Distribution of Plasmid Sequences in Avian and Mammalian Strains of *Chlamydia psittaci*

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Plasmid DNA from an avian strain of *Chlamydia psittaci* was purified and estimated to be 7.9 kb in size using restriction endonuclease analysis. A 5.9 kb fragment of this plasmid was cloned, mapped and used to screen a range of chlamydial strains. Hybridizing DNA was absent from ovine abortion and arthritis isolates and also from the Cal 10 strain but related sequences were detected in *C. psittaci* strains of feline pneumonitis, guinea-pig inclusion conjunctivitis, ovine conjunctivitis and *C. trachomatis* serovar L2. The plasmid DNA from the feline strain was shown to have a distinct restriction endonuclease profile. Similar plasmid sequences were detected in all avian isolates tested: thus the clone may have a useful diagnostic role for the detection of the pathogen in its natural host and in zoonotic episodes.

INTRODUCTION

The obligate intracellular parasite *Chlamydia psittaci* infects a variety of animal hosts, including man, and causes a wide spectrum of disease. There are no well-established typing systems for this pathogen but recent studies using microimmunofluorescence (Perez-Martinez & Storz, 1985) and restriction endonuclease analysis of chlamydial DNA (McClenaghan *et al.*, 1984) suggest that the species comprises many subtypes. In agarose gel analysis of undigested *C. psittaci* DNA from various strains, plasmid-like bands were observed in avian isolates but not in ovine strains (McClenaghan *et al.*, 1984). Ethidium bromide staining in agarose gels is a relatively insensitive technique which would not detect very low copy numbers of plasmid or plasmid DNA integrated into the chromosome. It was therefore necessary to use hybridization as a more sensitive method of detection. Because of the difficulties in preparing workable amounts of *C. psittaci* plasmid, we cloned a plasmid fragment and used this in hybridization assays to screen a range of *C. psittaci* strains for the presence of similar sequences.

METHODS

Chlamydial strains. Some of the strains of *C. psittaci* used have been described previously (McClenaghan *et al.*, 1984). They include eight isolates associated with outbreaks of ovine abortion, and one strain from a lamb with polyarthritis. In addition, a further recent ovine abortion isolate, 84/501, and a strain isolated from a case of ovine conjunctivitis, CH 1284, were examined. Purified preparations of elementary bodies of strains from guinea-pig inclusion conjunctivitis and feline pneumonitis were kindly supplied by Dr J. H. Pearce, Department of Microbiology, University of Birmingham, UK, and Dr S. J. Richmond, Department of Bacteriology and Virology, University of Manchester, UK, respectively. The L2 serovar of *C. trachomatis* LGV was a gift from Dr I.

Abbreviation: CsCl/EB, caesium chloride/ethidium bromide.

Table 1. *Avian isolates of C. psittaci*

Isolate designation	Source/passage level	Gel track in Fig. 2
352	Duck/not known	
ISN 1021	Collared dove/7	1
ISN 1057	Collared dove/4	2
ISN 1316	Budgerigar/4	3
ISN 1536	Budgerigar/4	4
ISN 1670	Budgerigar/3	5
ISN 1671	Budgerigar/4	6
ISN 1528	Parrot/5	7
ISN 1529	Parrot/4	8
ISN 1585	Parrot/5	9
ISN 1263	Parakeet/6	10
ISN 1264	Parakeet/5	11
ISN 1812	Cockatoo/3	12
ISN 1813	Java sparrow/2	13
ISN 1814	Munia (finch)/3	14
ISN 1815	Finch (pin-tailed parrot)/2	15
ISN 725	Cockatiel/11 (plasmid donor strain)	16
ISN 741	Wood pigeon/11	17

Smith, Department of Bacteriology, University of Edinburgh, UK. The avian isolates included in this study are described in Table 1.

Culture and purification of elementary bodies. Methods used were described by McClenaghan *et al.* (1984). Briefly, chlamydial strains were cultured in BHK-21 cells treated with 5-iodo-2'-deoxyuridine, except for avian isolates ISN 1021 to 1815 listed in Table 1, which were cultured in L929 cells treated with emetine (Bevan & Bracewell, 1986). Elementary bodies were purified by centrifugation through 30% (v/v) Urografin (Schering), followed by density gradient centrifugation (30–60%, v/v Urografin). All avian strains were grown and purified in biohazard laboratories.

