***Mycoplasma hominis* expresses two variants of a cell-surface protein, one a lipoprotein, and one not**

Søren A. Ladefoged and Gunna Christiansen

Author for correspondence: Søren A. Ladefoged. Tel: +45 89 42 17 46. Fax: +45 86 19 61 28.
e-mail: sorenl@biobase.dk

Department of Medical Microbiology and Immunology, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark

A protein similar to the previously characterized variable surface-exposed membrane protein P120 was identified (P120'), establishing that *Mycoplasma hominis* PG21 possesses a novel gene family. The gene, *p120'*, was sequenced and found to have some distinctive properties including a putative start codon of GTG, rather than the common ATG codon, and a coding region with a high G+C content, characteristic of essential housekeeping genes in mycoplasmas. No sequence homology was found to known proteins. The genomic locations of the *p120* and *p120'* genes were determined on the restriction map of five *M. hominis* strains by PFGE. The genes were localized in two separate regions separated by more than 6 kb. Genes as well as proteins corresponding to P120' were identified in 24/24 *M. hominis* isolates tested and no size variation was detected. P120' had a molecular mass of 98 kDa, 20 kDa smaller than P120 as estimated by SDS-PAGE. The protein was surface-exposed and associated with the mycoplasma membrane, but had predominantly hydrophilic characteristics upon Triton X-114 extraction. The N-terminal part of P120' had a hydrophobic leader sequence without the characteristics of a lipoprotein. This might explain the membrane association of the protein. Unlike P120, which is frequently recognized by sera of patients seropositive for *M. hominis*, P120' was only rarely recognized. The conserved nature of the P120 gene family indicates that it has an essential, although currently unknown, function.

Keywords: reiterated genes, chromosome mapping, membrane proteins, P120' protein, P120 protein

INTRODUCTION

In spite of their uniquely small chromosome (Himmelreich *et al.*, 1996; Fraser *et al.*, 1995; Bak *et al.*, 1969), and consequently limited biosynthetic capability, mycoplasmas have successfully adapted to a wide range of hosts. Mycoplasmas lack a cell wall, and the surface components of the single limiting cell membrane are thus essential for host adaptation. *Mycoplasma hominis* is an opportunistic pathogen causing gynaecological infections, and is increasingly detected in extragenital infections (Meyer & Clough, 1993). Surface antigens of *M. hominis* vary considerably between isolates (Christiansen, 1992; Christiansen *et al.*, 1994; Ladefoged *et al.*, 1990, 1995, 1996; Jensen *et al.*, 1995; Olson *et al.*,

1991b; Andersen *et al.*, 1987). Even in isolates derived successively from a single chronically infected patient some antigenic variation was observed (Olson *et al.*, 1991a). Three different surface-exposed membrane proteins have been characterized in detail in *M. hominis*. The Lmp family consists of two 135 kDa surface-exposed proteins, Lmp1 and Lmp3 (Ladefoged *et al.*, 1995, 1996; Jensen *et al.*, 1995). The corresponding genes, *lmp1* and *lmp3*, are separated on the chromosome and contain multiple tandem repeated elements of 0.5 kb. Two other genes, *lmp2* and *lmp4*, also containing the repeated element, are located immediately downstream of *lmp1* and *lmp3*, respectively, but are not translationally active in *M. hominis* PG21. *M. hominis* Lmp proteins are subject to mutational variation, due to the loss of repetitive elements resulting in size variation (Jensen *et al.*, 1995). A role in agglutination of cells has been suggested for this protein. A putative adhesin of *M. hominis*, the variable adherence-associated (Vaa) anti-

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gen, identified as a lipoprotein, also shows variation in size and antigenicity between isolates (Henrich *et al.*, 1996; Zhang & Wise, 1996). The size variation is caused by loss or gain of central repetitive elements and the antigenic variation is associated with sequence variation in the C-terminal portion of Vaa. A gene in *M. hominis* (*p120*) encoding a 120 kDa surface-exposed membrane protein (P120) with structural characteristics consistent with a lipoprotein has previously been characterized (Nyvold *et al.*, 1997). The gene has been identified in all *M. hominis* strains examined, while the gene product is absent in a minority of the strains examined. The protein displays antigenic variation due to the presence of one hypervariable and two semivariable regions. No size variation has been observed. The hypervariable portion of P120 is often recognized by patients seropositive for *M. hominis*. An open reading frame on the *M. hominis* chromosome (ORF445), the 3' end of which had been partly characterized previously (Ladefoged & Christiansen, 1994), was found to be similar to *p120*. The aim of this study was to characterize the remainder of ORF445 (the *p120'* gene), the *p120* gene family in 24 *M. hominis* isolates, and the product of the *p120'* gene.

