Chlamydia trachomatis clinical isolates identified as tetracycline resistant do not exhibit resistance in vitro: whole-genome sequencing reveals a mutation in porB but no evidence for tetracycline resistance genes

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Chlamydia trachomatis is the most common bacterial sexually transmitted infection worldwide and the leading cause of preventable blindness in developing countries. Tetracycline is commonly the drug of choice for treating C. trachomatis infections, but cases of antibiotic resistance in clinical isolates have previously been reported. Here, we used antibiotic resistance assays and whole-genome sequencing to interrogate the hypothesis that two clinical isolates (IU824 and IU888) have acquired mechanisms of antibiotic resistance. Immunofluorescence staining was used to identify C. trachomatis inclusions in cell cultures grown in the presence of tetracycline; however, only antibiotic-free control cultures yielded the strong fluorescence associated with the presence of chlamydial inclusions. Infectivity was lost upon passage of harvested cultures grown in the presence of tetracycline into antibiotic-free medium, so we conclude that these isolates were phenotypically sensitive to tetracycline. Comparisons of the genome and plasmid sequences for the two isolates with tetracycline-sensitive strains did not identify regions of low sequence identity that could accommodate horizontally acquired resistance genes, and the tetracycline binding region of the 16S rRNA gene was identical to that of the sensitive control strains. The porB gene of strain IU824, however, was found to contain a premature stop codon not previously identified, which is noteworthy but unlikely to be related to tetracycline resistance. In conclusion, we found no evidence of tetracycline resistance in the two strains investigated, and it seems most likely that the small, aberrant inclusions previously identified resulted from the high chlamydial load used in the original antibiotic resistance assays.

INTRODUCTION

Chlamydia is the most common bacterial sexually transmitted infection worldwide, but is often asymptomatic, resulting in serious sequelae in untreated women, such as pelvic inflammatory disease, ectopic pregnancy and infertility. Chlamydia trachomatis, the causative agent, is an obligate intracellular bacterium that has lost many biosynthetic pathways, instead obtaining its metabolic requirements from the host cell (Stephens et al., 1998). Mobile genetic elements are rare among the chlamydiae, as with most intracellular bacteria, due to the combined effects of reductive evolution, deletion bias and physical separation of the metabolically active reticulate bodies (RBs) from external mobile gene pools (Andersson et al., 2002; Bordenstein & Reznikoff, 2005). Therefore, access to mobile genetic elements, which may facilitate horizontal transmission of antibiotic resistance genes, is limited. This is probably a major factor in the sustained efficacy of clinically important antibiotics against C. trachomatis.

Tetracycline is one of the most commonly used antibiotics employed to treat chlamydial infections. Tetracycline

Abbreviations: EB, Elementary body; MOMP, major outer-membrane protein; RB, reticulate body; SNP, single nucleotide polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are: IU824 genome, HF562298; IU824 plasmid pIU824, HF562299; IU888 genome, HF562300; IU888 plasmid pIU888, HF562301.
works by binding to the 16S rRNA, preventing protein synthesis (Brodersen et al., 2000), and is used widely in the treatment of various bacterial infections of both humans and animals. This has selected for a large number of antibiotic resistance genes, many of which have been acquired by pathogens from commensal bacteria already present in the surrounding environments. These can be divided on the basis of the encoded mechanism, of which four are currently known to exist. Tetracycline efflux is the most common mechanism, mediated by the presence of one of at least 28 genes (as reviewed by van Hoek et al., 2011). Ribosomal protection is afforded by carriage of one of 10 tet genes or one otr gene, and enzymic inactivation is conferred by one of three tet genes. The fourth mechanism is by way of mutations in the 16S rRNA gene, which have been identified as lending tetracycline resistance to *Helicobacter pylori* (Gerrits et al., 2002), as well as the Gram-positive *Propionibacterium acnes* (Ross et al., 1998). The genes conferring the first three resistance mechanisms may reside upon horizontally transmissible elements such as plasmids and insertion elements, enabling their transfer among a wide range of bacterial species. Nonetheless, in the chlamydiaceae, tetracycline resistance is rare, with confirmed carriage of a known tetracycline resistance determinant, tetC, only in the pig pathogen *Chlamydia suis* (Dugan et al., 2004). There have also been two reports of phenotypic tetracycline resistance in *C. trachomatis*. The first was documented in Indianapolis, USA: 10 isolates from five patients were resistant to at least 8 µg tetracycline ml⁻¹, with some isolates surviving 64 µg ml⁻¹ (Jones et al., 1990; Dugan et al., 2004). The next occurrence was in Toulouse, France, where *C. trachomatis* was isolated from an asymptomatic woman who had previously been treated with tetracycline. The isolate was found to be resistant to over 64 µg tetracycline ml⁻¹ (Lefevre et al., 1997). However, growth was very poor in *vitro* even when subjected to a much lower tetracycline concentration, and it was suggested that the isolate had entered a persistent, metabolically inactive state. In both reports, repeated treatment failure preceded detection of the apparently resistant isolates, although reinfection was not ruled out in all cases.

