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Behind the chlamydial cloak: The replication cycle of chlamydiaphage Chp2, revealed

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ABSTRACT

Studying the replication of the chlamydiaphages presents significant challenges. Their host bacteria, chlamydiae, have a unique obligate intracellular developmental cycle. Using qPCR, immunochemistry, and electron microscopy, the life cycle of chlamydiaphage Chp2 was characterised. Chp2 infection has a dramatic inhibitory effect on bacterial cell division. The RB to EB transition is arrested and RBs enlarge without further division. There is a phase of rapid Chp2 genome replication 36 to 48 h post infection that is coincident with the expression of viral proteins and the replication of the host chromosome. The end stage of Chp2 replication is characterised by the appearance of paracrystalline structures followed by bacterial cell lysis. These data indicate that the Chp2 life cycle is closely coordinated with the developmental cycle of its bacterial host. This is a remarkable adaptation by a microvirus to infect and replicate in a bacterial host that has an obligate intracellular developmental cycle.

Introduction

Bacteriophages of free living hosts are the most highly characterised organisms on a molecular level but very little is known about phages that infect hosts with developmental cycles, especially if the host bacterium has an obligate intracellular replication phase. In recent years, several phages of chlamydiae (Garner et al., 2004) and *Bdellovibrio bacteriovorans* (Brentlinger et al., 2002), two obligate intracellular developmental bacteria, have been isolated. The phages, known as Gokushoviruses, are distantly related to the well-characterised single-stranded DNA microvirus ΦX174. Five chlamydiaphages (Chp1, Chp2, Chp3, CPAR39 and CPG1) (Richmond et al., 1982; Liu et al., 2000; Garner et al., 2004; Read et al., 2000; Hsia et al., 2000) have been described in detail; for our studies we have concentrated on Chp2 which was originally isolated from a strain of *Chlamydophila abortus* in Macedonia, Greece (Liu et al., 2000).

Unlike the large dsDNA phages, ΦX174 replication is extremely rapid, with progeny detected as early as 10 min post infection (Chen et al., 2007). Moreover, ΦX174 does not encode host shut off mechanisms (Fane et al., 2006) and lysis requires host cell division for the phage mediated block in cell wall synthesis to have an effect (Bernhardt et al., 2000, 2001). Thus, the phage life cycle is linked to the cell cycle of the host. As the chlamydiae and *Bdellovibrio bacteriovorans* are obligate intracellular parasites, the association between phage and host life cycles may be even more intimately linked, occurring only when the host bacteria have infected their respective target cells and are metabolically capable of replication. The obligate intracellular nature of chlamydial replication presents significant challenges to phage characterisation and the development of phage protocols. In the case of chlamydiaphages it would be disadvantageous to destroy the host during the first bacterial cell divisions. Therefore, our hypothesis is that chlamydiaphage replication is closely linked to the developmental cycle of the host.

The chlamydial developmental cycle commences when the infectious, but metabolically inert, extracellular form of the organism, the elementary body (EB), attaches to susceptible host cells (Ward, 1983; Rockey and Matsumoto, 2000). During uptake, EBs become enveloped within a host-derived intracellular membrane enclosed structure known as an 'inclusion'. Early in infection, EBs become reticulate bodies (RBs), which divide rapidly by binary fission, and lead to the expansion of the inclusion. Approximately 24–48 h post infection (h.p.i), depending on the species, the chromosomal DNA within RBs condenses to form EBs, which are then released from the infected host cells by lysis. Using a combination of quantitative PCR, immunochemistry, and electron microscopy, the life cycle of chlamydiaphage Chp2 was characterised, and the consequences on host development were defined. The results of this study suggest that Chp2 replication is closely coordinated with the developmental cycle of its bacterial host.

Results and discussion

Analytical and morphological studies were used to characterise the developmental cycle of *C. abortus*, *C. abortus* infected with Chp2, and the Chp2 viral life cycle.

