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Molecular Cloning and Characterization of an Adherence-Related Operon of *Mycoplasma genitalium*

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Adhesins and adhesin-related accessory proteins of pathogenic mycoplasmas are required for cytadherence and the subsequent development of disease pathology. The classic example has been *Mycoplasma pneumoniae*, which causes primary atypical pneumonia in humans. Mutants of *M. pneumoniae* defective in adhesins (P1 and P30) or in adherence-accessory proteins (HMW1 through HMW4) are unable to colonize host tissues and are avirulent. *Mycoplasma genitalium*, implicated in nongonococcal, nonchlamydial urethritis, pneumonia, arthritis, and AIDS progression, was found to encode a 140-kDa adhesin that shared both DNA and protein sequence similarities with P1, a major adhesin of *M. pneumoniae*. In this report, we show that *M. genitalium* possesses additional homolog sequences to well-characterized adherence-related genes and proteins of *M. pneumoniae*. The *M. genitalium* homologs are designated P32 and P69 and correspond to P30 and HMW3 of *M. pneumoniae*, respectively (J. B. Baseman, p. 243–259, *in* S. Rottem and I. Kahane, ed., *Subcellular biochemistry, vol. 20. Mycoplasma cell membranes*, 1993, and D. C. Krause, D. K. Leith, R. M. Wilson, and J. B. Baseman, Infect. Immun. 35:809–817, 1982). Interestingly, the operon-like organizations of P32 and P69 in the *M. genitalium* genome are similar to the organizations of P30 and HMW3 genes of *M. pneumoniae*, suggesting that the conservation of these adherence-related genes and proteins might have occurred through horizontal gene transfer events originating from an ancestral gene family.

The pathogenic human mycoplasmas, *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, have genome sizes of 500 and 400 MDa, respectively, with the latter mycoplasm considered the smallest self-replicating biological cell (2, 40). These pathogens possess terminal structures, or tip organelles, which mediate their adherence to target cells. The characterization of these cytadherence events has identified specific mycoplasma membrane adhesins and adherence-related accessory proteins as essential for successful surface parasitism (2). For example, the P1 (170-kDa) and P30 (30-kDa) adhesins of *M. pneumoniae* and the P140 (140-kDa) adhesin of *M. genitalium* have been shown through biochemical, genetic, immunological, and ultrastructural studies to be required for cytadherence (2). Mutants lacking these proteins are incapable of cytadherence and are avirulent, spontaneous cytadhering revertants regain the ability to synthesize these adhesins, antibodies reactive against these proteins block cytadherence, and immunoelectron microscopy has shown the adhesins to be localized and densely clustered at the specialized tip attachment organelles (2, 4, 8, 24, 29). The complexity of mycoplasma cytadherence has been demonstrated by the involvement of other *M. pneumoniae* proteins, including HMW1 through HMW4, which have been implicated in cytoskeleton-like functions, such as the maintenance and integrity of the tip structure and the clustering of the adhesin proteins at the specialized tip (2, 25, 39).

For several reasons, we have been interested in identifying additional proteins in *M. genitalium* that might be considered homologs of *M. pneumoniae* cytadherence-related proteins. *M. genitalium* was first isolated in 1980 from urethral specimens of patients attending a clinic for sexually transmitted disease and, more recently, was detected in urethral samples from approximately 25% of individuals with acute nongonococcal, nonchlamydial urethritis (21, 47, 48). Furthermore, *M. genitalium* has been isolated along with *M. pneumoniae* from nasopharyngeal throat swabs of patients with acute respiratory disease (3) and from synovial fluids from patients with arthritis (43, 45). Furthermore, *M. genitalium*, like other mycoplasmas, has been implicated as a cofactor in AIDS progression (31, 46). Evidence has been provided that the overlapping tissue tropism exhibited by *M. pneumoniae* and *M. genitalium* is manifested in sequence similarities among their adherence-mediating molecules (2, 9). In this case, high levels of DNA and protein sequence homology and immunological cross-reactivity between the P1 adhesin of *M. pneumoniae* and the P140 adhesin of *M. genitalium* exist (2, 9, 27, 32). Monoclonal antibodies directed against the P140 adhesin of *M. genitalium* bind to the P1 adhesin of *M. pneumoniae* and block cytadherence (32). Consistent with the isolation and characterization of P1-less mutants of *M. pneumoniae* (2), spontaneous hemadsorption-negative mutants of *M. genitalium*, lacking the P140 adhesin or with a defect in the processing of P140, failed to cytadhere to human lung fibroblasts (29). These similarities among the mycoplasma adhesins occur in spite of the substantial differences in the G+C content of *M. genitalium* (39.9%) versus *M. pneumoniae* (53.5%) adhesin genes and the preferential use of A and T rather than G and C in two of three codon positions by *M. genitalium*. The structural and functional relationships which exist among the mycoplasma adhesins were further reinforced by the identification of an adhesin-like gene in *Mycoplasma pirum* by using conserved regions within the adhesin genes of *M. pneumoniae* and *M. genitalium* as genetic probes (44).

