The plasmids of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents

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A 7-5 kbp cryptic plasmid is found in almost all isolates of *Chlamydia trachomatis*. Real-time PCR assays, using TaqMan chemistry, were set up to quantify accurately both the chlamydial plasmid and the single copy, chromosomal *omcB* gene in the infectious, elementary bodies (EBs) of *C. trachomatis* L1 440. Plasmid copy number was also determined in the EBs of six other lymphogranuloma venereum (LGV) isolates (serovars L1–L3), ten trachoma isolates (serovars A–C) and nine urogenital isolates (serovars D–J). The results indicated an average plasmid copy number of 4·0 ± 0·8 (mean ± 95 % confidence interval) plasmids per chromosome. During the chlamydial developmental cycle, up to 7·6 plasmids per chromosome were detected, indicating an increased plasmid copy number in the actively replicating reticulate bodies. Attempts to eliminate the plasmid from strain L1 440 using the plasmid-curing agents ethidium bromide, acridine orange or imipramine/novobiocin led to a paradoxical increase in plasmid copy number. It is speculated that the stress induced by chemical curing agents may stimulate the activity of plasmid-encoded replication (Rep) proteins. In contrast to *C. trachomatis*, only a single isolate of *Chlamydophila pneumoniae* bears a plasmid. *C. pneumoniae* strain N16 supports a 7·4 kbp plasmid in which ORF1, encoding one of the putative Rep proteins, is disrupted by a deletion and split into two smaller ORFs. Similar assay techniques revealed 1·3 ± 0·2 plasmids per chromosome (mean ± 95 % confidence interval) in EBs of this strain. These findings are in agreement with the hypothesis that the ORF1-encoded protein is involved in, but not essential for, plasmid replication and control of copy number.

**INTRODUCTION**

Members of the *Chlamydiaceae* are obligately intracellular, Gram-negative bacteria of significant importance in both human and animal pathogenesis. These organisms exhibit a unique developmental cycle, in which a metabolically active reticulate body (RB) gives rise to a dense, infectious elementary body (EB) (Rockey & Matsumoto, 2000). The family comprises two genera, *Chlamydia* and *Chlamydophila* (Everett et al., 1999), although this classification remains controversial (Schachter et al., 2001).

*Chlamydia pneumoniae* is an important human pathogen implicated in arterial disease (Saikku et al., 1992); infection of the respiratory tract causes an atypical pneumonia (Hahn et al., 2002). Although predominantly a human pathogen, *C. pneumoniae* strains have been isolated from animal species, e.g. equines (Everett et al., 1999; Storey et al., 1993; Wills et al., 1990) and koala (Wardrop et al., 1999).

*Chlamydia trachomatis* is not only the major infectious agent of preventable blindness in the developing world (Thylefors et al., 1995), but also the commonest cause of non-specific urethritis in developed countries (Burstein & Zenilman, 1999). Sexually transmitted disease caused by *C. trachomatis* is frequently undiagnosed in women, whereupon an ascending infection may lead to pelvic inflammatory disease, salpingitis and consequent infertility (Paavonen, 1998).

Extrachromosomal elements have been detected in several chlamydial species: both a cryptic plasmid (Lovett et al., 1980; Thomas et al., 1997) and a chlamydiphage of the microvirus family (Everson et al., 2003; Richmond et al., 1982) have been described. Almost all strains of *C. trachomatis* harbour the plasmid, but some plasmid-free isolates...
have been described (An et al., 1992; Farencena et al., 1997; Matsumoto et al., 1998; Peterson et al., 1990; Stothard et al., 1998, 1999). There is a marked conservation (<1% variation) of plasmid DNA sequences within this species (Black et al., 1989; Comanducci et al., 1988, 1990; Hatt et al., 1988; Sriprakash & MacAvoy, 1987b; Thomas & Clarke, 1992). The copy number of the plasmid has not been determined accurately, but isotopic methods suggest the presence of between seven and ten plasmid copies per bacterial chromosome (Palmer & Falkow, 1986; Tam et al., 1992).

