INTRODUCTION

Chlamydiae are obligate intracellular bacterial parasites of eukaryotic cells that cause a broad spectrum of clinically distinct diseases in animals and humans. A highly conserved plasmid of approximately 7.5 kb is present in almost all strains of *Chlamydia trachomatis*, with copy numbers estimated to range from 4 (Pickett et al., 2005) to 10 (Tam et al., 1992) copies per cell. The role of this plasmid is unknown. However, all eight predicted ORFs are expressed at the RNA level (Fahr et al., 1992; Pearce et al., 1991; Belland et al., 2003) and at least one of the plasmid-encoded proteins is expressed during infection (Comanducci et al., 1993). Naturally occurring plasmid-deficient clinical isolates are extremely rare; only three strains have been described (Peterson et al., 1990; Farenccna et al., 1997; Stothard et al., 1998). In contrast, Matsumoto et al. (1998) described the isolation and characterization of three plasmid-deficient *C. trachomatis* strains that occur naturally in tissue-culture-propagated cultures at an estimated rate of 1%. The derivatives identified by these authors appeared unable to accumulate glycogen within the intracytoplasmic inclusions that are formed by the bacteria as they grow. No other phenotypic changes were detected that could be attributed to the presence of the plasmid (Miyashita et al., 2000).

Currently, a genetic system for use in chlamydiae is lacking. A better understanding of the mechanisms by which the cryptic plasmid, a potential gene delivery vector, is maintained, and the ability to generate at high efficiency plasmid-deficient derivatives of the most highly characterized chlamydiae for use as recipient strains would advance progress in this area. We describe the curing of the mouse-virulent *Chlamydia muridarum* using novobiocin. Characterization of the cured strain revealed, in addition to a defect in the ability to accumulate glycogen, an in vitro attachment or uptake defect that could be partially overcome by centrifugation.

METHODS

Strains, cell lines and culture conditions. The chlamydial strains used in the course of this study are shown in Table 1. Cell culture media and reagents were purchased from Mediatech. The chlamydiae were routinely cultured in multiwell dishes (24- or 6-well) containing confluent monolayers of McCoy cells that were infected at an approximate m.o.i. of 0.5–1, unless otherwise stated, before being centrifuged at 1600 g for 1 h at 37 °C. The infective inoculum was then removed and replaced with 1 x Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 μg gentamicin ml⁻¹ and 0.1 μg cycloheximide ml⁻¹. Where indicated, novobiocin (Sigma) was added at the concentrations indicated (up to 250 μg ml⁻¹) at 4 h post-infection. Concentrations of novobiocin below 1000 μg ml⁻¹ are not cytotoxic for cells in culture (Hussy et al., 1986) and this was qualitatively confirmed by evaluating the ability of uninfected cells to exclude Trypan blue dye 48 h after treatment with the drug. Infected cells were then incubated at 37 °C, 5% CO₂ for 36 h before being harvested into SPG buffer (218 mM sucrose, 3-8 mM KH₂PO₄, 7-2 mM K₂HPO₄, 4-9 mM L-glutamate, pH 7-2), sonicated and stored at −80 °C. Bacteria were subsequently titrated by plaque assay or as inclusion-forming units (i.f.u.). Briefly, i.f.u. estimates were performed by infecting confluent McCoy cell monolayers growing in 96-well dishes with 10-fold dilutions of the chlamydial inoculum, centrifuging at 1600 g for 1 h at 37 °C before replacing the cell culture medium as described above. After approximately 30 h incubation at 37 °C, 5% CO₂, the cells were fixed with absolute methanol, then stained using a genus-specific fluorescently tagged monoclonal antibody (Bio-Rad) before inclusions were counted (Kelly et al., 1996).

