Nucleotide sequence and taxonomic value of the major outer membrane protein gene of *Chlamydia pneumoniae* IOL-207

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(Received 16 July 1990; revised 10 October 1990; accepted 25 October 1990)

Chlamydia pneumoniae IOL-207 genomic DNA was hybridized with a 1.5 kb labelled DNA probe containing the 3' region of the coding sequence for the major outer membrane protein (MOMP) of *C. trachomatis* serovar L1. An 8.5 kb *Bgl*II fragment containing the complete MOMP gene was cloned into λ EMBL3. Two hybridizing *Eco*RI fragments were sub-cloned into the λ ZAP II cloning vector and the resulting plasmids were used as templates for sequencing both strands of the *C. pneumoniae* MOMP gene. Computer taxonomic studies using the nucleotide and inferred amino acid sequence of the MOMP of *C. pneumoniae* IOL-207 and all known chlamydial MOMP sequences supported the designation of *C. pneumoniae* as a new species, but electron microscope studies suggested that the presence of pear-shaped elementary bodies (EBs) may not be a reliable taxonomic criterion.

Introduction

The Chlamydiae are obligate intracellular bacteria characterized by a unique growth cycle and responsible for a wide variety of diseases in man and animals. Three species of the genus *Chlamydia* are recognized: *C. trachomatis*, *C. psittaci* and the newly designated species *C. pneumoniae* (Grayston *et al.*, 1989a).

C. pneumoniae is an increasingly identified cause of pneumonia and acute respiratory disease in man (Grayston *et al.*, 1989b; Marrie *et al.*, 1987). A high proportion of adults from different countries had antibodies to *C. pneumoniae* (Forsey *et al.*, 1986; Grayston *et al.*, 1989b), implying a high prevalence of these infections. Justification for designating the TWAR agent as a new species of *Chlamydia* was based on the differing ultrastructural

morphology of the infectious elementary bodies (EBs) (Chi *et al.*, 1987; Kuo *et al.*, 1988), the lack of DNA homology (<10%) with either *C. trachomatis*, or *C. psittaci* (Cox *et al.*, 1988), the unique DNA restriction endonuclease patterns (Campbell *et al.*, 1987) and the demonstration by monoclonal antibodies (mAbs) of *C. pneumoniae* species-specific antigens (Kuo *et al.*, 1986).

Although *C. pneumoniae* is a common human respiratory pathogen (Grayston *et al.*, 1989b; Marrie *et al.*, 1987), very little is known about the immunochemistry of this organism. One problem is that *C. pneumoniae* is difficult to isolate in tissue culture. Immunochemical and genetic studies should lead to simple and non-culture-dependent diagnostic methodologies for this important organism, as has been the case for *C. trachomatis*. Moreover, comparative studies of the nucleotide sequences of key *C. pneumoniae* antigens with other *Chlamydia* species might give insights into the evolutionary and structural diversity arising within this genus.

The chlamydial antigen for which the structure is best known is the major outer membrane protein (MOMP). MOMP is the most abundant protein at the surface of both the infectious EB and the intracellular replicative reticulate body (RB). Disulphide bridging between

Abbreviations: BGMK, Buffalo Green Monkey Kidney; CDC, Center for Disease Control, Atlanta, GA, USA; EB, elementary body; mAb, monoclonal antibody; micro-IF, micro-immunofluorescence; MOMP, major outer membrane protein; ORF, open reading frame; PCR, polymerase chain reaction.

The nucleotide sequence data reported in this paper have been submitted to GenBank, and have been assigned the accession number M34942.

MOMP and other envelope proteins is considered important for maintaining the structural integrity of the infectious EB in the absence of cross-linking by peptidoglycan (Bavoil *et al.*, 1984). Disulphide interlinking between MOMP polypeptide chains is thought to play an important role in regulating MOMP porin function, nutrient ingress through the EB outer envelope and ultimately the initiation of chlamydial differentiation itself (Bavoil *et al.*, 1984). MOMP is an immunodominant antigen and antibodies to it form the basis of the serological classification of *C. trachomatis* (Stephens *et al.*, 1982). Moreover, antibody to MOMP has been reported to neutralize chlamydial infection *in vitro* and *in vivo* (Zhang *et al.*, 1987), making MOMP the principal candidate for the development of defined vaccines against *C. trachomatis* and *C. psittaci* and perhaps ultimately, *C. pneumoniae*.

Comparative analysis of deduced amino acid sequences for several *C. trachomatis* MOMPs shows that the protein has four variable sequences (VS 1–4) forming loops between five highly conserved regions. The serovar- and subspecies-specific epitopes, which have been associated with neutralization (Zhang *et al.*, 1987), are located in VSs 1, 2 or 4 (Baehr *et al.*, 1988; Conlan *et al.*, 1988; Stephens *et al.*, 1988). Nucleotide sequences have recently been published for the variable segments of 15 *C. trachomatis* serovars (Yuan *et al.*, 1989). In addition, complete nucleotide sequences are available for the MOMP genes of a smaller number of *C. trachomatis* serovars (Baehr *et al.*, 1988; Hamilton & Malinowski, 1989; Hayes *et al.*, 1990; Pickett *et al.*, 1987; Stephens *et al.*, 1986, 1987; Zhang *et al.*, 1990) and for four strains of *C. psittaci* (Herring *et al.*, 1989; Pickett *et al.*, 1988; Zhang *et al.*, 1989) but not for *C. pneumoniae*. This presumably reflects the difficulty of working with *C. pneumoniae* and the paucity of *C. pneumoniae* isolates now available.

We present here: (i) the complete nucleotide sequence of the MOMP gene of *C. pneumoniae* IOL-207; (ii) computer-derived phylogenetic maps of the evolutionary inter-relationships of MOMP nucleotide sequences in the genus *Chlamydia*; and (iii) a re-evaluation of the ultrastructural morphology of *C. pneumoniae*.