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The developmental cycle of the host bacterium C. abortus B577 in BGMK cells—EM studies

Thin-section transmission electron microscopy (TEM) was performed to define the developmental cycle of the host bacterium *C. abortus* (Fig. 1), which follows the well-characterised developmental cycle seen for *C. trachomatis* (Ward, 1983). However, in these control studies (without chlamydiaphage infection) the *C. abortus* development cycle took approximately 3 days to complete. This is slower than *Lymphogranuloma venereum* strains of *C. trachomatis* in BGMK cells (Lambden et al., 2006). Only small inclusions (data not shown) carrying at least eight RBs were visible by 24 h.p.i. At 48 h.p.i. *C. abortus* inclusions were characteristically compact and EBs were just beginning to form (Fig. 1A). RBs remained most abundant, comprising over 90% of the inclusion contents and dividing RBs were also present. By 72 h.p.i. eukaryotic host cell lysis had begun, EBs predominated and were released from lysed cells (Figs. 1B and C).

Infection of C. abortus B577 with Chp2—EM studies

Chp2 at a MOI of 3 was used in infection experiments. At 24 h.p.i. there were no observable differences between Chp2-infected *C. abortus* B577 inclusions and uninfected *C. abortus* B577 inclusions. At 48 h.p.i. the first effects of phage infection became visible (Fig. 1D). In contrast to *C. abortus* without Chp2 infection (Fig. 1A), inclusions at this stage contained no EBs. Some dividing RBs were present although many RBs had become enlarged, some with at least four times the diameter of the largest RBs found in uninfected *C. abortus*. At 72 h.p.i. most host eukaryotic cells had yet to lyse. Some RBs had lysed and around 50% of the intact RBs contained paracrystalline arrays of Chp2 (Figs. 1E and F).

