales replicative conditions (Bertelli et al., 2010; Bailey et al., 2007; Muschiol et al., 2006). The temporal expression of T3SS during a replicative cycle has only been studied in Chlamydiaceae including C. trachomatis, C. pneumoniae and C. psittaci. In this study, a complete characterization of the temporal expression of multiple T3SS components at the transcriptional and protein level has been performed in Parachlamydia acanthameobae replicating in its natural host cell Acanthamoeba castellanii. This study is the first description of the T3SS expression during the replicative cycle of a member of the Chlamydiales order not belonging to the Chlamydiaceae family. The replicative cycle of Parachlamydia within A. castellanii was separated in 3 phases, early, mid and late. The early-phase is characterized by a lag-phase when EBs are internalized in the host cell and differentiate into RBs. The mid-phase is characterized by exponential bacterial replication. The late-phase is starting when bacteria enter stationary phase where RBs redifferentiate into EBs which are then released in the extracellular milieu. This study showed that the temporal transcriptional expression of multiple genes encoding for T3SS components could be classified in 4 different temporal clusters depending on their pattern of expression during the early, mid and late-phases of an infectious cycle. Classical western blotting being of limited value for quantitative temporal assessment of protein expression of obligate intracellular bacteria, a new quantitative immunofluorescence approach allowing accurate measurement of protein expression was used in this study. The expression analysis of selected T3SS proteins encoded by genes belonging to different temporal clusters showed that a similar temporal pattern of transcriptional and protein expression was observed for each of these genes (copB, mcsc, sctJ, pahN, sctW).

Based on our experimental data, a model of temporal expression of T3SS during an infectious cycle can be proposed (Figure 9). The T3SS is a multi-component complex machinery containing protein with 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 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. Varying de novo rapid synthesis of T3SS
components depending of protein stability, quantity and functionality 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. Similarly, a
rapid modulation of host cell functions ensuring a fast maturation of the replicative vacuole is a pre-
request to trigger bacterial replication. Thus, the importance of a highly functional and efficient T3SS
during the early-phase of infection would likely confer a significant advantage for the intracellular
bacteria against the defense mechanisms of the host cell. 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 or only maintained in
bacteria being in direct contact with the inclusion membrane. Finally, increased de novo synthesis of
some T3SS components in the late-phase trigger the shutdown of the apparatus (SctW) and the
formation of a silent T3SS in EBs ready to be rapidly activated when requested.

A significant similarity between the transcriptional expression units of Parachlamydia and C.
trachomatis was observed, suggesting that T3SSs sequences, genetic organization and transcriptional
expression units are highly conserved among Chlamydiales bacteria (Hefty and Stephens, 2007).
However, several studies indicate that the temporal expression of these transcriptional units may vary
among Chlamydiales bacteria (Beeckman et al., 2008; Hefty and Stephens, 2007; Lugert et al., 2004;
Slepenkin et al., 2003). Some discrepancies may originate from several issues including chlamydial
species specificities, growth kinetics differences, host cell lines and variation of the limit of detection
of the molecular techniques used in the different experimental assets. The regulation of the T3SS expression in *Chlamydiales* is poorly understood. T3SS genes and operons are preceded by predicted *E. coli* σ70-like promoter elements but no transcriptional regulators have yet been identified (Hefty and Stephens, 2007). Similarly, the explanation of the separation of the *Chlamydiales* T3SS elements in distinct genomic clusters and transcriptional units expressed in different temporal classes remained to be determined. Transcriptional regulation of T3SS genes in other bacterial species such as *Yersinia* and *Salmonella* is mainly controlled by AraC-like transcriptional activators whose activities are regulated by several regulatory pathways responding to multiple environmental signals sensed during the infection process (Francis *et al.*, 2002). No gene encoding for an AraC-like transcriptional activators has been identified in *Chlamydiales* genomes, suggesting that *Chlamydiales* T3SSs are regulated by a different mechanism. The absolute requirement of T3SS for chlamydial survival and replication in host cells may suggest that T3SSs are controlled by the global regulatory network system of *Chlamydiales* bacteria via various transcriptional regulators and/or alternative regulatory systems. Recent data suggest that transcriptional expression of chlamydial T3SS operons is controlled by the degree of DNA supercoiling that varies temporally during a developmental cycle (Case *et al.*, 2010). Thus, the differences of sensitivity of the promoters to alteration of DNA supercoiling may define temporal transcriptional expression differences of T3SS operons and of other unrelated chlamydial genes.