Methods

Bacterial strains and growth conditions. *Chlamydia pneumoniae* IOL-207, isolated in 1967 from the conjunctiva of an Iranian child thought to be suffering from mild trachoma (Dwyer *et al.*, 1972), was propagated in the yolk sacs of 6–8-d-old embryonated hens' eggs. The inoculated eggs were incubated at $\geq 66\%$ relative humidity at 37°C and candled daily. Embryos were killed 20 d after infection by approved chilling at -20°C for 1 h. *C. pneumoniae* ATCC VR 1310 (CDC/CWL 029) was originally isolated from the throat of a patient with pneumonia. This

strain was obtained from the American Type Culture Collection and was grown in Buffalo Green Monkey Kidney (BGMK) cells (Flow Laboratories) in Dulbecco's Minimum Essential Medium (DMEM) supplemented with $1\text{ }\mu\text{g}$ emetine hydrochloride ml^{-1} (Sigma). *Escherichia coli* strain JM109 [*recA1 endA1 gyrA96 thi hsdR17* (r_{K} m_{K} supE44 , *relA1* $\lambda^- \Delta(\text{lac-proAB})$), (F' , *traD36 proAB lac Φ ZAM15*)], carrying a *PstI*–*EcoRI* insert in pUC9 coding for $\frac{3}{4}$ of the MOMP gene from *C. trachomatis* L1/440/LN, was grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) containing $50\text{ }\mu\text{g}$ ampicillin ml^{-1} (Sigma) or on plates of ampicillin supplemented LB with 1.5% (w/v) agar (Difco). *E. coli* strain Q358 (*hsd* r_{K} m_{K} $\text{supF } \phi 80r$) was used as a host for λ EMBL3 and was grown in either TB broth (5 g NaCl and 10 g Bacto-Tryptone per litre) supplemented with 0.2% (w/v) maltose and 10 mM-MgSO_4 , or on NZY (Sambrook *et al.*, 1989) 1.5% (w/v) agar plates. Growth and amplification of phage in *E. coli* Q358 was in NZY 0.7% (w/v) agarose overlays upon NZY 1.5% (w/v) agar plates. *E. coli* strain BB4, LE392.23, [F' *lac Φ ZAM15 proAB Tn10* (*tet R*)], obtained from Stratagene (Northumbria Biologicals) was used as the host for λ ZAP II and was grown in either TB broth supplemented with 0.2% (w/v) maltose, 10 mM-MgSO_4 and $15\text{ }\mu\text{g}$ tetracycline ml^{-1} as recommended by the manufacturer; or on NZY tetracycline 1.5% (w/v) agar plates. Growth and amplification of recombinant phage in *E. coli* BB4 was in NZY 0.7% (w/v) agarose overlays upon NZY 1.5% (w/v) agar plates. *E. coli* strain JM109 was grown either in TB broth supplemented with 0.2% (w/v) maltose and 10 mM-MgSO_4 or on NZY 1.5% (w/v) agar plates. *E. coli* JM109 cells carrying Bluescript SK(–) recombinant plasmids were grown on LB 1.5% (w/v) agar plates containing $50\text{ }\mu\text{g}$ ampicillin ml^{-1} .

Preparation of *C. pneumoniae* from infected yolk sacs. Embryonated eggs were used to overcome the difficulties associated with the bulk growth of *C. pneumoniae* in tissue culture. Yolk sacs were harvested aseptically and examined for the presence of chlamydial EBs. Four positive yolk sacs were suspended in Dulbecco's phosphate buffered saline (PBS) to give an approximate 20% (w/v) suspension. This suspension was homogenized for 2 min in a Sorvall-Omnimixer homogenizer and the resulting homogenate was centrifuged in a Sorvall HB4 rotor for 10 min at 1000 r.p.m. at 4°C . Eighteen millilitres of the middle layer was removed and centrifuged for 30 min. at 18000 r.p.m. and 4°C in a Sorvall SS34 swing-out rotor. Pellets were resuspended in 18 ml PBS and dispersed by vortexing with glass beads, cooled on ice and briefly ultra-sonicated. The chlorofluorocarbon Arklone (ICI) was added to the suspension at a ratio of $1:3$ and the mixture homogenized for 2 min on ice. The resulting emulsion was then centrifuged at 1000 r.p.m. in a Sorvall HB4 rotor for 5 min at 4°C . The upper white opalescent layer was removed and placed on ice whilst the sonication and extraction procedure was repeated on the residue. The final suspension was centrifuged at 1000 r.p.m. in a Sorvall HB4 rotor for 5 min at 4°C and the upper opalescent layer was removed and frozen at -20°C .

Preparation and extraction of *C. pneumoniae* DNA. The EBs from the thawed suspension were pelleted by centrifugation, resuspended in 5 ml of $0.2\text{ M-sodium acetate}$, $\text{pH } 6.9$ plus 10 mM-MgSO_4 and dispersed by vortexing with glass beads. To this suspension $50\text{--}100\text{ }\mu\text{l}$ DNAase I (9.5 mg ml^{-1}) (Sigma, molecular biology grade) was added and the mixture was incubated at 37°C for 1 h to digest remaining eukaryotic DNA. The reaction was stopped by the addition of EDTA to 50 mM and the EBs pelleted by centrifugation in a Sorvall SS34 rotor at 10000 r.p.m. at 4°C for 1 h. The pellet was resuspended in 2 ml saline Tris/EDTA (STE: 0.15 M-NaCl , 10 mM-Tris , $\text{pH } 7.4$, 1 mM-EDTA); RNAase A (Sigma, molecular biology grade) was added to $20\text{ }\mu\text{g ml}^{-1}$ and the mixture incubated at 37°C for 45 min. The EBs were then re-pelleted and lysed in 5 ml of 50 mM-Tris/HCl , $\text{pH } 7.5$, 20% (w/v) sucrose, 20 mM-EDTA , 25 mM-DTT , 1% (w/v) Sarkosyl and $200\text{ }\mu\text{g}$ proteinase K ml^{-1} at 55°C for 1–2 h or until clear. The nucleic acids

were extracted with phenol/chloroform and the aqueous phase dialysed for 24 h against STE and stored at 4 °C.

