The immunoreactive 116 kDa surface protein of *Mycoplasma pneumoniae* is encoded in an operon

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Sera from 10 patients infected with *Mycoplasma pneumoniae* were used in Western blot analysis of Triton-X-114-soluble protein preparations of *M. pneumoniae*. All 10 sera were reactive with a protein antigen of 116 kDa. Sera from another 17 patients were used in Western blot analysis of whole-cell *M. pneumoniae* proteins; 15 of these sera were reactive with the 116 kDa protein. Trypsin digestion of whole *M. pneumoniae* cells demonstrated the surface location of this protein. Sequencing of DNA which contained the gene for this protein identified an ORF of 3093 bp encoding a protein with a predicted molecular mass of 116 013 Da. The ORF for the 116 kDa protein had 99.8% nucleotide identity with the *M. pneumoniae* gene G07_orf1030 and 61% nucleotide identity with the *Mycoplasma genitalium* ORF MG075 of unassigned function. An ORF which was identified 5' to the 116 kDa protein ORF coded for a 16 kDa protein and had 99.8% nucleotide identity with the *M. pneumoniae* gene G07_orf135 and 58.4% nucleotide identity with the ORF MG074 of *M. genitalium*. Analysis of mRNA detected a 3.7 kb transcript with a single initiation site 5' to the ORF encoding the 16 kDa protein. The coding sequences for both the 16 kDa protein and the 116 kDa protein were present in this transcript, indicating that they were part of an operon and suggesting a possible functional relationship.

Keywords: *Mycoplasma pneumoniae*, 116 kDa surface protein, antigen, expression cloning, operon

INTRODUCTION

Mycoplasma pneumoniae is the primary cause of atypical pneumonia in young adults and children and has been associated with 18% of all cases of community-acquired pneumonia, second only to *Streptococcus pneumoniae* (responsible for 34% of cases) (Research Committee of the British Thoracic Society and the Public Health Laboratory Service, 1987). Infected patients often present with symptoms which resemble those of a persistent influenza infection. Although clinicians may suspect infections during epidemics (Levine & Lerner, 1978), misdiagnosis and subsequent inappropriate antibiotic treatment pose a threat to children and adults sensitized to *M. pneumoniae* antigens by previous infection (Tully & Baseman, 1991).

Diagnosis by culture can take weeks and complement-fixation tests are time-consuming and of low specificity (Jacobs, 1993). Rapid serodiagnostic tests are available but these employ crude antigen preparations and consequently their specificity is low (Thacker & Talkington, 1995).

As the complement-fixation test detects primarily an IgM response (Jacobs, 1993), an ELISA or a similar method employing cellular fractions of minimal cross-reactivity would be preferable. The advantage of using a pure preparation of a single protein as a serodiagnostic reagent as opposed to the crude preparations currently in use lies in the ability to increase the sensitivity of the test without sacrificing specificity.

M. pneumoniae membrane proteins of 100–115 kDa have been previously noted as antigens. A 105 kDa protein enriched in the detergent phase following Triton X-114 partitioning of *M. pneumoniae* appeared to be an abundant protein with hydrophobic domains (Hirsch-

Abbreviations: GST, glutathione S-transferase; RT, reverse transcriptase.

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berg *et al.*, 1989). Trypsin digestion and radioimmuno-precipitation have identified a 110 kDa protein as a surface antigen of *M. pneumoniae* (Leith *et al.*, 1983). A radiolabelled 110 kDa protein has been demonstrated to bind to hamster tracheal cells and this binding was competitively inhibited by unlabelled 110 kDa protein, indicating a possible role for the protein as an adhesin with a specific host receptor (Krause *et al.*, 1982). Immunoblotting revealed a 110 kDa antigen in 80% of strains investigated, with a high level of expression only in the high-passage type strain FH. However, all human convalescent sera tested reacted with the 110 kDa protein of the FH strain, indicating that the protein is universally expressed but that its expression may be down-regulated in field strains upon *in vitro* culture (Vu *et al.*, 1987).

The antigenicity of the 100–116 kDa polypeptides of *M. pneumoniae* and their possible role in adhesion to tracheal cells recommended them for detailed study. Specifically the following work was aimed at the identification of the gene encoding an antigenic 116 kDa protein, determination of its nucleotide sequence and characterization of the mRNA transcript used for its translation.

METHODS

Strains and culture conditions. *M. pneumoniae* strain FH (ATCC 15531) was grown in SP4 medium (Whitcomb, 1983) in glass bottles at 37 °C until the phenol red indicator changed colour to orange. Adherent organisms were scraped into the medium and harvested by centrifugation at 12 000 g at 4 °C for 30 min. The mycoplasmal pellets were washed three times with PBS.

Triton X-114 partitioning of *M. pneumoniae* cellular proteins. Proteins with hydrophobic domains were isolated in the detergent phase following Triton X-114 partitioning (Bordier, 1981). A 700 ml culture of *M. pneumoniae* was washed three times with PBS. The cell pellet was resuspended in 5 ml ice-cold 0.5% Triton X-114 in PBS, mixed, and incubated on ice for 60 min. Insoluble material was precipitated by centrifugation at 11 000 g at 4 °C for 35 min. The supernatant fraction was layered on 1 ml ice-cold 6% (w/v) sucrose containing 0.06% Triton X-114 in PBS, incubated at 37 °C for 9 min, and the detergent phase then precipitated by centrifugation for 7 min at 300 g at 37 °C. The supernatant fraction containing water-soluble proteins was aspirated to a separate tube and the detergent phase resuspended in 2 ml cold PBS. Total cell protein from a 700 ml culture was 16 mg, of which 210 µg was recovered in the detergent phase.

