Plasmid Diversity within the Genus *Chlamydia*

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Examination of 12 *Chlamydia psittaci* strains recovered from nine different host species (three avian and six mammalian) revealed the presence of a 7.5 kb plasmid in all isolates except two ovine abortion strains, the human strain IOL207 and the Cal 10 strain. Restriction mapping analysis distinguished four different plasmids that were associated with avian, feline, equine and guinea-pig *C. psittaci* isolates, respectively. The restriction maps of these four *C. psittaci* plasmid types all differed from that of the plasmid recovered from *C. trachomatis* L2/434. Despite this plasmid diversity, which is likely to be of taxonomic importance, all four plasmids identified within the species *C. psittaci* were found to share some sequence homology, which was mapped to two separate regions in the plasmid molecules. One region, which showed a high degree of homology between *C. psittaci* plasmids and also detectable homology with the *C. trachomatis* plasmid, may represent a common replication control region for plasmids of this genus.

INTRODUCTION

The genus *Chlamydia* contains two species, *Chlamydia trachomatis* and *Chlamydia psittaci*. With the exception of a single murine strain (Nigg, 1942), the species *C. trachomatis* is exclusively a human pathogen. The two human biovars include 15 serotypes which all contain a cryptic 7.5 kb plasmid (Palmer & Falkow, 1986). Restriction mapping of plasmids from these 15 serotypes (Palmer & Falkow, 1986) and direct DNA sequence analysis of plasmids from serotypes B (Sriprakash & MacAvoy, 1987), L1 (Hatt *et al.*, 1988) and L2 (Comanducci *et al.*, 1988) have shown this plasmid to be highly conserved within the species *C. trachomatis*.

The species *C. psittaci* on the other hand comprises a diverse group of chlamydial strains which infect a wide range of avian and mammalian hosts. Attempts to recognize subdivisions within this species have been made by plaque neutralization (Schachter *et al.*, 1974), inclusion morphology (Spears & Storz, 1979), microimmunofluorescence (Perez-Martinez & Storz, 1985; Wang & Grayston, 1971), restriction endonuclease analysis of chlamydial chromosomal DNA (McClenaghan *et al.*, 1984; Timms *et al.*, 1988) and monoclonal antibody reactivity (Fukushi *et al.*, 1987). Plasmids have been described in some, but not all, strains of *C. psittaci*; a 6.2 kb plasmid obtained from a Cal 10 strain was described by Joseph *et al.* (1986) and McClenaghan *et al.* (1988) characterized a fragment of plasmid present in all avian isolates they examined. Homology with plasmid DNA from *C. trachomatis* (Joseph *et al.*, 1986) and other *C. psittaci* strains (McClenaghan *et al.*, 1988) was reported. However, the latter workers were unable to detect a plasmid in their Cal 10 strain or in either ovine abortion or ovine arthritis isolates. Plasmids also appeared to be absent from TWAR strains described by Campbell *et al.* (1987).

In the present study cloned *C. psittaci* plasmids from a wide range of avian and mammalian hosts were characterized by restriction mapping and Southern-blot hybridization. Plasmid

Abbreviations: EB, elementary body; LMTA, low-melting-temperature agarose.

diversity was demonstrated in *C. psittaci* recovered from different host sources which may be of taxonomic value both to distinguish avian from mammalian *C. psittaci* and for subgrouping mammalian members of this diverse species.

METHODS

Chlamydial strains. Chlamydiae studied are listed and their origins referenced in Table 1. N352, one of the two *C. psittaci* isolates from domestic ducks, contains phage (Richmond *et al.*, 1982) but this was not detected serologically (Bacon *et al.*, 1986) or by DNA hybridization in the other duck strain, 360 (C. C. Storey, unpublished). Strains 6BC, RA1/56, GPIC, Cal 10, IOL207 and L2/434 were kindly donated by, respectively, Dr T. Hatch, University of Tennessee, Memphis, USA; Dr J. Strauss, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia; Dr J. Pearce, University of Birmingham, Birmingham, UK; Dr P. Wyrick, University of North Carolina at Chapel Hill, USA; Professor S. Darougar, Institute of Ophthalmology, London, UK; and Professor J. Schachter, University of California, San Francisco, USA. Strain N16 is a recent equine isolate obtained by Dr J. Wills, University of Bristol, UK. Dr Wills also supplied the ovine and feline isolates.