Preparation of chlamydial DNA. In most cases, total chlamydial DNA was extracted from purified elementary bodies by the method described by Wenman & Lovett (1982). However, with avian isolates, DNA was prepared from elementary bodies produced by the sucrose cushion method (McClenaghan *et al.*, 1984) or simply from infected cells.

Plasmid DNA was isolated by caesium chloride/ethidium bromide (CsCl/EB) density gradient centrifugation as described by Maniatis *et al.* (1982). The gradient fractions containing plasmid DNA were identified by agarose gel electrophoresis of small samples. Plasmid DNA for cloning was isolated by preparative gel electrophoresis; plasmid bands were excised from gels and the DNA was purified by electroelution, phenol extraction, ethanol precipitation and ion-exchange chromatography using an 'Elutip' mini-column (Schleicher and Schüll).

Gel analysis of DNA. Agarose gel analysis was done using standard techniques with either Tris/sodium phosphate buffer (36 mM-Tris, 30 mM-sodium dihydrogen phosphate, 1 mM-EDTA, pH 7.6) or Tris/acetic acid buffer (40 mM-Tris, 20 mM-sodium acetate, 1 mM-EDTA, pH 7.5) for the preparative experiments.

Continuous (7.5% w/v) polyacrylamide gels were used to analyse small DNA digest fragments. These gels were silver-stained by the method described by Herring *et al.* (1982) except that with this buffer system, the initial washing time in 10% (v/v) ethanol and 0.5% acetic acid was reduced to 10 min and the 0.01 M-silver nitrate wash to 20 min.

Restriction endonuclease digests were done under the conditions recommended by the manufacturers (BRL and Northumbria Biologicals).

Hybridization conditions. Southern transfer was by the method described by Maniatis *et al.* (1982), and dot blot filters were prepared by loading 0.1 µg amounts of alkaline-denatured DNA using a BRL 'Hybridot' manifold.

Purified preparations of plasmid DNA or the insert from clone pAV-1 were labelled by nick-translation to a specific activity of $1-2 \times 10^8$ c.p.m. µg⁻¹ (Rigby *et al.*, 1977). Filter hybridization was done by using the methods described by Maniatis *et al.* (1982); filters were then washed twice in $1 \times$ SSC (0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) with 0.1% (w/v) SDS for 15 min at room temperature, followed by two washes in $0.1 \times$ SSC in 0.1% (w/v) SDS at 42 °C.

Molecular cloning of chlamydial plasmid DNA. Cloning was by essentially standard methods (Maniatis *et al.*, 1982) using the pUC9 vector (Yanisch-Perron *et al.*, 1985) linearized at the *Hind*III site. Following dephosphorylation and ligation to *Hind*III-digested chlamydial plasmid DNA, ligated DNA was used to transform *Escherichia coli* strain JM83 made competent by the high-efficiency procedure of Hanahan *et al.* (1983).

Transformants were selected on ampicillin plates containing X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside) and IPTG (isopropyl β -D-thiogalactopyranoside), and screened by the method of Grunstein & Hogness (1975) using nick-translated chlamydial plasmid. Recombinant plasmid DNA was purified from the clone pAV-1 by the alkaline-lysis method (Maniatis *et al.*, 1982) followed by CsCl/EB density gradient centrifugation.

Restriction endonuclease site mapping. Mapping was achieved by conventional double digest techniques, and by using plasmid insert cleaved with *NciI* after end-labelling the *HindIII* sticky ends with the Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982). The large *NciI* fragment was purified by rate zonal density gradient centrifugation (Raafat El-Gewely & Helling, 1980) and mapped by partial digestion (Smith & Birnstein, 1976).

