The effect of penicillin on *Chlamydia trachomatis* DNA replication

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Chlamydia trachomatis L2 was used to infect BGMK cells at an m.o.i. of 1·0, and the developmental cycle was followed by transmission electron microscopy and quantitative PCR (QPCR) for both chromosomal and plasmid DNA. Samples were taken at sequential 6 h time points. Subsequent analysis by QPCR showed that there was an initial slow replication period (0–18 h), followed by a rapid phase (18–36 h) coinciding with exponential division when the DNA doubling time was 4·6 h. Chromosomal DNA was amplified 100–200-fold corresponding to 7–8 generations for the complete developmental cycle. Penicillin (10 and 100 units ml⁻¹) was added to cultures at 20 h post-infection (p.i.). This blocked binary fission and also prevented reticulate body (RB) to elementary body transition. However, exposure to penicillin did not prevent chromosomal or plasmid DNA replication. After a short lag period, following the addition of penicillin, chlamydial chromosomal DNA replication resumed at the same rate as in control *C. trachomatis*-infected cells. *C. trachomatis*-infected host cells exposed to penicillin did not lyse, but instead harboured large, aberrant RBs in massive inclusions that completely filled the cell cytoplasm. In these RBs, the DNA continued to replicate well beyond the end of the normal developmental cycle. At 60 h p.i. each aberrant RB contained a minimum of 16 chromosomal copies.

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INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium that replicates within a specialized cytoplasmic vacuole known as an 'inclusion'. After entry into the host cell, the infectious form, or elementary body (EB), differentiates into a reticulate body (RB) that subsequently undergoes several rounds of binary fission before differentiation back into EBs. The ultrastructural changes that take place during this developmental cycle have been well documented (Rockey & Matsumoto, 2000; Ward, 1983).

The normal, lytic chlamydial developmental cycle can be altered by a number of factors, including the use of antibiotics, growth in continuous culture, depletion of essential amino acids, depletion of iron and treatment of host cells with gamma interferon (IFN γ) (reviewed by Beatty *et al.*, 1994). Heat shock, infection of monocytes and bacteriophage infection also have similar effects (Hogan *et al.*, 2004). Under these various conditions chlamydiae lose infectivity and enter into a 'persistent' infection, where RB to EB transition is retarded. IFN γ treatment depletes the pools of available tryptophan by inducing host indoleamine 2,3-dioxygenase and thus mimics the effects of amino acid

deprivation in retarding the developmental cycle. Exposure of *C. trachomatis*-infected cells to penicillin *in vitro* results in a similar phenotype; the mechanism by which penicillin causes this effect is unknown. Recovery of viable chlamydia is possible if penicillin is removed early, as the normal developmental cycle is resumed, although this is both dose and time dependent (Matsumoto & Manire, 1970). Penicillin does not prevent EB to RB conversion (Barbour *et al.*, 1982), nor does it stop the growth of RBs; however, it does block their division and prevents their conversion into EBs. Exposure to β -lactam antibiotics results in the accumulation of large, aberrant RBs or 'penicillin forms' (Armstrong, 1967; Matsumoto & Manire, 1970). Such aberrant RBs, formed during exposure to penicillin, contain inner and outer membranes (Clark *et al.*, 1982; Johnson & Hobson, 1977; Kramer & Gordon, 1971; Phillips *et al.*, 1984). An enduring enigma is that chlamydiae do not contain detectable peptidoglycan (PG) yet are sensitive to the action of penicillin (Chopra *et al.*, 1998; McCoy & Maurelli, 2006; Moulder, 1993). Penicillin acts by binding irreversibly to penicillin-binding proteins (PBPs), blocking PG biosynthesis. Three PBPs have been clearly demonstrated in *C. trachomatis* (Barbour *et al.*, 1982; Storey & Chopra, 2001). It is possible that chlamydiae produce a PG sacculus in a single molecular layer and this may account for the inability to detect PG (Chopra *et al.*, 1998; Moulder, 1993). Alternatively it has been suggested that there is no structural need for PG; chlamydiae appear to lack a PBP

Abbreviations: BGMK, buffalo green monkey kidney; EB, elementary body; LGV, lymphogranuloma venereum; PBP, penicillin-binding protein; PG, peptidoglycan; p.i., post-infection; QPCR, quantitative PCR; RB, reticulate body; TEM, transmission electron microscopy.

with transglycosylase activity and thus might produce a glycan-less PG wall (Ghuysen & Goffin, 1999).