Culture and purification of chlamydial elementary bodies. Elementary bodies (EBs) of each strain of *Chlamydia* were prepared from heavily infected cell monolayers grown in 25 cm² or 75 cm² (basal area) plastic flasks in the presence of cycloheximide (Richmond *et al.*, 1985). Strains IOL207 and GPIC were grown in BGM cells; all other chlamydiae were propagated in McCoy cells. EBs were purified by centrifugation through 32% (v/v) Urografin 370 (Schering Chemicals) (Kuo *et al.*, 1977).

Preparation and cloning of chlamydial plasmid DNA. Plasmid DNA was prepared from purified EBs by the method of Birnboim & Doly (1979), except that lysozyme treatment was omitted. Plasmid DNA for use as hybridization probes was further purified by electrophoresis through low-melting-temperature agarose (LMTA) gel (Feinberg & Vogelstein, 1984); otherwise purified plasmid was used directly for cloning. All plasmid DNA preparations were analysed by agarose gel electrophoresis (Maniatis *et al.*, 1982).

Cloned plasmid DNA was prepared by standard techniques (Maniatis *et al.* 1982). Briefly, plasmid DNA preparations were digested with *Eco*RI or *Bal*I and ligated to pUC9 plasmid vector DNA that had been cleaved with *Eco*RI or *Sma*I and subsequently dephosphorylated (Yanisch-Perron *et al.*, 1985). Ligated DNA was used to transform *Escherichia coli* JM83 by the method of Kushner (1978). Transformed colonies were screened by colony blot hybridization (Grunstein & Hogness, 1975) using radiolabelled LMTA-purified native chlamydial plasmid as a probe. Recombinants were amplified in liquid culture prior to DNA isolation and purification by standard alkaline-lysis and CsCl/ethidium bromide gradient centrifugation (Maniatis *et al.*, 1982).

Restriction endonuclease site mapping. Restriction endonuclease cleavage site maps of cloned plasmid insert DNA were constructed by analysis of the products of single and mixed enzyme digests on agarose electrophoresis gels (Maniatis *et al.*, 1982). All restriction endonuclease and other enzyme incubations were performed under the conditions recommended by the manufacturer (Boehringer Mannheim, Pharmacia or BRL).

Hybridization conditions. Southern transfer and hybridization were carried out as described by Maniatis *et al.* (1982). LMTA-purified preparations of native or recombinant chlamydial insert DNA were radiolabelled to a

Table 1. *Plasmids identified in the genus Chlamydia*

Host species	Strain	Plasmid detected	Plasmid designation	Reference
<i>C. psittaci</i>				
Avian				
Duck	N352	+	pCpA1	Richmond <i>et al.</i> (1982)
Parakeet	6BC	+	pCpA2	Morgan & Wiseman (1946)
Duck	360	+	pCpA3	Bacon <i>et al.</i> (1986)
Black-headed gull	RA1/56	+	pCpA4	Strauss <i>et al.</i> (1957)
Mammalian				
Cat	Pring	+	pCpF1	Wills <i>et al.</i> (1984)
Cat	Cello	+	pCpF2	Cello (1967)
Horse	N16	+	pCpE1	
Guinea-pig	GPIC	+	pCpGP1	Murray (1964)
Human/ferret	Cal 10	—	—	Francis & Magill (1938)
Human	IOL207	—	—	Dwyer <i>et al.</i> (1972)
Ovine	A22	—	—	Stamp <i>et al.</i> (1950)
Ovine	S26	—	—	McClenaghan <i>et al.</i> (1984)
<i>C. trachomatis</i>				
Human	L2/434	+	pCtL2	Kuo <i>et al.</i> (1977)

specific activity of $1\text{--}2 \times 10^9$ c.p.m. μg^{-1} by the random-primed oligolabelling procedure of Feinberg & Vogelstein (1984). DNA that had been transferred to nylon filters (Hybond N; Amersham) was hybridized with radiolabelled probe overnight at 65°C then washed twice at 65°C in $2 \times \text{SSC}$ (SSC consisted of $0\cdot15$ M-NaCl, $0\cdot15$ M-trisodium citrate, pH 7.0). Filters were washed in $2 \times \text{SSC}$ and $0\cdot1\%$ SDS (low-stringency wash) or $0\cdot1 \times \text{SSC}$ and $0\cdot1\%$ SDS (high-stringency wash) at 65°C , and then exposed overnight to Fuji RX-G film.