RESULTS

Agarose gel analysis of undigested DNA preparations from avian strains of *C. psittaci* derived from cockatiel, pigeon and duck sources all showed extrachromosomal plasmid bands. Analysis of the total undigested DNA of the cockatiel strain 725 on CsCl/EB density gradients confirmed that these bands consisted of covalently closed circular DNA, since they were found at a higher density than the chromosomal DNA. The constraints imposed by the containment facilities limited the size of avian cultures produced for analysis. Therefore only small amounts (10–50 ng) of purified plasmid were available for restriction endonuclease studies to identify a suitable cleavage site with which to clone plasmid sequences. The largest fragment identified was a *HindIII* fragment of approximately 6 kb. However, PAGE of *HindIII* digests revealed that the enzyme also produced four smaller fragments as shown in Fig. 1 (track 1). Accordingly, a *HindIII* digest was ligated to *HindIII*-linearized pUC9 and the mixture used to transform competent *E. coli* cells. Several colonies were identified by screening with nick-translated plasmid and examined by plasmid extraction.

One colony, designated pAV-1, was shown to have an insert of 5.9 kb and was selected for further investigation. After gel purification and labelling, this sequence was shown to hybridize to the extrachromosomal bands of parental strain DNA in Southern blotting experiments (Fig. 2, track 16). The insert was digested with a range of restriction endonucleases and analysed using PAGE. *NciI*, *HaeIII* and *EcoRI* were found to cut at one, two and three sites respectively, and were used for mapping by double digest and end-labelling techniques. The orientation of the insert in the pUC9 vector was established by comparing *EcoRI* digests of the intact recombinant plasmid and the insert. The resultant map is shown in Fig. 3. Fragment sizes were consistent with data previously obtained for intact chlamydial plasmid digests with *HaeIII* and *EcoRI* (unpublished data).

The cloned insert was used as a hybridization probe to attempt to find a suitable enzyme which would linearize the chlamydial plasmid and allow cloning of the complete molecule. Purified plasmid was cleaved in the presence of λ bacteriophage DNA as a control for complete digestion, and analysed by Southern blotting. No restriction sites were found for *BamHI*, *PstI*, *SmaI* or *XhoI* while *TaqI* cut at multiple sites and, as yet, no linearizing enzyme has been identified.

The size of the intact chlamydial plasmid was estimated to be 7.9 kb from the insert size (5.9 kb) plus the four small *HindIII* bands (Fig. 1). This was consistent with the mobility of a faint band present in undigested chlamydial DNA in agarose gels which we presume to be a linear form of plasmid.

The insert from clone pAV-1 was used to screen a variety of *C. psittaci* isolates for plasmid sequences in dot blot and Southern blot hybridization assays. Fig. 2 shows the results of screening 13 separate avian isolates and the parent strain 725. Total infected cell DNA was used, so there was little control over the amount of chlamydial DNA in each sample; nevertheless, all isolates showed hybridization with extrachromosomal bands of identical mobility. Weak hybridization observed at the level of chromosomal DNA may indicate a low frequency of integration but may also be due to physical entrapment by the large amount of DNA in this band. The analysis was then extended to include a range of mammalian isolates (Fig. 4) and hybridization was observed with feline pneumonitis, guinea-pig inclusion conjunctivitis and ovine ocular isolates, and also with DNA from *C. trachomatis* LGV L2 strain. No hybridization

Fig. 1. PAGE of *Hind*III digests of chlamydial plasmid DNA. Track 1, plasmid from avian strain 725; track 2, plasmid from feline pneumonitis strain; track 3, *Hae*III digest of bacteriophage ϕ X174 replicative form DNA. Size standards in base pairs are as indicated. Faintly staining bands are arrowed.

was found with the ovine arthritis strain or with our Cal 10 isolate. When ovine abortion isolates were screened, no hybridization was observed with any of seven isolates as shown by the dot blot analysis in Fig. 5; the other mammalian and avian strains were included as positive controls. Additionally, two abortion isolates of low passage (H574 pass 6 and S507 pass 4) were screened by Southern blotting and were also found to be negative. A previous dot blot experiment showed that our probe did not hybridize with uninfected cellular DNA. The strong hybridization found with the feline pneumonitis strain suggested that this isolate may possess sequences identical to

Fig. 2. Southern blot analysis of total undigested DNA from cells infected with avian chlamydial isolates 1-17 (see Table 1 for details). Track 16 is the plasmid donor strain. Origin, chromosomal and plasmid bands as detected by ethidium bromide staining are indicated as O, Ch and P, respectively.