Whilst RB division is arrested soon after the addition of penicillin, RBs continue to grow and expand; yet it is not known what effect penicillin treatment has on the replication of chlamydial DNA, as no direct assay for DNA replication has been applied under these conditions. We have recently developed an accurate quantitative PCR (QPCR) assay for both the chromosomal and plasmid DNA of *C. trachomatis* (Pickett *et al.*, 2005). The aims of the current work were: (1) to apply these sensitive assays to characterize fully the replication of *C. trachomatis* DNA, and (2) to put the original, descriptive observations on the morphological effects of penicillin treatment into a molecularly quantifiable framework.

METHODS

Cell culture, chlamydial growth, EB purification and quantification of infectivity. Plaque purified, mycoplasma-free *C. trachomatis* L2/434/Bu (ATCC VR902B) was grown as previously described using BGMK cells (Pickett *et al.*, 2005). These cells are widely used for chlamydia culture as they grow rapidly and allow clear visualization of inclusions. BGMK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (DMEM/FCS). Cells were infected with chlamydial EBs in medium containing cycloheximide ($1 \mu\text{g ml}^{-1}$). For large scale production of purified EBs, infected cells were detached from flasks at 40 h post-infection (p.i.) with PBS containing trypsin/EDTA and pelleted by centrifugation in DMEM/FCS. Intracellular *C. trachomatis* were released using a Dounce homogenizer, and EBs purified by centrifugation through Urografin 370 (32%, v/v; Schering) as previously described (Skipp *et al.*, 2005). The infectivity of the EB preparation was titred by serial dilution in 96-well trays. Infected cells were incubated for 40 h and then fixed as described by Shemer & Sarov (1985). Inclusions were stained using an in-house monoclonal antibody specific to chlamydial LPS, mAb29. Bound mAb29 was detected by a polyclonal anti-IgG conjugated to β -galactosidase. Bound conjugate was detected using a standard histochemical β -galactosidase assay.

Electron microscopy studies. Confluent BGMK cell monolayers were infected with *C. trachomatis* L2/434/Bu at a m.o.i. of 1.0. At the required time points, the monolayers were examined by phase-contrast microscopy and then washed with PBS. Monolayers were fixed in 3% glutaraldehyde in 0.1% cacodylate buffer pH 7.4 for 4 days, and then prepared for transmission electron microscopy (TEM) as previously described (Liu *et al.*, 2000).

Time-course of infection and extraction of DNA for real-time PCR analyses. BGMK cells grown to confluence in 96-well trays were infected with purified *C. trachomatis* L2/343/Bu EBs at a m.o.i. of 1.0. EBs were allowed to adsorb to cells for 1 h at 37 °C; cells were then washed with PBS to remove any residual unadsorbed EBs. The infected cells were overlaid with 100 μl culture medium and incubated at 37 °C in 5% CO₂. For each time point, cells were infected in triplicate, and the infection was stopped at 6, 12, 18, 24, 30, 36 and 48 h p.i. by snap freezing at -80 °C. For penicillin-treated cultures, medium was removed at 20 h p.i. and replaced with media containing 10 and 100 units penicillin G ml^{-1} (Sigma). In these wells the infection was stopped at 24, 30, 36, 48 and 60 h p.i. Genomic and plasmid DNA were extracted in a microplate format

following the protocol described by Pickett *et al.* (2005). The residue was then resuspended in 100 μl nuclease-free water. Samples were diluted 1 in 100 prior to QPCR analysis.

ELISA. Individual wells from a 96-well microtitre tray containing BGMK cells infected with *C. trachomatis* were scraped and the cells resuspended in 10 μl carbonate/bicarbonate buffer (0.05 M) pH 9.6 and used to coat immunoassay trays at room temperature overnight. Wells were then washed four times in 0.9% sodium chloride/0.05% Tween (ELISA wash), blocked in 5% milk powder/PBS at 37 °C for 1 h, and then washed four times (in ELISA wash). Polyclonal antisera raised against purified recombinant *C. trachomatis* OmcB in two rabbits (Watson *et al.*, 1994) was diluted 1:1000 in 1% milk powder/PBS/0.05% Tween, and incubated at 37 °C for 3 h. Wells were washed four times, and bound antibody detected with mouse anti-rabbit horseradish-peroxidase conjugate (ISL) and tetramethylbenzidine substrate.