Eight major ORFs (>100 bases) have been assigned to the chlamydial plasmid (Black et al., 1989; Comanducci et al., 1988, 1990; Hatt et al., 1988; Sriprakash & MacAvoy, 1987b; Thomas & Clarke, 1992). The four 22-bp tandem repeats located in the intergenic region between ORF8 and ORF1, together with an upstream AT-rich region, are indicative of an origin of replication (Chattoraj, 2000). This location of the origin has been confirmed by electron microscopy (Tam et al., 1992). The position of ORF1 and ORF2 immediately downstream of the iteron-like, tandem repeats, together with the size and net positive charge of their hypothetical products (which share 32–35% amino acid identity), indicates that they may function as replication proteins (Thomas et al., 1997). In other systems, plasmid copy number is controlled by the cross-linking, or ‘hand-cuffing’, of iteron regions by such plasmid-encoded replication proteins (Park et al., 2001).

Investigation of the molecular biology of the Chlamydiaceae has been hampered by the lack of a gene transfer system. Consequently, there is no method available to introduce foreign DNA stably into these organisms. The chlamydiophage and plasmid are obvious candidates as vectors in such a system. Previous attempts to transform chlamydiae with a recombinant plasmid containing the chloramphenicol acetyltransferase gene inserted into ORF1 yielded only transient expression (Tam et al., 1994). Such a shuttle vector system must comprise a replication-competent vector with a selectable marker into which foreign DNA sequences may be inserted. The limited understanding of the function of plasmid ORFs and the lack of intergenic regions may restrict the placement of foreign DNA sequences. In addition, a gene transfer system must overcome potential restriction barriers and incorporate a host able to support replication of the vector. As plasmid-free strains of chlamydiae may not be able to support plasmid replication, the most suitable host would be a plasmid-containing strain cured of its plasmid.

In this work, quantitative PCR (QPCR) assays were used to determine accurately chlamydial plasmid copy number in both C. trachomatis and C. pneumoniae. Changes in plasmid copy number during the developmental cycle were also investigated. Finally, attempts were made to eliminate the chlamydial plasmid from the organism by using chemical curing agents.

**METHODS**

**Culture of chlamydiae and EB purification.** Strains used in this study are listed in Table 1. Chlamydiae were grown as previously described (Garner et al., 2004). Host buffalo green monkey kidney (BGMK) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal calf serum (FCS; 10% v/v). Cells were infected with chlamydial EBs in medium containing cycloheximide (1 µg ml⁻¹) and gentamicin (25 µg ml⁻¹). All strains except those belonging to the C. trachomatis lymphogranuloma venereum (LGV) biavar required centrifugation onto host cells for efficient infection. Infected cells were detached from the flask with PBS containing trypsin/EDTA and pelleted by centrifugation in DMEM/FCS. Intracellular chlamydiae were released using a Dounce homogenizer and EBs were purified by centrifugation through Urogafin 370 (32% v/v) or Triosil 440 (25% w/v; Nycomed).

**Use of plasmid-curing agents.** BGMK cells were cultured in the presence of increasing amounts of the plasmid-curing agents ethidium bromide (EtBr), acridine orange (AcOr), imipramine or novobiocin to determine their minimum cytotoxic concentrations (MCCs), assessed by cell rounding and detachment from the flask. In subsequent experiments, each agent was used at levels below its MCC. Initially, the agents were used at concentrations known to exhibit antiplasmid effects in free-living bacteria (Courtright et al., 1988; Moirnar & Foldek, 1989; Trens, 1986); these were 25 µg EtBr ml⁻¹, 2-5 µg AcOr ml⁻¹ or a mixture of 20 µg novobiocin and 20 µg imipramine ml⁻¹. Subsequently, a range of EtBr concentrations up to 80% of the MCC was used. C. trachomatis LI 440 was grown in BGMK cells for seven passages with plasmid-curing agent and infected cells were then harvested at 48 h post-infection (p.i.) for PCR analysis.

**Extraction of DNA for QPCR analysis.** Lysis of infected cells and/or chlamydiae was achieved by heating in the presence of ammonia using a modified method of Lanham et al. (2001). Infected cells or purified EBs were centrifuged at 12 000 g for 10 min. The supernatant was discarded and the pellet resuspended in PBS. The sample was centrifuged under the same conditions, the supernatant discarded and the pellet resuspended in 135 µl ammonia solution (2 M). Each sample was heated at 96°C in an open microfuge tube or microplate well until dryness (approx. 50 min). The residue was then resuspended in 50 µl high-quality water. Samples were diluted 1:1000 prior to QPCR analysis.