The detergent phase enriched for proteins with hydrophobic domains was methanol/chloroform precipitated (Wessel & Flugge, 1984) and the dried protein pellet resuspended in 4 M urea in PBS and examined by SDS-PAGE on a 10% polyacrylamide gel followed by Coomassie blue staining.

Western blotting with sera from patients infected with *M. pneumoniae*. *M. pneumoniae* whole-cell proteins and *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase were separated by SDS-PAGE on 10% gels, transferred to PVDF membrane and Western blotted (Jacobs *et al.*, 1986). Ten sera from *M. pneumoniae*-infected patients and two negative control sera were kindly supplied by Ms Vicki Peters, Department of Virology, The Royal Children's Hospital,

Melbourne. Diagnosis of infection had been based on in-house ELISA detection of anti-*M. pneumoniae* IgG. These sera were used at 1/100 dilution in Western blots to examine antibody reactivity with *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase. Bound human IgG was detected with rabbit anti-human IgG conjugated to horseradish peroxidase (Dako) at a dilution of 1/600.

Sera from a further 17 infected patients were kindly supplied by Dr Michael Catton and Ms Jennie Leydon, Victorian Infectious Diseases Laboratory, Fairfield Hospital, Fairfield, Victoria, Australia. Diagnosis was based on a gelatin particle agglutination assay with undefined membrane components of *M. pneumoniae* strain Mac as antigen (Serodia-Mycoll, Fujirebio). Typically diagnosis was based on a single serum sample with a positive antibody titre. Paired sera were available for four patients; of the remaining 13 patients, one was described as convalescent and the rest as acute. These sera were tested against whole-cell proteins of *M. pneumoniae* in Western blots at a dilution of 1/600. Bound human IgG was detected with rabbit anti-human IgG conjugated to horseradish peroxidase (Dako) at a dilution of 1/800.

Preparation of rabbit antiserum to the 116 kDa protein and glutathione S-transferase (GST). *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase were separated by SDS-PAGE through 10% gels. Strips from each side of the gels were marked, cut from the gel, Coomassie blue stained, destained in 7% acetic acid and then realigned. The region of the unstained gel containing the 116 kDa band was excised, the gel strips ground between glass plates and the macerated gel subsequently used to immunize rabbits intramuscularly (Harlow & Lane, 1988). Twenty micrograms of protein was used per inoculation. The initial inoculation of antigen was delivered in Freund's complete adjuvant and four subsequent inoculations were administered in Freund's incomplete adjuvant. The rabbit antiserum had a titre of 1/2700 by Western blotting. The optimal signal-to-background ratio was obtained at a dilution of 1/800 and this dilution was used in all subsequent Western blots.

Rabbit antiserum raised to GST was donated by Dr Heidi Drummer, Department of Veterinary Science, The University of Melbourne.

The rabbit anti-116 kDa protein antiserum was used to detect recombinant proteins by colony immunoblotting of transformed *Escherichia coli* strain DH5α. To remove reactivity with *E. coli* the rabbit anti-116 kDa protein antiserum was affinity purified by an adaptation of a previously described method (Beall & Mitchell, 1986). Rabbit anti-GST antiserum was purified by the same method. In brief, *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase, or purified GST, were transferred to PVDF membranes. The region of the membrane to which the 116 kDa protein, or GST, had bound was excised and incubated for 2 h with 200 µl antiserum diluted 1/10 in 20% (v/v) foetal calf serum in PBS containing 0.05% Tween 20. The membrane was washed three times for 10 min each with PBS containing 0.05% Tween 20 and the antibody eluted by incubation in 2 ml 0.15 M NaCl containing 0.1 M glycine, pH 2.6, for 3 min. The pH of the eluted antibody was immediately neutralized by addition of 210 µl 2 M Tris/HCl, pH 7.5. The affinity-purified antibody was then diluted in 4.4 ml 5% (v/v) foetal calf serum in PBS containing 0.05% Tween 20.

Trypsin digestion of cell-surface proteins. Adherent *M. pneumoniae* cells from a 50 ml culture were washed three times with PBS, resuspended in 2 ml PBS and divided into 0.5 ml aliquots, each of which contained 0.065 mg whole-cell

M. pneumoniae protein. The cells in one aliquot were harvested by centrifugation and resuspended in 0.5 ml 1% Nonidet P-40 in PBS. An aliquot of intact washed cells and of *M. pneumoniae* cells lysed with Nonidet P-40 were each incubated at 37 °C with 50 µg trypsin for 15 min and an intact cell suspension was incubated without trypsin for 15 min. Following incubation, 50 µg soybean trypsin inhibitor was added to each tube. The intact cells were recovered by centrifugation at 16000 *g* for 4 min and the cell pellets washed twice with 1 ml PBS prior to suspension in 60 µl Laemmli gel sample buffer. Equivalent proportions of each sample were examined by SDS-PAGE and Western blotting using undiluted affinity-purified rabbit anti-116 kDa protein antibodies as primary detection agent.