In this study we focus on two of the *C. trachomatis* strains isolated in the USA (Jones et al., 1990). Isolate IU888 was originally cultured from a 19-year-old woman 10 months after treatment with tetracycline and erythromycin for acute salpingitis caused by *C. trachomatis*. This strain was originally found to be resistant to concentrations of tetracycline as high as 64 µg ml⁻¹, as seen for the French isolate (Lefevre et al., 1997). IU888 was also resistant to a number of other antibiotics, including high-level resistance to erythromycin, clindamycin and sulphamethoxazole, whereas the French isolate was sensitive to these antibiotics (Lefevre et al., 1997). The second isolate investigated in this study, IU824, was cultured from the endometrium of a 27-year-old woman 5 months after tetracycline and 11 months after cephalosporin treatment for *C. trachomatis* (Jones et al., 1990). IU824 was found to be resistant to a lower tetracycline concentration (16 µg ml⁻¹) than was IU888, but was equally resistant to the other antibiotics tested. In both reports, resistance was said to be heterotypic, with less than 1% of the bacterial population exhibiting resistance. Furthermore, a large inoculum was usually needed to invoke resistance *in vitro*.

The existence of tetracycline resistance among *C. trachomatis* strains has been called into question (Wang et al., 2005; Sandoz & Rockey, 2010), but contrary experimental evidence has yet to be provided. Identification of tetracycline resistance loci through genome sequencing was previously an expensive and laborious task, largely inaccessible to researchers outside the few pioneering laboratories with such capabilities. However, the advent of high-throughput whole-genome sequencing technology in recent years has enabled the interrogation of a large number of *C. trachomatis* genomes (Harris et al., 2012) at a fraction of the cost. Using genome sequence analysis and tetracycline resistance assays we present data that contradict the previous evidence for tetracycline resistance in *C. trachomatis*.

**METHODS**

*C. trachomatis* strains. L2/434/Bu (ATCC VR-902B) was used as the tetracycline-sensitive control strain, as this was used as a tetracycline-sensitive control in the original article describing the tetracycline resistance of IU824 and IU888 (Jones et al., 1990).

McCoy cell culture. Mouse McCoy cells were grown in a 75 cm² tissue culture flask (T75) and incubated at 37 °C (5% CO₂) for 48 h in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), resulting in a confluent monolayer of cells. These were detached from the plastic by incubating in PBS containing 0.125% trypsin and 0.02% EDTA at room temperature for 5 min. Glass coverslips were placed in the wells of a 24-well microtitre tissue culture tray, overlaid with 2.5 × 10⁵ cells in 1 ml DMEM/FCS and incubated at 37 °C (5% CO₂) overnight. Two further trays were prepared in the same way, without coverslips.