**Preparation of DNA standards.** Laboratory strains of Escherichia coli harbouring the recombinant plasmids listed in Table 2 were grown in liquid culture at 37°C to late exponential phase in the presence of ampicillin (25 µg ml⁻¹). Plasmids were prepared by alkaline lysis using a Plasmid Midi kit (Qiagen) according to the manufacturer’s instructions. Estimates of plasmid purity were obtained by agarose gel electrophoresis and measurement of A260/ A280 ratios. Plasmid quantification was carried out by the measurement of A260, A280 and also using a fluorometric PicoGreen (Molecular Probes) assay in an SLT Fluostar (BMG Labtech), with a pBR322 (Promega) standard. To minimize errors during QPCR analysis, the C. trachomatis standard DNAs (pCT112A and pSR1A) were mixed prior to serial dilution in high-quality water containing pBR322 (100 µg ml⁻¹); the latter was used to block absorption of standard DNA to the polypropylene tubes. The C. pneumoniae standards (pH17 and pMWU1A) were co-diluted by the same method. Standard dilutions used in the QPCR ranged between 10⁶ and 10⁷ DNA molecules per reaction.

**Real-time QPCR.** The absolute number of chlamydial plasmids and omR genes in each sample was determined by performing 5'-exonuclease (TaqMan) assays using unlabelled primers and carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) probes.
dual-labelled probes. Due to the lack of sequence similarity between the genera, separate primer and probe sets were designed for *C. trachomatis* and *C. pneumoniae* (Table 3). For the *C. trachomatis* assay, primers and probes were selected from DNA sequence regions conserved between the serovars. A region within ORF2 of the chlamydial plasmid displays appropriate sequence conservation. A single copy of the *omcB* gene is located on the chlamydial chromosome, hence the ratio of chlamydial plasmid : *omcB* is equivalent to the number of plasmid copies per bacterium. Five microlitres of sample was added to 20 μl reaction mixture 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. The threshold cycle (Ct) for each sample is the PCR cycle number at which the threshold level of ΔRn is achieved. For each sample, the Ct approximates to the cycle at which fluorescence change is first detectable. Standard curves of Ct against the logarithm of the number of DNA molecules per reaction were plotted. The Ct value obtained for an unknown may then be used to interpolate the number of DNA molecules present.

### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serovar</th>
<th>Source and/or reference</th>
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<tbody>
<tr>
<td>115</td>
<td>L1</td>
<td>Hayes et al. (1994)</td>
</tr>
<tr>
<td>224</td>
<td>L1</td>
<td>Hayes et al. (1994)</td>
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<tr>
<td>2098</td>
<td>L1</td>
<td>J. D. Treharne, Institute of Ophthalmology, London, UK</td>
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<tr>
<td>12790</td>
<td>L1</td>
<td>J. D. Treharne, Institute of Ophthalmology, London, UK</td>
</tr>
<tr>
<td>440</td>
<td>L1</td>
<td>ATCC VR-901B; Schachter &amp; Meyer (1969)</td>
</tr>
<tr>
<td>434</td>
<td>L2</td>
<td>ATCC VR-902B; Schachter &amp; Meyer (1969)</td>
</tr>
<tr>
<td>404</td>
<td>L3</td>
<td>ATCC VR-903; Schachter &amp; Meyer (1969)</td>
</tr>
<tr>
<td>SA-1</td>
<td>A</td>
<td>Hayes et al. (1990)</td>
</tr>
<tr>
<td>Jali 5</td>
<td>B</td>
<td>Bailey et al. (1994)</td>
</tr>
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<td>B</td>
<td>Hayes et al. (1992)</td>
</tr>
<tr>
<td>Jali 13</td>
<td>B</td>
<td>Hayes et al. (1992)</td>
</tr>
<tr>
<td>Jali 16</td>
<td>B</td>
<td>Hayes et al. (1992)</td>
</tr>
<tr>
<td>Jali 20</td>
<td>B</td>
<td>Watson et al. (1989)</td>
</tr>
<tr>
<td>Jali 33</td>
<td>B</td>
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<tr>
<td>TW-5</td>
<td>B</td>
<td>Wang &amp; Grayston (1963)</td>
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<td>Bruce C</td>
<td>C</td>
<td>A. B. MacDonald, University of Massachusetts, USA</td>
</tr>
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<td>UW-1</td>
<td>C</td>
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<tr>
<td>IOL-1883</td>
<td>D</td>
<td>Watson et al. (1991)</td>
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<td>D</td>
<td>N. Lycke, University of Gothenburg, Sweden</td>
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<td>Clare</td>
<td>E</td>
<td>M. Tuffrey, MRC Clinical Research Centre, Harrow, UK</td>
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<tr>
<td>DK-20</td>
<td>E</td>
<td>J. D. Treharne, Institute of Ophthalmology, London, UK</td>
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<td>NI-1</td>
<td>E</td>
<td>Tuffrey et al. (1986)</td>
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<tr>
<td>93 M 128 – Bruce F</td>
<td>F</td>
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<tr>
<td>IOL-238</td>
<td>G</td>
<td>Watson et al. (1991)</td>
</tr>
<tr>
<td>UW-4</td>
<td>H</td>
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</tr>
<tr>
<td>UW-36</td>
<td>J</td>
<td>ATCC VR-886; Wang &amp; Grayston (1975)</td>
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</table>