METHODS

Strains and growth conditions. *M. hominis* was cultivated in BEA medium (Andersen *et al.*, 1987). The 24 *M. hominis* strains used in this study (PG21, P71, P7, P2, 4195, 93, 10, W2, 132, 183, SC4, M1449, V2785, DC63, 5503, 4712, 7357, 1893, 3105, 6188, 5941, 7488, 7808, 3449) have been described elsewhere (Christiansen & Andersen, 1988). *Escherichia coli* strain XL-1 Blue (Stratagene) was used as host for the phagemid pBluescript (Stratagene) and pGEX (Pharmacia LKB Biotechnology). *E. coli* was cultivated in Luria-Bertani (LB) broth or Terrific Broth (TB) (Sambrook *et al.*, 1989). *E. coli* INVαF' was used as host for the pCRII vector (Invitrogen Corporation) and cultivated as described by the supplier. A pGEX vector containing the hypervariable domain of P120 from *M. hominis* PG21 and expressing the corresponding hypervariable peptide as a GST fusion protein was supplied by C. Nyvold (Nyvold *et al.*, 1997).

Patient sera and monoclonal antibodies. Patient sera were obtained from the World Health Organization Reference Bank for Reproductive Immunology, Department of Medical Microbiology and Immunology, University of Aarhus, Denmark. The samples were originally collected for investigation of unexplained infertility (Mathur *et al.*, 1985). The collection contained 195 sera, 21 of which were found positive in an indirect haemagglutination assay for *M. hominis*. Twenty-one sera from the same collection which were found negative were used as controls. Monoclonal antibodies mAb 30.1.5, directed against *M. hominis* elongation factor Tu (anti-EF-Tu) (Ladefoged & Christiansen, 1991), mAb 43.2 directed against a surface-exposed integral membrane protein (Ladefoged *et al.*, 1990), and mAb 522, directed against the putative *M. hominis* adhesin Vaa (Zhang & Wise, 1996; Ladefoged *et al.*, 1990), and the polyclonal anti-P120 antibody pAb 121 (Nyvold *et al.*, 1997) were used as controls in the immunoblotting experiments and/or the immunofluorescence assay.

DNA manipulation and Southern hybridization. *M. hominis* DNA was prepared as described previously (Ladefoged & Christiansen, 1991). Plasmid DNA preparation by the alkaline lysis method, restriction endonuclease digestion and agarose

gel electrophoresis were done according to Sambrook *et al.* (1989). DNA transfer to Hybond-N membrane (Amersham), DNA fragment labelling with [α - 32 P]dATP by nick-translation, and Southern hybridization were carried out using standard methods (Sambrook *et al.*, 1989). For recovery of restriction fragments from agarose gels for cloning, electrophoresis was performed through a 1.0% low-melting-point agarose (SeaPlaque GTG Agarose, FMC Bio-Products). Bands were visualized by staining with ethidium bromide and examination by UV transillumination. Gel slices containing the appropriate restriction fragments were excised and melted at 62 °C before purification by phenol/chloroform extraction and ethanol precipitation. PFGE was performed by the contour-clamped homogeneous-electric field (CHEF) technique using the CHEF-DR II system (Bio-Rad) as described previously (Ladefoged & Christiansen, 1992). The genomic DNA was digested with *Sma*I, *Bam*HI, *Xho*I and *Sal*I (Boehringer Mannheim).

Nucleotide sequencing and sequence analysis. DNA sequencing of denatured double-stranded DNA was done by the methods of Hattori & Sakaki (1986), using Sequenase version 2 (United States Biochemical). The sequence data were compiled and analysed with the Genetics Computer Group's Sequence Analysis Software Package Version 7.1-UNIX (Devereux *et al.*, 1984).

Northern blotting. Total RNA was isolated by the single-step method of Chomczynski & Sacchi (1987). Northern-blot hybridization of *M. hominis* RNA was performed using the procedure described by Sambrook *et al.* (1989), with a 0.24–9.5 kb RNA ladder (Life Technologies) as molecular mass markers.