Preparation of DNA standards. Laboratory strains of *Escherichia coli* harbouring the recombinant plasmids pCTL12A (Hatt *et al.*, 1988) and pSRP1A (Clarke & Lambden, 1988) were grown, in liquid culture at 37 °C, to late exponential phase in the presence of ampicillin ($25 \mu\text{g ml}^{-1}$). Plasmids were prepared and quantified as previously described (Pickett *et al.*, 2005).

Real-time QPCR. The absolute number of chlamydial plasmids and *omcB* genes in each sample was determined by performing 5'-exonuclease (TaqMan) assays using unlabelled primers and carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) dual-labelled probes. Real-time PCR cycles were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Reactions were set up as previously described (Pickett *et al.*, 2005) using duplicate standard samples.

RESULTS

Optimization of growth conditions for *C. trachomatis*

Purified *C. trachomatis* L2 EBs were quantified by infectious-centre assay and then used to infect BGMK cells at different m.o.i. We used gradient-purified EBs and selected a m.o.i. of 1.0 for these experiments as this ensured that at least ~35% of cells were infected by a single EB. An m.o.i. in excess of 10 was toxic to cells, and an m.o.i. lower than 0.1 gave too few inclusions per field to allow the developmental cycle to be monitored by phase-contrast microscopy. A single round of the developmental cycle under these conditions takes 36–48 h to complete. Inclusions first became visible by approximately 18 h p.i., and then rapidly developed to fill the cytoplasm of infected cells. To calibrate the system described here, we examined infected cells by TEM at 24 h p.i., when inclusions typically contain 8–16 RBs (Fig. 1a), and at 48 h p.i., when mature inclusions containing several hundred mature EBs and RBs are visible (Fig. 1b). The characteristic apparent motion within inclusions is visible by phase-contrast microscopy from 20–40 h p.i.

Effect of penicillin on BGMK cells infected with *C. trachomatis*

In a parallel experiment to that described above, penicillin G (at a final concentration of 10 and 100 units ml^{-1} culture

Fig. 1. Electron micrographs showing the effect of penicillin on the *C. trachomatis* developmental cycle. Normal inclusions in untreated control BGMK cells are shown at (a) 24 h p.i., where the inclusion contains only RBs, and (b) 48 h p.i., where many mature EBs are evident, but RBs and dividing RBs (arrowed) are also present. Penicillin (100 units ml⁻¹) was added to infected cells at 20 h p.i. A typical inclusion at 48 h p.i. is shown (c) containing at least eight large aberrant RBs of different sizes. At 60 h p.i. (d) some aberrant RBs are beginning to form nucleoids where DNA has condensed. Bars, 1 µm.

media) was added to *C. trachomatis*-infected cells at 20 h p.i. Within 4 h of penicillin addition, all apparent motion in inclusions had ceased. At 48 h p.i. the penicillin-treated cultures still contained inclusions and there was little evidence for host cell lysis; in contrast, most infected cells from untreated cultures had lysed. TEM revealed that RB division had halted after penicillin addition, and indicated that the number of chlamydia within an inclusion remained constant whilst individual RBs continued to enlarge. The progress of infection was followed until 60 h (Fig. 1d), when there was the first indication of multiple nucleoid condensation within individual, aberrant RBs.

It has been reported that β -lactam antibiotic treatments cause repression of *omcB* gene expression (*omcB* encodes the cysteine rich 60 kDa outer-membrane protein) (Cevenini *et al.*, 1988; Sardinia *et al.*, 1988). To verify that penicillin treatment in our system was consistent with these observations, we followed the accumulation of the OmcB protein by ELISA using polyclonal antisera raised against *C. trachomatis* OmcB (Watson *et al.*, 1994). This confirmed that, late in the normal developmental cycle at 36–48 h p.i., OmcB could be detected, but that in penicillin-treated cultures OmcB accumulation was inhibited (Fig. 2). A *t*-test ($\alpha = 0.05$) validated the statistical significance of this result.