The *Chlamydiales* T3SS is rapidly activated upon contact with the host cell and remains probably functional until bacterial detachment from the inclusion membrane and RBs conversion into EBs (Fields, 2006; Clifton *et al.*, 2004; Fields *et al.*, 2003). Even though cell contact-dependent activation of T3SS is conserved among bacteria (Hueck, 1998), the exact mechanism governing the activation in *Chlamydiales* remains to be identified and marked differences compared to other bacterial species T3SS could be observed considering the peculiar developmental cycle of *Chlamydiae*. For instance, the *Chlamydiales* needle protein SetF contains cystein residues that are, like the proteins forming the envelope, linked by disulfide bridges in EBs (Betts *et al.*, 2008b) indicating that a reduction of these bonds could be required for a complete activation of the secretion. This hypothesis is supported by the
observation that the classical activation of T3SSs secretion obtained by Ca2+ depletion did only poorly stimulated the secretion of the Tarp effector from purified cell-free *C. trachomatis* EBs (Jamison and Hackstadt, 2008).

Based on T3SS studies in other bacteria, some components of the chlamydial T3SS likely exhibit secretion regulatory functions such as SctW (CopN), SctU and SctL. SctW contains domains similar to *Yersinia* YopN and TyeA which are negative regulators of T3SS secretion (Joseph and Plano, 2007; Goss et al., 2004). The secretion of effector proteins by the T3SS is partially controlled by the complex YopN/TyeA which interacts with the secretory apparatus to prevent secretion before activation of the system upon detection of the right stimuli by the needle-tip complex (Joseph and Plano, 2007). The breaking of the YopN/TyeA interaction triggered by the stimuli that activate the T3SS induce the secretion of YopN, which render the apparatus operational for effectors secretion. The homologous SctW protein of *Chlamydiales* could have a similar regulatory function in addition of its secreted effector activity that trigger cell cycle arrest by altering the microtubule cytoskeleton (Huang et al., 2008; Fields and Hackstadt, 2000). The expression of SctW observed in late-phase of the replicative cycle of *Parachlamydia* is compatible with the dual function of this protein. The expression and the recruitment of SctW to the secretory apparatus in late-phase coincide with deactivation of the T3SS and thus inhibition of secretion. During the early-phase of infection, SctW is rapidly secreted which renders again the T3SS secretion competent.

The cleavage of the SctU *Yersinia* homolog YscU is required to permit secretion of effector proteins likely by allowing the recruitment of the ATPase complex SctN/L to the apparatus (Riordan and Schneewind, 2008). The transcriptional expression of the *sctU* gene is highly reduced in the mid-phase of the replicative cycle of *Parachlamydia*, which suggest that once the T3SS apparatus is assembled and activated, the requirement and the expression of the this protein is possibly decreased as observed in this study, or that single SctU proteins might be shared by several T3SS apparatus when its requirement decreases.
Regulation of the T3SS during the replicative cycle might be operated through the controlled temporal expression of some components required to initiate protein-protein interactions or functionality. For instance, studies on the *Yersinia* T3SS indicate that the assembly of the cytosolic complex consisting of the ATPase SctN, the interacting protein SctL and the C ring component SctQ require the presence of all of its components (Diepold *et al.*, 2010; Jackson and Plano, 2000). Thus, in *Parachlamydia*, the late-phase cycle expressed proteins SctN and SctQ would not be active until the early-phase expression of maximal amounts of SctL initiates or increases the early formation of the SctN/SctL/SctQ complex, triggering its docking onto the IM ring and the subsequent full activation of the T3SS secretion system. Similarly, the early expression of the multiple cargo secretion chaperone Mcsc would initiate or increase a protein-protein interaction hub consisting of Mcsc-effector-SctQ complexes and activation of T3SS-dependent effectors secretion.