Expression and purification of fusion proteins. To make a fusion protein containing part of the P120' protein, the primers 5'-GGATCCGAGGAATTTCAACTGGTGTC-3' and 5'-CTCGAGCTGTTGTAATAGCATTAAG-3' were used to amplify a fragment of the gene by PCR. A *Bam*HI and an *Xho*I site were introduced into the 5' end of the upstream and downstream primers, respectively. PCR products were ligated to the vector pCRII and the ligated plasmid transformed into *E. coli* INVαF' according to the supplier's instructions (Invitrogen). The recombinant plasmid was digested with *Xho*I and *Bam*HI and the fragment containing part of the *p120'* gene ligated to the expression vector pGEX-5X-3 (Pharmacia LKB Biotechnology). The ligated plasmid was transformed into *E. coli* XL-1 Blue. The *p120'* fragment would thus be expressed fused to the 26 kDa glutathione S-transferase (GST). Overnight cultures of *E. coli* XL-1 Blue containing the recombinant plasmid were diluted in fresh medium and grown for further 2 h at 37 °C. IPTG was added to 0.4 mM and after a further 2 h of growth, the cells were pelleted and resuspended in PBS with 1% Triton X-100. Cells were lysed by sonication and, after centrifugation, the supernatant was mixed with glutathione-agarose beads (Sigma). The beads were transferred to spin-X tubes (Costar) and the fusion protein eluted by competition with free glutathione (Sigma).

Preparation of antiserum against GST-fusion protein. Antibodies against purified GST-fusion protein were raised in a New Zealand White rabbit. The rabbit was inoculated intramuscularly with 10 µg antigen emulsified in Freund's incomplete adjuvant (Difco) on days 1, 5, 7, 11, 13 and 18. Intravenous inoculations of 10 µg of antigen without adjuvant were given on days 39, 49, and 55. On day 68 the rabbit was bled, and serum (anti-P120') was stored at –20 °C.

SDS-PAGE and immunoblotting. Separation of antigens under

reducing conditions by SDS-PAGE and immunoblotting were performed as described by Andersen *et al.* (1987).

Triton X-114 extraction. Triton X-114 phase partitioning was performed essentially as described by Bordier (1981). *M. hominis* PG21 was grown to mid-exponential phase, harvested and washed in PBS. The cells were lysed on ice for 30 min in a buffer containing 1% Triton X-114, 10 mM Tris/HCl pH 7.4 and 150 mM NaCl (protein concentration was adjusted to 1 mg ml⁻¹). The lysate was centrifuged at 20000 *g* for 30 min at 4 °C to pellet insoluble material. The supernatant was heated to 37 °C, at which temperature micelles are formed. Micelles were separated from the aqueous phase by sedimentation through a sucrose cushion, leaving the aqueous phase as a supernatant. The distribution of proteins in the Triton X-114 and the aqueous phases was analysed by SDS-PAGE followed by immunoblotting.

Membrane and cytosol extraction. Separation of *M. hominis* cells into a membrane fraction and a cytosolic fraction was done according to Proft & Herrmann (1994). Briefly, *M. hominis* PG21 was grown to mid-exponential phase, harvested, washed in PBS, and resuspended in 1 ml H₂O. The cells were disrupted by sonication eight times for 10 s on ice. Undisrupted cells were removed by centrifugation at 6000 *g* for 10 min at 4 °C. The supernatant was centrifuged at 130000 *g* for 1 h at 4 °C. The supernatant from this high-speed centrifugation was the cytosolic fraction. The pellet was resuspended in 1 ml PBS and recentrifuged at 130000 *g* for 1 h at 4 °C. This pellet was the membrane fraction. The fractions were analysed by SDS-PAGE and immunoblotting.

Surface proteolysis of *M. hominis*. To detect surface-exposed proteins, proteolysis was performed with trypsin on intact *M. hominis* cells. Cells were grown to mid-exponential phase, harvested, washed, and suspended in 1 ml PBS. Two 250 µl samples were each diluted to 750 µl with PBS containing 500 µg (4700 U) of trypsin V-S (Sigma). After 15 or 30 min incubation at 37 °C the cells were pelleted at 15000 *g* for 5 min at 4 °C. The cells were lysed by adding 500 µl 1 × SDS sample buffer [1 × SDS sample buffer is 0.0625 M Tris/HCl pH 6.8, 2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.05% bromophenol blue]. As controls two 250 µl samples were disrupted by sonication three times for 10 s. Five hundred micrograms of trypsin V-S was added to one sample and both samples were incubated for 30 min at 37 °C followed by addition of 250 µl 2 × SDS sample buffer. The samples were analysed by SDS-PAGE and immunoblotting.