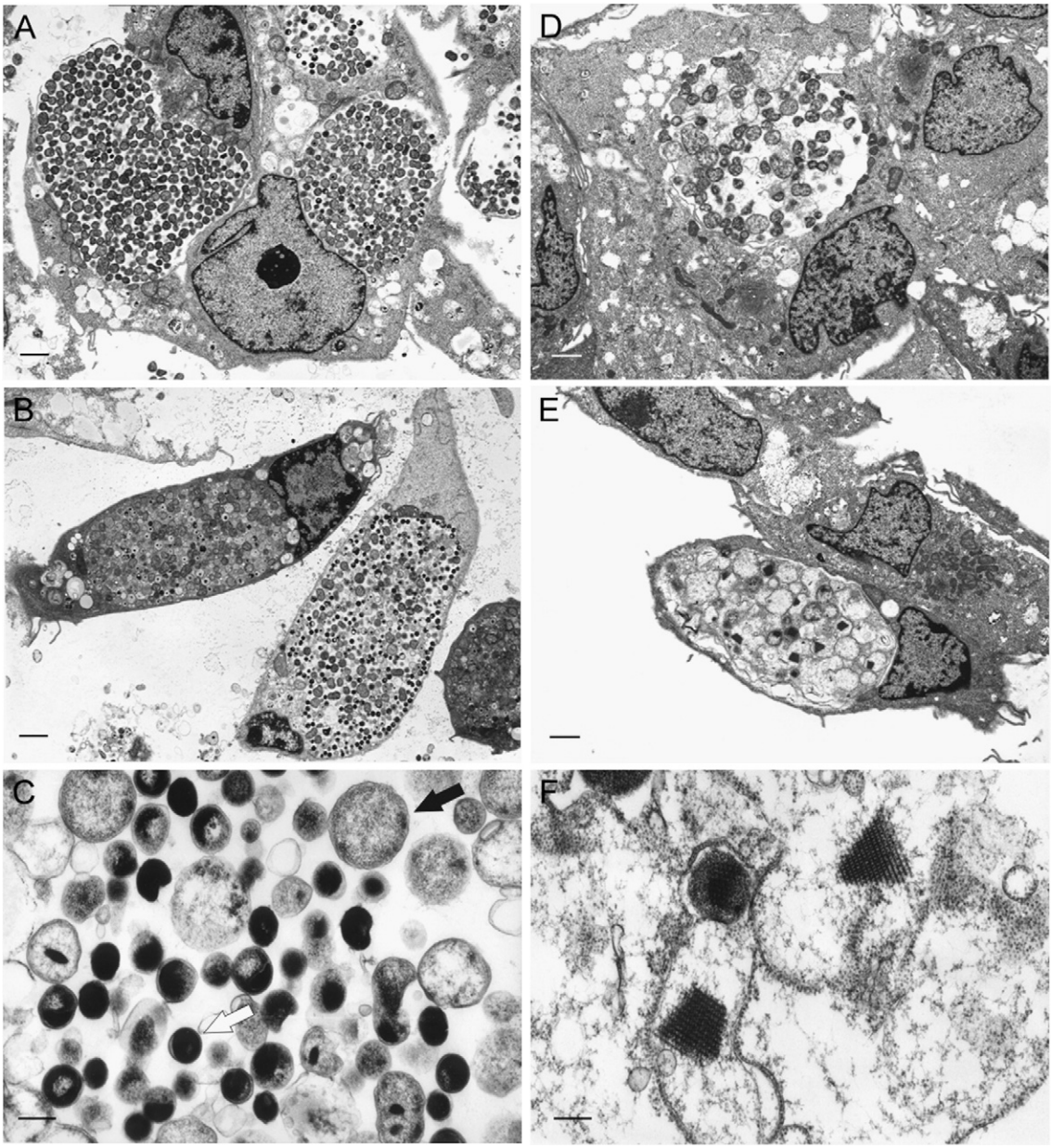

Fig. 1. Thin-section transmission electron micrographs of BGMK cells containing uninfected (A, B and C) and Chp2-infected (D, E and F) *C. abortus* strain B577. Uninfected chlamydiae-filled inclusions are well established by the 48 h time point and are mainly comprised of RBs, some of which are dividing (A). By the 72 h time point (B, and high mag/enlarged C) the inclusion is the predominant feature within the host cell and is dominated by EBs. In contrast to uninfected B577, inclusions at the 48 h.p.i. time point contain no EBs and enlarged RBs (D). By 72 h.p.i. RBs are still intact, have enlarged further and contain paracrystalline arrays of Chp2 (E and high mag/enlarged F). The scale bar represents 1.6 μm (A, B, D and E) and 0.3 μm (C and F). Examples of EBs and RBs in panel C are highlighted by the white and black arrows respectively.

Thus Chp2 infection has a profound morphological effect as it retards the developmental cycle and prevents both the division of RBs and inhibits their development into electron-dense EBs. These effects appear to mirror the results we have previously observed with *C. trachomatis* treated with penicillin which, administered at 20 h into the developmental cycle, retarded bacterial cell division and blocked maturation of RBs into EBs (Lambden et al., 2006). The mechanism(s) by which penicillin and chlamydiaphage exert these similar morphological effects is unknown.

Chromosomal replication in C. abortus and the effects of Chp2 infection

Real-time qPCR assays were applied to measure the chromosomal replication of *C. abortus* and to investigate the effects of Chp2 infection on chlamydial chromosomal replication. Previously we had developed highly specific and sensitive qPCR assays for both the *C. abortus* chromosome and for detecting Chp2 genomes (Clarke et al., 2004; Skilton et al., 2007).

The specificity of oligonucleotide primers and probes was confirmed by performing real-time qPCR reactions using chromosomal DNA purified from BGMK cells and *C. trachomatis* L2 EBs. Results were negative in both cases. In addition, the Chp2 real-time qPCR showed no reaction with *C. abortus* chromosomal DNA and the *C. abortus* qPCR did not react with Chp2 DNA. Amplification plots displayed a typical sigmoidal shape with the fluorescence change becoming detectable during the exponential phase of the reaction, followed by saturation due to depletion of the reactants. Both chlamydiaphage genome and *C. abortus* chromosomal assays consistently detected as few as ten molecules of target DNA.