Nucleic acid extraction. DNA was extracted from *M. pneumoniae* as described by Su *et al.* (1988). RNA was extracted from 350 ml *M. pneumoniae* culture. Following centrifugation the cells were immediately resuspended in 0.6 ml PBS and 10 ml 4 M guanidinium thiocyanate containing 25 mM sodium citrate and 0.1 M β -mercaptoethanol, pH 7, was added and the suspension homogenized for 5 s with a Polytron homogenizer (Kinematica). The subsequent procedure was as described by Chomczynski & Sacchi (1987). Briefly, 0.25 ml 20% (w/v) Sarkosyl, 1 ml 2 M sodium acetate, 10 ml water-saturated phenol and 2 ml 49:1 chloroform/isoamyl alcohol were added sequentially to the homogenized cells, and the solution was mixed and incubated on ice for 5 min. The solution was centrifuged at 10000 *g* for 20 min at 4 °C and the upper phase transferred to a fresh tube to which an equal volume of ice-cold 2-propanol was added. RNA was precipitated by incubation at -20 °C for 60 min followed by centrifugation at 10000 *g* for 20 min at 4 °C. RNA was resuspended in 3 ml 4 M guanidinium thiocyanate containing 25 mM sodium citrate and 0.1 M β -mercaptoethanol, pH 7, an equal volume of ice-cold 2-propanol added and RNA precipitated as described above. The precipitated RNA was washed with ice-cold 70% ethanol and precipitated in an Eppendorf centrifuge for 5 min at 4 °C. The precipitated RNA was vacuum dried and resuspended to 2.6 mg RNA ml⁻¹ in diethylpyrocarbonate-treated water.

DNA cloning and sequencing. *M. pneumoniae* DNA was digested with a series of dilutions of the restriction endonuclease *Sau3AI* (Sambrook *et al.*, 1989). Digestion was optimized to obtain an average fragment size of 1500 bp.

M. pneumoniae DNA partially digested with *Sau3AI* was ligated to the *Bam*HI-digested plasmid vector pGEX-1N (Pharmacia) and this used to transform *E. coli* DH5 α cells. Recombinant plasmids expressed the protein encoded by the DNA fragments as a fusion protein with the 26 kDa GST from *Schistosoma japonicum*.

E. coli clones containing recombinant plasmids expressing portions of the *M. pneumoniae* 116 kDa protein were detected by colony blotting using the previously described affinity-purified rabbit anti-116 kDa protein antibodies at a dilution of 1/3.

*Bam*HI-digested pGEX-1N, ligated and transformed into *E. coli* DH5 α cells, was used as a negative control for the colony blotting. A positive control for the colony blotting was provided by probing the expression library with a rabbit serum raised against whole cells of *M. pneumoniae* that had reactivity with *E. coli* removed by absorption with *E. coli* DH5 α -cells.

Plasmids from clones that were reactive with the affinity-purified rabbit anti-116 kDa protein antibodies were manually

sequenced using Deaza G/A ³²P Sequencing Mixes (Pharmacia). *Eco*RI-digested *M. pneumoniae* genomic DNA was probed by Southern blotting with ³²P-labelled plasmid DNA from clones reactive with the affinity-purified rabbit anti-116 kDa protein antibodies. Two DNA fragments that encoded the 116 kDa protein were identified in the *Eco*RI-digested *M. pneumoniae* genome. *Eco*RI-digested *M. pneumoniae* DNA was ligated to plasmid pUCBM20 (Boehringer Mannheim) and the resulting library screened with an [α -³²P]dCTP-labelled *Nhe*I-*Sma*I 3062 bp fragment excised from the recombinant plasmid pGEX-1N-MP8. Recombinant plasmid pUCBM20MP-1b, containing the 3.3 kb *Eco*RI fragment, and pUCBM20-16b, containing the 7.9 kb *Eco*RI fragment, were sequenced as for pGEX-1N-MP recombinant plasmids.

Nucleotide sequence analysis was performed using the software programs of the Genetics Computer Group.

Southern blotting. Restriction endonucleases *Bgl*II and *Eco*RI were used to digest genomic DNA from *M. pneumoniae* strain FH, *M. gallisepticum* strains ts-11 (Whithear *et al.*, 1990) and 6/85 (Abd & Kleven, 1993), and *M. synoviae* strains 86079/7NS (Morrow, 1990) and 90078/BC (isolated in the authors' laboratory in 1990 from the trachea of a chicken). DNA fragments were separated by electrophoresis through a 0.7% agarose gel and transferred to nylon membrane (Amersham Hybond-N⁺). The recombinant plasmids pGEX-1N-MP1, pGEX-1N-MP3, pGEX-1N-MP9, pGEX-1N-MP10 and pGEX-1N-MP11 were radiolabelled with [α -³²P]dCTP by random priming (Boehringer Mannheim). The *M. pneumoniae* DNA transferred to the membrane was hybridized with the radiolabelled plasmids using conditions described by Sambrook *et al.* (1989). The nylon membranes were then washed three times for 60 min in 0.1 $\times$ SSC at 55 °C.

Oligonucleotides for transcriptional analysis. Two oligonucleotides were designed as primers for the reverse transcriptase (RT) reaction: 16r, 5' CGCATTGCTAGCTCTTAATTTT-ACCACG 3', complementary to nucleotides 265-292 overlapping the start of the ORF for the 16 kDa protein; and 1165Pr, 5' TGTTCACACAGTACCAGCGA 3', complementary to nucleotides 831-850 within the ORF encoding the 116 kDa protein.

Two oligonucleotides were designed as forward primers for the RT-PCR: 165Pf, 5' GCGATTGAGATCCGCGACTC 3', spanning nucleotides 220-238 preceding the ORF encoding the 16 kDa protein, and 16kcon, 5' AGCGATTACTTCCGTTTGTTTA 3', spanning nucleotides 642-663 within the 3' end of the ORF encoding the 16 kDa protein.

Oligonucleotides (20 pmol) were radiolabelled using polynucleotide kinase (Boehringer Mannheim) in the presence of 1.48 MBq [γ -³²P]ATP (222 TBq mmol⁻¹) to a specific activity greater than 2.35 $\times$ 10⁵ c.p.m. pmol⁻¹.