Immunofluorescence assay. To detect surface-exposed proteins an indirect immunofluorescence assay was performed (Ladefoged *et al.*, 1990). *M. hominis* cells were immobilized on HeLa cells grown on coverslips. The medium used for cultivation was RPMI-1640 (GIBCO) containing 10 mM HEPES buffer, 10% newborn calf serum, 1 mM glutamine, and 100 IU penicillin ml⁻¹. Cells were incubated with primary antibodies for 30 min at room temperature with or without previous fixation in cold methanol. After a washing step, fluorescein-conjugated anti-rabbit or anti-mouse immunoglobulin (Dako), diluted 1:50 in growth medium containing 0.002% Evans Blue, was added and incubated for 30 min at room temperature. Cells not previously fixed were incubated with methanol. The coverslips were washed, air-dried and examined using a Leitz DMRBE fluorescence microscope (Leica Mikroskopie und Systeme) connected to a Sony 3CCD colour video camera and a SUN SPARC workstation. Washing procedures and dilution of antibodies were performed using growth medium. Prior to methanol fixation the coverslips were washed in PBS.

RESULTS

Cloning and DNA sequence analysis of *p120'*

The 3' end of ORF445 was found in the *gyrB* region. To characterize this *p120* homologue the region 5' to ORF445 was cloned and sequenced. By hybridization ORF445 was found on a 6.3 kb DNA *Bam*HI-*Xho*I fragment overlapping the previously sequenced *gyrB* region. From mapping studies it was known that *M. hominis* PG21 contained only three small *Bam*HI-*Xho*I fragments and only one of 6.3 kb (Ladefoged & Christiansen, 1992). The *Bam*HI-*Xho*I fragments were cloned into pBluescript and a clone with an insert of 6.3 kb was selected. A *Clal*-*Xho*I fragment containing the 5' end of ORF445 was subcloned from this plasmid. The subclone was sequenced bidirectionally by using subclones made from the available restriction sites, and by using synthetic oligonucleotide primers complementary to the characterized sequence. One large ORF of 2745 nucleotides was identified and designated *p120'*. The first codon in *p120'* was predicted to be GTG. The structural gene was preceded by a putative Shine-Dalgarno sequence (AGGA) located nine nucleotides upstream of the initiation codon. The region upstream of *p120'* contained, based on homology with the *E. coli* consensus promoter, a putative transcriptional element (-10, TATAAT; -35, TTGATT). Between the putative promoter and the predicted translational start a palindromic sequence of 14 bp was identified with the potential to form a stem-loop structure. The G+C content of *p120'* was 33.1 mol%. The G+C content across the gene varied between 24 and 44 mol% (using a window of 100 bp). The regions of highest G+C content were near the 5' end and in the 3' half of the gene. The predicted amino acid sequence was 915 amino acids, corresponding to a molecular mass of 104 kDa and a predicted isoelectric point of pH 5.9. The deduced protein sequence included 12 tryptophan residues, each encoded by TGA (Yamao *et al.*, 1985) and only one cysteine residue. Numerous potential trypsin cleavage sites were identified through the entire peptide, making trypsin a rational choice for proteolysis studies (see below). A typical N-terminal signal peptide was identified consisting of a stretch of primary hydrophobic residues preceded by three basic amino acids. Using the methods of von Heijne (1986) a signal peptidase I cleavage site was identified. Hydropathy analysis with the Kyte-Doolittle algorithm indicated that the protein is mainly hydrophilic and the signal peptide was the only domain which displayed significant hydrophobicity (data not shown). PSORT (available on the World Wide Web at <http://psort.nibb.ac.jp>) did not detect any potential transmembrane domains by the methods of Klein *et al.* (1985) other than the N-terminal signal sequence.

The P120 gene family

The deduced amino acid sequences of P120 and P120' were compared (Fig. 1). The overall similarity was 48%. The carboxyl-terminal 587 residues of P120' showed

Fig. 1. Alignment of deduced amino acid sequences of P120' and P120 (Nyvold *et al.*, 1997) from *M. hominis* PG21. The recombinant peptide expressed as a fusion protein in the pGEX vector (pG120-1) and used for immunization is underlined. Identical amino acids are shaded.

strong similarity to P120, with 58% identical amino acids and regions of up to 19 consecutive amino acids showing 100% identity. The first 328 amino acids of P120' displayed less similarity (28% identity). The larger

molecular mass of P120 compared to P120' was due to an 167 amino acid extension of P120 at the N-terminal end with no counterpart in P120'. A comparison of the 5' untranslated regions of the *p120* and *p120'* genes did

Fig. 2. (a) Northern blot of total RNA from *M. hominis* PG21. A probe derived from the 5' end of *p120'* (pG120-1; lane 1) and a probe derived from the 3' end of *p120'* (pE12H-23; lane 2) were used for hybridization. The positions of the standard markers are shown on the left. (b) Southern blotting of PG21 DNA cleaved with the restriction endonucleases indicated and probed with pG120-1. The positions of the standard markers are shown on the left.

not reveal any conservation in the primary structure. Apart from the homology to P120, database searching with the P120' sequence did not identify any significant similarities to known DNA or amino acid sequences.

