Immunoreactivity of the 60 kDa cysteine-rich proteins of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae* expressed in *Escherichia coli*

M. W. Watson, P. R. Lambden, J. S. Everson and I. N. Clarke

Author for correspondence: I. N. Clarke. Tel: +44 703 796975.

Molecular Microbiology,
Faculty of Medicine,
Southampton General
Hospital, Southampton, SO9
4XY, UK

The 60 kDa cysteine-rich proteins (CrPs) of *Chlamydia* are developmentally regulated outer envelope proteins synthesized late in the chlamydial growth cycle. These proteins, found only on the extracellular infectious elementary bodies, elicit major antibody responses in chlamydial infection. We have cloned and expressed in *Escherichia coli* the complete 60 kDa CrP genes from *Chlamydia trachomatis*, *C. psittaci* and *C. pneumoniae*. The recombinant products were expressed as either 'native' proteins or as fusions with the bacteriophage T7 gene 10 protein. Electron microscopy showed that recombinant proteins were produced as insoluble inclusions within the *E. coli* host cells. The recombinant 60 kDa CrPs were purified and used to raise high titre polyclonal antisera. In immunoblot analysis these antisera reacted with the 60 kDa CrPs from purified elementary bodies of all three chlamydial species in a genus-specific manner. Further molecular analysis allowed the genus-specific cross-reacting epitopes to be localized by using overlapping synthetic peptides covering the *C. trachomatis* 60 kDa CrP. Immunogold labelling experiments using purified infectious elementary bodies from the three chlamydial species indicated that the 60 kDa CrPs are not surface accessible to antibody binding.

Keywords: *Chlamydia* spp., cysteine-rich proteins, epitope, envelope proteins

INTRODUCTION

Three of the four recognized species of *Chlamydia* are human pathogens. *Chlamydia pneumoniae* is a recently defined human pathogen responsible for acute respiratory infections. *Chlamydia psittaci* infects a diverse range of host species and is a well known opportunistic pathogen in man causing mainly respiratory infections. *Chlamydia trachomatis* infections in humans are caused by two biovariants (biovars). The trachoma biovar infects mucosal epithelia of the eye and genital tract producing localized disease; the lymphogranuloma venereum biovar (LGV) infects mucosal genital epithelia but also causes more invasive disease.

Members of the genus *Chlamydia* have a unique intracellular life cycle which involves an infectious electron

dense elementary body (EB) and a replicative, non-infectious metabolically active reticulate body (RB). *Chlamydia* are Gram-negative bacteria but do not possess a defined peptidoglycan layer. The structural stability of the EB cell wall, in the absence of peptidoglycan, has been attributed to a unique set of cysteine-rich outer envelope proteins (Bavoil *et al.*, 1984). The EB outer envelope comprises the cysteine-rich major outer membrane protein (MOMP) and two additional cysteine-rich proteins (CrPs) of approximately 60 and 12 kDa molecular mass. The 12 kDa CrP has recently been shown to be an acylated protein translated from a short open reading frame of 88 amino acids (Everett & Hatch, 1991). Primer extension and Northern blot studies have shown the *C. trachomatis* L1 60 kDa CrP is closely linked on the chromosome to the gene for the 12 kDa lipid-modified CrP. Both open reading frames are transcribed together as a single operon and their expression is tightly synchronized with the chlamydial growth cycle, with maximum transcription occurring late in the growth cycle as RBs condense to form infectious EBs (Lambden *et al.*, 1990).

Abbreviations: CrP, cysteine-rich protein; MOMP, major outer membrane protein; EB, elementary body; RB, reticulate body; LGV, lymphogranuloma venereum biovar.