RESULTS AND DISCUSSION

Purified EBs prepared from 12 *C. psittaci* isolates and the *C. trachomatis* L2 strain were used to prepare chlamydial plasmid DNA. Faint bands of extrachromosomal DNA were seen following agarose gel electrophoresis of preparations from four avian, two feline, the GPIC strain and an equine isolate of *C. psittaci*, as well as the *C. trachomatis* L2 serotype (data not shown). Because yields of native plasmid DNA were small [approximately $10\text{--}100$ ng DNA (10^9 inclusion-forming units) $^{-1}$], molecular cloning of these plasmids was undertaken to facilitate further analyses. Initial restriction digests of native plasmid DNA showed that all plasmids identified in this study possessed single or multiple *EcoRI* sites. Accordingly, *EcoRI*-restricted plasmid DNA was ligated to *EcoRI*-digested pUC9 vector DNA and used to transform *E. coli* JM83 cells. Recombinant colonies were screened by colony blot hybridization using radiolabelled LMTA-purified plasmid DNA as a probe. Positive clones were used to prepare chlamydial insert DNA.

Digestion of *C. psittaci* plasmids with *EcoRI* revealed a considerable diversity of plasmids from different host species. Thus, the four avian *C. psittaci* plasmids (pCpA1–4) each yielded recombinant *EcoRI* fragments of 6.2, 0.62, 0.47 and 0.2 kb (Fig. 1a, lane 2), while both feline plasmids (pCpF1 and 2) demonstrated a single *EcoRI* fragment of 7.5 kb (Fig. 1a, lane 3). The equine plasmid (pCpE1) generated six *EcoRI* fragments of 4.1, 1.3, 1.2, 0.4, 0.3 and 0.15 kb (Fig. 1a, lane 4) and the guinea-pig plasmid (pCpGP1) was distinguished by four *EcoRI* fragments of 5, 1.5, 0.7 and 0.25 kb (Fig. 1a, lane 5). The *EcoRI* restriction profile of all four *C. psittaci* plasmid types differed from that obtained from the *C. trachomatis* plasmid clones, which showed *EcoRI* fragments of 4.5, 2.5 and 0.4 kb (Fig. 1a, lane 6).

Fig. 1. (a) Ethidium-bromide-stained agarose (1%, w/v) gel electrophoresis and (b) Southern blot hybridization of cloned *EcoRI* fragments of plasmid DNA loaded in equimolar concentrations. The Southern blot was probed with radiolabelled cloned 7.5 kb feline plasmid insert DNA and the filter was washed at low stringency. Lanes: 2, pCpA1; 3, pCpF1; 4, pCpE1; 5, pCpGP1; 6, *C. trachomatis* L2; 1 and 7, marker DNA (1 kb ladder; BRL). The arrowhead indicates the position of the 0.25 kb *EcoRI* fragment of pCpGP1.

As restriction endonuclease site mapping of the cloned *EcoRI* fragments revealed all chlamydial plasmids to have a unique *BalI* site, subsequent cloning experiments featured ligation of *BalI*-linearized plasmids to *SmaI*-digested pUC9. Recombinant *BalI* clones and *EcoRI*-cloned insert DNA were used to prepare restriction endonuclease cleavage maps of the identified plasmids (Fig. 2). Despite the diversity of the characterized plasmids, their overall size was similar (approximately 7.5 kb). Our restriction map of the *C. trachomatis* L2 plasmid showed good agreement with that predicted from the recently published nucleotide sequence of the L2 plasmid (Comanducci *et al.*, 1988).

The four avian *C. psittaci* strains, which include two recent isolates from domestic ducks and two strains isolated more than 30 years ago (Table 1), contained closely related plasmids. Southern blots showed all four cloned *EcoRI* fragments from the different avian plasmids to cross-hybridize with each other at high stringency. There was no apparent homology, however, between this plasmid and the phage present in N352 (Storey *et al.*, 1988). The 6.2 kb *EcoRI* fragments of the four plasmids showed conserved restriction sites for the enzymes *HpaII*, *HaeIII* and *XbaI* in addition to those shown for pCpA1 (Fig. 2). No cleavage of these fragments was obtained with the enzymes *ApaI*, *AvaII*, *BamHI*, *BclI*, *BglI*, *HpaI*, *HaeII*, *KpnI*, *PstI*, *PvuI*, *PvuII*, *SalI*, *Sau96I*, *SmaI*, *SphI*, *SstI* and *XhoI*. The restriction map of the 6.2 kb *EcoRI* fragment of the pCpA plasmids was similar, but not identical, to the 6.2 kb plasmid from Cal 10 described by Joseph *et al.* (1986). The 5.6 kb *HindIII* fragment of the pCpA plasmids also shared similarities in restriction sites with the 5.9 kb *HindIII* fragment of plasmid pAV-1 isolated from a cockatiel strain of *C. psittaci* by McClenaghan *et al.* (1988), which suggests that these plasmids are related. The latter group of workers also detected pAV-1-related sequences in Southern blots of plasmid DNA from 13 other avian *C. psittaci* isolates. These observations are consistent with the presence of a common plasmid conserved within avian strains of *C. psittaci*. This plasmid may, therefore, provide a marker which distinguishes avian *C. psittaci* from mammalian strains.