Tetracycline resistance assay. Elementary bodies (EBs) of IU824, IU888 and L2/434/Bu stored at −70 °C in a 1:1 ratio of 4 × sugar-phosphate buffer (4SP) with PBS were thawed at room temperature for 30 min, then five wells (containing glass coverslips) of a 24-well tissue culture plate were inoculated per strain with 5 µl of EBs (approx. 5 × 10⁵ EBs per well), leaving the sixth well in each row as a no-infection control. Trays were centrifuged at 750 g for 30 min, after which medium was replaced with fresh DMEM/FCS containing 1 µg cycloheximide ml⁻¹ and 1, 2, 4 and 8 µg tetracycline ml⁻¹, leaving the fifth well antibiotic-free for each strain as a positive infection control. Trays were incubated at 37 °C for 48 h (5% CO₂), after which cells were observed using phase-contrast microscopy (×400 magnification using a Nikon Eclipse TS100). Cells were then fixed for FITC staining (as below). To ascertain the infectivity after exposure to tetracycline, the cells were harvested from the 1 µg ml⁻¹ tetracycline-containing wells of a duplicate 24-well tissue culture plate prepared in an identical way but without the use of glass coverslips. These were passaged to fresh cells in a 24-well tissue culture tray, with and without 1 µg tetracycline ml⁻¹, and incubated at 37 °C for 48 h, followed by FITC staining. Additionally, cells in a third 24-well tray were incubated for 48 h, after which tetracycline-containing medium...
was removed and replaced with tetracycline-free medium and incubated for a further 48 h. Wells were observed using phase-contrast microscopy to identify whether the infectivity could be restored, which would be indicative of persistence.

**Erythromycin resistance assay.** Cells were grown in a 24-well plate and inoculated with IU824, IU888 and L2/434/Bu as described for the tetracycline resistance assay. After centrifugation, the medium was replaced with DMEM/FCS containing 1 μg cycloheximide ml⁻¹ and 1, 2, 4 and 8 μg erythromycin ml⁻¹, and the tray was incubated at 37 °C for 48 h (5% CO₂), after which cells were observed by eye using phase-contrast microscopy.

**FITC staining.** After incubation for 48 h at 37 °C, medium was removed from all wells, and cells were washed twice with PBS. Ice-cold methanol was then added to each well and the tray was incubated at −20 °C for 20 min. The cells were again washed with PBS, and stored at 4 °C in PBS. Coverslips were removed and placed face-down on droplets of a *Chlamydia* genus-specific, mouse/IgG3 monoclonal antibody (Acris Antibodies GmbH) diluted 1:2 with PBS, and incubated for 40 min at 37 °C (5 % CO₂). Coverslips were then washed three times with PBS. The FITC-conjugated goat anti-mouse monoclonal antibody (Immune Systems) was mixed (1:50) with Evans Blue (0.0025%) counterstain. Coverslips were placed face-down on drops of conjugated antibody/dye mixture and incubated for 30 min at 37 °C (5 % CO₂). Coverslips were then washed three times in PBS, followed by a final wash in double distilled water, and were mounted onto glass microscope slides using Vectashield (Vector Labs). Fluorescence was observed by phase-contrast microscopy using an external Nikon C-Shg mercury lamp illuminator as the UV light source.

**Growth curve experiment.** McCoy cells grown overnight in eleven 96-well tissue culture trays were inoculated with IU824 or IU888, in triplicate and incubated at 37 °C. A tray was removed and cells were fixed every 8 h using ice-cold methanol, as described above. Ten time points were taken, with the final being fixed after a total of 80 h. The X-Gal staining method was used to visualize inclusions, as described previously (Skitton et al., 2007). Briefly, *Chlamydia* genus-specific monoclonal antibody (2B Scientific) was added to each well, followed by incubation at 37 °C for 40 min. Cells were then washed three times in PBS, and second-affinity anti-mouse antibody conjugated with β-galactosidase (Calbiochem) was added to each well and incubated for 1 h at 37 °C. Cells were then washed in PBS, and 100 μl of staining solution (5.0 mM K₄Fe(CN)₆, 5.0 mM K₃[Fe(CN)₆]·3H₂O, 2.0 mM MgCl₂·6H₂O, 0.25 M X-Gal) was added to each well and trays were incubated for 4 h at 37 °C. The blue-stained *C. trachomatis* inclusions were then observed using light microscopy.

**Genome sequence analysis.** Extraction of DNA was performed on strains IU824 and IU888 as previously described (Seth-Smith et al., 2009). DNA was sequenced on the Illumina HiSeq platform using Illumina reads for IU824 and IU888, respectively. Illumina reads were assembled using Velvet v 1.0.12 (Zerbino & Birney, 2008), producing contiguous nucleotide sequences (contigs) which were scaffolded against the genome of strain E/SW3 (EMBL accession nos HE601870.1 and HE603212) and E/SW3 (Harris et al., 2012) using Artemis Comparison Tool (ACT) (Carver et al., 2005). Contigs which failed to scaffold against E/SW3 using ABACAS were compared against the GenBank database using BLAST to identify any tetracycline resistance genes which have not been present in the scaffolded contigs. The porB ompB gene was amplified using forward primer ompB_F2 (5′-TCTCCGTAGAGACGATGTG-3′) and reverse primer ompB_R2 (5′-TCGCTCCAGTGCAAGTTTT-3′). The PCR product was gel-purified using the Wizard SV gel and PCR cleanup system (Promega) and capillary-sequenced by SourceBioscience (Cambridge). Chromatograms were viewed using Chromas Lite v2.01 (Technelysis Pty Ltd).