*Chlamyphila pneumoniae*

| N16    | –       | Storey et al. (1993)    |

### Table 2. Recombinant plasmid standards

<table>
<thead>
<tr>
<th>Recombinant plasmid</th>
<th>Insert</th>
<th>Vector</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>pCTL12A</td>
<td><em>C. trachomatis</em> L1 440 plasmid (pLGV440)</td>
<td>pUC18</td>
<td>Hatt et al. (1988)</td>
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<tr>
<td>pSRP1A</td>
<td><em>C. trachomatis</em> L1 440 2-2 kb PstI fragment encoding N terminus of <em>omcB</em></td>
<td>pUC9</td>
<td>Clarke &amp; Lambden (1988)</td>
</tr>
<tr>
<td>pH17</td>
<td><em>C. pneumoniae</em> N16 plasmid (pCpnE1)</td>
<td>pUC8</td>
<td>Thomas et al. (1997)</td>
</tr>
<tr>
<td>pMWPU1A</td>
<td><em>C. pneumoniae</em> IOL-207 8-5 kb NheI fragment encoding <em>omcB</em></td>
<td>pBluescript SK (−)</td>
<td>Watson et al. (1990)</td>
</tr>
</tbody>
</table>
QPCR analyses carried out for the plasmid and *C. trachomatis*. Determination of real-time PCR (data not shown). Linearization did not affect the efficiency of detection by the ammonia/heat treatment used for cell lysis. Both circular and linearized plasmid standards were subjected to the reaction. The plasmid copy number per EB was calculated as $4.0 \pm 0.8$ (mean $\pm 95\%$ confidence interval).

### RESULTS

#### Real-time PCR assay for *C. trachomatis*

Assays were set up using recombinant plasmids as DNA standards (Fig. 1). The specificity of oligonucleotide primers and probes for *C. trachomatis* was confirmed by performing real-time PCRs using total DNA derived from cultured human cells and *C. pneumoniae*. Results were negative in both cases (data not shown). Amplification plots (Fig. 1a, c) show typical sigmoid shape, with the fluorescence change becoming detectable during the exponential phase of the reaction, followed by saturation due to depletion of reactants. As expected, an increase in the starting amount of DNA allows an earlier detectable fluorescence change. The total change in fluorescence throughout the reactions is greater for the plasmid (approx. 1·0 relative unit) than for *omcB* (approx. 0·6 relative units). The standard curves of $C_t$ against log (DNA molecules per reaction) are linear, with correlation coefficients $r^2$=0·91 for both plasmid and *omcB* assays. The slope of the standard curve is proportional to the efficiency of the PCR and indicates an efficiency of 0·84 for the plasmid assay and 0·81 for *omcB*, where an efficiency of 1·0 would represent a doubling of total DNA amount every cycle. Both assays consistently detected as few as ten molecules of target DNA, but replicates were unreliable when there were fewer than ten target molecules present in the reaction.

Both circular and linearized plasmid standards were subjected to the ammonia/heat treatment used for cell lysis. Linearization did not affect the efficiency of detection by real-time PCR (data not shown).

#### Determination of *C. trachomatis* plasmid copy number

Purified *C. trachomatis* EBs were lysed as described and QPCR analyses carried out for the plasmid and *omcB* gene.