Enzymes and vectors. Restriction endonucleases, T4 DNA ligase, calf intestinal phosphatase and the digoxigenin DNA labelling kit were obtained from Boehringer Mannheim. *rTaq* DNA polymerase was obtained as AmpliTaq from Perkin Elmer Cetus. The λ EMBL3 and undigested λ ZAP II cloning kits and Gigapack II Gold packaging extract were obtained from Stratagene. Sequenase Version 2.0, containing the additional nucleotide analogue dITP, and 7-deaza-dGTP sequencing kits were obtained from United States Biochemical Corporation.

DNA probe production. (a) *5' MOMP gene probe.* Oligonucleotides were synthesized by β -cyanoethyl phosphoramidite chemistry on a model 381A automated DNA synthesizer (Applied Biosystems). Separate oligonucleotide primers starting at 57 bases upstream, 5' CCGCCAGAAAAAGATAG 3', on the coding strand and at 403 bases downstream, 5' ACATCAAAACGATCCCA 3', on the complementary strand from the methionine start codon of the *C. trachomatis* L1/440/LN MOMP DNA sequence, plus *rTaq* DNA polymerase, digoxigenin nucleotide and a *C. trachomatis* A/SA1/OT genomic DNA template were used to prepare a labelled, 460 bp polymerase chain reaction (PCR) product, using a Perkin Elmer Cetus DNA thermal cycler.

(b) *3' MOMP gene probe.* A large-scale alkaline-lysed preparation of the recombinant plasmid pMP148 was purified (Sambrook *et al.*, 1989). Following complete *Pst*I and *Eco*RI digestion of the plasmid the $\approx$ 1.5 kb insert was separated by agarose gel electrophoresis. The liberated fragment, containing 898 bp of the coding sequence of *C. trachomatis* L1/440/LN MOMP, was excised from the gel, purified by GeneClean (Stratagene Scientific) and labelled with digoxigenin.

Genomic library construction. Restriction endonuclease digests of the *C. pneumoniae* IOL-207 DNA were separated by gel electrophoresis. Fragments were transferred from the gel to Hybond-N nylon membrane (Amersham) and baked at 80 °C for 90 min. A single 8.5 kb *Bgl*II fragment hybridized, at 65 °C under standard conditions, with the digoxigenin-labelled *Pst*I-*Eco*RI 3' MOMP gene probe. Therefore, *Bgl*II-digested DNA was ligated into *Bam*HI-digested λ EMBL3 and packaged using Gigapack II Gold with *E. coli* Q358 as host. Plaques were transferred to Hybond-N membranes, fixed, then hybridized with the *Pst*I-*Eco*RI 3' MOMP probe.

λ phage DNA production and cloning of the *C. pneumoniae* IOL-207 MOMP gene into λ ZAP II. Using *E. coli* Q358 as host, an amplified preparation of a single hybridizing recombinant plaque was used for the large-scale production (Sambrook *et al.*, 1989) of λ phage DNA. DNA was purified and concentrated with an Elutip-d Minicolumn from Schleicher & Schüll (Anderman & Co.) then digested with restriction endonucleases specific to the unique cloning sites of λ ZAP II. Using duplicate agarose gels, restriction fragments were separated, blotted and hybridized at 65 °C with either the PCR-generated 5' MOMP or the *Pst*I-*Eco*RI 3' MOMP probe.

Hybridizing *Eco*RI fragments corresponding to the 5' and 3' ends of the *C. pneumoniae* MOMP gene were excised from a preparative agarose gel and purified by GeneClean. Each of these fragments was ligated into separate *Eco*RI-digested, dephosphorylated preparations of λ ZAP II.

Separate preparations of λ phage containing the *Eco*RI fragments were packaged using Gigapack II Gold with *E. coli* BB4 as the host. Amplified preparations of recombinant plaques hybridizing with either the PCR or *Pst*I-*Eco*RI MOMP probes were made in *E. coli* BB4. Recombinant pBluescript SK(-) plasmids were prepared by *in vivo* excision with R408 helper phage. *E. coli* JM109 cells carrying

pBluescript SK(-) recombinant plasmids were grown on LB 1.5% (w/v) agar plates containing 50 μ g ampicillin ml⁻¹.

DNA sequencing. Large-scale preparations of the recombinant plasmids carrying *Eco*RI restriction fragments were extracted by alkaline lysis from *E. coli* JM109, and purified by chromatography on NACS-PREPAC columns (BRL). Templates were prepared (Zhang *et al.*, 1988) and sequenced by the dideoxynucleoside chain-termination method using [α -³²S]dATP and modified T7 DNA polymerase (Sequenase). Nucleotide analogues dITP and 7-deaza-dGTP were used to resolve adjacent band compression areas.

Computer taxonomy. Nucleotide and inferred amino acid sequences of the MOMP of *C. pneumoniae* IOL-207 were compared with those of the other *Chlamydia* strains listed in Table 1, using PHYLIP version 3.2 computer software (Felsenstein, 1980). Programs used within PHYLIP were: the protein parsimony program PROTPARS; the DNA parsimony program DNAPARS and the program DNADIST, which computes the 'distance' between pairs of species from DNA sequences which are then utilized by the distance matrix programs FITCH and KITSCH. Sequences were first aligned with the multiple alignment program CLUSTAL (Higgins & Sharp, 1988) so that they contained the same number of characters including padding characters. A small program was written to convert the CLUSTAL output into the format required for the PHYLIP programs. Alignment of the 15 protein sequences required 403 sequence positions (including padding characters); the 15 nucleotide sequences required 1220 residues. In general the sites of insertion of padding characters were similar for both protein and DNA sequences.