Fig. 3. Restriction site map of the insert of clone pAV-1. H, *Hind*III; N, *Nci*I; R, *Eco*RI; h, *Hae*III.

Fig. 4. Southern blot analysis of undigested DNA from purified elementary bodies of various mammalian strains. Ar, ovine arthritis; GP, guinea-pig inclusion conjunctivitis; Oc, ovine conjunctivitis; LG, lymphogranuloma venereum; FP, feline pneumonitis; C, Cal 10; L, λ bacteriophage *Hind*III digested size markers; Av, plasmid donor strain 725. DNA (1 μ g) was loaded in each track with the exception of the Oc and GP samples where amounts were too low for quantification, and the 725 strain which received 0.5 μ g. Other abbreviations are as in Fig. 2.

Fig. 5. Dot blot analysis of mammalian strain DNA. Each 'dot' was loaded with 100 ng DNA except for Oc and GP (see Fig. 4). Ovine abortion strains were as follows: OA1, 84/501 (pass 3); OA2, ZC 31; OA3, ZC 26; OA4, ZC 25; OA5, ZC 19; OA6, S26/3 (pass 9); OA7, A22 vaccine strain (pass 25). The other abbreviations used are as in Fig. 4, the avian reference strains Av1 and Av2 representing isolates 725 and 741 respectively.

the avian plasmid. However, when plasmid DNA was purified from this strain by CsCl/EB density gradient centrifugation, digested with *Hind*III and compared with avian plasmid DNA by PAGE, a clearly different profile was obtained as shown in Fig. 1.

DISCUSSION

The presence of small 6.6 kb plasmids in chlamydiae was first reported by Lovett *et al.* (1982) for both *C. trachomatis* and *C. psittaci* (meningopneumonitis strain). More recently, Hyypiä *et al.* (1984) confirmed that the plasmid is present in all 15 serovars of *C. trachomatis* and suggested that this property could be used to detect chlamydiae in clinical specimens. The plasmid from the meningopneumonitis strain (Cal 10, a laboratory strain of uncertain origin and passage history; Francis & Magill, 1938) was cloned by Joseph *et al.* (1986) and found to be 6.2 kb in size. The smaller size and the single restriction site for *Eco*RI distinguishes that plasmid from the avian plasmid we have partially cloned. However, both the Cal 10 plasmid and our avian plasmid share sequence homology with the *C. trachomatis* plasmid which suggests a common ancestry. Plasmids of the same compatibility grouping often share homology at the origin of replication (Broda, 1979). That plasmid DNA may well show more sequence variation than genomic DNA has been suggested by Peterson & De La Maza (1984) who demonstrated differences in the restriction endonuclease profiles of plasmids from LGV serovars I and II from that of serovar III. We have demonstrated that the plasmid from the feline pneumonitis strain has a clearly different restriction endonuclease profile from that of our parent avian strain, but shows strong hybridization with the cloned 5.9 kb fragment. The genomic restriction endonuclease profile of the feline pneumonitis strain was found to differ substantially from those of avian or ovine strains (A. J. Herring, unpublished data).

Both Joseph *et al.* (1986) and Clarke & Hatt (1986) undertook *in vitro* transcription and translation of cloned *C. trachomatis* plasmid DNA and identified plasmid-encoded proteins. Plasmid conservation in a wide variety of strains in both species argues that it confers some selective advantage, but our finding that certain *C. psittaci* strains lack this class of plasmid DNA suggests it is not essential. It is possible that plasmids may be lost in long-term culture as appears to have happened with our strain of Cal 10, but our consistent failure to find plasmid sequences in nine ovine abortion isolates of varying passage level and identical restriction endonuclease profile make it unlikely that any abortion strains have this class of small plasmid.

Recently, it has been reported that the TWAR strains, which are associated with human respiratory disease, also lack plasmid bands discernible by conventional agarose gel electrophoresis (Campbell *et al.*, 1987). However, the newly introduced pulsed field electrophoretic techniques for larger DNA molecules are ideally suited to reveal if other, larger plasmid forms exist in chlamydiae.

The presence of plasmid in all avian isolates tested from a diversity of species means that clone pAV-1 may well have potential as a diagnostic reagent for the detection of this widespread infection of birds (Grimes, 1985), and in identifying zoonotic episodes.