Fig. 2. Effect of penicillin on the expression of *omcB* in *C. trachomatis*. Replicate cell cultures were infected with *C. trachomatis*. At 20 h p.i., penicillin G was added to one set of infected cells, and culturing continued for a further 40 h. At regular intervals, samples were taken from both cultures for OmcB protein estimation by ELISA. ●, No penicillin; ○, penicillin (100 units ml⁻¹).

Real-time QPCR assay

DNA was extracted from quadruplicate samples of *C. trachomatis*-infected cells at 6, 12, 18, 24, 30, 36 and 48 h p.i., and from penicillin-treated cells at 24, 36, 48 and 60 h p.i. QPCR assays (Pickett *et al.*, 2005) were applied to these samples to measure the amounts of chromosomal and plasmid DNA. The results (Fig. 3) show that plasmid and chromosomal DNA replication follow a typical sigmoidal amplification curve during normal growth. There is an approximately 200-fold increase in chromosomal DNA relative to the input inocula. The mid-log period of chlamydial replication had a DNA doubling time of 4.6 h.

Statistical analysis by ANOVA ($\alpha=0.01$) reveals that the addition of penicillin to the culture medium causes an initial slight inhibition of chromosomal, but not plasmid, replication; this soon recovers and the amplification of both plasmid and chromosomal DNA follows closely to that of the untreated culture.

DISCUSSION

C. trachomatis LGV strain L2 has been adopted as a popular model system for studies; at the end of the developmental cycle the yield of infectious *C. trachomatis* L2 EBs is typically 200–300 per inclusion (Mathews *et al.*, 1999). We routinely use BGMK cells to culture *C. trachomatis* because these cells show consistent growth properties, giving reproducible chlamydial growth characteristics, inclusions are easy to visualize by light microscopy and the cells lyse when the developmental cycle is complete. In all cell cultures the chlamydial developmental cycle becomes asynchronous,

Fig. 3. Replication of *C. trachomatis* genomic and plasmid DNA during the developmental cycle. Infected cells were removed for QPCR analysis at 0, 6, 12, 18, 24, 30, 36 and 48 h p.i., additional samples were taken at 60 h for the penicillin-treated cultures. The relative number of chlamydial genomes, determined using the *omcB* assay (a), and the relative numbers of plasmids (b) during the chlamydial developmental cycle are shown. ○, No penicillin; □, 10 units penicillin ml⁻¹; △, 100 units penicillin ml⁻¹.

thus there is no clear end point, and, in the case of LGV strains (in BGMK cells), released EBs can infect cells to begin a second round of replication in the 36–48 h period. Based on the assumptions that RBs divide by binary fission and that individual RBs give rise to only a single infectious EB at the end of the developmental cycle, the complete *C. trachomatis* developmental cycle involves no more than eight bacterial cell divisions.

We used *C. trachomatis* L2 in conjunction with BGMK cells treated with cycloheximide prior to infection to study the replication of the chlamydial chromosome and plasmid. A key feature of our experimental design was the use of highly purified and carefully quantified EBs. After adsorption, cell cultures were washed to remove EBs that had not initiated an infection. In BGMK cells, characteristic *C. trachomatis* L2 inclusions are easy to visualize by phase-contrast microscopy and inclusions containing RBs only become visible at 18–20 h p.i. Fig. 1(a) shows a typical TEM image of infected

cells at 24 h p.i.; at this time point inclusions contain 8–16 RBs and thus represent the third or fourth generation after infection. By 48 h the developmental cycle is complete, and mature inclusions contain approximately 200 EBs together with RBs and partially differentiated RBs (Fig. 1b).

QPCR of chromosomal and plasmid DNA, performed in quadruplicate, and sampled at six hourly intervals through the developmental cycle up to 48 h, shows a typical sigmoidal curve for DNA replication. We believe that the initial reduction in DNA levels at the first time point after infection (6 h) indicates that a proportion of the EBs taken up by the cell, fail to replicate and are consequently destroyed by lysosomal action and DNase activity. There is then a period of slow DNA replication (6–18 h), corresponding to the ‘eclipse’ period when few viable chlamydia can be isolated (Shaw *et al.*, 2000). During this phase EBs have to enter the host cell, establish an inclusion by converting to RBs and become metabolically active before division can occur. From 18 h p.i. DNA replication follows an exponential phase until 36 h when mature inclusions are forming and replication slows. Plasmid and chromosomal DNA both replicate at the same rate giving a doubling time of 4.6 h during the period of exponential growth (18–36 h); the complete replication profile closely mirrors that described using a LightCycler genomic assay for *C. trachomatis* L2 (Mathews *et al.*, 1999). Although the generation time is longer in our system than in previous estimates of *C. trachomatis* L2 generation times (2.6–3.0 h), those experiments used HEp-2 host cells and were based on the assumption that 16S rRNA transcription is directly proportional to genome replication (Mathews *et al.*, 1999; Wilson *et al.*, 2004).