**RESULTS**

**Antibiotic resistance assays**

Neither strain IU824 nor strain IU888 developed inclusions at any concentration of tetracycline or erythromycin, consistent with the sensitive control strain L2/434/Bu. The positive control wells grown without antibiotics contained inclusions with normal morphology for all three strains. The tetracycline resistance assay was further investigated by staining the infected monolayer with FITC-conjugated antibody, giving more sensitive detection of inclusions. Strongly fluorescing inclusions were observed in the positive control wells of all strains grown without antibiotics. Fluorescence, significantly lower than the positive control wells, was observed in the tetracycline-treated wells for both IU824 and IU888, as well as the L2/434/Bu control strain, but this appeared to be from cell debris and was not concentrated in inclusions. No fluorescent signal was apparent in the control wells (cells grown in the absence of infection or tetracycline) (Fig. 1). To identify whether any infectious EBs were produced from the first passage of IU824 and IU888, one well of each strain, which had been grown under 1 μg tetracycline ml⁻¹ in the first passage, was used to inoculate fresh McCoy cells without the addition of tetracycline. These were observed daily for 1 week, but inclusions were not observed in any of the wells by phase-contrast microscopy or fluorescent staining (data not shown).
Growth curve experiment

There was no significant difference between the growth rates of the two isolates, both of which peaked at 32 h post-infection.

Genome sequence analysis

The genomes of both strains, IU824 and IU888, were sequenced and assembled. Analysis of the \textit{ompA} gene encoding the major outer-membrane protein (MOMP) showed that IU824 and IU888 both belong to genotype E, and are near-identical, with just one single nucleotide polymorphism (SNP) differentiating them.

L2/434/Bu was used as the tetracycline-sensitive control strain, as previously (Jones \textit{et al.}, 1990), and shows no evidence of tetracycline resistance either in the literature or in antibiotic resistance assays performed in our lab. Genome comparisons of IU824 and IU888 with strain L2/434/Bu identified three deletions in the L2/434/Bu genome relative to the IU824 and IU888 genomes, which are explained by genotype-specific differences between L2/434/Bu and E, and are not implicated in antibiotic resistance.

Mapping the sequence data against reference genomes indicated that the genomes of IU824 and IU888 were most closely related to that of E/Bour, with only 26 (IU888) or 27 (IU824) SNPs distributed throughout the genomes identified in relation to this reference genome (Table 1, Fig. 2). E/Bour has been shown to be susceptible to the synthetic tetracycline antibiotic, doxycycline (Roblin \& Hammerschlag, 2000; Donati \textit{et al.}, 2010). More distantly related, but still in the same subclade, is strain E/SW3, with 1039 SNPs separating the genome of this strain from that of IU824; this is also sensitive to tetracycline (Unemo \textit{et al.}, 2010). The genome of strain E/SW3 has been annotated, and this annotation was used as a basis to transfer annotation to IU824 and IU888, after which manual checking of the annotation was performed.

Among the SNPs distinguishing the genomes of IU824 and IU888 from E/Bour was a unique SNP in the \textit{ompB}/\textit{porB} gene of IU824 (but not IU888), resulting in a premature stop codon at codon 81. This base change from C to T (at position 826442 relative to the E/Bour genome) was verified by capillary sequencing of \textit{porB} (Fig. 2b, c).

Inactivation of \textit{porB} has not been described previously, but this is unlikely to be involved in tetracycline resistance. Additionally, both copies of the 23S large subunit rRNA gene contain a C (in E/Bour) to T (in both IU824 and IU888) mutation (nucleotide position 858503 and 880494 of the E/Bour genome; with \textit{E. coli} numbering this is nucleotide position 1840 of the gene), which has not been documented before in \textit{C. trachomatis}. All SNPs in relation to the reference genome E/Bour are shown in Table 1.