The CopB protein identified in *Parachlamydia* is poorly homologous to the translocator proteins CopB and CopB2 of *Chlamydiaceae* bacteria (Chellas-Gery *et al.*, 2011). Similar to *sctU*, the expression of CopB is reduced during the mid-phase. Even though no sequence homologies are observed, CopB of *Parachlamydia* exhibit more similarities with the predicted secondary structures of the *C. trachomatis* CopB2 than with CopB (data not shown). In addition, no co-localisation of CopB with the inclusion membrane of *Parachlamydia* has been observed in this study suggesting that this protein is possibly secreted in the host cell cytoplasm similarly to CopB2 of *C. trachomatis* (Chellas-Gery *et al.*, 2011).

In conclusion, the differential temporal expression of T3SS components during the replicative cycle of *Parachlamydia* suggests that the maximal expression and activation of T3SS injectisomes occurs during the early to mid-phase of the infectious cycle, which corresponds to a critical phase during which the intracellular bacterium has to evade and/or block lytic effectors of the amoebal host cell, to differentiate from EBs to RBs and to modulate the maturation of its replicative vacuole to create replicative condition able to sustain efficient bacterial replication. Based on several studies mainly performed in other bacteria than *Chlamydiales*, the functions of several T3SS components are well described whereas for other components, no functionality has yet been attributed. In addition of a
described enzymatic, regulatory or structural function, a temporal activity remains to be characterized for many proteins that compose the chlamydial T3SS machinery. The T3SS machinery is likely a dynamic structure which can fluctuate during a replicative cycle to modulate its functionality. The expression of each component of the T3SS during the replicative cycle depends of several molecular criteria including function, activity, stoechiometric ratio, stability and turnover of each protein. In addition, some proteins might be shared between different apparatus of the cell. Finally, recent data strongly suggest that unlike other described bacterial species, the Chlamydiales T3SS is likely deeply involved in additional roles than solely protein secretion and translocation (Peters et al., 2007). For instance, the attachment to inclusion membranes and the differentiation of RBs into EBs may be controlled by the T3SS. Thus, differential temporal expression of various components of the chlamydial T3SS may be required to modulate these multiple regulatory activities.

Experimental procedures

Bacterial strain. P. acanthamoebae strain Hall’s coccus were grown at 32 °C within Acanthamoeba castellanii in 75-cm² cell culture flasks (Corning, New-York, USA) with 30 ml of peptone-yeast extract-glucose (PYG) broth (Greub and Raoult, 2002). After 5 days of incubation, cultures were harvested and the broth was filtered through a 5µm pore to eliminate both amoebal trophozoites and cysts and to collect bacteria in the flow through. A. castellanii ATCC 30010 was cultured in 75-cm² cell culture flasks at 25°C.

Infection procedure. Amoebae harvested from 24h fresh culture were centrifuged at 200 × g for 5 min, resuspended in RPMI-HEPES supplemented with 200 mM L-glutamine (Gibco-BRL, Life Technologies, Paisley, Scotland) and 10% fetal calf serum (Gibco-BRL). Then, 10⁵ amoeba cells/ml were incubated for 2h at 25 °C in 24-well cell culture plates (Corning). Non-adherent cells were washed and the remaining adherent cells were incubated for 16h at 25°C.

The amount of bacteria after 5 days of amoebal co-culture was equal to about 10⁸ cells per ml as measured by quantitative PCR of the 16S rDNA. Host cell lysis assay following infection of A.
*castellanii* with limiting serial dilution of a bacterial filtrate confirmed that most collected bacteria are living cells.

Wells containing $10^5$ amoebae were infected with a $10^6$ bacteria/ml suspension corresponding to a multiplicity of infection (MOI) of about 10. Plates were centrifuged at $1790 \times g$ for 10 min at room temperature. After 30 min of incubation at $32 {}^\circ C$, amoebal cells were washed with RPMI-HEPES and further incubated for different periods at $32 {}^\circ C$ in RPMI-HEPES supplemented with 200 mM L-glutamine and 10% fetal calf serum.