Northern blotting. Total RNA (20 µg) was separated through a 1.5% (w/v) agarose gel containing 0.22 M formaldehyde and transferred to Hybond-N⁺ membrane (Amersham). The nylon membrane was incubated with Church buffer (Church & Gilbert, 1984) for 4 h at 50 °C. Radiolabelled 1165Pr (0.27 pmol ml⁻¹) was hybridized with the membrane for 4.25 h at 50 °C. The membrane was washed twice for 10 min at 50 °C with 0.1 $\times$ SSC containing 0.5% (w/v) SDS.

RT-PCR. RNA was prepared for reverse transcription by mixing 5 µg total RNA with 20 U RNase-free DNase I (Boehringer Mannheim), 5 µl 5 $\times$ avian myeloblastosis virus RT buffer (Promega) and either 20 U RNAGuard RNase inhibitor (Promega) or 10 µg RNase A in a total volume of 10 µl. Nucleic acid was digested at 37 °C for 30 min followed

Fig. 1. Western blot of solubilized whole cells of *M. pneumoniae* (lane A) and of *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase (lanes B–K) probed with sera from infected patients. Lanes A and G were probed with serum from the same patient. *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase were also probed with a negative serum (lane L). The 116 kDa protein of *M. pneumoniae*, indicated by labelled arrows, is the most prominent immunoreactive protein soluble in the Triton X-114 detergent phase for all but two sera (lanes E and K). The positions of Amersham Rainbow coloured protein molecular mass markers are indicated to the left of the panels. This image was captured using a Nikon Touchscan and Adobe Photoshop.

by heat inactivation of DNase I at 70 °C for 10 min. In the reaction which had not been treated with RNase, RNA was protected by adding 30 U RNAguard RNase inhibitor. Reverse transcription was initiated by adding 54 pmol oligonucleotide 1165Pr, 0.25 mmol DTT, 25 nmol of each of the four deoxyribonucleotide triphosphates and 15 U avian myeloblastosis virus RT in a final volume of 20 µl. The mixture was incubated at 42 °C for 60 min, then stopped by heating to 99 °C for 5 min.

PCR amplification of cDNA was performed in a reaction volume of 50 µl. The reactions contained 50 mM KCl, 10 mM Tris/HCl, (pH 9), 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM deoxyribonucleotide triphosphates, 0.25 mM forward and reverse primers, 1 U *Taq* DNA polymerase (Promega) and 8 µl cDNA template from the RT reactions. The amplification cycle for the primer pair 165Pf and 1165Pr (94 °C for 30 s, 56 °C for 30 s and 72 °C for 20 s) was repeated 25 times with a final extension at 72 °C for 5 min. The amplification cycle for the primer pair 16kcon and 1165Pr was as above but annealing was performed at 53 °C. A 15 µl sample from each reaction was analysed by electrophoresis through a 1.5% agarose gel.

Primer extension. RT reactions were performed with 1.5 pmol and 3 pmol primer oligonucleotides 16r and 1165Pr, radio-labelled as described above, and 19 µg total RNA. The procedure was as described for RT-PCR with the exceptions that 20 U avian myeloblastosis virus RT was employed and the 5 × avian myeloblastosis virus RT buffer was added after the annealing step. As this buffer contains DTT, no additional DTT was required.

Plasmid pUCBM20-MP1b, containing the 3.3 kb *Eco*RI fragment of *M. pneumoniae* that ends at nucleotide 2728, was used as the template for dideoxynucleotide sequencing ladders. The reactions were performed with T7 DNA polymerase and short sequencing mixes (Pharmacia) using 3 pmol radiolabelled oligonucleotides 16r and 1165Pr as primers.

RESULTS

Identification of a Triton X-114 detergent phase soluble 116 kDa protein of *M. pneumoniae*

M. pneumoniae cells were partitioned with Triton X-114 to achieve enrichment of integral membrane proteins in the detergent phase (Bordier, 1981). The *M. pneumoniae* proteins extracted in the Triton X-114 detergent phase included a protein of approximately 116 kDa that was enriched when compared to a whole-cell lysate (data not shown). Sera from 10 patients diagnosed as infected with *M. pneumoniae* reacted with the 116 kDa protein in Western blots. The 116 kDa protein was the most prominent immunoreactive antigen soluble in the Triton X-114 detergent phase for eight out of ten sera (Fig. 1). The two other sera (Fig. 1, lanes E and K) were still reactive with the 116 kDa protein but to a lesser extent. A human serum negative for anti-*M. pneumoniae* antibodies weakly bound a number of proteins soluble in the Triton X-114 detergent phase including the 116 kDa protein (Fig. 1, lane L) but the low level of

Fig. 2. SDS-PAGE and Western blotting of trypsin-treated *M. pneumoniae* cells and of trypsin-treated Nonidet P-40 lysates of *M. pneumoniae*. Intact *M. pneumoniae* cells were incubated with trypsin for 15 min (lane labelled 15 min) or incubated in the absence of trypsin (0 min). *M. pneumoniae* cells were also lysed with 1% Nonidet P-40 and then incubated with trypsin for 15 min (NP-40). (a) SDS-PAGE of proteins from whole-cell lysate. Arrows indicate protein bands of approximately 160 kDa and 100 kDa that were reduced in intensity following trypsin treatment of intact cells and would co-migrate with P1 and the 116 kDa protein, respectively. (b) Western blot probed with affinity-purified rabbit anti-116 kDa protein antibodies; the position of the 116 kDa protein of *M. pneumoniae* is indicated by an arrow. The positions of New England Biolabs prestained protein markers are indicated to the left of both panels. This image was captured using a Nikon Touchscan and Adobe Photoshop.

this reactivity could be easily distinguished from the reactivity of all the sera positive for anti-*M. pneumoniae* antibodies.