The feline, equine and guinea-pig strains of *C. psittaci* each contained a distinct, hitherto uncharacterized plasmid. The plasmids pCpF1 and pCpF2 recovered from the two feline *C. psittaci* strains were found to be highly homologous by Southern blot hybridization analysis of restricted plasmid DNA (data not shown). Both plasmids had identical arrays of sites for the enzymes *EcoRV*, *HaeII*, *HpaI*, *HpaII* and *XmnI* in addition to those shown in Fig. 2, while they lacked sites for *ApaI*, *AvaII*, *BclI*, *BglI*, *KpnI*, *MspI*, *NciI*, *PstI*, *PvuI*, *SmaI* and *XhoI*. The close similarity of the plasmids isolated from these two feline *C. psittaci* strains [one of which was isolated in the USA (Cello, 1967) and the other in the UK in 1980 (Wills *et al.*, 1984)] suggests that this plasmid is highly conserved within feline *C. psittaci* strains. The three different chlamydial plasmids we have identified in feline, equine and guinea-pig strains of *C. psittaci* may provide useful taxonomic markers for the three distinct mammalian *C. psittaci* strains in which they were found.

Plasmids were not detected in DNA preparations from the four remaining *C. psittaci* strains analysed by agarose gel electrophoresis or Southern blots probed with the five plasmids characterized in this work. Despite the previous isolation of a plasmid from Cal 10 reported by Joseph *et al.* (1986), neither we nor other groups (McClenaghan *et al.*, 1988; Dr Ian Clarke, personal communication) have been able to detect plasmids in our respective Cal 10 strains. The absence of a plasmid in the two ovine abortion strains (A22 and S26) is in agreement with the observations of McClenaghan *et al.* (1988) and the lack of a plasmid in the human *C. psittaci* isolate IOL207 reinforces the belief in the similarity between this organism and the TWAR strain described by Campbell *et al.* (1987).

Despite the distinct restriction maps of the four *C. psittaci* plasmids, Southern blots of cloned *EcoRI* fragments probed reciprocally with each of the plasmids showed that they shared partial DNA homology (Fig. 1*b*). Additional Southern blot analysis of homologous *EcoRI* fragments after digestion with appropriate restriction enzymes showed the hybridization to be confined to two separate regions present in each of the identified plasmids (Fig. 2). One region of low sequence homology (approximately 1.5–2.4 kb long) consisted of a cluster of *HindIII/XmnI/EcoRI* restriction fragments of plasmid DNA which cross-hybridized under conditions of low stringency (region 1, Fig. 2). A second region, of high homology (which under the high-

stringency conditions used corresponded to a minimum sequence of approximately 200 bp), was located within a 0.75 kb *Cla*I fragment in the avian plasmid, a 0.7 kb *Pst*I/*Cla*I fragment in the GPIC plasmid, a 1 kb *Sst*I/*Bal*I fragment in the feline plasmid and a 0.45 kb *Hind*III fragment in the equine plasmid (region 2, Fig. 2). This region also hybridized under conditions of low stringency with a 200 bp *Xmn*I fragment of the *C. trachomatis* plasmid. Analysis of the nucleotide sequence of the equivalent *Xmn*I fragment of the *C. trachomatis* L2 plasmid

determined by Comanducci *et al.* (1988) revealed that it consisted of an intergenic region of sequence which included the putative origin of replication for the plasmid. This region of the L2 plasmid was characterized by four tandem repeat sequences, 22 bp long, each of which contained a central *AccI* site. Our observation that homology region 2 in *C. psittaci* plasmids also contains a conserved *AccI* site (Fig. 2) further suggests that this region includes the origins of replication. Despite the plasmid diversity revealed by restriction enzyme analysis in this work, the origin of replication of all *Chlamydia* plasmids may be similar.

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