Fig. 1. FITC staining of strains IU824, IU888 and control strain L2/434/Bu: (a) IU824 positive control (no tetracycline); (b) IU824 1 \(\mu\)g tetracycline ml\(^{-1}\); (c) IU888 positive control; (d) IU888 1 \(\mu\)g tetracycline ml\(^{-1}\); (e) L2/434/Bu positive control; (f) L2/434/Bu 1 \(\mu\)g tetracycline ml\(^{-1}\). Scale bars, 20 \(\mu\)m.
The \textit{tetC} gene, known to confer resistance to tetracycline in \textit{C. suis} (Dugan \textit{et al.}, 2004), was not identified within the assembled genome and plasmid sequences of IU824 or IU888, and none of the sequence data mapped to this gene, indicating that it is not present in these strains.

Comparison of the plasmids from IU824 and IU888 revealed that they are identical to that of E/Bour.

**DISCUSSION**

The emergence of new antibiotic resistant bacteria is a burden on healthcare organizations the world over. \textit{C. trachomatis} is already the leading cause of treatable sexually transmitted infections in the Western world and of preventable blindness in developing countries, so resistance to tetracycline antibiotics could present a serious threat to public health. Previous reports identified clinical isolates that were apparently resistant to tetracycline (Jones \textit{et al.}, 1990, Lefevre \textit{et al.}, 1997), but no recent observations of tetracycline resistance in \textit{C. trachomatis} have been made, and the present study offers empirical evidence to the contrary.

Isolates IU824 and IU888, previously described as being tetracycline resistant (Jones \textit{et al.}, 1990), were found in this study to be phenotypically sensitive to tetracycline, with no inclusions developing at any tetracycline concentration. Fluorescence microscopy was used to more easily identify the small inclusions that were previously described by Jones and co-workers, but only revealed low-level fluorescence (relative to the positive controls) at all tetracycline concentrations for both isolates, and also in the sensitive control strain L2/434/Bu, highly suggestive that this was not caused by the presence of resistant chlamydial inclusions. To investigate this further, cells grown in the lowest tetracycline concentration medium for each strain were used to reinfect fresh monolayers in the presence and absence of tetracycline. If the fluorescence observed was caused by the presence of tetracycline-resistant \textit{C. trachomatis}, the number of inclusions would have increased upon passage. Instead, no inclusions were observed during the second passage of either strain, suggesting that the fluorescence was not caused by the presence of tetracycline-resistant \textit{C. trachomatis}. Moreover, the UV-fluorescent properties of tetracycline have been long recognized and utilized in medicine (Milch \textit{et al.}, 1958), so it seems likely that the low-level fluorescence seen in these experiments can be explained simply by the intrinsic fluorescence of tetracycline rather than the presence of FITC-stained \textit{C. trachomatis}.

### Table 1. SNPs in strains IU824 and IU888 in comparison to the reference genome E/Bour

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<th>Position in Bour</th>
<th>SNP</th>
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<th>Predicted gene or product</th>
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It is possible that persistence rather than antibiotic resistance might explain the aberrant inclusions described by Jones and co-workers, as suggested before (Lefevre et al., 1997). Persistence is a well-studied phenomenon, particularly in relation to the effects of beta-lactam antibiotics on C. trachomatis (Skilton et al., 2009), but little information is available on this response to tetracycline. When C. trachomatis enters a persistent state, the reproductive cycle is slowed dramatically and differentiation of RBs to EBs is halted. Therefore, ever decreasing numbers of inclusions will be observed upon serial passage until extinction, consistent with the previously published findings. In the present study, tetracycline-containing medium (in a parallel experiment) was removed 2 days post-infection and replaced with tetracycline-free medium to investigate the possibility that the isolates had entered a persistent state. However, no recovery of infectivity was seen after 1 week, so tetracycline-induced persistence is not implicated here. Although little is known about chlamydial persistence in response to tetracycline treatment, it has been noted that persistence to one antibiotic may impact upon the susceptibility of Chlamydia to another. For example, it has been found that when persistence is induced by penicillin treatment, azithromycin resistance can result, despite a previously high degree of susceptibility (Wryick & Knight, 2004). Incidentally, the patients carrying IU824 and IU888 had both previously been treated with other antibiotics (erythromycin and cephalosporin in addition to tetracycline) for their respective infections, so this might have contributed to the in vivo tolerance of these isolates to tetracycline treatment.