In these experiments Chp2 was used to infect *C. abortus* at a MOI of 3.0. To measure chromosomal and Chp2 DNA these infections were performed in quadruplicate in 96 well trays, with samples collected for analysis at 0, 12, 24, 36, 48, 60 and 72 h.p.i. A parallel set of samples of *C. abortus* strain B577 (previously shown to be chlamydiaphage-free by qPCR and immunofluorescence) that had not been infected with Chp2 were also collected for direct comparison.

The results of qPCR for host chromosomal copies in both Chp2-infected and uninfected *C. abortus* strain B577 are displayed in Fig. 2A. These plots show a typical sigmoid replication curve, and by 36 h.p.i. the yield of chromosomal DNA was similar for Chp2 infected and uninfected *C. abortus* with 100 fold increase in relative copies. However, from this time point the rate of increase in Chp2 infected *C. abortus* was slightly slower. This was coincident with the first observable signs of Chp2 infection by TEM at 48 h.p.i. The chlamydial chromosome, in both Chp2 infected and uninfected cells, continued to replicate and by 72 h.p.i. chromosomal copies had increased 10^3 fold. Thus, the complete developmental cycle of *C. abortus* takes 10 bacterial cell divisions and gives a 2–4 fold higher yield than *C. trachomatis* grown in the same eukaryotic host cells (Lambden et al., 2006). The higher yield reflects the more densely packed mature inclusions of *C. abortus* infection compared to *C. trachomatis* inclusions. Chp2 infection slows chromosomal replication slightly, but this process still continues in the absence of cell division. *C. abortus* is arrested at approximately the eighth bacterial cell division by Chp2 infection. From this point chromosomal DNA replication continues and whilst there is no more cell division RBs continue to expand in size.

'Burst size' of Chp2 infection

The qPCR profile of Chp2 genome replication is shown in Fig. 2B. For the first 24 h there is very limited Chp2 genome replication, which is coincident with limited host chromosomal replication. Thus it appears that the lag phase before viral genome replication is most likely due to the host bacterial cells in their own developmental cycle reaching a metabolic state that supports DNA synthesis. This phase is followed by rapid Chp2 genome replication. Using the ss (single-stranded) DNA replication cycle of the phage ΦX174 as a paradigm, the modest increase in Chp2 genome number (0–24 h) reflects Stage I DNA replication, in which ssDNA is converted to a dsDNA replicative form, and stage II DNA replication, the limited amplification of replicative form DNA. Whilst stage I DNA synthesis is conducted exclusively by host cell proteins (Shlomai et al., 1981), stage II DNA synthesis requires the viral protein A and the host cell rep helicase (Eisenberg et al., 1976). The rapid replication of the Chp2 genome at 24–48 h.p.i. most likely represents Stage III DNA replication, during which ss genomic DNA is exclusively synthesized and packaged into procapsids.