Whole-cell *M. pneumoniae* preparations were probed by Western blotting with sera taken from 17 infected patients (data not shown). Sera from only two of the 17 patients were non-reactive with the 116 kDa protein.

Demonstration of the surface location of the 116 kDa protein of *M. pneumoniae*

Intact *M. pneumoniae* cells were incubated with trypsin, then washed and prepared for analysis by SDS-PAGE and Western blotting. When probed with affinity-purified rabbit anti-116 kDa protein antibodies a band of 116 kDa was observed in the control profile (Fig. 2b, lane 0 min) but its abundance was greatly reduced by trypsin treatment (Fig. 2b, lane 15 min), suggesting its surface location on intact mycoplasmas. Examination of the protein profile by Coomassie blue staining showed that most cell proteins were not affected by trypsin treatment (Fig. 2a). That the trypsin treatment did not cause cell lysis is demonstrated by the far more extensive digestion of protein from *M. pneumoniae* cells lysed with 1% (v/v) Nonidet P-40 prior to incubation with

trypsin (Fig. 2a, lane NP-40) (Layh-Schmitt & Herrmann, 1992).

Identification of the gene encoding the 116 kDa protein of *M. pneumoniae*

M. pneumoniae DNA partially digested with *Sau3AI* was ligated to the plasmid vector pGEX-1N and this was used to transform *E. coli*. Eleven positive clones were isolated from the pGEX-1N-*Sau3AI* *M. pneumoniae* expression library by colony blotting with the affinity-purified rabbit anti-116 kDa protein antibodies. Western blots demonstrated the lack of reactivity of the affinity-purified rabbit anti-116 kDa protein antibodies with *E. coli* proteins (Fig. 3b, lane GST m) and their reduced reactivity with *M. pneumoniae* proteins other than the 116 kDa protein (Fig. 3b, lanes Mp m and Mp c). Complete nucleotide sequencing of clones pGEX-1N-MP3, pGEX-1N-MP9 and pGEX-1N-MP10, and partial nucleotide sequencing of clone pGEX-1N-MP8, revealed that pGEX-1N-MP9, pGEX-1N-MP3 and pGEX-1N-MP10 contained non-overlapping regions of sequence and that all three of these regions were contained in clone pGEX-1N-MP8 (Fig. 3a).

Southern blots of *M. pneumoniae* genomic DNA dig-

Fig. 3. (a) Map of the ORFs encoding the 16 kDa and 116 kDa proteins of *M. pneumoniae*. Shaded regions indicate non-coding DNA. Depicted above the ORFs are the non-overlapping regions of the 116 kDa protein gene that were contained in plasmids pGEX-1N-MP9, pGEX-1N-MP3 and pGEX-1N-MP10 and the region of the 116 kDa protein gene contained in the plasmid pGEX-1N-MP8, which spans the three non-overlapping plasmids. Below the ORF map the arrows labelled S indicate the position of *Sau*3AI sites and the arrow labelled E indicates the position of the *Eco*RI site. (b) Western blots of whole-cell lysates of *E. coli* containing different pGEX-1N-MP plasmids. The left lane of each pair was probed with affinity-purified rabbit anti-116 kDa protein antiserum (lanes m) and the right lane with affinity-purified rabbit anti-GST antiserum (lanes g). The expression clones were pGEX-1N-MP3 (lanes MP3), pGEX-1N-MP10 (lanes MP10), pGEX-1N-MP9 (lanes MP9), pGEX-1N-MP1 (lanes MP1), and pGEX-1N-MP8 (lanes MP8). Small arrows indicate the positions of the *M. pneumoniae* 116 kDa protein derived polypeptides encoded by the plasmids. *E. coli* transformed with pGEX-1N and thus expressing GST but no *M. pneumoniae* polypeptides was also probed with the affinity-purified rabbit antibodies (lanes GST). The position of GST is indicated by an arrow. Solubilized whole *M. pneumoniae* cells (lanes Mp) were probed with affinity-purified rabbit anti-116 kDa protein antibodies (lane Mp m) and untreated rabbit anti-116 kDa protein antiserum (lane Mp c). The position of New England Biolabs prestained protein molecular mass markers are indicated to the left of the panels. This figure was generated using Nikon Touchscan with Adobe Photoshop.

ested with *Bgl*II and *Eco*RI were probed separately with radiolabelled plasmid DNA from the three non-overlapping clones expressing polypeptides reactive with the affinity-purified rabbit anti-116 kDa protein antibodies (data not shown). Each plasmid probe hybridized with a single *Bgl*II fragment of 10.3 kb. The probe pGEX-1N-MP3 hybridized to two *Eco*RI fragments, of 7.9 and 3.3 kb, whilst probe pGEX-1N-MP9 bound only to the 3.3 kb fragment and probe pGEX-1N-MP10 bound only

to the 7.9 kb fragment. Two other plasmid probes, pGEX-1N-MP1 and pGEX-1N-MP11, whose *M. pneumoniae* DNA inserts overlapped with those of plasmids pGEX-1N-MP9 and pGEX-1N-MP3, bound to the 10.3 kb *Bgl*II fragment and both the 3.3 kb and the 7.9 kb *Eco*RI fragments. The fact that the three non-overlapping expressed sequences all bound the same *Bgl*II fragment suggested that they were all part of the same gene.

