Plasmid diversity in *Chlamydia*

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Chlamydiae exhibit low interspecies DNA homology and plasmids from different chlamydial species can be readily distinguished by Southern blot analysis and restriction enzyme profiling. In contrast, available plasmid sequence data from within the species *Chlamydia trachomatis* indicate that plasmids from human isolates are highly conserved. To evaluate the nature and extent of plasmid variation, the complete nucleotide sequences were determined for novel plasmids from three diverse non-human chlamydial isolates: pCpA1 from avian *Chlamydia psittaci* (N352); pCpnE1 from equine *Chlamydia pneumoniae* (N16); and pMoPn from *C. trachomatis* mouse pneumonitis. Comparison of the sequence data did not identify an overall biological function for the plasmid but did reveal considerable sequence conservation (> 60%) and a remarkably consistent genomic arrangement comprising eight major ORFs and four 22 bp tandem repeats. The plasmid sequences were close to 7500 nucleotides in length (pCpA1, 7553 bp; pMoPn, 7502 bp) however the equine *C. pneumoniae* plasmid was smaller (7362 bp) than all other chlamydial plasmids. The reduced size of this plasmid was due to a single large deletion occurring within ORF 1; this potentially generates two smaller ORFs. The disruption of ORF 1 is the only significant variation identified amongst the chlamydial plasmids and could prove important for future vector development studies.

Keywords: chlamydia, plasmid

INTRODUCTION

Chlamydia spp. are intracellular pathogens responsible for a wide range of important medical and veterinary diseases. With the addition of *Chlamydia pecorum* (Fukushi & Harai, 1992), four species are currently recognized within the genus *Chlamydia*. *Chlamydia trachomatis* and *Chlamydia pneumoniae* are predominately human pathogens, although recently strains of both species infecting other mammals have been described, while *Chlamydia psittaci* and *Chlamydia pecorum* are mainly pathogenic to birds and mammals. *Chlamydia* have an absolute requirement for ATP from the host cell and cannot be grown on synthetic media. *Chlamydia* have no near eubacterial relatives and are themselves highly diverse, with interspecies genomic DNA homology as low as 10% (Kingsbury & Weiss,

1968). Despite this lack of DNA homology and extensive differences in host range and pathogenicity, all chlamydiae share a number of biological features. Most notable is their unique developmental life-cycle involving two alternating forms; the larger reticulate bodies multiply by binary fission until, at the end of the life-cycle they condense to form the smaller metabolically inert but infectious elementary bodies (EBs; Ward, 1988).

A physical and genetic map of the *C. trachomatis* chromosome describes a genome of 1045 kb (Birkelund & Stephens, 1992). This is one of the smallest prokaryotic genomes and it is likely that there are many genetic factors absent from the chlamydial genome relative to other bacteria (Hatch, 1988). In addition to the genome, extrachromosomal DNA elements have been identified in isolates from all four chlamydial species. Plasmids were initially described in both *C. trachomatis* and *C. psittaci* by Lovett *et al.* (1980). Also, a 22 nm diameter icosahedral bacteriophage, later called chlamydiophage, was first observed by thin section electronmicroscopy specifically from duck isolates of

Abbreviations: EB, elementary body; MoPn, mouse pneumonitis.

The EMBL accession numbers for the plasmid sequences reported in this paper are X62475, X82078 and X76707 for pCpA1, pCpnE1 and pMoPn, respectively.

C. psittaci (Richmond *et al.*, 1982). Following these early reports, there have been many studies describing the molecular characterization of chlamydial plasmids and the chlamydia phage, which have been reviewed by Lusher *et al.* (1991). The plasmid and the phage are unrelated and there is no sequence homology between them (Storey *et al.*, 1989).

Several complete plasmid sequences have been published for human *C. trachomatis* isolates, from serovars B (Sriprakash & MacAvoy, 1987), L2 (Comanducci *et al.*, 1988; Black *et al.*, 1989), L1 (Hatt *et al.*, 1988; Thomas & Clarke, 1992) and D (Comanducci *et al.*, 1990). This work showed that all plasmids from human *C. trachomatis* isolates are highly conserved (less than 1% nucleotide sequence variation), almost uniform in size at 7500 nucleotides and encode eight significant ORFs greater than 100 amino acids. Database searches have indicated putative functions for five of the eight ORFs, in addition ORF 5 encodes a protein of 28 kDa (pgp3) which has been expressed in *Escherichia coli* (Comanducci *et al.*, 1993). This protein may be a marker for current chlamydial infection and can be clearly identified by staining inclusions in chlamydia-infected cells (Comanducci *et al.*, 1994). However, the possible role of pgp3 in the chlamydial life-cycle and the overall biological function of the plasmid remain unknown.