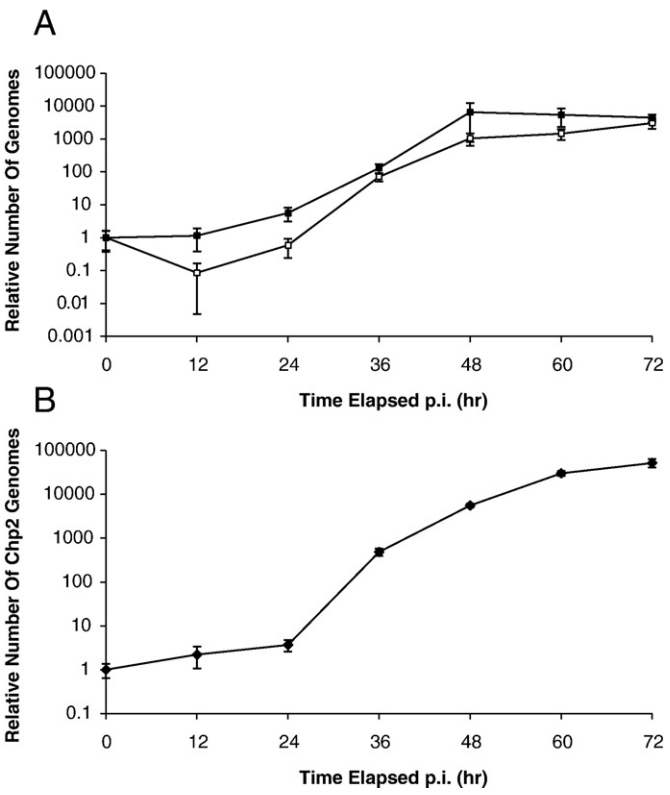

Fig. 2. (A) Chromosomal replication during the developmental cycle of uninfected (■) and infected (□) *C. abortus* strain B577. Cells were removed for qPCR analysis at 0, 12, 24, 36, 48, 60 and 72 h.p.i. The relative number of chlamydial genomes was determined using the *uvrD* TaqMan assay. (B) Genome replication of Chp2 during infection of *C. abortus* strain B577. Infected cells were removed for qPCR analysis at 0, 12, 24, 36, 48, 60 and 72 h.p.i. The relative number of Chp2 genomes was determined using a previously described Chp2 TaqMan assay.

Although Chp2 infection slows chromosomal replication slightly, this process still continues in the absence of cell division. The EM studies show that cell division is arrested at approximately the 7th/8th bacterial cell division. Thus it is not possible to calculate a 'burst size' (phage/cell) in the classical use of the term. However, as seen in Fig. 2 chromosomal DNA is replicated 10^3 fold while the relative yield of phage is 5×10^5 fold, thus the yield of Chp2 genome per *C. abortus* genome equates to a 'developmental cycle size' of 500. Counts of Chp2 particles in TEM samples fit closely to this number.

Production of procapsids and the switch to stage III DNA synthesis

In ΦX174, single-stranded genomes are synthesized and concurrently packaged into procapsids. This stage of DNA synthesis, stage III, cannot occur in the absence of procapsids (Aoyama et al., 1981). In Chp2, VP3 has been previously demonstrated to be the viral scaffolding protein, found exclusively in procapsids (Clarke et al., 2004). In phage ΦX174, the viral protein C acts as an inhibitor of stage II DNA replication

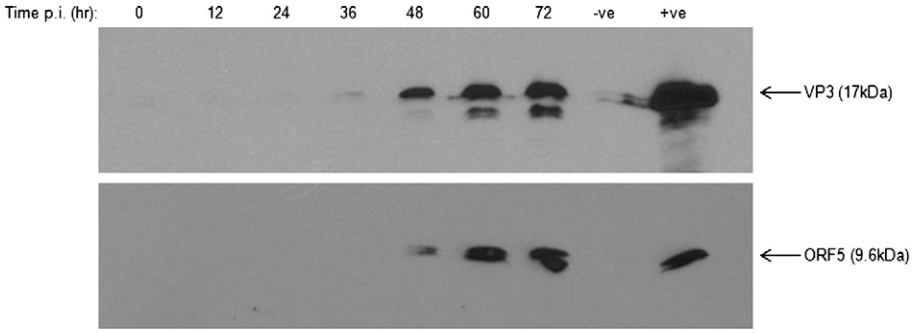

Fig. 3. Expression of structural (VP3) and non-structural (ORF5) proteins during the developmental cycle of Chp2-infected *C. abortus* strain B577. Infected cells were removed at 0, 12, 24, 36, 48, 60 and 72 h.p.i. for analysis by Western blot. Uninfected B577 at the 72 h time point and Chp2-infected *C. abortus* strain MA were used as negative and positive controls respectively.