In this study of cytadherence-related gene sequences in *M. genitalium*, we searched for the homolog of the P30 adhesin of *M. pneumoniae* by using the 5′ end of the P30 gene as hybridization probe. The results revealed that the P30 gene probe identified a region of *M. genitalium* chromosomal DNA which contained four open reading frames (ORFs). One of the

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ORFs, ORF2, encoded a 32-kDa protein (P32) which shared significant homology with the P30 adhesin of *M. pneumoniae*. ORF3, located downstream of ORF2, encoded a 69-kDa protein that shared significant homology with the HMW3 protein of *M. pneumoniae*. The adhesin genes (P30 and HMW3 of *M. pneumoniae* and P32 and P69 of *M. genitalium*) were found to be transcribed in a nonoverlapping manner on different reading frames; i.e., the reading frames for the deduced product of each ORF differed. The sequence homologies and organizational similarities among these adhesin genes reinforce the concept that a superfamily of adherence-related genes among the pathogenic mycoplasmas exists.

MATERIALS AND METHODS

Organisms and growth conditions. *M. genitalium* G37, five other clinical isolates of *M. genitalium*, and *M. pneumoniae* B10 were kindly supplied by J. Tully of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. These strains were grown in 50 to 100 ml of SP-4 medium in tissue culture bottles (32 oz [ca. 940 ml]) at 37°C under 5% CO_2. Adherent mycoplasmas were washed three times with sterile phosphate-buffered saline (PBS) (pH 7.2), scraped, and resuspended in PBS for DNA or protein extraction.

Reagents. All enzymes were obtained from Bio-Rad Laboratories (Richmond, Calif.), New England Biolabs (Beverly, Mass.), and Promega (Madison, Wis.). Erase-a-base kits were purchased from Stratagene (La Jolla, Calif.), and reagents for DNA sequencing were purchased from United States Biochemical (Cleveland, Ohio). Mycoplasma SP-4 medium components were purchased from Difco (Detroit, Mich.), agarose was purchased from Gibco-BRL (Gaithersburg, Md.), and IPTG (isopropyl-β-D-thiogalactopyranoside), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and other chemicals were purchased from Sigma (St. Louis, Mo.). Radionucleotides were purchased from Dupont/NEN (Boston, Mass.). Peroxidase and alkaline phosphatase-conjugated secondary antibodies were obtained from Zymed (San Francisco, Calif.). A synthetic peptide containing 12 amino acids corresponding to a deduced protein sequence of the putative P32 adhesin of *M. genitalium* and its keyhole limpet hemocyanin (KLH) conjugate were prepared by Peninsula Laboratories (Belmont, Calif.). Rabbit polyclonal antibody reagents were generated by the University of Texas Health Science Center Institutional Immunology Facility. For this, 2.5-kg New Zealand White male rabbits were immunized with 0.5 mg of the KLH-coupled P32 synthetic peptide or KLH-irrelevant peptide emulsified in Freund's incomplete adjuvant. On days 24, 43, and 59, rabbits received boosters of 300 μg, 400 μg, and 3.5 mg, respectively, of coupled peptides resuspended in Freund's incomplete adjuvant. All immunizations were administered in multiple sites subcutaneously.

Mycoplasma genomic DNA extractions and DNA-DNA hybridizations, cloning, subcloning, and other routine molecular biological techniques, including preparation of mycoplasma proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblots, were performed as described before (8, 9, 28).

Construction of DNA probes. The 900-bp *Hin*dIII fragment includes all but the 5′ end of the P30 gene of *M. pneumoniae*, as well as the 5′ end of the adjacent gene for HMW3 (8, 35). The DNA sequence that encodes the proline-rich carboxy terminus of the P30 gene is highly repetitious and shares homology with parts of the P1 adhesin gene and multicopy regions of the *M. pneumoniae* chromosome (8). In order to increase the specificity of the P30 gene probe and avoid unwanted hybridization signals, the C terminus of the P30 gene along with the HMW3-related gene sequences was removed by digestion with *Hpa*II. The resultant 520-bp *Hin*dIII-*Hpa*II fragment, carrying only the 5′ end of the P30 gene, served as the probe in Southern hybridization experiments. Labeling of the probe with [^{32}P]dATP or with [^{32}P]dCTP by nick translation (Bio-Rad Laboratories) or by the random-primer (Promega) method was performed according to the manufacturer's recommended instructions.

Construction of the *M. genitalium* gene bank. Genomic *M. genitalium* DNA was digested to completion with *Eco*RI, and the restriction fragments were separated on agarose gels. The fragments, ranging from 10 to 20 kb in size, were electrophoresed onto NA45 membranes (Schleicher & Schuell), eluted with high concentrations of salt, and extracted with phenol-chloroform (9). These restriction fragments were ligated to the *Eco*RI-digested vector pUC18, and then *Escherichia coli* DH5-alpha cells were transformed. Ampicillin-resistant white colonies were picked from Luria-Bertani solid medium supplemented with ampicillin (50 μg/ml), IPTG (1 mM), and X-Gal (40 μg/ml).