Plaque-forming assay. Dilutions of the freshly harvested, novobiocin-treated bacteria were inoculated by centrifugation at 1600 g.
for 1 h at 37 °C onto confluent monolayers of McCoy cells grown in 6-well tissue culture dishes. The infective inoculum was then removed and the monolayers were overlaid with 1 × DMEM, 10% FBS, 0.25% agarose, 0.01 µg cycloheximide ml⁻¹, and incubated at 37 °C, 5% CO₂ for 5–7 days, to allow plaques to form. To visualize plaques, the solid overlay was removed and the monolayer stained with 0.025% neutral red in 1× PBS (130 mM NaCl, 0.8 mM Na₂HPO₄, 0.2 mM KH₂PO₄, pH 7.4). Plaque purifications were carried out by picking individual plaques into 200 µl SPG buffer. The resulting plaque suspensions were then sonicated to lyse the cells and appropriate dilutions replated as described above. Expansion of plaques arising after novobiocin treatment or during sequential plaque purification was achieved by the picking of individual plaques as described, then using this material to infect, via centrifugation, individual wells of a 24-well dish containing confluent monolayers of McCoy cells for a single passage (~40 h) before harvesting in SPG buffer. An additional passage was then performed in an identical manner using ~1/10th of the well harvest as inoculum. Two sequential rounds of infection usually resulted in stocks of ~1–2 × 10⁹ I.f.u. ml⁻¹ that were then used for further analysis. Plaque efficiency under different culture conditions was evaluated by the titration of individual strains by a plaque-forming assay, with indicated modifications, in parallel with titration to estimate I.f.u. (Kelly et al., 1996). The efficiency of plating (e.o.p.) is calculated as p.f.u. ml⁻¹/1.f.u. ml⁻¹.

Iodine staining of chlamydia-infected cells. McCoy cells cultured on glass cover slips in multiwell plates were infected as described above. After 40 h incubation, the culture medium was removed and the monolayer rinsed several times with 1× Hanks’ balanced salt solution (HBSS) before being fixed and stained with iodine (Schachter & Dawson, 1978) in order to detect the presence of accumulated glycopen. Stained cells were immediately photographed using a digital camera system and SPOT version 4.0.6 software from Diagnostic Instruments.

Detection of chlamydial plasmid or chromosome by PCR and Southern blotting. Genomic material was routinely prepared for use as PCR template by boiling chlamydial suspensions containing 10⁷ I.f.u. ml⁻¹ for more for 10 min, before diluting 10-fold in distilled H₂O. PCR amplifications were performed using 5 µl of this preparation in a 20 µl reaction volume. Template preparations were amplified as follows: 95 °C, 30 s; 50 °C, 30 s; 72 °C, 30 s for 40 amplification cycles using the primer pairs described in Table 2. Genomic DNA for Southern hybridization analysis was purified from chlamydia-infected cells using a kit obtained from Epicentre Biotechnologies. A plasmid-specific DIG-labelled (Roche Diagnostics) probe was generated by labelling, via random hexamer priming, the 7.5 kb PstI fragment of pCT01 (O’Connell & Maurelli, 1998) which comprises the entire cryptic plasmid of C. trachomatis strain L2/434/Bu.

RESULTS

Effect of novobiocin treatment on replication of chlamydiae

Treatment with sublethal doses of novobiocin can influence the extent of DNA supercoiling (Luttinger, 1995), affecting gene expression and rendering the antibiotic an effective plasmid-curing agent (Gado et al., 1987; Hooper et al., 1984; Wolfson et al., 1983). Thus we were interested in investigating the possibility that novobiocin treatment might enhance the rate of plasmid loss from C. trachomatis. We titrated the impact of increasing amounts of novobiocin on chlamydial growth using C. muridarum Nigg (MoPn) (Nigg, 1942), a strain that previously had not been demonstrated to be curable. Increasing amounts of freshly diluted novobiocin were added to the cell culture medium 4 h post-infection to ensure that normal uptake and differentiation to the reticulate body (RB) form were not inhibited by antibiotic treatment. The yields of novobiocin-treated bacteria were measured by plaque assay (Fig. 1) at harvest (~40 h after infection). Although the impact of drug treatment on the recovery of infectious chlamydiae was minimal at the lowest concentrations assayed, a novobiocin concentration of 62.5 µg ml⁻¹ reduced the relative infectious yield of bacteria by at least 100-fold. By extrapolation from studies with other micro-organisms (Gado et al., 1987; Hooper et al., 1984; Wolfson et al., 1983), we anticipated that plasmid curing would be most effective at concentrations where ~99% of bacterial growth was inhibited by the antibiotic.

Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. muridarum Nigg</td>
<td>Wild-type, mouse virulent</td>
<td>Nigg (1942)</td>
</tr>
<tr>
<td>C. muridarum CM972</td>
<td>Plasmid-deficient derivative of strain Nigg</td>
<td>This study</td>
</tr>
<tr>
<td>C. trachomatis 25667R</td>
<td>Plasmid-deficient clinical isolate, serovar L2</td>
<td>Peterson et al. (1990)</td>
</tr>
<tr>
<td>C. trachomatis CT599</td>
<td>Plasmid-deficient clinical isolate, serovar E</td>
<td>Stothard et al. (1998)</td>
</tr>
</tbody>
</table>