Electron microscopy. Infected cell monolayers on 24-well tissue culture trays were processed as previously described (Ward & Murray, 1984). Briefly, infected cell monolayers in 24-well trays were fixed in glutaraldehyde, post-fixed in osmium tetroxide, stained with 1% (w/v) aqueous uranyl acetate and dehydrated in a graded ethanol series. The intact cell sheet was then detached from the plastic substratum by brief treatment with 2-epoxy propane and flat-embedded in Spurr low-viscosity epoxy resin. After ultra-thin sectioning with a diamond knife, the preparations were examined in a Hitachi H7000 transmission electron microscope.

Results

Molecular cloning of *C. pneumoniae* IOL-207 MOMP gene

The $\approx$ 1.5 kb fragment liberated from pMP148 by digestion with *Pst*I and *Eco*RI was labelled with digoxigenin and used as a probe (Fig. 1, line *a*) for the 3' region of the *C. pneumoniae* MOMP gene. The hybridization of digested *C. pneumoniae* IOL-207 DNA with the 3' *Pst*I-*Eco*RI probe suggested that the MOMP gene was most likely contained within an 8.5 kb *Bgl*II fragment (Fig. 2*a*). This fragment was therefore cloned into *Bam*HI-digested λ EMBL3 DNA. Successful cloning was confirmed by hybridization of the resulting plaques with the 3' *Pst*I-*Eco*RI probe. Digestion of the recombinant λ phage DNA with various restriction endonucleases was followed by separate hybridizations with both the digoxigenin-labelled 3' *Pst*I-*Eco*RI (Fig. 1, line *a*) and the 5' PCR-generated (Fig. 1, line *b*) probes. A 1.4 kb *Eco*RI fragment hybridized with the 3' *Pst*I-*Eco*RI probe

Fig. 1. Digoxigenin-labelled hybridization probes constructed from the MOMP genes of *C. trachomatis*: (a) 3' 1.5 kb *PstI*–*EcoRI* restriction fragment of L1/440/LN; (b) 5' 460 bp PCR-synthesized fragment of A/SA1/OT. The hatched box marks the position of the complete MOMP gene. P1, primer 1; P2, primer 2.

(Fig. 2b, panel B) and a 3.3 kb fragment hybridized with both probes (Fig. 2b, Panels A and B). This suggested that these two restriction fragments carried the whole MOMP gene between them; the 3.3 kb fragment containing the region bounded by the 5' start codon and the *EcoRI* site, whilst the 1.4 kb fragment contained the region between the *EcoRI* site and the 3' stop codon. These two restriction fragments were therefore sub-cloned into separate *EcoRI*-digested, dephosphorylated preparations of λ ZAP II. Successful cloning of the two *EcoRI* inserts was confirmed by hybridization of their respective plaques in *E. coli* BB4 with either the 3' *PstI*–*EcoRI* or the 5' PCR-generated probe. λ ZAP II recombinants were then converted to pBluescript SK(–) plasmids using R408 helper phage.

The *C. pneumoniae* IOL-207 MOMP gene sequence

Plasmids carrying the 1.4 and 3.3 kb *EcoRI* inserts were purified and used as templates for DNA sequencing. Forward Universal primer, used in the first sequencing reactions of both plasmid templates, provided open reading frames (ORFs) of the MOMP gene. Alignments were found between the translated product of the ORFs of both inserts and the MOMP gene sequence of *C. trachomatis* L1. Orientation of the aligned inserts confirmed that the 5' and 3' regions of the *C. pneumoniae* IOL-207 MOMP gene were contained within the 3.3 and the 1.4 kb fragments, respectively. Both strands of the *C. pneumoniae* IOL-207 MOMP gene were then sequenced, giving an ORF of 1167 bp (389 amino acids). This sequence is shown in Fig. 3. A conserved *XbaI* restriction site was then used to align and compare the restriction maps of the MOMP genes of *C. pneumoniae* IOL-207 and *C. trachomatis* serovar L1 (Fig. 4).

Fig. 2. Southern blots of *C. pneumoniae* IOL-207 DNA. (a) Genomic DNA was digested with restriction endonucleases (A, *BglII*; B, *SacI*; C, *Sall*; D, *XhoI*; E, *SpeI*), separated by agarose gel electrophoresis, Southern blotted and probed with the digoxigenin-labelled 3' *PstI*–*EcoRI* MOMP gene probe. (b) Recombinant λ EMBL3 DNA digested with restriction endonucleases (B, *BglII*; E, *EcoRI*), separated, Southern blotted and probed with either (panel A) digoxigenin-labelled 5' PCR-synthesized MOMP gene probe or (panel B) 3' *PstI*–*EcoRI* MOMP gene probe. Tracks labelled L contain a digoxigenin-labelled 1 kb ladder.