and is also required for DNA packaging (Aoyama and Hayashi, 1986). The ORF5 protein of Chp2 is a small acidic protein that is believed to be the functional equivalent of protein C (Liu et al., 2000). Fig. 3 shows that both VP3 and ORF 5 proteins first become detectable at 36 and 48 h post infection, respectively, which corresponds with the 7th/8th chlamydial chromosomal division. As previously demonstrated, the monoclonal antibody against VP1, the major coat protein, only recognizes coat proteins assembled into virions and procapsids (Everson et al., 2002). In ELISA assays (Fig. 4) assembled coat protein was also first detected during this time interval, but after the commencement of rapid Chp2 genome replication. As protein detection by antibodies is a function of protein concentration and the efficacy of recognition, they are less sensitive than qPCR assays. Thus, the relative concurrent detection of viral proteins 1, 3, and 5 with the rapid increase in Chp2 chromosome copy number suggests that the rapid increase in DNA synthesis represents the synthesis of ssDNA. Thus it appears that the chlamydiaphages take advantage of the developmental cycle of their host because the morphological effects of Chp2 infection are only apparent at approximately 48 h.p.i. which equates to the 7th/8th bacterial division during the developmental cycle. This is curious since the prototype microvirus ΦX174 is a lytic bacteriophage that destroys its host within a single cycle of bacterial cell division. Our results also show that the Chp2 genome can be used as a stable replicon and is inherited by daughter cells at division. These data are crucial in learning how to develop the chlamydiaphage as a vector. The overall burst size of Chp2, measured by genome equivalents, is 500 phage genomes produced for each chlamydial chromosome replicated and this is similar to the burst size of ΦX174 (Fane et al., 2006).

The coordination of chlamydiaphage Chp2 life cycle to the developmental cycle of its host *C, abortus* is remarkable, yet fundamentally different compared to the phylogenetically related coliphage ΦX174. Control of gene expression in chlamydiae appears to operate in part through the regulation of promoters by different sigma factors (Yu and Tan, 2003; Schaumburg and Tan, 2003) and we speculate that differentially expressed sigma factors control promoters on the Chp2 genome, in this way the host bacterium has been exploited by the phage to grow and divide to provide an optimal environment for Chp2 replication. Future work will involve defining Chp2 promoters and investigating their regulation during the developmental cycle.

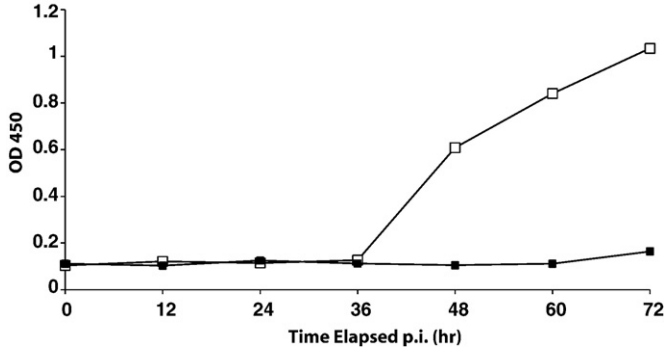

Fig. 4. Expression of the major coat protein VP1 during the developmental cycle of Chp2-infected *C. abortus* strain B577. BGMK cells infected with *C. abortus* but not Chp2 (■) and BGMK cells infected with *C. abortus* carrying Chp2 (□) were removed at 0, 12, 24, 36, 48, 60 and 72 h.p.i. for analysis by ELISA. The presence of assembled Chp2 coat protein was detected using Mab 55.

Materials and methods

Cells and chlamydia

BGMK cells were used to propagate *C. abortus*. Chlamydiae were tested for mycoplasma contamination by fluorescence microscopy using Hoechst no. 33258 staining and by using a VenorGem® Mycoplasma PCR Detection Kit (Minerva Biolabs, Berlin, Germany) according to the manufacturer's instructions. The BGMK cells were grown in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% foetal calf serum. Cells were infected with *C. abortus* by centrifugation at 1000× *g* for 1 h in medium containing cycloheximide (1 μg/ml) and gentamicin (25 μg/ml). Infected monolayers were detached with PBS containing 0.125% trypsin/0.02% EDTA and EBS harvested in DMEM containing 10% FCS at 3000× *g* for 10 min. The infected cell pellet was suspended in PBS: H2O (1:10) and homogenised in a Dounce homogeniser to break open cells and release the EBs. Cell debris was removed by centrifugation at 250× *g* for 5 min and the supernatant containing partially purified chlamydiae was mixed with an equal volume of phosphate buffer containing 0.4 M sucrose (2SP), which was stored at −80 °C and used for the subsequent Chp2 infection time course studies.