Table 2. PCR primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tufA F4 (sense)</td>
<td>5’-CACTACGCTCAGCTTGAGAGCTG-3’</td>
<td>593</td>
</tr>
<tr>
<td>tufA R4</td>
<td>5’-CTCCTCGAGCACGTTTCTCTG-3’</td>
<td></td>
</tr>
<tr>
<td>pMoPn F2 (sense)</td>
<td>5’-CTGCCAGGCTTGTCCTTAA-3’</td>
<td>317</td>
</tr>
<tr>
<td>pMoPn R2</td>
<td>5’-CTATGCTGCAAGGAGGTAA-3’</td>
<td></td>
</tr>
</tbody>
</table>
To examine whether novobiocin treatment had cured the plasmid, individual plaques were picked at random from the 62.5 µg ml⁻¹ plate, where we had observed <90% inhibition and thus might expect optimal plasmid curing. The plaques were then sequentially plaque purified three to five times, before being amplified to high titre. Each of the plaque-purified isolates was then screened by PCR for the presence of the cryptic plasmid. A chromosome-specific target, the tufA gene encoding the elongation factor EF-Tu involved in protein biosynthesis, was used as control. In two independent experiments, the frequency of novobiocin-treated isolates for which a plasmid-specific PCR product was not detected ranged from 4 to 30% (1 of 24 and 8 of 24) (Fig. 2a). An identical screen was performed on 73 plaque isolates that were picked from untreated control plates. No plasmid-deficient isolates were identified within this group (0 of 55 and 0 of 18). A single representative of the plasmid-deficient isolates was selected for continued analysis and designated C. muridarum strain CM972. The absence of plasmid in CM972 was confirmed by Southern hybridization analysis using the entire cryptic plasmid from C. trachomatis L2/434/Bu as probe (Fig. 2b).

To confirm the parental background of strain CM972, we performed immunofluorescent staining of infected McCoy cells using the strain-specific mouse monoclonal antibody M40, obtained from Dr Ellena Peterson (University of California, Irvine). This antibody is directed against a VD1 epitope of the major outer-membrane protein (MOMP) expressed by C. muridarum Nigg. The bacteria within inclusions formed by both the Nigg and the plasmid-deficient CM972 strains stained positively (Fig. 2c, d). The genome of C. muridarum Nigg has been sequenced (Read et al., 2000) so we further confirmed the identity of the CM972 by amplifying and sequencing a region of ompA, the gene that encodes MOMP, using the primer set BII and FII (Dean et al., 1995). The DNA sequence of the amplified fragment was identical to both the parental Nigg strain and the published sequence (data not shown).

Plasmid-deficient C. muridarum is defective in accumulation of glycogen within inclusions

Previously, the three plasmid-deficient strains isolated by Matsumoto et al. (1998) were shown to be unable to accumulate glycogen, as detected by iodine stain, within the intracytoplasmic inclusion formed by the growing bacteria. We used iodine staining of 40 h infected McCoy cells to determine the relative glycogen content of inclusions containing the Nigg or the CM972 strain of C. muridarum and found that the plasmid-minus CM972 strain was severely deficient in the accumulation of glycogen compared to the parental strain.
to the plasmid-containing Nigg strain (Fig. 3a, b). Interestingly, the clinical isolates *C. trachomatis* 25667R and CT599, known to lack the cryptic plasmid, were also found to be deficient in their ability to accumulate glycogen (Fig. 3c, d). Novobiocin treatment of Nigg-infected McCoy monolayers resulted in variable iodine staining of inclusions, ranging from non-staining inclusions to inclusions that appeared to contain only patchy depositions of iodine to inclusions that resembled those formed by untreated bacteria (data not shown). Analysis of the 48 plaque-purified isolates derived from the initial drug-treated bacteria screens revealed that only the plasmid-cured isolates stained negatively with iodine, indicating that glycogen accumulation may be regulated from the plasmid.