Transcription of the *p120'* gene in *M. hominis*

A transcript of 3.2 kb had been identified previously (Ladefoged & Christiansen, 1994) using a hybridization probe (pE12H-23) derived from the 3' region of *p120'*, which is similar to that of *p120* (Fig. 2a, lane 2). To confirm that the hybridizing RNA fragment was a *p120'*, and not a *p120* transcript (the length of which has also been determined to be 3.2 kb; Nyvold *et al.*, 1997), Northern blot analysis was carried out using the *p120'*-specific hybridization probe pG120-1. The probe detected an mRNA of about 3.2 kb, a size in accordance with earlier results and correlating with the length of the coding region, implying a monocistronic organization (Fig. 2a, lane 1). The Northern blotting indicated some degradation of the mRNA transcript during preparation. A control experiment demonstrating the specificity of the *p120'* probe pG120-1 is shown in Fig. 2(b). pG120-1 hybridized exclusively to a *Bgl*II fragment of 8.6 kb, an *Eco*RI fragment of 0.77 kb, an *Eco*RV fragment of 20 kb, and two *Hind*III fragments of 1.8 kb and 0.2 kb, as expected from the restriction mapping data obtained from the cloning and subsequent sequen-

Fig. 3. Immunoblotting of *M. hominis* preparations with anti-P120'. Proteins were separated on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-P120'. (a) A cytosolic and a membrane fraction were isolated by ultracentrifugation. Lanes: 1, membrane fraction; 2, cytosolic fraction; 3, cell lysate. (b) *M. hominis* cells were subjected to Triton X-114 phase fractionation. Aqueous (lane 2) and Triton X-114 (lane 1) phases were separated by SDS-PAGE. Total *M. hominis* cell lysate (lane 3) was analysed together with the protein preparations.

cing of the region (the 0.2 kb *Hind*III fragment was too small to be detected on the gel shown in Fig. 2b).

Detection of P120' by monospecific polyclonal antibodies

Part of the *p120'* gene was cloned into the expression vector pGEX, and the fusion protein expressed was purified and used to raise antibodies. The location of the expressed region, indicated in Fig. 1, extended from the first to the second TGA codon in *p120'*. Expression of part of P120' as a glutathione *S*-transferase fusion protein (GST-P120') was achieved, and analysis by SDS-PAGE revealed the expression of a 44 kDa protein in accordance with the predicted size. The fusion protein was purified and examination by SDS-PAGE demonstrated limited degradation during purification, leaving the majority of the product intact. To identify P120' in *M. hominis*, rabbit antibodies were raised to the GST-P120' fusion protein. The antiserum (anti-P120') specifically recognized a protein of 98 kDa in *M. hominis* PG21 (Fig. 3a, lane 3). The molecular mass determined by SDS-PAGE (98 kDa) was in good agreement with the mass deduced from the amino acid sequence (104 kDa).

Detection of P120' in different *M. hominis* strains and *Mycoplasma* species

Immunoblots of whole-cell proteins of 24 *M. hominis* strains were probed with the rabbit anti-P120' serum. The serum detected a 98 kDa band in all *M. hominis*

Fig. 4. Surface proteolysis of *M. hominis* with trypsin. Intact *M. hominis* cells were treated with trypsin for 15 or 30 min. *M. hominis* cells disrupted by sonication before trypsin treatment and cells disrupted by sonication but without trypsin treatment served as controls. Proteins were separated on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes and probed with (a) the polyclonal anti-P120' antibody, (b) the polyclonal anti-P120 antibody, (c) the mAb 43.2, and (d) the mAb anti-EF-Tu. Lanes (same designation in each panel): 1, whole cells, trypsin 30 min; 2, whole cells, trypsin 15 min; 3, sonicated cells, trypsin 30 min; 4, sonicated cells, no trypsin.