Penicillin was added to the chlamydial cultures at 20 h p.i. This timepoint was carefully chosen as it represents the start of the most rapid phase of chromosomal and plasmid DNA replication; thus, any effects on DNA levels will be maximized. Two final concentrations of penicillin were used (10 and 100 units ml⁻¹); the effects on the chlamydial developmental cycle were similar at both concentrations. By 4 h after the addition of penicillin the characteristic movement observed in chlamydial inclusions had stopped; this is likely to represent the time taken for penicillin to reach a critical concentration within RBs. Nevertheless, inclusions continued to expand throughout the experiment until by 60 h the inclusions completely filled the host cell cytoplasm (Fig. 1d). TEM examination revealed that the RBs had stopped dividing, thus each inclusion contained only a maximum of 8–16 RBs by 48 h (Fig. 1c). Addition of penicillin had little effect on plasmid replication, but initially retarded the start of chromosomal replication as shown by the 24 h time point. However, by the next time point (30 h) the rate of chromosomal DNA replication had increased and was similar to that of untreated chlamydia. Chromosomal and plasmid DNA then continued to accumulate exponentially up to 60 h p.i. The slight lag in chromosomal replication relative to plasmid replication

gives rise to a rapid change in the plasmid to chromosome ratio and indicates an increase in plasmid copy number of up to fivefold in the presence of penicillin at both 10 and 100 units ml⁻¹. This indicates that the plasmid and chromosome may use independent mechanisms for controlling their replication.

It is clear that β -lactam antibiotics have high affinity for chlamydial PBPs and their effects on the short developmental cycle of LGV strains of *C. trachomatis* are rapid and dramatic (Barbour *et al.*, 1982; Storey & Chopra, 2001). Chlamydial chromosomal and plasmid DNA replication are little affected by the addition of penicillin, yet there is a major morphological effect on cell division. A key factor in bacterial cell division is the formation of a septum, initiated by expression of *ftsZ* (Errington *et al.*, 2003). Unlike most other bacteria the chlamydial genome does not encode an FtsZ equivalent (McCoy & Maurelli, 2006; Stephens *et al.*, 1998). However, in *C. trachomatis* L2, a septation antigen that cannot be immunoblotted and which forms a ring-like structure during RB division has been described (Brown & Rockey, 2000). Apart from its role in conferring structural integrity for bacteria, PG also forms a scaffold during initiation of the septation process (Scheffers & Pinho, 2005). It has been speculated that the primary role of chlamydial PG is in RB division (Chopra *et al.*, 1998) and this is supported by our results. In the absence of an FtsZ-initiated septation mechanism it is possible that PG subsumes the key role of this protein, as removal of penicillin from treated cultures allows rescue of the normal developmental cycle (Matsumoto & Manire, 1970). How septum formation and cell division occurs in this situation will be the subject of future study.

A key element of successful bacterial growth is the need for chromosomal segregation to be linked to the replication cycle by making use of the cell division process (Errington *et al.*, 2003). The addition of penicillin to chlamydia-infected cells uncouples DNA replication from cytokinesis during the exponential phase of RB division, suggesting a central role for PG biosynthesis in this process. Following penicillin exposure, and thus in the absence of septum formation, it might be expected that chromosomal DNA would follow an arithmetic rather than exponential amplification. However, this is not the case and thus the mechanism for the segregation of chlamydial chromosomal DNA is not necessary for its replication. Removal of penicillin (and hence release from inhibition of PG biosynthesis) allows some recovery of infectious EBs (Matsumoto & Manire, 1970), but whether these EBs contain multiple chromosome copies or whether efficient segregation of single chromosomes occurs is not known. A secondary effect, evident from our electron microscopy studies of the later stages of penicillin exposure is the inhibition of chromosomal DNA condensation, which is an important step in RB to EB transition. Discovering the location of PG remains a major goal to understanding the potential role of this structure in chlamydial development.

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