Chp2 preparation and purification

Monolayers of BGMK cells in 25 cm^2 tissue culture flasks (T-25) were infected with the *C. abortus* (strain MA) bearing the Chp2 chlamydiaphage by centrifugation as described above. At 72 h.p.i. the culture medium was replaced with a small volume of phosphate-buffered saline (PBS) and the flasks frozen at −70 °C. To produce a batch of Chp2, one hundred T-25 flasks were prepared, stored frozen and processed together. Individual flasks were frozen and thawed three times to lyse any residual host cells together with chlamydial RBs and release the Chp2 particles. Lysates were pooled and this suspension was centrifuged at 2000× *g* for 15 min to sediment cell debris. The supernatant was passed through a 0.45 μm filter followed by a 0.22 μm filter. The filtrate was centrifuged at 100,000× *g* in a Beckman SW28 rotor for 3 h and the resultant pellet washed with PBS and centrifuged at 80,000× *g* for 40 min. The pellet was finally suspended in PBS, vortexed with glass beads and stored at −70 °C.

Time course of Chp2 infection and Chp2 titration

For monitoring DNA replication, ELISA and immunoblotting for protein expression (where multiple samples were required for each time point) BGMK cells were grown in 96 well trays. ELISA assays with Chp2 specific monoclonal antibody 55 were performed as previously described (Everson et al., 2002). For transmission EM studies (TEM) BGMK cells infected with *C. abortus* were grown in 24 well trays and were fixed with 3% gluteraldehyde in 0.1% cacodylate buffer and processed for negatively stained thin-section grids (Liu et al., 2000), and for immunofluorescence cells were grown on sterile 13 mm coverslips in 24 well trays. In each case, an inoculum of Chp2 was titrated with purified EBs of the chlamydiaphage-free *C. abortus* strain B577 for 30 min at room temperature as previously described (Skilton et al., 2007). Chlamydiaphage binding was performed in DMEM containing FCS (10%), cycloheximide (1 μg/ml) and gentamicin (25 μg/ml). The series of dilutions of Chp2-infected *C. abortus* was used to infect BGMK cells by centrifugation at 1000× *g* for 1 h. Cells were infected in parallel with the same dilution series of unchallenged control *C. abortus* B577. For measuring DNA replication, trays were set up for time points of 0, 12, 24, 36, 48, 60 and 72 h. At each time point a tray was transferred to −70 °C and stored for subsequent nucleic acid extraction.

Extraction of DNA for qPCR analysis

Microplate cultures of *C. abortus* were taken from freezer storage and allowed to thaw at room temperature. Aliquots (10 μl) of 0.880 SG ammonia were added to each culture well containing 100 μl of tissue culture medium. Each microplate was placed unsealed onto a pre-warmed heating plate at 98 °C for a total of 55 min and the contents of wells were evaporated to dryness. The microplate was immediately removed from heat and cooled to room temperature. Dried residues in relevant wells were resuspended with 100 μl of ultra pure water and mixed. Wells were sealed with parafilm and the microplates stored at 4 °C.