strains tested. No specific reactions were observed between anti-P120' and other *Mycoplasma* species pathogenic for humans (*M. buccale*, *M. pneumoniae*, *M. orale*, *M. fermentans*, *M. salivarium* or *M. genitalium*)

Topology of the P120' molecule

M. hominis lysate was separated into a cytosolic and a membrane fraction by differential ultracentrifugation. Each fraction was subjected to SDS-PAGE followed by immunoblotting with anti-P120'. P120' was found solely in the membrane fraction (Fig. 3a, lane 1). The monoclonal antibodies mAb 30.1.5, directed against the cytoplasmic protein elongation factor Tu, and mAb 43.2, directed against a surface-exposed integral membrane protein, were used in control experiments and recognized proteins of the expected size primarily in the cytosolic and membrane fractions, respectively.

Following Triton X-114 partitioning, proteins from the aqueous phase and the Triton X-114 phase were separated by SDS-PAGE and immunoblotted with anti-P120'. P120' was primarily partitioned into the hydrophilic phase (Fig. 3b, lane 2). In control experiments mAb 30.1.5 and mAb 43.2 reacted with proteins of the expected size exclusively in the aqueous phase and the Triton X-114 phase, respectively.

The potential surface exposure of P120' was analysed by surface proteolysis of intact mycoplasmas using trypsin. Immunoblot analysis of trypsin-treated intact cells with anti-P120' antiserum showed partial degradation of

P120', indicating surface exposure of the protein (Fig. 4a). Trypsin treatment of sonicated cells resulted in the complete degradation of P120' (Fig. 4a). When mAb 43.2 and anti-P120 were used in parallel experiments as positive controls degradation was seen in analysis of trypsin-treated intact cells, as expected from the surface exposure of the corresponding antigens (Fig. 4b, c). In each of the experiments (Fig. 4a–c) the antigens in intact cells degraded to varying extents, leaving varying amounts of antigen intact. The sonicated and subsequently trypsin-treated antigens were far more thoroughly degraded. This indicates a varying degree of trypsin accessibility to the proteins in the intact cell membrane. The cytosolic elongation factor Tu was not degraded by trypsin treatment of intact *M. hominis* cells but was completely degraded when cells were disrupted by sonication, as expected from its intracellular location (Fig. 4d).

Direct evidence for the association of P120' with the cell membrane and exposure of anti-P120' epitopes on the surface was obtained by immunofluorescence microscopy (Fig. 5). The bacteria showed positive immunofluorescence with anti-P120' on fixed (Fig. 5a) as well as unfixed cells (Fig. 5b), indicating surface exposure of the N-terminal part of P120' recognized by anti-P120'. When mAb 522 and anti-p120 were assessed as positive controls, immunofluorescence was observed on both fixed and unfixed cells. The reactivity of anti-P120' and mAb 522 was of equal intensity while the reactivity of anti-P120 was slightly stronger. Anti-EF-Tu showed immunofluorescence on methanol-fixed (and consequently permeabilized) cells (Fig. 5c) but not on

Fig. 5. Immunofluorescence analysis of *M. hominis* PG21. Monolayers of HeLa cells were infected with *M. hominis* and reacted with anti-P120' with (a) or without (b) prior fixation with methanol. Control experiments with (c) or without (d) fixation were performed with a monoclonal antibody reacting with the cytosolic protein EF-Tu. Bar, 10 μ m.

Fig. 6. Location of *p120'* and *p120* on the 100 kb *Smal* A fragment of the *M. hominis* PG21 chromosome. Restriction nuclease sites for *Smal*, *Bam*HI, *Xho*I, and *Sal*I are shown. The locations of the ribosomal RNA operon (*rrnB*) and the *gyrB*, *licA*, and *p120'* genes are indicated together with three ORFs (ORF268, ORF499, and ORF219) without functional assignment. The position of *p120* is indicated as precisely as possible given the resolution of the restriction map.

unfixed (intact) cells (Fig. 5d), as expected for a cytosolic protein. Preimmune serum gave a faint reaction on fixed as well as unfixed cells. From these data we conclude that P120' is a membrane protein associated with the exterior surface of the membrane.

Humoral immune response in humans to P120'

To determine whether P120' was an antigen recognized *in vivo*, the recombinant P120' peptide was tested against human serum samples by immunoblotting. Of 21 serum samples positive by an indirect haemagglutination assay for antibodies against *M. hominis*, one (sample designated 169) gave a strong positive reaction, while the remaining 20 sera gave no reaction (data not shown). All 21 serum samples negative for antibodies against *M. hominis* by indirect haemagglutination gave no reaction.