Both the 60 kDa CrP and the lipid-modified 12 kDa CrP can be extracted in a highly cross-linked form only from EBs. The MOMP, which is found in both EBs and RBs, is thought to act as a porin (Bavoil *et al.*, 1984) and has a role in the attachment and uptake of EBs by host cells. It is also recognized as an exposed surface molecule possessing infection-neutralizing epitopes (Collett *et al.*, 1989; Zhang *et al.*, 1989; Conlan *et al.*, 1989; Conlan *et al.*, 1990; Peterson *et al.*, 1990). Polyacrylamide gel analysis of *C. trachomatis* proteins has shown that the 60 kDa CrP occurs as a singlet in the trachoma biovar and as a 60/62 kDa doublet in the LGV biovar (Batteiger *et al.*, 1985). The *C. trachomatis* 60/62 kDa doublet appears to undergo two-site post-translational processing of its amino terminal signal peptide (Allen & Stephens, 1989). Only the *C. psittaci* avian isolate 6BC has been subjected to detailed analysis of its 60 kDa CrPs. This isolate, which is closely related to the *C. psittaci* strain used in our study, produces a 60/62 kDa doublet (Hatch *et al.*, 1984; Hatch *et al.*, 1986). In *C. pneumoniae* strain AR-39 the 60 kDa CrPs also occur as a doublet (Melgosa *et al.*, 1993). The complete nucleotide sequence of the 60 kDa CrP genes has been determined for ten chlamydial strains: *C. trachomatis* serovars LGV1 (Clarke *et al.*, 1988), LGV2 (Allen & Stephens, 1989), LGV3, E (strain Bour), C (De La Maza *et al.*, 1991), B (Watson *et al.*, 1989), E (strain DK20) (Coles *et al.*, 1990); *C. psittaci* avian strains EAE A/22M (Watson *et al.*, 1990a), 6BC (Everett & Hatch, 1991); *C. pneumoniae* IOL 207 (Watson *et al.*, 1990b). The evolutionary relationships of these sequences have been analysed and a phylogenetic tree for chlamydia proposed (Fitch *et al.*, 1993) which shows three distinct arms each representing the *C. trachomatis*, *C. psittaci* and *C. pneumoniae* species.

Major antibody responses to 60 kDa proteins in chlamydial infection have been reported (Newhall *et al.*, 1982; Batteiger & Rank, 1987; Brunham *et al.*, 1987; Wagar *et al.*, 1990). Preliminary studies using lactoperoxidase-catalysed radioiodination of EBs suggested the 60 kDa CrP might be surface exposed (Batteiger *et al.*, 1985; Newhall *et al.*, 1982). These observations coupled to the higher net positive charge seen for the LGV biovar and *C. psittaci* 60/62 kDa (doublet) CrPs compared to the singlet 60 kDa CrP from trachoma biovars of *C. trachomatis* have led to the suggestion that this set of proteins may have a role in enhancing chlamydial attachment to host cells (Batteiger *et al.*, 1985). Other workers have suggested, on purely hypothetical grounds, that the 60 kDa CrP might be associated with the cytoplasmic face of the inner membrane (Everett & Hatch, 1991).

In the only experimental study of 60 kDa CrP epitope localization, surface exposure of the *C. trachomatis* 60 kDa CrPs was not demonstrated (Collett *et al.*, 1989). However, this study was limited because it used three relatively uncharacterized monoclonal antibodies to the 60 kDa CrP, and a single polyclonal mouse antiserum of undefined molecular specificity.

Thus, despite extensive serological surveys, the role of the 60/62 kDa CrPs in human immunity to chlamydial

infection remains unresolved. Also it is still unclear whether these proteins are surface exposed on EBs (Fitch *et al.*, 1993). Therefore, the purpose of this work was to investigate the immunoreactivity of the chlamydial 60 kDa CrPs. Our approach was to attempt expression in *E. coli* of the intact 60/62 kDa CrP from *C. trachomatis*, *C. psittaci* and *C. pneumoniae* to obtain recombinant 60 kDa CrPs free from all other chlamydial proteins. These products could then be used to prepare mono-specific high titre polyclonal antisera to the 60 kDa CrPs. We wished to use these resources to perform a comprehensive analysis of the immunological relationships between the 60 kDa CrPs from the three chlamydial species and also to address the important unresolved question of 60 kDa CrP surface localization by immunogold labelling of purified EBs from all three chlamydial species.

METHODS

Organisms. *C. trachomatis* serovar B (strain B/Jali 20/OT), *C. psittaci* (strain EAE A22/M) and *C. pneumoniae* (strain IOL 207) were grown and purified as described previously (Salari & Ward, 1981; Carter *et al.*, 1991). The *C. psittaci* strain has been described by Pickett *et al.* (1988). Chlamydia were stored at -80°C to preserve their structure and viability. *E. coli* strain K12 JM109 was used for all genetic manipulation work.