The small aberrant inclusions observed by Jones and co-workers only occurred when cells were inoculated with a large titre of Chlamydia. These strains were therefore suggested to exhibit heterotypic resistance, with the large inoculum facilitating the observation of a very small but highly resistant minority. Heterotypic resistance has been described in various bacterial species, and a number of underlying genetic causes are known. For example, Staphylococcus aureus may exhibit heterotypic resistance to beta-lactam antibiotics due to mutations and genetic rearrangements in a small proportion of a population, and upon subculturing under antibiotic selection this resistance becomes homotypic (Finan et al., 2002). However, in Chlamydia, antibiotic selection does not increase the size of penicillin treatment, azithromycin resistance can result, despite a previously high degree of susceptibility (Wryick & Knight, 2004). Incidentally, the patients carrying IU824 and IU888 had both previously been treated with other antibiotics (erythromycin and cephalosporin in addition to tetracycline) for their respective infections, so this might have contributed to the in vivo tolerance of these isolates to tetracycline treatment.

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the tetracycline-resistant component (Jones et al., 1990; Lefevre et al., 1997). Rather, chlamydial inclusions decrease with each passage, so an underlying genetic cause is not implicated. Heterotypic resistance in Chlamydia has been described as a purely phenotypic response of bacterial communities to antibiotic stress (Sandoz & Rockey, 2010); in this case it seems likely that what is perceived to be heterotypic resistance is actually explained by stochastic escape of Chlamydia from antibiotic activity due to the inoculum effect (Brook, 1989; Kenny et al., 1989; Kang et al., 2004; LaPlante & Rybak, 2004).

Antibiotic susceptibility testing has not yet been standardized for C. trachomatis, hampering the definition of an accurate MIC or minimum chlamydicidal concentration (MCC). For example, differences in cell line, m.o.i., stage of antibiotic addition and passage number of the host cells and the Chlamydia may have a varying degree of effect on MIC determination (as reviewed by Wang et al., 2005). Such discrepancies between protocols may account for the different observations of this study in comparison to previous work (Jones et al., 1990). Other studies have conducted tetracycline resistance assays on IU824 (Jones et al., 1990; Solomon et al., 2005). In one study, the tetracycline MIC for this strain was recorded as being 2 μg ml⁻¹ (Solomon et al., 2005). This is significantly lower than that recorded by Jones and co-workers, and higher than that recorded here, exemplifying the current irregularity of MIC testing in Chlamydia. Until a standardized methodology has been defined, reports of antibiotic-resistant Chlamydia should be viewed with caution.

Although the isolates investigated in this study were phenotypically sensitive to tetracycline, it is possible for antibiotic resistance genes to be present but not expressed, due to gene silencing. For example, in one study, 52 E. coli isolated from pigs were found to harbour the resistance plasmid pVE46 carrying bla (beta-lactam resistance), aadaA1 (streptomycin resistance), sul1 (sulphonamide resistance) and tetA (tetracycline resistance), but reduced or complete loss of resistance to tetracycline was recorded for all isolates, despite retention of the resistance plasmid; this was suggested to be due to chromosomally located regulatory genes (Enne et al., 2006). The clinical significance is that isolates such as these may revert to a resistant phenotype upon activation of the gene by the presence of the antibiotic (Enne & Bennett, 2010). Through whole-genome analysis this study found that neither IU824 nor IU888 contained any known tetracycline resistance determinants, or any regions indicative of acquired resistance genes, consistent with the tetracycline-sensitive phenotype. Notably, the tetC gene, known to encode a tetracycline efflux pump, thereby lending tetracycline resistance to the pig pathogen C. suis (Dugan et al., 2004), was absent. No whole-genome insertions were identified in comparison to the genomes of the sensitive control strains, suggesting that if a hitherto unidentified, silent tetracycline gene exists in these clinical isolates, they must also exist in the closely related E. coli serovar genomes analysed here. This also applies to sulphonamide, erythromycin and clindamycin resistance. However, in many species of bacteria, macrolide resistance is provided by mutations in the 23S large subunit rRNA gene, altering the antibiotic target (as reviewed by Vester & Douthwaite, 2001). Interestingly, an SNP in both copies of the 23S large subunit rRNA gene appears to be unique to IU824 and IU888 out of the genomes that have been sequenced to date. Double and triple SNPs in the erythromycin-binding V domain of the 23S rRNA gene have previously been found to confer erythromycin resistance on Chlamydia (Misyurina et al., 2004). The single SNP identified in this study was located outside of the erythromycin-binding sequence, so was unlikely to affect erythromycin sensitivity. However, to further investigate this, an erythromycin resistance assay was performed. Both isolates were sensitive to erythromycin (MIC <1 μg ml⁻¹), so we conclude that this 23S rRNA gene SNP does not confer erythromycin resistance.