Fig. 4. (a) Primer extension of whole *M. pneumoniae* RNA with 1.5 pmol (lane 1) and 3 pmol (lane 2) of the oligonucleotide 16r. Primer extension products are indicated by an arrow. A control primer extension was performed with 3 pmol 16r in the absence of RNA (lane 3). The *T marked in the sequencing ladder corresponds to the endpoint of reverse transcription and is complementary to the A residue at nucleotide position 85, the transcription initiation site. (b) RT-PCR across the intergenic region between the ORFs encoding the 16 kDa and 116 kDa proteins. All RT reactions were performed on RNase-free DNase I-treated whole *M. pneumoniae* RNA with the oligonucleotide 1165Pr. PCR with the oligonucleotides 165Pf and 1165Pr generated a product of 631 bp (lane A), PCR with the oligonucleotides 16kcon and 1165Pr generated a product of 209 bp (lane B); lane C, RNA treated with RNase A prior to reverse transcription and PCR with the oligonucleotides 165Pf and 1165Pr; lane D, RNA treated with RNase A prior to reverse transcription and PCR with the oligonucleotides 16kcon and 1165Pr. Bacteriophage λ gt11 digested with *Hind*III and pUC18 digested with *Hae*III were used as molecular mass markers. (c) Northern blot of whole *M. pneumoniae* RNA (lane B) and adult mouse liver RNA (lane A) hybridized with oligonucleotide 1165Pr. The arrow indicates *M. pneumoniae* RNA of about 3.7 kb that hybridized with 1165Pr. RNA markers (Promega) were used as molecular mass markers. This figure was generated using Nikon Touchscan with Adobe Photoshop.

Analysis of nucleotide sequence data revealed that all 11 clones reactive with the rabbit affinity-purified anti-116 kDa protein antibodies by colony blotting contained regions of the 116 kDa protein ORF. Alignment of the sequences suggested that clone pGEX-1N-MP10 was the most 3' of the clones, but it contained no stop codon. The 3.3 and 7.9 kb *Eco*RI fragments of *M. pneumoniae* were therefore cloned and used to obtain the entire sequence of the ORF for the 116 kDa protein.

Whole-cell proteins from clones expressing regions of the 116 kDa protein gene were probed in Western blots with affinity-purified rabbit anti-116 kDa protein antibodies and with affinity-purified rabbit anti-GST antibodies. Only pGEX-1N-MP3 and pGEX-1N-MP10 expressed regions of the 116 kDa protein gene as GST fusion proteins, of 54 kDa and 32.3 kDa, respectively. Both of these fusion proteins were reactive with the affinity-purified rabbit anti-116 kDa protein antibodies and with the affinity-purified rabbit anti-GST antibodies (Fig. 3b). The other recombinant plasmids contained the ATG start codon of the 116 kDa protein ORF and so these clones expressed regions of the 116 kDa protein gene independent of the GST protein. Thus the affinity-

purified rabbit anti-116 kDa protein antibodies were reactive with polypeptides of 79.5 kDa, 110.5 kDa and 52.2 kDa that were expressed by clones pGEX-1N-MP1, pGEX-1N-MP8 and pGEX-1N-MP9, respectively, but the affinity-purified rabbit anti-GST antibodies were reactive only with the 26.6 kDa GST protein from the same clones (Fig. 3b).

The 116 kDa protein was encoded by a 3093 bp ORF. It was preceded by a 408 bp ORF that was predicted to encode a 16 kDa protein. The two ORFs were separated by 105 nucleotides. Both ORFs commenced with ATG start codons and there were no other possible start codons 3' to the upstream, in-frame stop codons.

Identification of an operon and the site of its transcriptional initiation

The oligonucleotide 16r, which is complementary to nucleotides overlapping the start of the ORF encoding the 16 kDa protein, was used in primer extension and identified the transcription initiation site of the 16 kDa protein gene as the A residue at nucleotide position 85 (Fig. 4a). No extension product was detectable for the reverse transcription reaction which employed the

Fig. 5. Truncated nucleotide sequences and their predicted translations showing strain variations. (a) Comparison of the ORF encoding the 16 kDa protein of *M. pneumoniae* strain FH with ORF G07_orf135 of *M. pneumoniae* strain M129 (Himmelreich *et al.*, 1996). (b) Comparison of the ORF encoding the 116 kDa protein of *M. pneumoniae* strain FH with ORF G07_orf1030 of *M. pneumoniae* strain M129 (Himmelreich *et al.*, 1996). Numbers to the left of the sequences refer to the position of adjacent residues relative to the start codon. Lines indicate identity between aligned nucleotides. This figure was generated using the programs GAP and PUBLISH of the Genetics Computer Group.

oligonucleotide 1165Pr to extend past the start codon of the gene for the 116 kDa protein. When the radiolabelled oligonucleotide 1165Pr was used as a probe on a Northern blot of whole *M. pneumoniae* RNA it hybridized with a band of about 3.7 kb (Fig. 4c).

RT-PCR across the intergenic region established that the genes encoding the 16 kDa and 116 kDa proteins were transcribed as a polycistronic mRNA (Fig. 4b). A reverse transcription reaction was first performed using *M. pneumoniae* mRNA as a template and oligonucleotide 1165Pr as a primer. The entire intergenic region between the 16 kDa and 116 kDa protein genes and some or all of the 16 kDa protein coding sequence would thus be copied but only if the 16 kDa and 116 kDa protein genes were cotranscribed. The ssDNA product obtained was then subjected to PCR amplification using the oligonucleotides 16kcon and 1165Pr, resulting in the amplification of the 53 nucleotides at the 3' end of the 16 kDa protein ORF, the intergenic region and the first 50 nucleotides of the 116 kDa protein ORF. The predicted PCR product of 209 bp was produced (Fig. 4b). To show that this product was due to mRNA rather than *M. pneumoniae* DNA contaminating the primary (RT) or secondary (PCR) templates, DNase treatment of the RNA sample was conducted prior to reverse transcription. This treatment had no effect upon the generation of the 209 bp product. In contrast, RNase treatment prior to reverse transcription prevented synthesis of the 209 bp PCR product.