### Table 3. Real-time PCR primer and probe sequences

<table>
<thead>
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<th>Species</th>
<th>Oligonucleotide</th>
<th>Name</th>
<th>Sequence (5′–3′)</th>
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<tbody>
<tr>
<td><strong>Chlamydial plasmid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. trachomatis</em></td>
<td>Forward primer</td>
<td>Ctrachplas1F</td>
<td>cagcttgtagctctgcttgtaaga</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>Ctrachplas19R</td>
<td>caagatactacgtaacagaga</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>Ctrachplas57T</td>
<td>FAM-ccccacattttccggagga-TAMRA</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td>Forward primer</td>
<td>N16P5080F</td>
<td>acagtgccctggcgttagt</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>N16P5226R</td>
<td>tgcctcaagaagataacggag</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>N16P5117T</td>
<td>FAM-tccttgcttttattttgatactcctggat-TAMRA</td>
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<tr>
<td><strong>omcB</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>C. trachomatis</em></td>
<td>Forward primer</td>
<td>Ctrach60k127F</td>
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<td></td>
<td>Reverse primer</td>
<td>Ctrach60k232R</td>
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<td>Probe</td>
<td>Cpneum60k907P</td>
<td>FAM-agaccttgcttagaaataaaccagttgaacaa-TAMRA</td>
</tr>
</tbody>
</table>

#### Plasmid copy number variation during the developmental cycle

BGMK cells infected with *C. trachomatis* L1 440 were removed for ammonia lysis and QPCR analysis at 0, 16, 24, 41 and 48 h p.i. The level of *omcB* detected in each sample (Fig. 2a) reflects the amount of chlamydial genome (and hence chlamydiae) in each aliquot. An increase in *omcB* is first detected at 24 h p.i. Copies of chlamydial genome then increase exponentially – a doubling time of approximately 3 h is indicated. Lysis of host cells was observed approximately 40 h p.i. Fig. 2(b) indicates that, during the infectious cycle, there is an initial increase in plasmid copy number, which reaches a maximum at 16 h p.i. Thereafter, a decrease in plasmid copy number is observed.

#### Plasmid copy number in other *C. trachomatis* serovars

The plasmid copy number in 25 other strains from 12 serovars of *C. trachomatis* was determined by QPCR. All strains had a similar plasmid content to L1 440; copy number variation was statistically not significant (ANOVA).

#### Effect of plasmid-curing agents on BGMK cells and *C. trachomatis*

The MCCs of the curing agents for BGMK cells were 250 μg ml$^{-1}$ (EtBr), 25 μg ml$^{-1}$ (AcOr), 25 μg ml$^{-1}$ (novobiocin) and 25 μg ml$^{-1}$ (imipramine). When an imipramine/novobiocin mixture was used for chlamydial plasmid curing, these agents were present in the cell culture medium at all times. However, if EtBr or AcOr was present at the time of infection, no chlamydial inclusions were produced, suggesting chlamydicidal properties associated with these two agents. Therefore, in subsequent
experiments, EtBr or AcOr was added 16 h p.i. When C. trachomatis was cultured in the presence of AcOr or EtBr alone, or a mixture of novobiocin and imipramine, the ratio of plasmid : omcB did not decrease (Fig. 3a). This indicated that these agents did not have a curative effect on the chlamydial plasmid. Paradoxically, the ratio of plasmid : omcB increased. To investigate this effect further, we used EtBr at increasing concentrations and again determined the plasmid : omcB ratio. The results (Fig. 3b) indicate that, as the concentration of EtBr increases, the plasmid copy number rises. TaqMan assay sensitivity was not affected by the addition of EtBr (200 ng ml⁻¹) or AcOr (20 ng ml⁻¹) to the reaction (data not shown).

Real-time PCR assay for C. pneumoniae

Due to the lack of sequence similarity between the C. trachomatis and C. pneumoniae plasmids, discrete primers and probes were designed for the QPCR assay of plasmid and omcB of the latter organism. Recombinant plasmids containing the C. pneumoniae N16 plasmid or omcB gene were used as assay standards. Serial dilutions of these plasmids were used as templates to produce amplification plots and standard curves as described above (results not shown). The efficiency of the C. pneumoniae PCR, calculated from the slope of the standard curves, was 0·90 for the plasmid and 0·92 for omcB. The sensitivity of the two assays differs slightly. The plasmid assay reproducibly detects as few as ten DNA molecules, a similar sensitivity to the C. trachomatis assays. However, the threshold sensitivity for the omcB assay is between ten and 100 DNA molecules, with only the latter being detected reproducibly.