Oligonucleotide synthesis and PCR reactions. Oligonucleotides for use as sequencing or polymerase chain reaction (PCR) primers were synthesized on a model 381A automated DNA synthesizer (Applied Biosystems) using β -cyanoethyl phosphoramidite chemistry. PCR reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler. The reaction conditions were as recommended by Perkin-Elmer Cetus; KCl 50 mM; 10 mM Tris/HCl (pH 8.8); MgCl_2 15 mM; Triton X-100 1% (v/v); 0.2 mM each dNTP; 1 μM each primer; 10–100 ng template DNA; 1–2.5 units of *Taq* DNA polymerase.

Molecular cloning of the 60 kDa CrP genes for expression in *E. coli*. The recombinant plasmids used in this study together with details of their expressed products are summarized in Table 1. The entire 60 kDa CrP gene of *C. trachomatis* serovar B/Jali-20/OT was sub-cloned as a 1.8 kbp *SpeI* fragment from the pBluescript vector (pJ3B) described by Watson *et al.* (1989) into a modified pGEMEX 1 vector (Promega). Plasmid pGEMEX1 was modified by insertion of a *SpeI* restriction site (TCGAGACTAGTC) at the *XbaI* site to form vector pGEMSPE. Plasmid clones in both possible orientations [pJGEM(a), positive orientation for expression and pJGEM(b), inverse orientation] were identified using restriction endonuclease digestion with *XbaI* and agarose gel electrophoresis.

A 2.95 kbp *XbaI* genomic fragment containing the 60 kDa CrP of *C. psittaci* (Watson *et al.*, 1990a) was cloned into the *XbaI* site of pBluescript SK $^{-}$ using the λ Zap II vector system (Stratagene) to yield recombinant plasmids pPS8C (Fig. 1b) and the opposite orientation insert pPS9C.

A 60 kDa CrP gene cassette from *C. pneumoniae* was generated by PCR using two synthetic, restriction-site-containing oligonucleotides, IOLNXho (5' ACCCAAATCTCGAGGGTAAGAGTTTACAAA3') and IOLCXba (5' TATCGCTCTGCTCTAGAATAATTCCTTAGA3'), based on the sequences described by Watson *et al.* (1990b). The 60 kDa CrP gene was amplified from genomic DNA of IOL-207, repaired with T4 DNA polymerase and ligated into *SmaI* cut, dephosphorylated pSP73 (Promega) and transformed into *E. coli* JM109 to give

Table 1. Recombinant plasmids

Source of DNA	Recombinant plasmid	Host vector	Expression of recombinant protein
<i>C. trachomatis</i>	pJ3B	pBlueScript	None
<i>C. psittaci</i>	pPS8C	pBlueScript	'Native' 60 kDa CrP
<i>C. trachomatis</i>	pJGEM	pGEMEX-1 (<i>SpeI</i> linker)	Gene 10 60 kDa CrP fusion
<i>C. pneumoniae</i>	pPUNAT	pSP73	'Native' 60 kDa CrP
<i>C. pneumoniae</i>	pPUGEM	pGEMEX-1	Gene 10 60 kDa CrP fusion

Fig. 1. Molecular details of recombinant plasmids. (a) *C. trachomatis* pJ3B (in pJ4B the 1.8 kbp *SpeI* insert is in the opposite orientation), (b) *C. psittaci* pPS8C (pPS9C opposite orientation), (c) *C. trachomatis* pJGEM, (d) *C. pneumoniae* pPUNAT, (e) *C. pneumoniae* pPUGEM.

pPUNAT (Fig. 1d). The *C. pneumoniae* 60 kDa CrP was also expressed as a fusion protein in pGEMEX1. Synthetic oligonucleotides were used to generate a PCR product containing the *C. pneumoniae* 60 kDa CrP gene flanked by suitable restriction sites. The oligonucleotides used were IOLFNXho (5'CTT-TGCCAGCGGGGTCTCGAGGCCGCTGT3') and IOLF-CXhoHind (5'CTCGAGCGGTACTTCTTTGAAAGCTTT-CCTAAA3'). The PCR fragment was digested with *XhoI* and *HindIII*, purified by GeneClean and ligated into *XhoI*/*HindIII* cut, dephosphorylated pGEMEX1 (Promega) to give pPUGEM (Fig. 1e).