The serum sample positive in immunoblotting when tested against the recombinant P120' peptide was negative when tested against the hypervariable domain of P120 from *M. hominis* PG21 expressed as a GST-fusion protein in the pGEX vector, excluding any cross-reaction with the GST portion of the fusion protein.

Localization of the *p120'* gene in *M. hominis* strains

A probe cloned from the 5' end of *p120'* hybridized to the genomic DNA of all 24 strains of *M. hominis* under high-stringency conditions, indicating the presence of a highly conserved region in these strains. Some degree of restriction fragment length polymorphism was observed in the *p120'* gene as well as in the flanking regions. Several genes have been mapped on the genome of five *M. hominis* strains (*M. hominis* PG21, 132, 4195, 93 and

7488) by PFGE (Ladefoged & Christiansen, 1992). The *p120'* and a *p120*-specific probe were hybridized to chromosomal DNA digested with *Sma*I, *Bam*HI, *Xho*I and *Sal*I and the genes were localized on the maps of these strains. The *p120'*, *p120* and *gyrB* genes were found to have the same relative positions in all strains. Part of the chromosomal map of *M. hominis* PG21 is shown in Fig. 6. In all strains *p120'* and *p120* were placed on homologous *Sma*I fragments (fragment *Sma*I A, ranging in size from 100 to 106 kb, having homologous positions on the map of the five strains). In each strain *p120'* was positioned on a 4 kb *Xho*I–*Sal*I fragment within the *Sma*I A fragment close to the *gyrB* gene. In each strain *p120* and *gyrB* lay on the same *Bam*HI fragment (fragment *Bam*HI A). Given the resolution of the restriction maps the distance between *p120* and *p120'* was estimated to be at most 80 kb in *M. hominis* PG21, 44 kb in *M. hominis* 4195, 84 kb in *M. hominis* 132, 86 kb in *M. hominis* 93, and 46 kb in *M. hominis* 7488.

DISCUSSION

In order to understand the pathogenesis of mycoplasmas we need to understand the interactions between the proteins on the cell surface and the host. In this study we have identified and characterized a 98 kDa surface protein, P120', of *M. hominis*, and the corresponding gene, *p120'*.

Features of the *p120'* gene

In the completely sequenced genome of *Mycoplasma genitalium* 7% of the identified ORFs have a proposed GTG start codon, with the remaining ORFs starting with ATG (Dalphin *et al.*, 1997). A GTG start rather than an ATG start in *p120'* has been established with considerable certainty. The GTG start predicts a protein with a molecular mass (104 kDa) in accordance with the mass determined by SDS-PAGE (98 kDa) while an ATG start predicts a much smaller protein (79 kDa). Additionally, the polyclonal anti-P120' antibody identifying P120' in *M. hominis* has been established by raising antibodies to a peptide derived from the region between the proposed GTG start and the first ATG codon in the ORF.

In many bacteria with a low genomic G + C content, including mycoplasmas, it is known that the functional importance of genes is correlated with the G + C content of the coding regions, the housekeeping genes having the highest G + C content (Muto & Osawa, 1987). *M. hominis* has a low genomic G + C content (28–29 mol %; Neimark, 1971) and the housekeeping genes sequenced so far (*tuf*, Ladefoged & Christiansen, 1991; *ftsY*, Ladefoged & Christiansen, 1997; *gyrB*, Ladefoged & Christiansen, 1994; *lysS*, Ozkokmen *et al.*, 1994; *arcC*, Harasawa *et al.*, 1992; *licA*, Ladefoged & Christiansen, 1994; *oppB*, EMBL X99740; *deoC*, EMBL Z27121; *upp*, EMBL Z27121) all have a G + C content higher than the average for the genome. The high G + C content of *p120'* (33.1 mol %) might thus reflect a conserved amino

acid sequence necessary to maintain a prominent function in the cell, or recent acquisition from an exogenous source.

Gene duplication in *M. hominis*

The *p120* and *p120'* genes were located in two separate regions on the chromosome of *M. hominis* PG21. The genes were found in equivalent positions in *M. hominis* PG21, 4195, 132, 93 and 7488, confirming the conserved gene order observed earlier in *M. hominis* strains (Ladefoged & Christiansen, 1992). The *p120* and *p120'* genes have probably evolved by gene duplication followed by diversification and are thus serving similar but not identical functions. From Southern hybridization data it is known that the *gyrB* gene downstream of *p120'* and the region immediately upstream of *p120'* are not duplicated, suggesting that only the *p120* genes and not a larger block of the genome has been duplicated.