15 30 45 60 75 90 105
 ATG AAA AAA CTC TTA AAG TCG GCG TTA TTA TCC GCC GCA TTT GCT GGT TCT GTC GGC TCC TTA CAA GCC TTG CCT GTA GGG AAC CCT TCT GAT CCA AGC TTA TTA ATT GAT GGT ACA
 Met Lys Lys Leu Leu Lys Ser Ala Leu Leu Ser Ala Ala Phe Ala Gly Ser Val Gly Ser Leu Gln Ala Leu Pro Val Gly Asn Pro Ser Asp Pro Ser Leu Leu Ile Asp Gly Thr
 120 135 150 165 180 195 210 225
 ATA TGG GAG GGT GCT GCA GGA GAT CCT TGC GAT CCT TGC GCT ACT TGG TGC GAC GCT ATT AGC TTA CGT GCT GGA TTT TAC GGA GAC TAT GTT TTC GAC CGT ATC TTA AAA GTA GAT
 Ile Trp Glu Gly Ala Ala Gly Asp Pro Cys Asp Pro Cys Ala Thr Trp Cys Asp Ala Ile Ser Leu Arg Ala Gly Phe Tyr Gly Asp Tyr Val Phe Asp Arg Ile Leu Lys Val Asp
 240 255 270 285 300 315 330 345
 GCA CCT AAA ACA TTT TCT ATG GGA GCC AAG CCT ACT GGA TCC GCT GCT GCA AAC TAT ACT ACT GCC GTA GAT AGA CCT AAC CCG GCC TAC AAT AAG CAT TTA CAC GAT GCA GAG TGG
 Ala Pro Lys Thr Phe Ser Met Gly Ala Lys Pro Thr Gly Ser Ala Ala Ala Asn Tyr Thr Thr Ala Val Asp Arg Pro Asn Pro Ala Tyr Asn Lys His Leu His Asp Ala Glu Trp
 360 375 390 405 420 435 450 465
 TTC ACT AAT GCA GGC TTC ATT GCC TTA AAC ATT TGG GAT CGC TTT GAT GTT TTC TGT ACT TTA GGA GCT TCT AAT GGT TAC ATT AGA GGA AAC TCT ACA GCG TTC AAT CTC GTT GGT
 Phe Thr Asn Ala Gly Phe Ile Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly Ala Ser Asn Gly Tyr Ile Arg Gly Asn Ser Thr Ala Phe Asn Leu Val Gly
 480 495 510 525 540 555 570 585
 TTA TTC GGA GTT AAA GGT ACT ACT GTA AAT GCA AAT GAA CTA CCA AAC GTT TCT TTA AGT AAC GGA GTT GTT GAA CTT TAC ACA GAC ACC TCT TTC TCT TGG AGC GTA GGC GCT CGT
 Leu Phe Gly Val Lys Gly Thr Thr Val Asn Ala Asn Glu Leu Pro Asn Val Ser Leu Ser Asn Gly Val Val Glu Leu Tyr Thr Asp Thr Ser Phe Ser Trp Ser Val Gly Ala Arg
 600 615 630 645 660 675 690
 GGA GCC TTA TGG GAA TGC GGT TGT GCA ACT TTG GGA GCT GAA TTC CAA TAT GCA CAG TCC AAA CCT AAA GTT GAA GAA CTT AAT GTG ATC TGT AAC GTA TCG CAA TTC TCT GTA AAC
 Gly Ala Leu Trp Glu Cys Gly Cys Ala Thr Leu Gly Ala Glu Phe Gln Tyr Ala Gln Ser Lys Pro Lys Val Glu Glu Leu Asn Val Ile Cys Asn Val Ser Gln Phe Ser Val Asn
 705 720 735 750 765 780 795 810
 AAA CCC AAG GGC TAT AAA GGC GTT GCT TTC CCC TTG CCA ACA GAC GCT GGC GTA GCA ACA GCT ACT GGA ACA AAG TCT GCG ACC ATC AAT TAT CAT GAA TGG CAA GTA GGA GCC TCT
 Lys Pro Lys Gly Tyr Lys Gly Val Ala Phe Pro Leu Pro Thr Asp Ala Gly Val Ala Thr Ala Thr Gly Thr Lys Ser Ala Thr Ile Asn Tyr His Glu Trp Gln Val Gly Ala Ser
 825 840 855 870 885 900 915 930
 CTA TCT TAC AGA CTA AAC TCT TTA GTG CCA TAC ATT GGA GTA CAA TGG TCT CGA GCA ACT TTT GAT GCT GAT AAC ATC CGC ATT GCT CAG CCA AAA CTA CCT ACA GCT GTT TTA AAC
 Leu Ser Tyr Arg Leu Asn Ser Leu Val Pro Tyr Ile Gly Val Gln Trp Ser Arg Ala Thr Phe Asp Ala Asp Asn Ile Arg Ile Ala Gln Pro Lys Leu Pro Thr Ala Val Leu Asn
 945 960 975 990 1005 1020 1035 1050
 TTA ACT GCA TGG AAC CCT TCT TTA CTA GGA AAT GCC ACA GCA TTG TCT ACT ACT GAT TCG TTC TCA GAC TTC ATG CAA ATT GTT TCC TGT CAG ATC AAC AAG TTT AAA TCT AGA AAA
 Leu Thr Ala Trp Asn Pro Ser Leu Leu Gly Asn Ala Thr Ala Leu Ser Thr Thr Asp Ser Phe Ser Asp Phe Met Gln Ile Val Ser Cys Gln Ile Asn Lys Phe Lys Ser Arg Lys
 1065 1080 1095 1110 1125 1140 1155 1170
 GCT TGT GGA GTT ACT GTA GGA GCT ACT TTA GTT GAT GCT GAT AAA TGG TCA CTT ACT GCA GAA GCT CGT TTA ATT AAC GAG AGA GCT GCT CAC GTA TCT GGT CAG TTC AGA TTC TAA
 Ala Cys Gly Val Thr Val Gly Ala Thr Leu Val Asp Ala Asp Lys Trp Ser Leu Thr Ala Glu Ala Arg Leu Ile Asn Glu Arg Ala Ala His Val Ser Gly Gln Phe Arg Phe *

Fig. 3. Nucleotide and inferred amino acid sequence of the open reading frame of the MOMP gene of *C. pneumoniae* IOL-207. The cleavage site of the signal peptide is indicated by an arrow. The underlined nucleotide sequence indicates the *EcoRI* restriction site.

Fig. 4. Restriction endonuclease maps of the MOMP genes of *C. pneumoniae* IOL-207 and *C. trachomatis* L1/440/LN. E, *EcoRI*; P, *PstI*; V, *EcoRV*; A, *AsnI*; X, *XbaI*; B, *BamHI*; S, *StyI*.