Freshly transformed *E. coli* containing appropriate expression vectors were grown to an OD₆₀₀ of 0.5 in Luria broth containing 100 µg ampicillin ml⁻¹ before addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 5 mM. The culture was grown with shaking for a further 3 h, after which the cells were harvested by centrifugation at 4000 g. Cells were used immediately and assessed by SDS-PAGE for expression of 60 kDa CrPs.

Preparation of *E. coli* outer membranes. *E. coli* outer membranes were prepared essentially by the method of Osborn & Munson (1974). Briefly, spheroplasts were lysed and after removal of cell debris by low speed centrifugation, the supernatant was centrifuged at 100 000 g for 2 h. The resultant membrane pellet was resuspended in 2 ml cold 25% (w/v) sucrose/5 mM EDTA, pH 7.5, and layered onto a 55% to 30% sucrose step gradient and centrifuged at 100 000 g for 18 h. Gradient fractions containing outer membranes were collected and assayed for protein using the bicinchoninic acid technique (Smith *et al.*, 1985).

Purification of inclusion bodies. A 250 ml culture of recombinant *E. coli* was induced, harvested by centrifugation and resuspended in 15 ml TAS, a mixture of Tris/acetate (10 mM), sucrose (0.75 M) and sodium azide (0.05%) on ice. Lysozyme (20 mg) was added and the bacteria stirred gently for 5 min before addition of 30 ml 1.5 mM EDTA (pH 7.5). Resultant spheroplasts were lysed by sonication and cell debris removed by centrifugation at 4000 g for 10 min. Inclusion

bodies were sedimented by centrifugation at 10000 *g* for 15 min and washed in 20 ml TAS containing 5% Triton X-100. The pellet of inclusion bodies was resuspended in 5 ml freshly prepared sodium azide (0.05%)/phenylmethylsulphonyl-fluoride (1 mM)/EDTA (1 mM). Where required inclusion bodies were further purified by preparative SDS-PAGE. Protein bands were recovered by electroelution into TE buffer, containing Tris/HCl (10 mM, pH 7.5) and EDTA (1 mM, pH 8.0). Eluted protein was precipitated by dialysis against saturated ammonium sulphate and pelleted by centrifugation at 12000 *g* for 5 min.

Antisera. A pool of monoclonal antibodies to the 60 kDa CrP of *C. trachomatis* (Newhall & Basinski, 1986) were obtained from Dr J. Newhall (CDC, Atlanta, GA, USA) for use as immunoblotting probes.

Polyclonal antisera to the recombinant 60 kDa CrPs from *C. psittaci*, *C. trachomatis* and *C. pneumoniae* were raised in female New Zealand White rabbits. Pre-immune serum was obtained from each animal followed by subcutaneous immunization on day one with 100 µg protein in Freund's complete adjuvant and subsequent immunizations with 100 µg protein in Freund's incomplete adjuvant on days 15, 29, 43, 57, 67, 71 and 85. Antisera were raised to the following recombinant proteins: *C. pneumoniae* 60 kDa CrP 'native protein'; *C. trachomatis* 60 kDa CrP 'fusion protein'; *C. pneumoniae* 60 kDa CrP fusion protein; *C. psittaci* 60 kDa CrP native protein; T7 gene 10 protein; *E. coli* JM109/DE3 total cell extract. Cross-reacting antibodies were removed by adsorption with proteins obtained from sonic extracts of *E. coli* JM109/DE3.

SDS-PAGE and immunodetection of proteins. SDS-PAGE was performed using the discontinuous buffer system method of Laemmli (1970) with 10% acrylamide gels (acrylamide:bisacrylamide 38.5:1, w/w). Electrophoresis to nitrocellulose sheets was performed in transfer buffer containing Tris/HCl (25 mM), glycine (192 mM), SDS (0.1%, w/v) and methanol (20%, v/v) using a Trans-Blot SD 'semi-dry' blotter (Bio-Rad). Following blotting the nitrocellulose membrane was washed in TTBS blocking solution, containing TBS [NaCl (0.5 M) and Tris/HCl (20 mM); pH 7.5] and Tween-20 (0.05%, v/v) at 22 °C. The blocking solution was discarded and primary antibody (1/200) in TTBS/1% (w/v) dried skimmed milk (Marvel) was added to the tube (10 ml) for between 2 and 16 h. After washing in TTBS, membranes were treated with the secondary antibody-alkaline phosphatase conjugate. Alkaline phosphatase-antibody conjugates were detected using a BCIP/NBT (bromochloroindolyl phosphate/nitroblue tetrazolium salt) redox chromogenic reaction catalysed by alkaline phosphatase in a carbonate buffer (NaHCO₃, 0.1 mM/MgCl₂, 1.0 mM; pH 9.8). Greater sensitivity was achieved when micellar [3'-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxetane]AMPPD (Lumi-phos; Cambridge Research Biochemicals) was used to detect bound alkaline phosphatase.