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REFERENCES

- BEVAN, B. J. & BRACEWELL, C. D. (1986). Chlamydiosis in birds in Great Britain. 2. Isolation of *C. psittaci* from birds sampled between 1976 and 1984. *Journal of Hygiene* **96**, 453–458.
- BRODA, P. (1979). *Plasmids*. Oxford & San Francisco: W. H. Freeman.
- CAMPBELL, L. A., KUO, C.-C. & GRAYSTON, J. T. (1987). Characterization of the new *Chlamydia* agent, TWAR, as a unique organism by restriction endonuclease analysis and DNA–DNA hybridization. *Journal of Clinical Microbiology* **25**, 1911–1916.
- CLARKE, I. N. & HATT, C. (1986). *In vitro* transcription/translation analysis of cloned plasmid DNA from *Chlamydia trachomatis* serovar L1. In *Chlamydial Infections*, pp. 85–88. Edited by D. Oriel, G. Ridgway, J. Schachter, D. Taylor-Robinson & M. Ward. Cambridge: University Press.
- FRANCIS, T. & MAGILL, T. P. (1938). An unidentified virus producing acute meningitis and pneumonia in experimental animals. *Journal of Experimental Medicine* **68**, 147–160.
- GRIMES, J. E. (1985). Enigmatic psittacine chlamydiosis: results of serotesting and isolating attempts, 1978 through 1983, and considerations for the future. *Journal of the American Veterinary and Medical Association* **186**, 1075–1079.
- GRUNSTEIN, M. & HOGNESS, D. (1975). Colony hybridisation: a method for the isolation of cloned DNAs that contain a specific gene. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 3961–3965.
- HANAHAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557–580.
- HERRING, A. J., INGLIS, N. F., OJEH, C. K., SNODGRASS, D. R. & MENZIES, J. D. (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver stained polyacrylamide gels. *Journal of Clinical Microbiology* **16**, 473–477.
- HYYPÄ, T., JALAVA, A., LARSEN, S. H., TERHO, P. & HUKKANEN, V. (1985). Detection of *Chlamydia trachomatis* in clinical specimens by nucleic acid spot hybridization. *Journal of General Microbiology* **131**, 975–978.
- JOSEPH, T., NANO, F. E., GARON, C. F. & CALDWELL, H. D. (1986). Molecular characterization of *Chlamydia trachomatis* and *Chlamydia psittaci* plasmids. *Infection and Immunity* **51**, 699–703.
- LOVETT, M., KUO, C. C., HOLMES, K. & FALKOW, S. (1980). Plasmids of the genus *Chlamydia*. In *Current Chemotherapy and Infectious Disease 2*, pp. 1250–1252. Edited by J. D. Nelson & C. Grassi. Washington, DC: American Society for Microbiology.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- McCLENAGHAN, M., HERRING, A. J. & AITKEN, I. D. (1984). Comparison of *Chlamydia psittaci* isolates by restriction endonuclease analysis. *Infection and Immunity* **45**, 384–389.
- PEREZ-MARTINEZ, J. A. & STORZ, J. (1985). Antigenic diversity of *Chlamydia psittaci* of mammalian origin determined by microimmunofluorescence. *Infection and Immunity* **50**, 905–910.
- PETERSON, E. M. & DE LA MAZA, L. M. (1983). Characterization of *Chlamydia* DNA by restriction endonuclease cleavage. *Infection and Immunity* **41**, 604–608.
- RAAFAT EL-GEWELY, M. & HELLING, R. B. (1980). Preparative separation of DNA–ethidium bromide complexes by zonal density gradient centrifugation. *Analytical Biochemistry* **102**, 423–428.
- RIGBY, P. W. J., DIECKMANN, M., RHODES, C. & BERG, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–251.
- SMITH, H. O. & BERNSTEIL, M. L. (1976). A simple method for DNA restriction site mapping. *Nucleic Acids Research* **3**, 2387–2398.
- WENMAN, W. M. & LOVETT, M. A. (1982). Expression in *E. coli* of *Chlamydia trachomatis* antigen recognized during human infection. *Nature, London* **296**, 68–70.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains. Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.