Plasmid-deficient *C. muridarum* form smaller plaques at reduced efficiency

While attempting to determine the effectiveness of novobiocin as a plasmid-curing agent, we observed that when the drug-treated *C. muridarum* were plated in a plaque assay using manual agitation, e.g. rocking (Banks et al., 1970), to inoculate the monolayers, only iodine-positive, plasmid-containing isolates were recovered. In contrast, if the bacteria were inoculated into the plaque assay via centrifugation, both plasmid-containing wild-type and plasmid-deficient, iodine-negative isolates could be recovered. Furthermore, attempts to plaque the clinical isolates *C. trachomatis* 25667R and CT599 without centrifugation were unsuccessful. We reasoned that loss of the cryptic plasmid might be associated with a reduced ability to form a plaque. To test the efficiency with which *C. muridarum* Nigg and strain CM972 were able to form plaques, the strains were titrated in parallel experiments by plaque assay with or without centrifugation. The results of this experiment are shown in Table 3. It is clear from these data that, while the e.o.p. for both strains approached 1 when the inoculum was applied to the monolayers during centrifugation, the ability of strain Nigg to form plaques was reduced ~35-fold when the inoculum was applied in the absence of centrifugation. An even more striking difference was observed for strain CM972, whose ability to plaque was reduced an additional ~150-fold when the strain was applied to the monolayers without centrifugation. In addition, plaques formed by strain CM972 were approximately one-half the diameter of those of the parental strain in parallel experiments, even when the bacteria had been centrifuged onto the monolayers (Fig. 4). To exclude the possibility that the differences in plaque size were due to a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Efficiency of plating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centrifugation</td>
</tr>
<tr>
<td>Nigg</td>
<td>$4.95 \times 10^{-1} \pm 3.28 \times 10^{-1}$</td>
</tr>
<tr>
<td>CM972</td>
<td>$6.02 \times 10^{-1} \pm 2.35 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Fig. 3. Glycogen accumulation within inclusions is not observed in the plasmid-deficient *C. muridarum* CM972, or in the plasmid-deficient clinical isolates *C. trachomatis* 25667R and CT599. (a–d) In a representative parallel experiment, chlamydia-infected McCoy cells were fixed and stained with iodine 40 h post-infection before being photographed at 1000× magnification. (a) *C. muridarum* Nigg; (b) *C. muridarum* CM972; (c) *C. trachomatis* 25667R; (d) *C. trachomatis* CT599. Arrows indicate the location of chlamydial inclusions.
reduced ability to replicate within McCoy cells, we compared, in parallel experiments, the infectious yield of both strains when grown in culture with an m.o.i. of 0.5 and no difference was observed (Nigg: 3.45 \times 10^7 \pm 6.39 \times 10^6 i.f.u. ml^{-1} vs strain CM972: 4.24 \times 10^7 \pm 7.58 \times 10^6 i.f.u. ml^{-1}; means \pm SE), suggesting that once inside the cell, both strains replicate and form infectious progeny equally well.

**DISCUSSION**

This paper describes the isolation of plasmid-deficient derivatives of *C. muridarum* strain Nigg, using novobiocin as a curing agent. Plasmid-deficient derivatives created by this method resemble naturally occurring plasmid-deficient strains in their inability to accumulate glycogen within intracytoplasmic inclusions. In addition we describe a novel phenotype, poor plaquing efficiency, which was observed for all plasmid-deficient strains of *C. trachomatis*.

**Novobiocin is an effective curing agent for chlamydiae**

The ability to cure *C. muridarum* strain Nigg would suggest that, at appropriate concentrations of novobiocin, plasmid replication is sufficiently inhibited that plasmid-deficient isolates can be recovered; this is in contrast to previous reports that at low concentrations of the drug (20 \mu g ml^{-1} in conjunction with 20 \mu g imipramine ml^{-1}), plasmid copy number increases (Pickett et al., 2005). The apparently elevated concentrations of novobiocin required to suppress chlamydial replication were striking. However, each of the chlamydial strains for which genome sequencing has been completed [*C. trachomatis* strain D/UV-3Cx (Stephens et al., 1998), *C. trachomatis* strain A/HAR-13 (Carlson et al., 2005) and *C. muridarum* strain Nigg (Read et al., 2000)] carries phospholipase D family orthologues (CT154-158; CTA_0163-0167 and TC0432-436; TC0440, respectively). In *Escherichia coli*, phospholipase D is encoded by *cls*. The *cls* gene has been recognized as identical to the wild-type form of the *nov* allele (Tropp et al., 1995) and mutations in *nov* are associated with greatly enhanced novobiocin sensitivity as a consequence of changes in outer-membrane lipid structure which affect permeability. Thus it is possible that the amount of novobiocin that was required to trigger plasmid loss by chlamydiae may reflect a level of intrinsic resistance due to the relative impermeability of the chlamydial outer membrane. Nonetheless, the effective concentration of novobiocin required for plasmid curing was not cytotoxic, enabling the enrichment of plasmid-deficient bacteria. The use of novobiocin as a plasmid-curing agent is highly effective and allows the recovery of plasmid-deficient derivatives at high frequency. This technique should be generally applicable to other *C. trachomatis* strains or other plasmid-containing *Chlamydia* species.