Characterization of plasmid functions and of chlamydial genes in general has been greatly impeded by the lack of a genetic system for *Chlamydia*. Electron microscopical examination of plasmid DNA (Tam *et al.*, 1992) has shown that the plasmid origin of replication is located close to the 22 bp tandem repeats and that replication is unidirectional. Since the plasmid of *C. trachomatis* may be under positive selection and is found in most isolates of this species, it appears the most promising candidate for vector development. The cryptic plasmid has been used for the construction of a preliminary chlamydial 'vector' using a promoterless chloramphenicol acetyltransferase cassette inserted into ORF 1 (Tam *et al.*, 1994). However, transformation was inefficient and transformants were unstable, surviving a maximum of only four passages in cell culture.

The principal difficulty of using the cryptic plasmid as a cloning vehicle is in identifying non-essential parts of the sequence that may be replaced or modified during vector construction. It is clear that the plasmid is not essential for chlamydial growth as one strain of *C. trachomatis* (Peterson *et al.*, 1990) and possibly some clinical isolates have been described that are plasmid-free (An *et al.*, 1992). In addition, several *C. psittaci* (Lusher *et al.*, 1991) and all human *C. pneumoniae* (Campbell *et al.*, 1987; Lusher *et al.*, 1989) isolates so far analysed do not have the cryptic 7.5 kb plasmid. The purpose of this work was to define the extent of natural variation between potentially diverse plasmids from three chlamydial species and thereby identify non-essential regions of the plasmid. We have chosen plasmids from the avian (duck) strain N352 of *C. psittaci*, the equine isolate N16 which is the only plasmid-carrying strain of *C. pneu-*

moniae (Storey *et al.*, 1993; Black *et al.*, 1994) and the plasmid from the mouse pneumonitis (MoPn) strain of *C. trachomatis* (Nigg, 1942). In Southern blotting experiments this latter plasmid has been reported not to share homology with the other *C. trachomatis* plasmids and to be several hundred bases larger (Palmer & Falkow, 1986). The complete nucleotide sequences of these three plasmids were determined and compared with the sequence of the serovar L1 plasmid, which is representative of the human *C. trachomatis* plasmids.

METHODS

Growth of chlamydiae and purification of EBs. The chlamydial strains employed in this study and the designations of the native plasmids are shown in Table 1. Chlamydiae were propagated in BGMK cell monolayers in plastic culture flasks (Flow Laboratories) in the presence of cyclohexamide. The host cells were grown in Dulbecco's modified essential medium (Flow Laboratories), supplemented with 10% (v/v) foetal calf serum, 1% non-essential amino acids and 20 µg gentamicin ml⁻¹ (Sigma). EBs were prepared by centrifugation through 32% (v/v) Urografin 370 (Schering) or through 25% (w/v) Triosil 440 (Nycomed).

Preparation and cloning of plasmid DNA. Plasmid DNA was prepared from purified *C. psittaci* and *C. pneumoniae* EBs by the method of Birnboim & Doly (1979), except that the lysozyme treatment was omitted. Plasmid DNA preparations were digested with *EcoRI*. Four *EcoRI* restriction fragments of 6.2, 0.6, 0.4 and 0.2 kb were obtained for pCpA1 and five *EcoRI* restriction fragments of 4.2, 1.3, 1.2, 0.4 and 0.3 kb were obtained for pCpnE1. These fragments were ligated into pUC vector DNA cleaved with *EcoRI* and used to transform *E. coli* JM83. Additionally pCpA1 and pCpnE1 were cloned via their unique *BalI* restriction sites into *SmaI*-cleaved pUC9.

Total DNA was extracted and purified from *C. trachomatis* MoPn EBs by incubation at 37 °C with 1.4% (w/v) Sarkosyl (Sigma) and 200 ng Proteinase K ml⁻¹ followed by sequential extraction with (i) phenol, (ii) 50% (v/v) phenol/chloroform and (iii) chloroform. This DNA preparation was digested with *EcoRI* and ligated into lambda Ziplox *EcoRI* arms (D'Alessio *et al.*, 1992). The bacteriophage vector was assembled by an *in vitro* packaging system (Life Technologies) and mixed with *E. coli* strain Y1090 (ZL) in Luria-Bertani broth (LB) containing 0.2% (w/v) maltose. The resulting phage library was screened by standard hybridization techniques (Sambrook *et al.*, 1989) using the complete cryptic plasmid from *C. trachomatis* serovar L1 (pLGV/440) as a probe (Thomas & Clarke, 1992).