Solid phase peptides and epitope mapping. Solid phase peptides were synthesized on polyethylene pegs using the method of Geysen *et al.* (1987). A nested set of overlapping (five residues) solid phase synthetic peptides (decamers) covering the entire sequence of the mature 60 kDa CrP of *C. trachomatis* serovar L1 were constructed in duplicate on polyethylene pegs. Pegs were blocked in 'supercoktail' a mixture containing 1% (w/v) ovalbumin (Sigma, grade II) and 1% (w/v) BSA in TPBS (0.145 M NaCl/60 mM Na₂HPO₄/28 mM NaH₂PO₄/0.05% (w/v) Tween-20; pH 7.4) in 96-well microtitre plates (150 µl per well) for 2 h at room temperature. Rabbit antisera were diluted 1:300 in 'supercoktail' and reacted with the pegs overnight at 4 °C. Unbound antibody was removed with four 10 min washes

in 1 × TPBS. Immunoreactive peptides were detected by treatment with goat anti-rabbit horse radish peroxidase conjugate and colour development in flat-bottomed microtitre plates using 150 µl ABTS substrate, containing Na₂HPO₄ (0.1 M), citric acid (0.08 M, pH 4.0), ABTS [2,2'-azino-di(3-ethylbenz-thiazoline sulphonic acid)] (Sigma, 0.5 mg ml⁻¹) and H₂O₂ (120 vol., 0.3 µl ml⁻¹) per well. Absorbance was monitored at 402 nm in a Titertek Twinreader colorimeter (Flow Laboratories). Trays of pegs were processed for further rounds of ELISA by sonication for 30 min at 60 °C in disruption buffer containing NaH₂PO₄ (0.1 M), 2-mercaptoethanol (0.1%, v/v) and SDS (1%, w/v), pH 7.2, using a Camlab Transsonic T700/H sonicator to remove bound antibody. Disruption buffer was removed by four washes with 60 °C water before placing the peg trays into boiling methanol for 2 min. Pegs were then air dried and stored desiccated at 4 °C or blocked immediately for another round of ELISA.

Electron microscopy. *E. coli* cells expressing the chlamydial 60 kDa CrPs were examined by electron microscopy as described by Pickett *et al.* (1988). For immunogold studies purified elementary bodies were incubated with rabbit antisera at 37 °C for 1 h. Unbound antibody was removed by three washes with TBS. EBs were then resuspended in 50 µl of a 1 in 25 dilution of protein-G-gold conjugate (15 nm particle size; BioCell) in TBS and incubated at 37 °C for 1 h. Unbound gold conjugate was removed by washing in TBS and the EBs resuspended in 50 µl water. Drops of EB suspensions (5 µl) were placed on nickel Formvar electron microscopy grids coated with poly-lysine. The grids were blotted dry and viewed using a Hitachi H7000 transmission electron microscope.

RESULTS AND DISCUSSION

Molecular cloning and expression of chlamydial 60 kDa CrPs

We have previously encountered problems of stability when cloning the amino terminus of the 60 kDa CrP gene of *C. trachomatis* L1 into plasmid vectors (Clarke & Lambden, 1988). Thus our alternative approach was to attempt high level expression of the 60 kDa CrPs of *C. trachomatis* (B/Jali 20/OT) serovar B as a fusion protein using the tightly regulated pGEMEX vector system (Promega). In this system recombinant proteins are fused to the bacteriophage T7 capsid gene 10 protein and are under control of the T7 RNA polymerase promoter (Studier & Moffatt, 1989). The *C. trachomatis* serovar B 60 kDa CrP had previously been shown by Southern blot and sequence analysis to be almost entirely contained within a 1.8 kbp *SpeI* restriction endonuclease fragment (Watson *et al.*, 1989) which was cloned in pBluescript to give pJ3B (Fig. 1a). This DNA fragment, missing only the 5' terminal 48 bp of the 60 kDa CrP open reading frame, was ligated into the modified pGEMEX vector pGEMSPE to generate pJGEM (Fig. 1c).