**Plasmid-deficient chlamydiae are less able to form plaques in cell culture**

It would appear that a reduced ability to form plaques in cell culture is associated with plasmid loss from *C. trachomatis*. Previously identified plasmid-deficient clinical isolates (Peterson et al., 1990; Stothard et al., 1998) that are available for study also failed to plaque in our assay (data not shown). However, the absence of an isogenic, plasmid-containing parental control for these clinical isolates made the significance of these observations difficult to interpret. Our observations that plasmid-deficient isolates were only recovered after novobiocin treatment if the bacteria were passed via centrifugation at all steps are consistent with those of Matsumoto et al. (1998), who reported the recovery of plasmid-deficient derivatives of *in vitro*-cultured strains with little difficulty. It is noteworthy that centrifugation of the inoculum onto the cells was an integral component of all screening and passage steps in their protocols. The parental strain Nigg demonstrates a marked reduction in plaquing efficiency if applied to monolayers without centrifugation. The significance of this observation is not clear; it may reflect strain-specific or receptor-specific variations in attachment or uptake into cells as has been described for other chlamydial strains (Chen & Stephens, 1997; Davis et al., 2002; Carabeo & Hackstadt, 2001). However, a more profound deficiency in the ability to plaque under these conditions is demonstrated by strain CM972; this suggests...
that plasmid-deficient derivatives may be selected against under some culture conditions, and particularly when the plaque assay protocols that are used for isolation and screening do not involve centrifugation of the monolayer. We also noted that plaques formed by CM972 were one-half the diameter of the plaques of the parent strain. This is likely a further reflection of the attachment/uptake defect manifested by this strain because plaque size may reflect the relative efficiency with which the bacteria undergo subsequent rounds of uptake and amplification in the host cells immediately adjacent to the focus of infection.

Plaque size is highly variable between experiments and is known to be influenced by many other factors including the overall quality of the cell monolayer, the culture medium and the length of time the plaque assays are maintained in culture. This may affect the rate at which plasmid-deficient isolates are recovered from a novobiocin-treated population, possibly leading to variable estimates of curing efficiency: low if overall plaque size is small; higher if overall plaque size is larger. It was not possible to estimate the rate of natural plasmid loss by C. muridarum strain Nigg because we failed to identify any naturally cured derivatives.

**The role of the cryptic plasmid in chlamydiae**

Loss of the plasmid is associated with two distinct and possibly unrelated phenotypes (glycogen accumulation, efficiency of plaque formation), raising the possibility that a regulator or co-factor for genes important for these phenotypes may be encoded on the plasmid. Nonetheless, the significance of these phenotypes to chlamydial infection is unclear. We have observed that a variety of environmental factors can suppress the accumulation of glycogen within inclusions, including ampicillin treatment and substrate limitation (unpublished). Furthermore, it should be noted that neither *Chlamydiophila psittaci* nor *Chlamydiophila pneumoniae* accumulate glycogen within their inclusions, although the majority of *C. psittaci* and some *C. pneumoniae* strains carry the plasmid (Lusher et al., 1989; Thomas et al., 1997; McClenaghan et al., 1988) and the glycogen metabolic genes have been retained on the chromosome (Read et al., 2000, 2003; Kalman et al., 1999). It would be highly desirable to reintroduce the cryptic plasmid into *C. muridarum* and demonstrate restoration of both intra-inclusion glycogen accumulation and plaquing efficiency. However, an effective gene transformation system has yet to be developed for the chlamydiae.

Although it has been noted that centrifugation of the chlamydial inoculum onto cell monolayers can increase the efficiency with which cells may become infected (Rota, 1977; Lee, 1981; Pearce et al., 1981), it is difficult to envisage how this manipulation might mimic a relevant *in vivo* condition. Our isolation of a plasmid-deficient variant of the mouse-virulent *C. muridarum* Nigg will enable us to investigate whether loss of the cryptic plasmid affects the ability of the bacterium to establish infection. In addition, it is possible that the plaquing defect that we have observed may ultimately be used as the basis for a positive selection for transformants.

**ACKNOWLEDGEMENTS**

The authors gratefully acknowledge the generous gift of M40 monoclonal antibody by Dr Elleneta Peterson. The authors also wish to thank Drs Roger Rank, Tony Darville, Uma Nagarajan, Marie Chow and Ming Tan for helpful discussions. Catherine O’Connell also wishes to recognize the encouragement and support of You-xun Zhang and Peter Rice MD during the initial phases of this project. This work was supported by grants from NIH/NIAID, AI45565 and A138515.

**REFERENCES**