To confirm the conclusion that the 16 kDa and 116 kDa protein coding sequences were part of the same operon, a different pair of oligonucleotides, 165Pf and 1165Pr, were also used as PCR primers for the amplification of

ssDNA. The entire 16 kDa protein ORF, the intergenic region and the first 50 nucleotides of the 116 kDa protein ORF were amplified, generating a 631 bp product (Fig. 4b).

Comparison with homologous genes in *M. pneumoniae* strain M129 and *M. genitalium*

After we completed the sequencing work described in this paper, the genome sequence of *M. pneumoniae* strain M129 was published (Himmelreich *et al.*, 1996). This allowed comparison of the 16 kDa protein gene and the 116 kDa protein gene from *M. pneumoniae* strain FH with the equivalent genes from *M. pneumoniae* strain M129. Between the 16 kDa protein gene of *M. pneumoniae* strain FH and the gene G07_orf135 of *M. pneumoniae* strain M129 there is only one variant nucleotide, which does not affect the translation (Fig. 5a). Between the 116 kDa protein gene of *M. pneumoniae* strain FH and the gene G07_orf1030 of *M. pneumoniae* strain M129 there are five variant nucleotides, three of which result in amino acid differences (Fig. 5b). The genomic sequence of *M. genitalium* (Fraser *et al.*, 1995) contained contiguous ORFs corresponding to the 16 kDa and 116 kDa protein ORFs of *M. pneumoniae*. The *M. genitalium* ORF MG074 had 58.4% nucleotide sequence identity and 37.3% amino acid sequence identity to the gene for the 16 kDa protein. The *M. genitalium* ORF MG075 had 61% nucleotide sequence identity and 52% amino acid sequence identity to the gene for the 116 kDa protein of *M. pneumoniae*. No function has been assigned to either ORF MG074 or ORF MG075, but the gene product of ORF MG075 may be a surface protein on the basis of this work.

DISCUSSION

Identification of the gene encoding a 116 kDa membrane protein of *M. pneumoniae*

The entire band excised from the 116 kDa region of SDS-PAGE of *M. pneumoniae* proteins soluble in the Triton X-114 detergent phase was used to immunize rabbits. The resulting affinity-purified rabbit anti-116 kDa protein antibodies identified multiple clones in a genomic expression library and all such clones contained fragments of the same gene. These observations were strong evidence that the prominent band at 116 kDa (Fig. 1) was a single, homogeneous polypeptide.

Predicted features of the 16 kDa/116 kDa protein operon

Nucleotide sequencing revealed two ORFs encoding a 16 kDa protein and a 116 kDa protein in the order 5'-16 kDa ORF-116 kDa ORF-3'. Northern blot analysis and RT-PCR amplification of the intergenic region established that the ORFs encoding the 16 kDa and 116 kDa proteins were transcribed as a single mRNA. The oligonucleotide 1165Pr, complementary to nucleotides 831-850 within the ORF encoding the 116 kDa

protein, hybridized in Northern blots with a discrete band of about 3.7 kb. From the experimentally determined initiation of transcription to the end of the first putative transcription terminator is 3848 nucleotides, while from the start codon for the 116 kDa ORF to the putative transcription terminator is only 3134 nucleotides. As the second-largest species to which 1165Pr hybridized in the Northern blot was 1.75 kb, and thus too small to contain the complete 3093 bp 116 kDa protein ORF, the 116 kDa protein ORF could not be transcribed independently of the 16 kDa protein ORF. The smaller species of RNA to which oligonucleotide 1165Pr bound in Northern blots are probably breakdown products of the full-length transcript. This effect in Northern blot analysis of *M. pneumoniae* RNA has been previously noted and attributed to the short half-life of the transcript (Inamine *et al.*, 1988).

Comparison of the 50 nucleotides preceding the transcriptional start of the operon encoding the 16 kDa and 116 kDa proteins with *E. coli* promoter sequences (Hawley & McClure, 1983) identified the sequence ATGGCTT, which begins at nucleotide 49 (-34 relative to the transcriptional start) (Fig. 6) as a possible -35 promoter sequence. The sequence CAAAAC,

Fig. 6. Putative transcriptional and translational regulatory nucleotide sequence motifs of the operon encoding the 16 kDa and 116 kDa proteins of *M. pneumoniae* and salient features of their predicted translations. Translations for the proteins are shown below the nucleotide sequence. All motifs of interest are underlined. Predicted -35 and -10 promoter sequences and ribosome-binding site (RBS) for the 16 kDa protein are labelled. The transcriptional initiation site at nucleotide 85 is labelled TI. The putative prolipoprotein signal sequence for the 16 kDa protein is labelled SPaseII. The sequence with homology to the novel translation initiation region of *M. genitalium* (Loechel *et al.*, 1991) preceding the 116 kDa protein gene is labelled TIR. The putative transcriptional terminator is labelled TERM. The positions of the oligonucleotides 165Pf, 16kcon and 1165Pr that were used in transcriptional analysis are also labelled and underlined.

which begins at nucleotide -13 relative to the transcriptional start, was identified as a possible -10 promoter sequence.

M. pneumoniae lacks a rho termination factor gene (Himmelreich *et al.*, 1996). A possible rho-independent termination sequence was identified 3' to the ORF encoding the 116 kDa protein from nucleotides 3955 to 3968 with a Gibbs free energy of $-19.2 \text{ kJ mol}^{-1}$ (Tinoco *et al.*, 1973). This region is followed by a G residue and three T residues in the genomic sequence. No other candidate transcriptional termination sequences could be detected in the 350 nucleotides 3' to the gene G07_orf1030, the *M. pneumoniae* strain M129 116 kDa protein gene equivalent (Himmelreich *et al.*, 1996).