Real-time qPCR for Chp2 genome and C. abortus chromosomal DNA

The absolute number of Chp2 genomes and *uvrD* genes (chromosomal copies) in each sample was determined by performing 5′-exonuclease (TaqMan) assays using unlabelled primers and carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) dual-labeled probes. Two separate reaction mixes were prepared. Briefly, the *C. abortus* chromosomal DNA assay was comprised of ABI Universal Mix, uvrD 1385 F primer (5′-AACAACTCGAACATGCTTATGCAA-3′), a uvrD 1463R primer (5′-TGAAAATATCCCGTGATGCGT-3′), and a uvrD sequence-specific probe 1410T (FAM-CCTTCCTCTGAATGAGTTTGT-TGTCGCTAC-TAMRA). The primers and probe for the Chp2 genomic DNA assay have been described previously (Clarke et al., 2004) and was comprised of 1931F primer, Chp2 2044R primer and Chp2 probe 1957T. A dual standard curve of serial ten-fold co-dilutions of puvrD (representing *C. abortus* chromosomal DNA) and pChp2 (chlamydiaphage genomic DNA) was prepared in ultra pure water containing pBR322 (Skilton et al., 2007). Resuspended contents from each culture well (5 μl), or standard curve dilution, were added to 20 μl of reaction mix in suitable 0.2 ml PCR tubes. These were subjected to a 40 cycle real-time qPCR in an ABI Prism 7700 Sequence Detection System. PCR products were quantified in comparison with puvrD and pChp2 standard curves.

A single copy of the *uvrD* gene is located on the *C. abortus* chromosome (Thomson et al., 2005), hence the ratio of Chp2/*uvrD* is equivalent to the number of Chp2 genome copies per bacterium. Five microlitres of sample were added to each 20 μl reaction mix containing forward primer (300 nM), reverse primer (300 nM), probe (100 nM) and TaqMan Universal PCR Master Mix (Applied Biosystems). Real-time PCR cycles were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Amplification plots showing the relative change in fluorescence (ΔRn) during the PCR were assessed and an arbitrary threshold level of ΔRn set close to, but above, baseline levels. For each sample, the Ct approximated to the cycle at which fluorescence change was first detectable. Standard curves of Ct against the logarithm of the number of DNA molecules/reaction were plotted. The Ct value obtained for an unknown was used to interpolate the number of DNA molecules present.

Expression of ORF 5 protein in E. coli, polyclonal sera and immunoblotting

Chp2 ORF 5 was cloned and expressed using the Xpress™ system (Invitrogen life technologies) which allows expression of the recombinant protein fused at the N-terminus to a six-histidine tag, facilitating purification. An ORF5-specific primer pair, ORF5F: 5′-GCATAC**GAGCTC**ATGAAAGTTTTTACAGTG-3′ and ORF5R: 5′-GAGCG**TGGTACC**C-TACTGTTTGTGCTTAAAATC-3′ (engineered restriction sites, (*Sac I* and *Kpn I*) are shown in bold, preceding the complementary recognition sequence), were used to amplify the ORF5 gene using Chp2 genome as template in a PCR reaction containing Bio-X-Act thermostable DNA polymerase (Bioline, UK) over 30 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 30 s. The 225 bp amplicons were gel purified, cleaved with *SacI* and *Kpn I* and cloned into pRSETA (Invitrogen). Soluble purified ORF5 protein, purified using the Xpress™ system (Invitrogen) according to the manufacturer's instructions, was used to raise polyclonal rat sera as previously described. For immunoblotting samples of crude cell lysate from infected/uninfected B577 were run on a 14% SDS PAGE gel and the proteins electroblotted onto Hybond™-C extra nitrocellulose membrane (Amersham) for 1 h at 20 V. The membrane was incubated in a solution of phosphate-buffered saline supplemented with 0.05% Tween-20 (PBS-T) and 10% dried milk for 30 min at room temperature. Rat polyclonal antisera directed against the structural VP3 and non-structural ORF5 proteins were used at a dilution of 1 in 500 and 1 in 100, respectively, in a solution of PBS-T supplemented with 1% dried milk, and incubated with the membrane for 1 h at room temperature. Following extensive washing with PBS-T the membrane was incubated for 30 min at room temperature with a horseradish peroxidase-conjugated goat anti-rat antibody (Sigma) at the recommended dilution. After further washing with PBS-T the membrane was incubated in ECL reagent (Amersham) as per the manufacturer's instructions, and exposed to Kodak BioMax XAR film which was subsequently developed.

To re-probe, the membrane was incubated in a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH6.7) for 30 min at 50 °C then washed in PBS-T. The membrane was re-probed as described.

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