Further characterization of the regions is needed to prove this hypothesis. Several examples of duplicated genes exist among mycoplasmas but the most extreme is found in *Mycoplasma gallisepticum*, where the pMGA gene encoding a cell-surface haemagglutinin is estimated to be present in 70 copies, accounting for up to 16% of the genome size in one strain (Baseggio *et al.*, 1996). The pMGA family is believed to be part of a surface antigen variation system. In contrast to the *p120* family only one pMGA gene is assumed to be expressed in each cell (Glew *et al.*, 1995).

Cellular location of P120'

In our efforts to localize the P120' protein in *M. hominis* we performed experiments which are generally accepted to elucidate membrane association and surface exposure of bacterial proteins. P120 is considered to be a lipid-modified membrane protein due to the presence of lipoprotein consensus sequence elements and its hydrophobic characteristics on Triton X-114 extraction (Nyvold *et al.*, 1997; Christiansen *et al.*, 1994). The results of the Triton X-114 extraction, membrane extraction, surface proteolysis, and immunofluorescence assays using P120'-specific antisera suggest that P120' is a surface-exposed membrane-associated protein, which in contrast to P120 is without lipid modifications. The mechanism by which P120' associates with the membrane surface is as yet unclear. The sequence data combined with the biochemical properties suggest a model for P120' either as a class II integral membrane protein anchored to the plasma membrane by an uncleaved N-terminal signal peptide (von Heijne & Gavel, 1988), or as a peripheral membrane protein bound non-covalently to the membrane either between the peripheral protein and the lipid polar headgroups or to charged amino acids on other membrane protein constituents (McElhaney, 1992; Singer & Nicolson, 1972). According to the amino acid sequence, P120' contains a signal peptidase I cleavage site, but since neither this enzyme nor the corresponding gene has been identified in the completely sequenced mycoplasma

chromosomes (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996) the actual fate of the signal peptide is uncertain. P120' only contains a single cysteine residue, making it unlikely that disulphide bonds are of importance in anchoring of the protein to the membrane.

Immune responses against P120 and P120'

Human serum samples positive for antibodies against *M. hominis* have previously all been found to react with several proteins, including proteins with a molecular mass equivalent to P120', in immunoblotting using *M. hominis* lysate as antigen (Nyvold *et al.*, 1997). Using the recombinant GST-P120' as antigen, P120', although only detected in one case, was found to be recognized by the human immune system, giving rise to a strong reaction upon immunoblotting. Only a minor part of the P120' antigen was expressed in the recombinant protein and it thus remains to be established whether the mature P120' protein is recognized by a larger proportion of seropositive samples.

Significance of multigene families in the mycoplasmas

The number of lipoproteins in mycoplasmas is substantially higher than in bacteria with cell walls, implying that mycoplasmas in many instances require lipid modification in order to secure these otherwise hydrophilic proteins to the membrane (Wieslander *et al.*, 1992). In the completely sequenced genome of *M. pneumoniae*, 36 of a total of 46 proposed lipoproteins are found in six gene families containing from 4 to 16 members (Himmelreich *et al.*, 1996, 1997). The lipoprotein families also include 20 proteins with sequence similarities to lipoproteins but without the functional lipoprotein signal peptide. At present only four translational products from these lipoprotein gene families have been identified in *M. pneumoniae* (R. Herrmann, 1997: http://zmbh.uni-heidelberg.de/M_pneumoniae). In *M. hominis* two lipoproteins have been identified, P120 and Vaa. In contrast to P120, Vaa is encoded by a single-copy gene. Only a few functions have been attributed to lipoproteins in mycoplasmas. Besides a few enzymic functions proposed from sequence homology (Himmelreich *et al.*, 1996; Fraser *et al.*, 1995; Dudler *et al.*, 1988; Gilson *et al.*, 1988), lipoproteins are supposed to protect organisms from the humoral response by adaptive mutational systems creating extensive antigenic diversity at the cell surface (Citti *et al.*, 1997; Bhugra *et al.*, 1995; Lysnyansky *et al.*, 1996; Markham *et al.*, 1994). P120 and P120' display no size variation and are expressed, with few exceptions, in all *M. hominis* strains tested, indicating a function incompatible with extensive alterations.

Because of the small *M. hominis* genome it is reasonable to suppose that each gene confers a selective advantage for the cell. Further studies are required to determine the functional role of P120 and P120' and the significance of the highly similar regions in the primary structure of these proteins.

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