Southern blotting and restriction analysis of chromosomal DNA purified from *C. psittaci* EAE A22/M indicated that the 60 kDa CrP gene was located on a unique 2.95 kbp *XbaI* restriction endonuclease fragment (data not shown). This DNA fragment was cloned into the *XbaI* site of λZAP II and released as a pBluescriptSK⁻ recombinant plasmid pPS8C (Fig. 1b) as an intermediate sub-cloning step. Surprisingly, gel analysis of the recombinant proteins (Fig. 2a) indicated expression of the 60 kDa CrP

Fig. 2. (a) PAGE blue-stained SDS-polyacrylamide gel (10%) of *E. coli* proteins extracted from various expressing recombinants. Lanes: 1, *E. coli* JM109 (DE3) induced with IPTG; 2, *E. coli* JM109 (DE3) uninduced; 3, pGEMEX1 in JM109 (DE3) induced with IPTG; 4, pGEMEX1 in JM109 (DE3) uninduced; 5, pPS9C in JM109 (DE3) induced with IPTG; 6, pPS9C in JM109 (DE3) uninduced; 7, pPS8C in JM109 (DE3) induced with IPTG; 8, pPS8C in JM109 (DE3) uninduced; 9, pPUGEM in JM109 (DE3) induced with IPTG; 10, pPUGEM in JM109 (DE3) uninduced; 11, pJGEM in JM109 (DE3) induced with IPTG; 12, pJGEM in JM109 (DE3) uninduced; 13, pPUNAT in JM109 (DE3) induced with IPTG; 14, pPUNAT in JM109 (DE3) uninduced; S, standard molecular mass markers. (b) Immunoblot of a duplicate gel to (a) with identical lane loading order. Molecular mass standards on blots were stained with amido black. A pool of monoclonal antibodies to *C. trachomatis* 60 kDa CrPs (Newhall & Basinski, 1986) was used to detect expressed recombinant proteins.

Fig. 3. Transmission electron micrographs of inclusion bodies seen in IPTG-induced *E. coli* [JM109 (DE3)]. (a) no plasmid, (b) pPS8C (*Chlamydia psittaci*, native 60 kDa CrP), (c) pJGEM (*Chlamydia pneumoniae*, gene 10–60 kDa CrP fusion protein). Bar, 2 µm.

Fig. 4. *C. pneumoniae* purified EBs separated by SDS-PAGE and probed with antisera against (lanes): 1, *C. psittaci*, native 60 kDa CrP; 2, *C. pneumoniae*, native 60 kDa CrP; 3, *C. pneumoniae*, gene 10–60 kDa CrP fusion; 4, *C. trachomatis*, gene 10–60 kDa CrP fusion; 5, control *E. coli* JM109 (DE3); 6, control *E. coli* JM109 (DE3) containing pGEMEX-1. Lane S, amido black stained molecular mass markers; Lane M, *C. pneumoniae* EBs separated on a different gel and probed with the pool of 60 kDa CrP monoclonal antibodies (Newhall & Basinski, 1986).

from both T7 and *lac* promoters for the two possible orientations of the insert (pPS8C and pPS9C).

The *C. pneumoniae* 60 kDa CrP gene did not have useful restriction endonuclease cleavage sites for cloning; therefore a pair of oligonucleotide primers based on the published flanking nucleotide sequences of the *C. pneumoniae* 60 kDa CrP gene (Watson *et al.*, 1990b) were used in the PCR to generate a 60 kDa CrP gene cassette. The PCR product was ligated into the *Sma*I site of the cloning vector pSP73 to give pPUNAT (Fig. 1d). This construct under control of the T7 promoter expressed the 60 kDa CrP at approximately half the levels of the *C.*

psittaci recombinant (pPS8C) as judged by SDS-PAGE. In order to increase expression of the *C. pneumoniae* 60 kDa CrP a second pair of primers were synthesized. These primers incorporate unique *Xba*I and *Hind*III restriction sites to facilitate directional cloning of the PCR products into pGEMEX 1 to give pUGEM (Fig. 1e).