For preparation of sequencing templates, the required recombinants were grown in liquid culture and plasmid DNA isolated by standard alkaline lysis followed by purification with either CsCl/ethidium bromide gradient centrifugation (Sambrook *et al.*, 1989) or by nucleic acid chromatography system (NACS) from Life Technologies.

Nucleotide sequence determination. Purified recombinant plasmid DNA from pCpA1 was used either directly for sequencing reactions (Hattori & Sakaki, 1986) or purified insert DNA was subcloned into M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985) after partial digestion with restriction enzymes: *AluI*, *BglII*, *DraI*, *HindIII*, *Sau3A* or *TaqI*. Sequence determination of pCpA1 was by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977), using M13 universal primers or synthetic oligonucleotide

Table 1. Chlamydial strains and plasmids

Species	Host	Strain	Plasmid	Reference
<i>C. trachomatis</i>	Human	L1/440/LN	pLGV440	Schachter & Meyer (1969)
<i>C. trachomatis</i> *	Murine	MoPn	pMoPn	Nigg (1942)
<i>C. psittaci</i>	Avian	N352	pCpA1	Richmond <i>et al.</i> (1982)
<i>C. pneumoniae</i>	Equine	N16	pCpnE1	Wills <i>et al.</i> (1990)

* *C. trachomatis* (mouse pneumonitis).

primers. Sequencing reactions involved incorporation of [³⁵S]dATPαS followed by resolution on 6% polyacrylamide buffer gradient gels and the sequence was visualized by autoradiography. Using the cloned restriction fragments as templates, the pCpnE1 and the pMoPn nucleotide sequences were determined on 6% polyacrylamide gels using a model 373A automated DNA sequencer (Applied Biosystems) with fluorescently labelled dideoxynucleotide terminators. Standard *Taq* cycle sequencing was performed on double-stranded DNA purified from recombinant clones. For each plasmid the complete nucleotide sequence of both strands was determined and the sequence data assembled and analysed using PCGENE and DNASTAR software.

RESULTS AND DISCUSSION

Molecular cloning and sequencing of the chlamydial plasmids

Plasmids are widely distributed throughout the genus *Chlamydia* and have been successfully cloned and characterized from a broad range of isolates (Girjes *et al.*, 1988; Hugall *et al.*, 1989; Joseph *et al.*, 1986; Kahane & Sarov, 1986; McClenaghan *et al.*, 1988; Lusher *et al.*, 1989; Palmer & Falkow, 1986; Timms *et al.*, 1988). However, complete nucleotide sequences have only been determined for plasmids from human *C. trachomatis* biovars, limiting direct comparison between plasmids from different chlamydial species to restriction enzyme profiling and Southern blot analysis. Therefore the cloning and sequencing of three novel and potentially divergent plasmids will help to define the degree of natural variation between plasmids and to identify important regulatory and replication elements.

The plasmid from *C. trachomatis* mouse pneumonitis biovar (pMoPn) was cloned as three *Eco*RI fragments (3.7, 2.0 and 1.8 kb) into the bacteriophage vector lambda Ziplox. DNA preparations of pCpA1 and pCpnE1 were purified and cloned directly into a plasmid vector (pUC) as *Eco*RI fragments and additionally as a 'full-length' ~7.5 kb *Bal*I fragment. A set of uni-directional nested deletions were also constructed using an Erase-a-Base system (Promega) to determine the sequence of one strand of the largest 4.2 kb *Eco*RI fragment of pCpnE1. Synthetic oligonucleotide primers were used to fill in gaps remaining in the sequence and to confirm the sequence spanning the cloning junctions by sequencing from full-length plasmid DNA template. As pMoPn was not available as a full-length clone for this purpose, the sequence of each cloning junction was

determined directly from PCR products amplified from native plasmid template spanning each junction.

Analysis showed the plasmid sequences varied in size from 7362 bp (pCpnE1) to 7553 bp (pCpA1). Contrary to a previous report (Palmer & Falkow, 1986), we found the 7502 bp MoPn plasmid is very similar in length to the highly conserved human *C. trachomatis* plasmids. With the exception of several incomplete recombinant *C. psittaci* avian plasmid clones, all chlamydial plasmids characterized by restriction profiling are also around 7.5 kb (Girjes *et al.*, 1988; Hugall *et al.*, 1989; Joseph *et al.*, 1986; McClenaghan *et al.*, 1988; Lusher *et al.*, 1989; Palmer & Falkow, 1986; Timms *et al.*, 1988). This remarkable size conservation suggests there may be some physical constraint on plasmid size.