Determination of C. pneumoniae plasmid copy number

Purified EBs of C. pneumoniae N16 EBs were lysed and quantitative plasmid and omcB PCRs were performed. The ratio of plasmid to omcB is equivalent to the plasmid copy number and was calculated as 1·3 ± 0·2 plasmids per EB (mean ± 95% confidence interval).
DISCUSSION

We describe the use of real-time QPCR assays to determine plasmid copy number accurately in both genera of the Chlamydiaceae. Of the chemistries available (Wittwer et al., 1997), the 5′-exonuclease (TaqMan) assay was selected. Unlike SYBR Green I chemistry, TaqMan utilizes a complementary oligonucleotide probe internal to the PCR primers, affording the assay an additional level of specificity. This was considered important when assaying total DNA extracted from infected cells rather than from purified EBs. Separate oligonucleotide primer and probe sets were selected for C. trachomatis and C. pneumoniae, as neither their plasmids (Thomas et al., 1997) nor their omcB genes (Watson et al., 1991) display sufficient DNA sequence similarity to enable the design of pan-Chlamydiaceae assays. Plasmid and omcB assays were performed in separate tubes, as multiplexing requires an optimization process to limit primer concentrations (Livak, 2001). Such limitation reduces assay sensitivity. Absolute quantification using standard curves was chosen for the following reasons: (i) only this method permits the determination and comparison of analytical sensitivities, (ii) recombinant plasmids were available that could be used as assay standards and (iii) the assay could be used in future infectious dose and/or diagnostic studies. The source of standard DNA for QPCR may be the whole organism (Apfalter et al., 2003; van Doornum et al., 2003) or recombinant plasmid. The use of both closed, circular (Coker et al., 2003; Weidmann et al., 2003) and linearized (Pusterla et al., 1999; Weidmann et al., 2003) plasmid standards has been described in the literature. There may be different degrees of supercoiling in the chlamydial plasmid, chlamydial genome and plasmid standards, and the effect of this on PCR efficiency was considered. Although linearization of a plasmid has been found to affect significantly its functioning as a PCR target of analytical sensitivities, (ii) recombinant plasmids were available that could be used as assay standards and (iii) the assay could be used in future infectious dose and/or diagnostic studies. The source of standard DNA for QPCR may be the whole organism (Apfalter et al., 2003; van Doornum et al., 2003) or recombinant plasmid. The use of both closed, circular (Coker et al., 2003; Weidmann et al., 2003) and linearized (Pusterla et al., 1999; Weidmann et al., 2003) plasmid standards has been described in the literature. There may be different degrees of supercoiling in the chlamydial plasmid, chlamydial genome and plasmid standards, and the effect of this on PCR efficiency was considered. Although linearization of a plasmid has been found to affect significantly its functioning as a PCR target of analytical sensitivities, (ii) recombinant plasmids were available that could be used as assay standards and (iii) the assay could be used in future infectious dose and/or diagnostic studies. The source of standard DNA for QPCR may be the whole organism (Apfalter et al., 2003; van Doornum et al., 2003) or recombinant plasmid. The use of both closed, circular (Coker et al., 2003; Weidmann et al., 2003) and linearized (Pusterla et al., 1999; Weidmann et al., 2003) plasmid standards has been described in the literature. There may be different degrees of supercoiling in the chlamydial plasmid, chlamydial genome and plasmid standards, and the effect of this on PCR efficiency was considered. Although linearization of a plasmid has been found to affect significantly its functioning as a PCR target of analytical sensitivities, (ii) recombinant plasmids were available that could be used as assay standards and (iii) the assay could be used in future infectious dose and/or diagnostic studies. The source of standard DNA for QPCR may be the whole organism (Apfalter et al., 2003; van Doornum et al., 2003) or recombinant plasmid. The use of both closed, circular (Coker et al., 2003; Weidmann et al., 2003) and linearized (Pusterla et al., 1999; Weidmann et al., 2003) plasmid standards has been described in the literature. There may be different degrees of supercoiling in the chlamydial plasmid, chlamydial genome and plasmid standards, and the effect of this on PCR efficiency was considered. Although linearization of a plasmid has been found to affect significantly its functioning as a PCR target.
(Pogożelski et al., 2003), Tasker et al. (2003) reported that chromosomal and plasmid templates were amplified by PCR with similar efficiencies. This result was confirmed in the present study by submitting identical amounts of (i) supercoiled, closed circular and (ii) linearized, standard plasmid template for TaqMan PCR assay. Presumably, any topological constraints (due to supercoiling) affecting polymerase access to its template would be significant only in the first few PCR cycles; subsequently, linear product would represent the major template species. Efficiencies of the four TaqMan assays described in this work compare favourably with those reported for other real-time PCR assays (Tichopad et al., 2003). Analytical sensitivities of 10–100 molecules were observed; these are similar to those observed in other TaqMan assays (Apfalter et al., 2003; Weidmann et al., 2003). Poor reproducibility below ten molecules per reaction is probably due to sampling errors, as previously predicted for low concentrations of PCR target (Coupland, 1994).