Amino acid sequence

The deduced amino acid sequences of the MOMP gene of *C. pneumoniae* IOL-207 and of 10 strains representing six serovars of *C. trachomatis* and four strains of *C. psittaci* (Table 1) were aligned. For clarity, Fig. 5 shows the alignments of the MOMP gene sequences for only *C.*

pneumoniae, *C. trachomatis* A/SA1/OT, B/Jali20/OT and L1/440/LN, *C. psittaci* ovine abortion agent A22/M and guinea pig inclusion conjunctivitis strain 1. The signal peptide of *C. pneumoniae* MOMP consisted of 23 amino acids in contrast to the 22 amino acids of the other Chlamydiae. Homology with other amino acid sequences in the signal peptide was 15/23 (*C. trachomatis*), 16/23 (*C. psittaci* A22/M) and 15/23 (*C. psittaci* GPIC). Fig. 5 also shows the characteristic four variable regions of MOMP for *C. pneumoniae*. Cysteine residues are considered crucial for MOMP porin function; *C. pneumoniae* and all other chlamydial MOMP sequences, with the exception of B/TW5/OT (Stephens *et al.*, 1987), contained seven conserved cysteine residues. *C. pneumoniae* had a unique cysteine residue at position 333 and there was an additional cysteine at position 212 which was also present in *C. trachomatis* but not *C. psittaci* (Fig. 5). Overall, there was 67.2–67.9% homology between the *C. pneumoniae* MOMP nucleotide sequence and *C. trachomatis* sequences and 71.5–72.4% homology with the *C. psittaci* sequences (Table 1).

Table 1. Nucleotide and amino acid homology of the MOMP gene of *C. pneumoniae* IOL-207 with strains of *C. trachomatis* and *C. psittaci*

Species	Serovar	Strain	Percentage homology		Reference of strain
			Nucleotide	Amino acid	
<i>C. trachomatis</i>	A	A/Har13/OT	67.2	63.5	Baehr <i>et al.</i> (1988)†
	A	A/SA1/OT	67.3	64.7	Hayes <i>et al.</i> (1990)
	B	B/TW5/OT	67.6	64.1	Baehr <i>et al.</i> (1988)†
	B	B/TW5/OT	67.4	63.8	Stephens <i>et al.</i> (1987)
	B	B/Jali20/OT	67.9	64.3	Hayes <i>et al.</i> (1990)
	C	C/TW3/OT	67.5	64.1	Stephens <i>et al.</i> (1987)
	F	IC-Cal-3	67.4	63.6	Zhang <i>et al.</i> (1990)
	H	*	67.9	65.1	Hamilton & Malinowski (1989)
	L1	L1/440/LN	67.6	64.2	Pickett <i>et al.</i> (1987)
<i>C. psittaci</i>	L2	L2/434/Bu	67.4	64.1	Stephens <i>et al.</i> (1986)
		A22/M	71.8	70.2	Pickett <i>et al.</i> (1988)
		S26/3	72.4	75.9	Herring <i>et al.</i> (1989)
		GPIC 1	72.1	73.9	Zhang <i>et al.</i> (1989)
<i>C. pneumoniae</i>		IOL-207	100.0	100.0	Zhang <i>et al.</i> (1989)

* Strain unknown. † Personal communication.

Fig. 5. Comparative alignment of the amino acid sequence of the MOMP gene of *C. pneumoniae* IOL-207 with the MOMP genes of *C. trachomatis* A/SA1/OT; B/Jali 20/OT; L1/440/LN, *C. psittaci* ovine abortion agent A22/M and guinea pig inclusion conjunctivitis strain 1 (GPIC 1). Dots indicate identity and dashes indicate deletions. Conserved cysteine residues and VS 1-4 are boxed. The signal peptide is marked as SP. Numbers refer to *C. pneumoniae* IOL-207 MOMP.

Fig. 6. The rooted tree obtained by computer taxonomy for 14 nucleotide sequences coding for the MOMP genes of *C. psittaci*: meningopneumonitis Cal 10 (Mn); ovine abortion A22/M (OA1); ovine abortion strain S26/3 (OA2); guinea pig inclusion conjunctivitis strain 1 (GP); *C. pneumoniae* IOL-207 (IOL); *C. trachomatis*: C/TW3/OT (C); serovar H strain unknown (H); A/SA1/OT (SA1); A/Har13/OT (A); L2/434/Bu (L2); B/TW5/OT (B); B/Jali 20/OT (JB); L1/440/LN (L1); F/IC-Cal-3/GU (F). In this figure the length of the lines connecting nodes is proportional to the relative evolutionary time between each of them.

Phylogenetic classification

When protein and DNA sequences are used to develop a phylogenetic relationship the initial result is an unrooted tree, i.e. a tree where the branching order, intermediate nodes and the relative distances between each node have been identified but where the ancestral node (the root of the tree) has not been identified. In order to identify the root, additional assumptions must be made. Usually it is sufficient to assume that there is a constant evolutionary clock which affects all sequences equally. Protein parsimony studies in which the order of presentation of the complete protein sequences was randomized always gave the same results: an unrooted tree with three minor variations in one location. These variations arose through the uncertainty of determining whether there are one or two intermediate nodes between the sequences for the MOMPs of serovars H and C.

More detailed studies were conducted using the nucleotide data as it is possible to estimate the relationships (distance) between nucleotide sequences with less ambiguity than for amino acid sequences. The program DNAPARS confirmed that the unrooted trees obtained using the nucleotide sequences were equivalent to those obtained using the protein sequences. Two trees were identified, again differing as to whether there are one or two intermediate nodes between the MOMPs of *C. trachomatis* serovars H and C.

In order to produce a rooted tree, the program DNADIST was used to calculate the distances between each pair of DNA sequences. The Kimura '2-parameter' model (Kimura, 1980) was used; this allows a difference

between transition and transversion rates. Unfortunately, this program could not be used for all 15 sequences, as two of the *C. trachomatis* serovar B sequences had only four nucleotide differences between them; this degree of similarity caused a numerical error condition and the program DNADIST terminated prematurely. However, removing one of these sequences permitted calculation of the distance matrix for the remaining 14 sequences. The program KITSCH, which employs a Fitch-Margoliash least-squares approach, was used to identify a single rooted tree. KITSCH assumes that the sequences available were all obtained at the same relative evolutionary time from the origin (i.e. that they are all present-day isolates). This implies, therefore, since there is also an assumption of a constant evolutionary clock, that the distance from the root to each tip representing an extant species must be the same. With these assumptions a rooted tree was obtained (Fig. 6).