Plasmid organization and putative function

It was not possible to identify a unique restriction site that is conserved in all plasmids for numbering the sequences (Lusher *et al.*, 1989), however the 22 bp tandem repeats are the most highly conserved region common to all chlamydial plasmids. Therefore the three plasmid sequences determined in this study, as well as the revised pLGV/440 sequence (Thomas & Clarke, 1992), are numbered from the start of these tandem repeats. The computer-generated ORFs are numbered so that ORF 1 follows directly after the origin of replication.

The genomic organization of each plasmid is strikingly conserved, with eight major ORFs identified greater than 100 amino acids and short non-coding regions between some but not all ORFs (Fig. 1). Both strands of the plasmid contain coding sequences and in every case ORF 2 is located on the complementary strand. Differences in the previously published genetic maps of some human *C. trachomatis* plasmids may be explained by minor errors in sequencing that alter the apparent initiation or termination codon of the predicted ORFs (Thomas & Clarke, 1992). The computer-predicted initiation codon for each ORF is also conserved between plasmids: ATG for ORFs 1, 3, 4, 5 and 6, and GTG for ORFs 7/8. There is no consensus ATG codon between the plasmid sequences for ORFs 7/8, however the position of GTG as potential translation initiation codon is conserved and under some circumstances tRNA^{f-met} will bind and initiate synthesis at GUG as well as AUG codons on bacterial mRNA. Until N-

Fig. 1. Alignment of chlamydial plasmid genome maps showing pLGV/440, pMoPn, pCpnE1 and pCpA1. For ease of comparison the maps have been linearized and start with the direct repeats (black box) at nucleotide number 1. Only the eight major ORFs are shown, with the ORF number indicated in each box. The boxes representing ORFs starting with a GTG initiation codon are shaded. All ORFs are transcribed from the same strand, except ORF 2, where the direction of transcription is indicated by an arrow.

Table 2. Translation products of chlamydial plasmid ORFs

ORF no.	No. of amino acids			
	pLGV440	pMoPn	pCpnE1	pCpA1
1	305	305	83(A), 102(B)*	307
2	332	330	334	335
3	451	451	456	458
4	354	353	345	347
5	264	264	267	264
6	102	102	101	102
7	243	254	261	223
8	247	246	256	246

* A and B refer to ORFs 1A and 1B, respectively, in pCpnE1.

terminal sequences are obtained for the polypeptide products of the computer-predicted ORFs the actual initiation codons will remain unknown. The initiation codon for ORF 2 is ATG for all human *C. trachomatis* plasmids; by sequence comparison we have predicted GTG as the initiation codon for ORF2 from pMoPn, pCpA1 and pCpnE1. Although earlier computer-predicted translation initiation sites within the same reading frame were possible for the pCpnE1 ORF 2 and ORFs 1, 4 and 6 of pCpA1, these were excluded from our predictions on the grounds of sequence alignment with the corresponding ORF from other chlamydial plasmids and the lack of a clearly distinguishable ribosome-binding site.

The sizes of the chlamydial plasmid ORFs are summarized and compared in Table 2. The most striking

feature apparent from sequence comparisons is variation in the length of ORF 1. In the human L2 plasmid sequence, ORF 1 terminates after 260 amino acids (Comanducci *et al.*, 1988), while this ORF is between 305 and 307 amino acids for the avian and all other *C. trachomatis* plasmids. The truncation of ORF 1 in serovar L2 is caused by a single nucleotide deletion which makes a reading frame shift. ORF 1 in the equine plasmid is significantly different and is divided into two smaller ORFs (designated ORFs 1A and 1B) around 200 nucleotides apart (Fig. 2). ORF 1B displays continuous homology with the last 100 amino acids of the full-length ORF 1 sequence. However, when aligned with the pCpA1 ORF 1 sequence, ORF 1A is split into two homologous regions separated by a gap of around 150 nucleotides. This gap represents a deletion that accounts for the reduced size of the equine plasmid compared to other chlamydial plasmids. To guard against the possibility that this deletion in ORF 1 of pCpnE1 was an artefact of cloning, its presence in the native plasmid was confirmed by PCR. *C. pneumoniae* N16 EB was used as template with a primer pair that straddles the deletion. The PCR product generated from the native pCpnE1 was the same size as that from the cloned pCpnE1 (data not shown). The PCR products from native pCpnE1 were sequenced confirming the deletion and two stop codons identified in cloned pCpnE1 ORF1.