High level expression of the 60 kDa CrP was achieved for all of the chlamydial species as assessed by gel analysis of total cell proteins (Fig. 2a). Growth curves for all recombinants showed a significant reduction in growth rates following induction with IPTG (data not shown). Interestingly, recombinant bacteria expressing the *C. psittaci* 60 kDa CrP (pPS9C) clumped on induction with IPTG (as visualized by phase contrast microscopy), causing a rapid reduction in optical density of the culture supernatant.

Properties of recombinant 60 kDa CrPs

Comparison of the deduced amino acid sequence from the *C. psittaci* 60 kDa CrP of strain 6BC and the 60 kDa CrP EAE/A22/M used in this study has shown only two amino acid differences (Everett & Hatch, 1991). The intact 60 kDa CrP from *C. psittaci* 6BC was expressed in *E. coli* and was reported to form a doublet, leading to the suggestion that it had undergone two-site post-translational cleavage (Everett & Hatch, 1991). The implication of this observation is that the protein was processed in a similar manner to its native chlamydial host and was located to the *E. coli* cell envelope. We were therefore interested to see if the two intact recombinant 60 kDa CrPs were transported to the *E. coli* cell envelope. Localization to the outer membrane may alter cell surface properties which could account for the aggregation of cells seen in the recombinant containing pPS9C after induction with IPTG.

E. coli cell envelope preparations were purified from each recombinant expressing chlamydial 60 kDa CrPs. The outer membrane preparations were analysed by polyacrylamide gel electrophoresis and showed the expected *E. coli* outer membrane proteins and LPS but no

Fig. 5. Reactivity of polyclonal 60 kDa CrP antisera with solid phase peptides of the 60 kDa CrP of *C. trachomatis*. Antisera are indicated at the top of each chart. The reactivities are the mean of two duplicate reactions with reactivities of pre-immune serum subtracted [in all cases pre-immune serum and serum raised to *E. coli* JM109 (DE3) containing the bacteriophage T7 gene 10 capsid protein showed no significant reaction with the peptide pegs]. Each nested decamer differs by five amino acids and is labelled between 1 and 103, corresponding to amino acids $(n \times 5) - 5$ to $(n \times 5) + 5$ (where n is the decamer number) of the mature 60 kDa CrP of *C. trachomatis* serovar L1/440/LN (amino acid number 41 onwards). Reactivity is measured as A_{402} at the end of the ELISA assays.

additional proteins (data not shown). Concentrated samples of growth media obtained from expressing cultures were similarly analysed and contained no 60 kDa CrPs. These results indicate that the 60 kDa CrPs were not secreted from *E. coli* nor was there any evidence for cell lysis under inducing conditions. We also conclude that *E. coli* does not localize the chlamydial 60 kDa CrPs to its outer membrane when expressed from these vectors.

Transmission electron microscopy on thin sections of IPTG-induced *E. coli* showed that all the recombinant 60 kDa CrPs were expressed as electron-dense insoluble cytoplasmic inclusions. Examples of the *C. psittaci* 'native' 60 kDa CrP and *C. pneumoniae* gene 10 60 kDa CrP fusion are shown in Fig. 3(b, c). It was possible to recover the large cytoplasmic inclusions of recombinant 60 kDa CrPs by a simple cell lysis procedure followed by sedimentation of insoluble inclusion bodies. This approach yielded large amounts of crude recombinant chlamydial protein. The yield of crude protein from inclusion body preparations was 0.2–0.4 g protein (g wet

wt *E. coli*)⁻¹ for non-fusion protein recombinants and 0.4–0.62 g protein (g wet wt *E. coli*)⁻¹ for fusion proteins. Higher yields of protein were produced using freshly transformed host cells. Inclusions prepared in this way were contaminated with some *E. coli* host proteins. The inclusion bodies were completely soluble only in 2% SDS in the presence of 5% 2-mercaptoethanol. Solubilization of the inclusions in urea or guanidine and re-precipitation of the protein by dilution removed little of the contaminating *E. coli* proteins. Therefore the recombinant 60 kDa CrPs were further purified using preparative SDS-PAGE and electroelution.