Electron microscopy

One of the taxonomic criteria suggested for *C. pneumoniae* is the presence of pear-shaped EBs (Grayston *et al.*, 1989a; Kuo *et al.*, 1988). However, we have also observed occasional aberrant pear-shaped EBs in preparations of *C. trachomatis* and *C. psittaci* and were thus concerned to establish whether pear-shaped EBs are a reliable characteristic of all *C. pneumoniae* strains. Figs 7(a) and 7(b) are transmission electron micrographs of *C. pneumoniae* strains IOL-207 and ATCC VR 1310 respectively. Representative *C. trachomatis* and *C. psittaci* strains whose ultrastructural morphology is well

Fig 7. Transmission electron micrographs of *C. pneumoniae* in tissue culture. (a) Low-power picture of IOL-207 in BGMK cells. The EBs appear as small dark dots within the inclusions. Note the relatively small number of infectious EBs despite the maturity of the inclusions. Bar, 5 μm . (b) ATCC VR 1310. Higher magnification picture to show the morphology of the EBs. The EBs of both *C. pneumoniae* strains were round, occasionally crenellated but rarely pear-shaped. These EBs could not reliably be distinguished from EBs of *C. trachomatis* or *C. psittaci* in material processed in parallel. Bar, 0.5 μm .

known were also processed in parallel (data not shown) as a control for possible fixation artifacts. Fig. 7(a) shows that excellent growth of *C. pneumoniae* was achieved in BGMK cells treated with 1 μg emetine ml^{-1} . However, although large mature inclusions were obtained, remar-

kably few infectious EBs were present. Those *C. pneumoniae* EBs which were observed were round rather than pear-shaped (Fig. 7b), occasionally crenellate, and could not reliably be distinguished from EBs of *C. trachomatis* or *C. psittaci*.

Discussion

Strains of *C. pneumoniae* were formerly known as TWAR agents, an acronym derived from the fact that the first isolate was from Taiwan and that subsequent isolates were associated with acute respiratory infection (Grayston *et al.*, 1986). Species designation was attained as recently as 1989 (Grayston *et al.*, 1989a). The type strain, TW 183, was not available to us at the commencement of this study. We therefore chose to work with IOL-207, the second known *C. pneumoniae* isolate (Dwyer *et al.*, 1972; Grayston *et al.*, 1989b). Both strains were originally isolated from the conjunctivae of children in trachoma-endemic areas; TW 183 was isolated in Taiwan in 1965 whilst IOL-207 was isolated in London from an Iranian child with mild trachoma in 1967. Both organisms share the same fastidious growth characteristic of *C. pneumoniae* (Grayston *et al.*, 1989b). In 1972 TW 183 and IOL-207 were found to be immunologically identical using a two-way micro-immunofluorescence (micro-IF) typing test (Dwyer *et al.*, 1972). During the course of this study a *C. pneumoniae* strain was deposited by CDC in the American Type Culture Collection (ATCC VR 1310) and thus became available to us. mAb to IOL-207 reacts with TW 183 in the micro-IF test (J. D. Trehanne, unpublished observation) and with ATCC VR 1310 on dot blot. Conversely, *C. pneumoniae*-specific mAb to TW 183 also reacts on micro-IF with IOL-207 (J. D. Trehanne, unpublished observation) and with ATCC VR 1310 (Carolyn Black, CDC, personal communication). Neither of these mAbs reacts with *C. psittaci* or *C. trachomatis*. We have also sequenced the gene for the 60 kDa cysteine-rich, outer membrane protein of IOL-207; oligonucleotide probes derived from this sequence reacted in a species-specific manner in the PCR reaction with genomic DNA from *C. pneumoniae* ATCC VR 1310 (M. W. Watson and others, unpublished observations). Finally our computer study (see below) confirmed that the IOL-207 MOMP sequence is taxonomically distinct from known MOMP sequences for *C. trachomatis* or *C. psittaci*. The eclectic association of the above findings would suggest that there can be little doubt that IOL-207 is a genuine *C. pneumoniae* isolate.

The MOMP gene of *C. pneumoniae* shows all the characteristics of a chlamydial MOMP. The signal peptides for MOMP in all three species of *Chlamydia* are virtually identical, with a classic Leu-Glu-Ala protease cleavage site. The *C. pneumoniae* signal peptide has 23 rather than 22 amino acids and there are a few minor substitutions for amino acids of similar properties. The N-terminus of the MOMP for all three species is also similar, with the first six amino acids identical (LPVGNP). We speculate that this region of the protein is conserved in order to ensure correct

anchorage in the membrane. The other conserved feature between the MOMP sequences is the position of seven conserved cysteine residues, which are probably important in the structural and functional role of this protein.

The four variable regions of MOMP are of major interest for vaccine development (Taylor-Robinson & Ward, 1989) and are also present in *C. pneumoniae*. VS 1, 2 and 4 of *C. trachomatis* are of particular interest because they are hydrophilic, have high beta turn potential, and are thought to form loops at the surface of chlamydial EBs accessible to neutralizing serovar- or subspecies-specific antibodies (Zhang *et al.*, 1987). VS 2 and VS 4 are topographically juxtaposed on the chlamydial surface, forming a discontinuous epitope which can be spanned by a single mAb paratope (Conlan *et al.*, 1990). Both these exposed loops may function in a co-ordinated manner to promote chlamydial adhesion (Su *et al.*, 1990). VS 1 of the C complex of *C. trachomatis* contains immunodominant subspecies- and type-specific epitopes (Baehr *et al.*, 1988; Stephens *et al.*, 1988) whose critical binding sites, in the case of A/SA1/OT, have been localized to the peptides VAGL and EKD respectively (Hayes *et al.*, 1990). As might be expected, there are no analogous peptides to these in the VS 1 of *C. pneumoniae*. In the B complex of *C. trachomatis*, by contrast, it is VS 2 which so far appears to contain the dominant type epitope (Baehr *et al.*, 1988; Conlan *et al.*, 1988; Stephens *et al.*, 1988). In particular in *C. trachomatis* B/Jali20/OT and L1/440/LN the critical binding sites for type-specific antibody are the peptides GTFVP and DA-VP (Conlan *et al.*, 1988; Hayes *et al.*, 1990). Interestingly, in all known chlamydial MOMP sequences there are two or three variable amino acids followed by a hydrophobic amino acid (L, Y or V) and proline, a computer-predicted turn-generating amino acid. We speculate that the unique peptide ANELP in the equivalent position in *C. pneumoniae* MOMP is also surface exposed and this may represent a type-specific region. In VS 4 of the B complex serovars of *C. trachomatis* there are two known subspecies- and one species-specific epitope (Baehr *et al.*, 1988; Conlan *et al.*, 1988, 1989). We are unable to identify a clear counterpart in *C. pneumoniae*.