Bacterial DNA was released from infected cells and chlamydiae by heating in the presence of ammonia, followed by the removal of alkali by evaporation for a fixed length of time (Lanham et al., 2001). Using the procedure as described produced PCR results with unacceptable replicate variance (results not shown). Residual ammonia was detected in some of the extracted DNA, and it was postulated that this was affecting the QPCR to varying extents. Residual ammonia was described produced PCR results with unacceptable replicate variance (results not shown). Residual ammonia was detected in some of the extracted DNA, and it was postulated that this was affecting the QPCR to varying extents. Although the spectral properties of the TaqMan probe fluor, FAM and TAMRA, should be unaffected by pH values from 7 to 10, the efficiency of the PCR will be altered (Payne et al., 1995). Therefore, in this study, the procedure of Lanham et al. (2001) was modified by heating the samples to dryness to ensure the complete removal of ammonia.

The QPCR methodology described above was used to determine the plasmid copy number in EBs of C. trachomatis L1 440. The result was $4.0 \pm 0.8$ plasmid copies per chromosome in the EB. Previously, C. trachomatis LGV2 EBs have been reported to contain 10 plasmid copies per chromosome, by a method using the incorporation of $^3H$-adenosine and liquid scintillation counting (Palmer & Falkow, 1986). This higher figure may be due to the preferential loss of chromosomal DNA during harvesting after its separation from plasmid DNA by CsCl gradient centrifugation or agarose gel electrophoresis. In addition, supercoiled plasmid DNA may be less prone to degradation by contaminating nuclease during the separation of plasmid and chromosome. The QPCR procedure does not require separation of these DNA species. There is less than 1% nucleotide sequence variation in the plasmids of human isolates of C. trachomatis (Black et al., 1989; Comanducci et al., 1988; Hatt et al., 1988; Sriprakash & MacAvoy, 1987b; Thomas & Clarke, 1992) — a significant factor in the laboratory diagnosis of C. trachomatis infections. Diagnostic methods targeting a multicopy plasmid display an increase in sensitivity compared with those that target a single copy gene. Kits that target the chlamydial plasmid are commercially available and use either PCR (Amplicor CT/NG; Roche Diagnostics) or strand displacement amplification (ProbeTec ET; Becton Dickinson). Significant plasmid DNA sequence similarity also suggests that the same plasmid replication and partition control mechanisms are in operation in different strains of C. trachomatis. The analysis of 26 strains of this species indicated no significant differences in plasmid copy number.

Bacterial plasmid copy number has been demonstrated to vary with both growth rate (Kapralek et al., 1998; Lin-Chao & Bremer, 1986; Sayadi et al., 1989) and growth phase (Coker et al., 2003). The plasmid copy number in chlamydial RBs has been previously estimated as between 7 and 10 copies per chromosome (Tam et al., 1992), using filter lysis and radioactive probes to plasmid and chromosome as described previously (Shields et al., 1986). However, the method used was insufficiently sensitive to detect small changes in copy number during the developmental cycle. QPCR revealed that there is an increase in plasmid to chromosome ratio during the first 15 h of the developmental cycle. At 15 h p.i., light microscopy indicated that all infecting EBs had differentiated to RBs within inclusions. Thereafter, when RBs are observed to differentiate into EBs, the ratio of plasmid to chromosome decreases. These results suggest that the plasmid copy number in chlamydial RBs is almost twice that found in EBs.

The plasmid copy number determined for C. trachomatis indicates that the plasmid is a low-copy-number replicon and that its replication is tightly controlled. As plasmid-free isolates of C. trachomatis are rare, low plasmid copy number also suggests the presence of an efficient partitioning system. Interestingly, plasmid ORF7 and ORF8 share several features with SopA/B and ParA/B operons (Riccio et al., 1995; Thomas et al., 1997). DNA sequence analysis has shown that iteron binding and/or inhibitor targeting mechanisms may operate to control chlamydial plasmid copy number (Thomas et al., 1997). There is significant similarity between ORF1 and ORF2 of the C. trachomatis plasmid, and it was suggested that both ORFs encode products that function as replication proteins. Control by twin replication proteins has been described in other plasmid systems (Basu et al., 2002). Although there is only 60% nucleotide sequence identity between the C. pneumoniae and C. trachomatis plasmids, their genomic organization is remarkably similar (Thomas et al., 1997). It is interesting to note that the C. pneumoniae N16 plasmid ORF1 contains a 150 bp deletion and is split into two separate ORFs, 1A and 1B. Therefore, the ORF2 product may be the sole replication protein in the C. pneumoniae plasmid. As it is possible that plasmid replication control might be damaged in C. pneumoniae N16, we also set up assays to determine plasmid copy number in this strain. Results showed that the C. pneumoniae N16 plasmid copy number is significantly lower than that of C. trachomatis. It is possible that the ORF1 product is not essential for...
plasmid replication, but stimulates the ORF2 product in its origin-binding activity. Such a mechanism has been postulated for the mycobacterial plasmid pAL5000 (Basu et al., 2002). This is an encouraging observation for vector development as it suggests that ORF1 is not essential for plasmid maintenance.