A weak Shine-Dalgarno sequence, GAG, starts 9 nucleotides 5' to the start codon of the gene for the 16 kDa protein. The gene for the 116 kDa protein of *M. pneumoniae* lacks a consensus Shine-Dalgarno sequence. However, with the exception of an additional cytidine residue at nucleotide 788, the sequence TTAA-CCAA starting at nucleotide 784 has identity to sequence involved in translation initiation of the *M. genitalium* *tuf* gene (Loechel *et al.*, 1991).

Lack of a consensus ribosome-binding site is not without precedent, as no ribosome-binding site was detected 5' to ORF-5 or ORF-6 or the ORF MP1-11 of the P1 operon (Inamine *et al.*, 1988) or the ORF *hmu3* of *M. pneumoniae* (Ogle *et al.*, 1992).

Features of the 16 kDa and 116 kDa proteins

Analysis of the predicted amino acid sequence of the 16 kDa protein of *M. pneumoniae* revealed a hydrophobic signal sequence with potential signal peptidase I cleavage sites between amino acids 22 and 23 and between amino acids 25 and 26 (von Heijne, 1989). Alternatively, the 16 kDa protein predicted amino acid sequence, $^{21}\text{FASLSFKLISC}^{31}$ (Fig. 6), with the exception of the lysine at amino acid position 27, conforms to the consensus for a prokaryotic prolipoprotein signal sequence cleaved by signal peptidase II.

The amino-terminal 26 amino acids of the 116 kDa protein constituted the most hydrophobic region according to a hydropathy plot (Kyte & Doolittle, 1982). Amino acids 6-22 were predicted by the program PSORT (available on the World Wide Web at <http://psort.nibb.ac.jp>) (Nakai & Kanehisa, 1991) to form a transmembrane region, and amino acids 1-12 were predicted to form an α -helix of hydrophobic amino acids (Chou & Fasman, 1978). These predictions, the surface location of the 116 kDa protein as demonstrated by tryptic digestion, and the absence of signal peptidase I or signal peptidase II cleavage sites in the hydrophobic N-terminal amino acid sequence, suggest that the hydrophobic amino terminus is involved in anchorage to the membrane.

The molecular masses of proteins expressed from the pGEX-1N-MP clones as determined by Western blotting were similar to those predicted from sequence data.

Two TGA codons were present in the ORF for the 116 kDa protein at nucleotides 3686 and 3701 (Fig. 6). However, none of the pGEX-1N-MP clones spanned this region of the ORF. Thus, the smaller peptides detected by affinity-purified rabbit anti-116 kDa protein antibodies in pGEX-1N-MP1, pGEX-1N-MP3, pGEX-1N-MP10 and pGEX-1N-MP8 were probably breakdown products of the expressed proteins.

Correlation with previous studies of *M. pneumoniae* proteins

The 116 kDa protein gene of *M. pneumoniae* strain FH has been identified as the equivalent of the *M. pneumoniae* strain M129 gene G07_orf1030 (Himmelreich *et al.*, 1996). Previous work identified 234 nucleotides of this gene (EMBL accession no. Z32666; Proft & Herrmann, 1994); however, the insertion of an A residue at nucleotide position 186 resulted in an incorrect predicted translation of the Z32666 sequence. Rabbit antiserum raised to the fusion protein FP G7-4A, expressed from the Z32666 sequence, bound two *M. pneumoniae* proteins, P100 (100 kDa) and P50A (50 kDa) (Proft & Herrmann, 1994). In our studies, rabbit anti-116 kDa protein antibody bound a 116 kDa band and an approximately 45 kDa band in Western blots of *M. pneumoniae* whole-cell proteins (Fig. 3b, lane Mp c).

Genomic locations of the operon and its *M. genitalium* homologue

Availability of the published genomes for both *M. pneumoniae* (Himmelreich *et al.*, 1996) and *M. genitalium* (Fraser *et al.*, 1995) has allowed comparison of the positions of the *M. pneumoniae* genes G07_orf1030 and G07_orf135 with their *M. genitalium* homologues, MG074 and MG075. In both species the genes are adjacent, in the same order, and in the same transcriptional orientation.

Specificity of the 116 kDa antigen

A single protein antigen would have greater specificity than the undefined cellular fractions currently used in serodiagnosis of *M. pneumoniae* infection. One candidate, the immunodominant P1 molecule, contains epitopes cross-reactive with its *M. genitalium* homologue (Jacobs, 1993). Cross-reactivity with *M. genitalium* could lead to false positive diagnosis of *M. pneumoniae* infection in patients with respiratory disease and concomitant urogenital *M. genitalium* infection. The low amino acid sequence identity of 52% between the ORF encoding the 116 kDa protein and its *M. genitalium* homologue MG075 suggested that they may have low immunological cross-reactivity. Preliminary experiments in this laboratory suggest that affinity-purified rabbit anti-116 kDa protein antibodies react with a 116 kDa protein from *M. pneumoniae* strains FH, PI1428 and M129 but do not react with any protein from *M. genitalium* strain G37 (data not shown). The possibility that the 116 kDa protein of *M. pneumoniae* is

not immunologically cross-reactive with its *M. genitalium* homologue is currently being investigated and suggests that this protein may have some future promise as a specific serodiagnostic reagent.

The finding that the ORFs encoding the 16 kDa and 116 kDa proteins are part of the same operon indicates that their functions may be linked. Knowledge of the full sequence of the 16 kDa and 116 kDa proteins and the availability of affinity-purified antibodies and cloned and expressed regions of the 116 kDa protein gene should facilitate future functional studies.

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