**Confocal microscopy.** Infected amoebae were washed with PBS, fixed with ice-cold methanol for 4 min. Cells were then washed three times with PBS and were then blocked and permeabilised for 1h in a blocking solution (PBS/0.1% Saponin/10% fetal calf serum). Coverslips were incubated with primary mouse antisera directed against different specific proteins (CopB, SycD1, Mesc, SetW, SetJ, PahN) or against total whole cells of *Parachlamydia* for 1h at room temperature in blocking solution. After washing three times with PBS/0.1% saponin, coverslips were incubated for 1h with secondary anti-mouse antibodies in blocking solution containing concanavalin A (Invitrogen, Basel, Switzerland) and DAPI (dilactate, D3571, Molecular Probes, OR, USA) to counterstain amoebae and label DNA, respectively. After two washing in PBS/0.1% saponin, two in PBS and one with deionised water, the coverslips were mounted onto glass slides using Mowiol (Sigma-Aldrich, MO, USA). Cells were observed on a confocal fluorescence microscope (Zeiss LSM710 Meta, Jena, Germany). Files were analysed using Image J for microscopy (www.macbiophotonics.com) softwares. All the assays were performed at least in triplicate.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR).** Amoebae were infected in 24-wells plates as described before. Samples were harvested at different times post-infection by scraping amoeba cells from the well surface and by resuspending the samples in RNAprotect bacteria reagent (Qiagen GmbH, Germany). Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen GmbH, Germany) as described by the manufacturer. The RNA samples were treated with the DNA-free kit (Ambion, Applied Biosystems, California, USA) to remove contaminating DNA from the RNA preparation.
The analysis of mRNA quantification was performed by a one step reverse transcription polymerase chain reaction using the One step MESA GREEN qRT-PCR MasterMix Plus for SYBR® assay (Eurogentec, Seraing, Belgium). The design of the specific primers listed in table 1 was done with the Primer3plus software (http://primer3.sourceforge.net/webif.php). The qRT-PCR assay was performed in a total volume of 20 µl with 10 µl of 2x reaction buffer, 100nM forward primer, 100nM reverse primer, 0.25U/µl Euroscript reverse transcriptase, 0.1U/µl RNase inhibitor and RNAse-free water. The thermocycling conditions were set up as follow: (i) reverse transcription, 30min, 48°C, (ii) Meteor Taq activation and Euroscript inactivation, 5min, 95°C, (iii) 40 cycles, 15sec, 95°C, 1min, 60°C, (iv) melt curve analysis, temperature increment of 0.3% from 60 to 95°C. The analysis of the 16S rRNA was used as an internal control to normalize the quantification of every specific mRNA collected at different times during an infectious cycle. The results are presented as a relative quantification taking samples at time 0 (EBs) as a reference of 1. Water was used as a negative control and the qRT-PCR reaction without reverse transcriptase was used as a control to measure DNA contamination in the extracted RNA samples. All measurements were performed at least in triplicates.

**Protein purification and generation of antisera.** *P. acanthamoebae* sctJ, sctW, copB, msc, pah-N and sycD (scc2) ORFs were amplified from purified *P. acanthamoebae* genomic DNA using primers listed in table 2 using the high fidelity Phusion polymerase (Finnzymes, Espoo, Finland). Using the Champion pET directional TOPO Expression Kits (Invitrogen), sctJ and msc were cloned in frame with a N-terminal and C-terminal peptide containing a polyhistidine (6xHis) tag to generate the plasmids pET200/D-TOPO-sctJ and pET101/D-TOPO-msc, respectively (Table 2). The four bases CACC were added to the forward primers to allow directional in-frame cloning of the PCR fragments into plasmids pET200/D-TOPO and pET101/D-TOPO. Following digestion of the PCR amplicons and the plasmids with NcoI/HindIII, copB (nt 1-960) and pahN were ligated into the plasmid pBADmycHis (Invitrogen) to generate the plasmids pBADmycHis-copB and pBADmycHis-pahN encoding for C-terminal polyhistidine tag (6xHis) recombinant proteins. Similarly, copN and scc2 amplicons and the plasmid pET28a were digested with NdeI/BamHI to generate by ligation the plasmids pET28a-copN and pET28a-scc2 encoding for N-terminal polyhistidine tag recombinant proteins. The proteins were expressed in BL21 star (DE3) *E. coli* strains following 0.5mM IPTG
(pET200/D-TOPO-sctJ / pET101/D-TOPO-mesc / pET28a-copN / pET28a-scc2) induction at 37°C or in TOP10 E. coli strains following 0.02% arabinose (pBADmycHis-copB / pBADmycHis-pahN) induction at 37°C. The BL21 and TOP10 E.coli strains expressing the protein of interest were lysed with the FastBreak cell lysis solution (Promega) and recombinant His-tagged proteins were purified in native or denaturing conditions using the MagneHis protein purification system (Promega) according to the manufacturer’s recommendation. Purified recombinant His-tagged proteins were used to conduct mouse immunization programs at the Eurogentec animal facility (Eurogentec, Seraing, Belgium) to elicit the production of specific mouse antisera.