All human C. pneumoniae isolates (Campbell et al., 1987; Lusher et al., 1989) and several isolates of Chlamydocphila psittaci (Lusher et al., 1991) lack the cryptic plasmid. Although it has been suggested that the C. trachomatis plasmid encodes an essential protein (Sriraksh & MacAvoy, 1987a, b), plasmid-free isolates have been reported. These data suggest that the cryptic plasmid is not essential for chlamydial viability and prompted our investigation into the effect of chemical curing agents on C. trachomatis. The intercalating agents EtBr (Crameri et al., 1986) and AcOr (Mesas et al., 2004) have been used successfully as plasmid-curing agents in other organisms. The DNA gyrase inhibitor novobiocin (McHugh & Swartz, 1977) and the antidepressant tricyclic drug imipramine (Molnar et al., 1978) have also been used for this purpose, both alone and in combination (Molnar & Foldesak, 1989). However, the successful curing of plasmids in obligately intracellular organisms has not been reported, and no reduction in plasmid copy number was observed when these agents were added to chlamydial cell culture medium. Even in free-living bacteria, the effective concentration of curing agent varies during the first PCR cycle) may occur. This is unlikely for the following reasons. (i) The concentrations of EtBr and AcOr present in the PCRs in this work are no greater than 200 ng ml$^{-1}$ and 20 ng ml$^{-1}$, respectively, and did not affect the TaqMan assays. Nath et al. (2000) found that 10 µg EtBr ml$^{-1}$ was required to inhibit the reaction. (ii) The DNA gyrase inhibitor novobiocin does not intercalate and, furthermore, its induction of positive supercoiling inhibits duplex melting (Sioud et al., 1988) and would be expected to decrease the chlamydial plasmid’s availability as a template. (iii) The non-planar molecule imipramine also does not intercalate and has not been reported to inhibit PCR or affect the supercoiling of plasmids (Molnar et al., 1978).

The paradoxical increase in plasmid copy number may be explained by the induction of chlamydial stress responses. In other bacteria, both the SOS response and the heat-shock response are induced by nutrient starvation (Janion et al., 2002; Zhang & Griffiths, 2003). As homologous genes for both these regulons have been identified in the chlamydial genome (Stephens et al., 1998), and chlamydiae are auxotrophic for most nucleotides (Tipples & McClarty, 1993), it is feasible that the detrimental effect of curing agent on host-cell metabolism induces stress responses. The chlamydial recA gene homologue encodes an active protein (Hintz et al., 1995; Zhang et al., 1995), which may regulate an SOS response within the organism. It is known that the SOS response affects plasmid copy number in other systems (Bertrand-Burggraf et al., 1989); indeed, in E. coli, it has been implicated as an additional control mechanism of plasmid copy number (Ingmer et al., 2001). In chlamydiae, heat-shock protein expression is regulated by the HrcA repressor/CIRCE operator (Wilson & Tan, 2004). Chlamydial heat-shock proteins may induce plasmid replication by promoting the binding of Rep protein to the plasmid origin, as described for plasmid P1 in E. coli (Sozhamannan & Chattoraj, 1993).

The assays described in this work have allowed the true plasmid copy number, i.e. plasmids per chromosome, to be determined accurately in chlamydiae. They may be used to investigate further the molecular biology of the plasmid in strains of C. trachomatis and C. pneumoniae grown in mutant host-cell lines deficient in, for example, specific enzymes of nucleotide or energy metabolism. The ability to detect small quantities of plasmid will aid future chlamydial transformation and shuttle vector studies. The high sensitivity of the method described will allow the estimation of plasmid copy number directly in clinical specimens, without cell culture of the organism. Plasmid-free isolates will be readily detected, and plasmid copy number correlated with bacterial virulence and other pathogenic traits.

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**REFERENCES**