Immunological studies

The gel-purified recombinant 60 kDa products were used to immunize rabbits to produce high titre hyperimmune polyclonal antisera. Immunoblot analysis of these high titre sera with chlamydial proteins from purified EBs showed genus-reactive properties with the three chlamydial species and the heterologous recombinant

antigens detectable at serum dilutions up to 1:10 000. Preimmune sera were negative in these blots as were antiserum against the *E. coli* host strain (JM109) and control antiserum to purified T7 gene 10 inclusions. Fig. 4 shows an example of the cross-reactivity of the four antisera to the different 60 kDa CrP recombinant products and control antisera to reduced and denatured *C. pneumoniae* EB antigens. These results also clearly demonstrate that the 60 kDa CrPs of *C. pneumoniae* IOL-207 are expressed as a 60/62 kDa 'doublet'.

The molecular specificity of the various antisera was investigated by epitope mapping studies. A series of 110 solid phase decameric peptides overlapping by five amino acids covering the coding sequence for the mature *C. trachomatis* L1 60 kDa CrP were synthesized. The results obtained with each antiserum are shown in Fig. 5. All recombinant antisera demonstrated some areas of reactivity interspersed with areas of lower or no reactivity. Reactions with the *C. trachomatis* fusion antisera showed six peptides (26, 43, 44, 65, 94 and 101) with significant sero-reactivities.

The degree of cross-reactivity of the *C. psittaci* 60 kDa CrP antiserum can be seen in Fig. 5(b). As expected from their different primary amino acid sequences, cross-reactivity of *C. psittaci* 60 kDa CrP antiserum with the *C. trachomatis* peptides was reduced compared to the homologous system. The only significantly reactive peptide common to both *C. psittaci* and *C. trachomatis* antisera was decamer 101 (GEAILSSDTL), which is conserved across the genus. The antisera raised to the two recombinant forms of the *C. pneumoniae* IOL-207 60 kDa CrP were reacted with the *C. trachomatis* 60 kDa CrP decameric peptides; the results are shown in Fig. 5(c, d). The two antisera demonstrated very similar patterns of reactivity, indicating the minimal effect of the N-terminal fusion with the T7 gene 10 protein on the antibody responses elicited to these proteins. Interestingly, the two *C. pneumoniae* antisera reacted similarly to the *C. psittaci* antisera with decamers 100 and 101. Control hyperimmune antisera to bacteriophage T7 gene 10 and to *E. coli* JM109 showed no reactivity with the 60 kDa CrP decameric peptides.

Surface accessibility of 60 kDa CrP epitopes

The possibility that the 60 kDa CrPs were surface exposed was investigated by immunogold labelling and transmission electron microscopy. Purified chlamydial preparations from each of the three species that were a mixture of EB with intermediate bodies and a few reticulate bodies were used in these studies. An antiserum raised to *C. trachomatis* recombinant MOMP expressed in *E. coli* as insoluble cytoplasmic inclusion bodies (Conlan *et al.*, 1990) was used as a positive control to demonstrate the technique. The results indicated that none of the antisera to the various 60 kDa CrPs showed any surface labelling in either heterologous or homologous combinations.

Thus the chlamydial 60 kDa CrPs are potent immunogens in rabbits and possess genus-reactive epitopes as assessed by immunoblot analysis. Further dissection of the anti-

body responses using synthetic peptides has indicated a common immunogenic linear peptide (decamer 101) of the 60 kDa CrP which exhibits genus-reactive properties. This study also shows that the epitopes recognized by the 60 kDa CrP antisera are not accessible on the EB surface in any of the three chlamydial species. To locate the precise position of the 60 kDa CrP within the chlamydial cell envelope, immunogold labelling experiments will have to be performed on cell sections of EBs using transmission electron microscopy. The technology is currently limited by the quality of embedding resins. Immunogold labelling can be performed on frozen sections although the quality of tissue preservation is still poor, with only the outermost regions of frozen tissue being perfectly preserved. Future work will involve transmission electron microscope studies on frozen sections of purified EBs and the recombinant antisera described here to locate the 60 kDa CrPs in the chlamydial EB envelope.

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