**Western blot.** For Immunoblot analysis, protein samples were separated by SDS-PAGE, transferred to 0.45 µm nitrocellulose membranes and blocked with 5% non-fat powder milk in TBST (50mM Tris-Base, 150mM NaCl, 0.2% Tween pH7.4). The nitrocellulose membranes were incubated with mouse primary antibodies (anti-SctJ, SetW, CopB, Mcsc, SctF) diluted in 0.5% milk-TBST, washed 3 times, incubated with secondary antibody conjugated to horseradish peroxidase diluted in 0.5% milk-TBST and detected by chemiluminescence. The density of the bands on western blots was measured with the gel analysis method of the ImageJ software as described by Luke Miller ([http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/](http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/)). Western blot have been done at least in triplicates for each analyzed proteins.

**Quantitative immunofluorescence.** Proteins were labeled by immunofluorescence staining with specific mouse polyclonal antibodies as described in confocal microscopy. 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 was generated. The region of interest (ROI) including only the signal of intensity emitted by the bacteria was defined by applying a median filter (2x2.0 pixels) and by adjusting the Isodata threshold setup. A histogram of the ROI composed of the signal intensity on the x-axis and of the frequency on the y-axis was generated. A mean value and a standard deviation of the histogram of the frequency distribution of fluorescence intensity were also generated. The results were then exported and analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). The signal intensity for each frequency was normalized by using a value of 100 for the highest intensity.
Several images from one experiment performed at least in triplicates have been analyzed for each protein.

References


### Table 1. List of primers used in qRT-PCR

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Tm calculation: thermodynamic parameters and salt correction formula: SantaLucia JR, 1998
### Table 2. Cloning primers and plasmids

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

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

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 proteinase 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 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.

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 set 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 tetraicopeptide repeat 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.

**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 bioinformatic 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*.

**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.

**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.

**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).
**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.

**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 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. Finally, increased de novo synthesis of some T3SS components in the late-phase trigger the shutdown of the apparatus (SctW) and the formation of a silent T3SS in EBs ready to be rapidly activated when requested. IM: Inner membrane.

OM: Outer membrane. RVM: Replication vacuole membrane

Supplementary figure 1. Proteinase K treatment is required for complete EBs lysis during DNA extraction. Bacterial growth quantified by DNA copy number in a logarithmic (A) and linear (B) scale measured by qPCR during a replicative cycle of Parachlamydia within A. castellanii from DNA extracted with and without proteinase K treatment. A significant difference is observed when extracted samples contain a bacterial population composed of a majority of EBs demonstrating the absolute requirement of proteinase K treatment for complete bacterial lysis of both EBs and RBs.

Supplementary figure 2. Estimation of protein expression during a replicative cycle by western blot. Total bacterial number was detected with an antisera directed against whole bacterial cells (α-Para). The relative expression of CopB, SctW (CopN) and SctJ at different times of bacterial growth was estimated by calculating the ratio between the intensity of the bands corresponding to the different
proteins and the intensity of the signal obtained with total anti-*Parachlamydia* antisera. The results were normalized to 1 at 8h post-infection.
Acknowledgments

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