Genome sequence analysis revealed a mutation in the porB gene of strain IU824, unique amongst all currently sequenced C. trachomatis strains, which would result in truncation of the expressed protein. PorB is a surface-exposed protein with low nucleotide sequence identity to ompA, but a similar mass (38 000 Da in comparison to the 40 000 Da of MOMP) and isoelectric point (4.9 versus 5 of MOMP) (Kubo & Stephens, 2000). Whereas MOMP is a generalized porin, facilitating the uptake of various molecules including amino acids and carbohydrates, PorB specifically enables the uptake of dicarboxylates; these may feed into the TCA cycle, which is incomplete in C. trachomatis and requires exogenous 2-oxoglutarate or glutamate from the host cell (Kubo & Stephens, 2001). The high intraspecific conservation of this gene implies that this has an important role in the survival of C. trachomatis. Nevertheless, the existence of a strain with an inactivating mutation in porB suggests that PorB is non-essential, supported by the lack of any effect on the growth rate of strain IU824 in comparison to IU888.

It is possible that this mutation has arisen in vitro, as both strains have been passaged several times since their isolation. This may also be significant with regard to the tetracycline susceptibility of these strains. If the tetracycline resistance initially identified was encoded by a gene carried on a plasmid, transposon or insertion sequence, serial passages in the absence of selection may have resulted in the loss of the gene. However, Jones et al. (1990) found that after passaging strain IU824 eight to 10 times in antibiotic-free medium, no loss of the perceived resistance was seen, so this seems unlikely to account for the tetracycline sensitivity of the isolates described here.

Although the tetC gene was not detected in the two isolates investigated here, future acquisition of the tetC genomic island by C. trachomatis from the pig pathogen C. suis remains a threat. Tetracycline is used heavily in pig farming to control a number of infectious diseases and is often administered indiscriminately to entire herds. Tetracycline,
among other antibiotics, was also used in livestock at subtherapeutic concentrations for over 50 years in Europe (a practice that continues in the USA) due to its growth-promoting side effects (Cromwell, 2002). Continuous exposure to this antibiotic has selected tetC-bearing *C. suis*, with diverse elements identified among various strains, indicating multiple acquisition events (Dugan et al., 2004). The tetC island contains an insertion sequence element that enables both intra- and interspecific mobilization, and its horizontal transmission has been demonstrated in crossing experiments between *C. suis* and *C. trachomatis* (Suchland et al., 2009), so the acquisition of tetC by *C. trachomatis* is a real possibility. Interspecific fusion of *C. suis* and *C. trachomatis* L2/434/Bu inclusions has been observed (Lenart et al., 2001), so it seems that the only barrier to acquisition of tetC by *C. trachomatis* is the fidelity of *C. suis* and *C. trachomatis* to their respective hosts. Human infections caused by *C. suis* have not yet been documented; however, *C. suis* infection has been identified in other domestic animals, demonstrating the species’ potential to infect multiple hosts (Pantchev et al., 2010).

In the clinic, it seems that repeated detection of *Chlamydia* despite antibiotic intervention results from a combination of factors that are not yet fully understood, but may include poor patient cooperation and adherence to antibiotic treatment regimens, or reinfection. The results of this study indicate that antibiotic resistance was not the cause of the failure to resolve the IU824 and IU888 infections, and at present a combination of other factors is more likely to have involved in the persistence of genitourinary tract *C. trachomatis* infections; however, increasing evidence for horizontal gene transfer within the chlamydiae (Wang et al., 2011; Harris et al., 2012) warns of future challenges in the battle against this globally important sexually transmitted infection.

**REFERENCES**


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