All chlamydial plasmids have four 22 bp tandem repeats in the intergenic region between ORFs 8 and 1. In plasmids from other bacterial species, tandem repeats or iterons are involved in plasmid replication, control of copy number and incompatibility (Novick, 1987). The tandem repeats have now been sequenced from several different chlamydial plasmids and the importance of this region for plasmid maintenance is reflected in the degree of sequence conservation between all chlamydial plasmids. Generally the region upstream of the tandem repeats is more AT-rich than the remainder of the plasmid and this region of the *C. trachomatis* human and MoPn plasmid contains a cluster of AT repeats. The avian and equine plasmids also have markedly AT-rich regions but these are not present as AT repeats. All *C. psittaci* plasmids have identical repeat sequences including those from avian (Hugall *et al.*, 1989; this study), feline (Lusher *et al.*, 1991) and guinea-pig inclusion conjunctivitis (GPIC) (Lusher *et al.*, 1991) isolates. This 22 bp sequence is conserved in the equine *C. pneumoniae* plasmid and in the plasmid from the koala urogenital strain. The repeats are also identical to those from the plasmid of the sporadic bovine encephalomyelitis isolate of *Chlamydia* which is the type strain of *C. pecorum* (Hugall *et al.*, 1989). Among human *C. trachomatis* plasmids, the repeats are identical but show two nucleotide substitutions from the conserved *C. psittaci* repeat sequence, with the MoPn plasmid differing by a further novel base substitution (Fig. 3).

The functions of some putative plasmid ORFs have been inferred from database searches to identify homology with proteins of known function. The most extensive

Fig. 4. Dendrogram based on alignment of full-length chlamydial plasmid sequences. The phylogenies are rooted assuming a biological clock, with the sequence conservation (%) between plasmids shown in parentheses.

now been identified in three *C. trachomatis* LGV isolates (Fahr *et al.*, 1992; Ricci *et al.*, 1993; Thomas & Clark, 1994) and in the MoPn biovar (data not shown) and may be a common regulatory element in all chlamydial plasmids.

Phylogenetic analysis

Several different chlamydial plasmid 'types' have been identified by restriction mapping (Lusher *et al.*, 1989), and computer-derived restriction maps from each of the plasmid sequences determined in this study were all significantly different from each other (data not shown). However, alignments using the Jotun-Hind program (Hein, 1990) demonstrated that with at least 60% inter-species DNA sequence identity, chlamydial plasmids are more closely related than the corresponding chromosomal DNA. The MoPn and L1 plasmid sequences (80% identity) and the pCpA1 and pCpnE1 sequences (69% identity) are more closely related to each other than the avian *C. psittaci* and the *C. trachomatis* L1 plasmids (60% identity). This explains why extensive cross-hybridization between *C. psittaci* and the *C. trachomatis* plasmids has only been observed under conditions of low stringency (Joseph *et al.*, 1986; Hugall *et al.*, 1989).

A phylogenetic tree based on an alignment of all available full-length plasmid sequences suggests that the MoPn plasmid is more closely related to human *C. trachomatis* plasmids than to those of other chlamydial species, while equine and avian plasmids are more closely related to each other (Fig. 4). These findings are consistent with other phylogenetic studies based on total genomic DNA and major outer-membrane protein sequences (Carter *et al.*, 1991; Fitch *et al.*, 1993; Kaltenboeck *et al.*, 1993).

Determination of three novel chlamydial plasmid sequences has revealed extensive sequence conservation between them suggesting that the plasmids have arisen by evolution from a common ancestor. The similarity in size and genomic organization strongly suggests that the mechanisms for plasmid maintenance and general

properties are shared. Although sequence analysis did not identify an overall plasmid function, and the existence of plasmid-free strains shows that it is not essential for chlamydial growth, the plasmid could influence the growth or virulence characteristics of its host strain (Comanducci *et al.*, 1994), as bacterial pathogenicity factors are often encoded by plasmids (Eberhard, 1989).

The plasmid genomic organization and the presence of tandem repeats and countertranscripts provide evidence for involvement of both iteron-binding and inhibitor targeting mechanisms in the regulation of plasmid replication. Possibly a combination of both mechanisms could be involved, which has been previously observed for the *E. coli* plasmid R6K (Patel & Bastia, 1987).

Molecular research into this important intracellular pathogen has been severely hindered by the lack of a suitable gene transfer system. Recently, transformation of chlamydial EBs has been described (Tam *et al.*, 1994) and the cryptic plasmid is the ideal vector to exploit this advance. To date, the highly conserved and compact nature of the plasmid genome, with little non-coding sequence, has precluded the identification of a suitable cloning site for rational vector development. Comparative plasmid sequence studies have now proven useful in identifying a potential region for the insertion of foreign DNA. Future studies will concentrate on the development of the equine plasmid as a vector for the transformation of chlamydiae.

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