Chlamydiae have no other porin proteins and therefore MOMP is presumed essential for chlamydial survival. From a taxonomic viewpoint MOMP is in many ways an ideal protein to study; there must inevitably be large regions of structure which have been conserved throughout evolution. However, there are also immunodominant variable regions which, at least for *C. trachomatis*, can be related to the serological classification and which are also epidemiologically associated with different patterns of clinical disease. Moreover, with the publication of the *C. pneumoniae* sequence, there

are now considerable data on MOMP gene structure for all *Chlamydia* species.

Computer taxonomy provides an objective method of applying generalized taxonomic criteria to the particular problem of evolutionary relationships amongst Chlamydiae; the program which we used was developed for plant taxonomy (Felsenstein, 1980). The same classification tree resulted irrespective of whether we used nucleotide or inferred amino acid sequence data. This implies that there are no dramatic differences in codon preference amongst the Chlamydiae. Hybridization studies using whole genomic DNA found less than 10% homology between *C. pneumoniae* and the other two chlamydial species (Cox *et al.*, 1988). This led to the suggestion that the chlamydial species might be an example of convergent evolution from divergent ancestors. This has never seemed to us a convincing argument given the uniqueness of the genus *Chlamydia*; it is certainly not supported by the high degree of homology amongst the chlamydial MOMP genes. Comparisons of the MOMP gene sequences of *C. pneumoniae* IOL-207 with those of other *Chlamydia* species gave homologies ranging from 67.2 to 72.4% for the nucleotides and from 63.5 to 75.9% for the amino acid sequences. This is reflected in the computer taxonomy, which shows all three species arising from a common ancestor, i.e. divergent evolution. Moulder (1988) suggests that the protochlamydiae, chlamydia-like organisms parasitic in invertebrates, are present-day descendants of a possible common ancestor.

The computer analysis classifies the present known MOMP sequences into three main branches. One branch consists of the four *C. psittaci* sequences, with a strong similarity between the sequence of *C. psittaci* A22/M ovine abortion agent and *C. psittaci* MnCal-10. The second branch consists of the single *C. pneumoniae* IOL-207 sequence. The distinction made for this branch supports separate species status for this organism whilst its origin amongst *C. psittaci* suggests that *C. pneumoniae* diverged from *C. psittaci*. This concept is supported by the fact that neither *C. psittaci* nor *C. pneumoniae* produce inclusions with an iodine-staining reserve carbohydrate (Grayston *et al.*, 1989b). *C. trachomatis* is classified by the computer analysis into two broad groups which correspond with the serogroups related to either serovar B or serovar C. Each of these serogroups is formed by the presence of a distinct subspecies epitope on MOMP (Conlan *et al.*, 1988; Hayes *et al.*, 1990). This subspecies epitope forms only a tiny portion of the MOMP variable sequences; it is remarkable that the computer analysis was able to consistently discriminate between them. Serovar F is shown as being distinct from either of these complexes. Similarly, in the serological classification of *C. trachomatis* by micro-IF, serovar F does not belong clearly to either group (Wang & Grayston, 1970).

One of the taxonomic criteria used to give species status to *C. pneumoniae* was the presence of unusual, pear-shaped EBs (Chi *et al.*, 1987). It has been suggested that the tip of these EBs might be a specialized attachment structure (Kuo *et al.*, 1988). We have seen similar structures in immature *C. trachomatis* EB and therefore re-examined *C. pneumoniae* ultrastructure ourselves. The electron micrographs clearly showed that the majority of EBs of both IOL-207 and ATCC VR 1310 were round. However, inclusions of *C. pneumoniae* contained a much lower proportion of fully developed EBs than those of *C. trachomatis* or *C. psittaci*. This may explain the greater difficulty in passaging *C. pneumoniae* in cell culture, as only mature EBs are infectious. We accept that there may be unknown technical reasons to explain our failure to observe significant numbers of pear-shaped EBs in this organism. Perhaps IOL-207 and VR 1310 differ in morphology from TW 183. In that case further work is required to ascertain the general validity of the ultrastructural criteria chosen for this new species.

Our work on the MOMP gene of IOL-207 and its taxonomic status fully supports species designation for *C. pneumoniae*. This is corroborated by the distinct clinical syndromes with which this organism is associated. One of the most controversial of clinical reports is the apparent association between antibody to *C. pneumoniae* and myocardial infarction (Saikku *et al.*, 1988). One of the key problems in defining the clinical syndromes associated with this fastidious organism is the lack of specific diagnostic probes. We hope that the *C. pneumoniae* MOMP gene sequence presented here will facilitate the development of improved, non-culture-dependent, diagnostic reagents for this newly recognized human pathogen.

The authors thank Dr M. A. Pickett for synthesis of the oligonucleotides, Mr M. W. Watson for his advice, and Mr Watson and Miss S. J. Cooke for help in preparing the manuscript. M. W. Carter is in receipt of a Medical Research Council research studentship. This investigation received financial support from the Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization.

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