Cloning adhesin-related genes of *M. genitalium*. Cells carrying recombinant plasmids were transferred to nitrocellulose filters for colony hybridization as reported previously (9, 28). Southern hybridization was performed with the P30 gene probe at 37°C (9). Positive colonies were compared and picked from master plates, and plasmid preparations were made for physical mapping and subcloning. Further determination of the location of P30-homologous gene was achieved by subcloning and DNA hybridization.

Sequencing. The dideoxy chain termination method of DNA sequencing was employed with the Sequenase sequencing kit supplied by United States Biochemical. Overlapping restriction fragments of the DNA insert were subcloned in pUC18 and sequenced from both ends with universal forward and reverse primers. Synthetic primers were used when necessary. Additional sequencing was performed with fluorescent terminators by cycle sequencing (with an Applied Biosystems model 373 DNA sequencer) by the institutional DNA facility. Generated sequences were read on a Beckman Gelmate and directly entered in the computer. Both DNA and protein sequences were analyzed with the PCGENE program (Intelligenetics, Mountainview, Calif.) and compared with those in the current NCBI, GenBank, and EMBL databases.

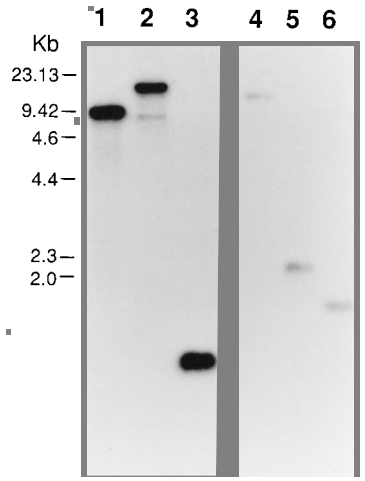

FIG. 1. Southern hybridizations between *M. pneumoniae* and *M. genitalium* genomic DNAs and the P30 gene probe of *M. pneumoniae*. Lanes 1 to 3, *M. pneumoniae* genomic DNA digested with *Eco*RI, *Xba*I, and *Hin*dIII, respectively; lanes 4 to 6, *M. genitalium* genomic DNA digested with *Eco*RI, *Xba*I, and *Hin*dIII, respectively. Values on the left represent the molecular sizes (in thousands) of *Hin*dIII-digested phage lambda genomic DNA.

Protease sensitivity assay. Preparations of intact *M. genitalium* cells at protein concentrations of 400 μg in 500 μl of PBS were treated with 5 or 50 μg of trypsin, chymotrypsin, papainase, and *Streptococcus griseus* protease for 30 min at 37°C. To stop the proteolytic digestion, protease inhibitors (phenylmethylsulfonyl fluoride, TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone], and TPCK [*N*-tosyl-L-phenylalanine chloromethyl ketone]) were added and chilled in ice for 10 min. Mycoplasmas were washed once with cold PBS and lysed prior to SDS-PAGE (4).

Nucleotide sequence accession number. The 3,745-bp nucleotide sequence (see Fig. 3) has been deposited with the National Center for Genome Resources, Santa Fe, N.Mex., under the accession no. L43097.

RESULTS

Identification, cloning, and mapping of the P30 homolog gene of *M. genitalium*. When the 5′ end of the *M. pneumoniae* P30 adhesin gene probe was used to identify hybridizing fragments of *M. genitalium* genomic DNA digested with various restriction enzymes, fragments of 14 kb (*Eco*RI), 2.1 kb (*Xba*I), and 1.4 kb (*Hin*dIII) were detected (Fig. 1). Identical patterns

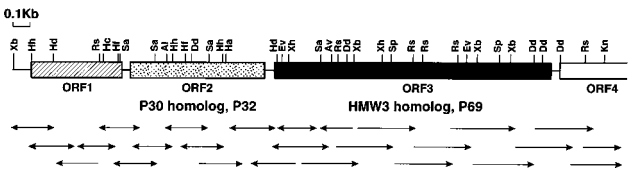

FIG. 2. Restriction map and sequencing strategies of *M. genitalium* genomic DNA containing adhesin-related genes. Restriction sites: Al, *Alu*I; Av, *Ava*II; Dd, *Dde*I; Ev, *Eco*RV; Ha, *Hae*II; Hc, *Hin*cII; Hd, *Hin*dIII; Hf, *Hin*fI; Hh, *Hha*I; Kn, *Kpn*I; Rs, *Rsa*I; Sa, *Sau*3A; Sp, *Spe*I; Xb, *Xba*I; Xh, *Xho*I. The arrowheads indicate the sequence directions, and the lengths of the arrows indicate the sizes of the sequences. Unidirectional arrows indicate sequencing by an automated sequencer with custom primers. Bidirectional arrows indicate sequencing of cloned fragments by universal primers.

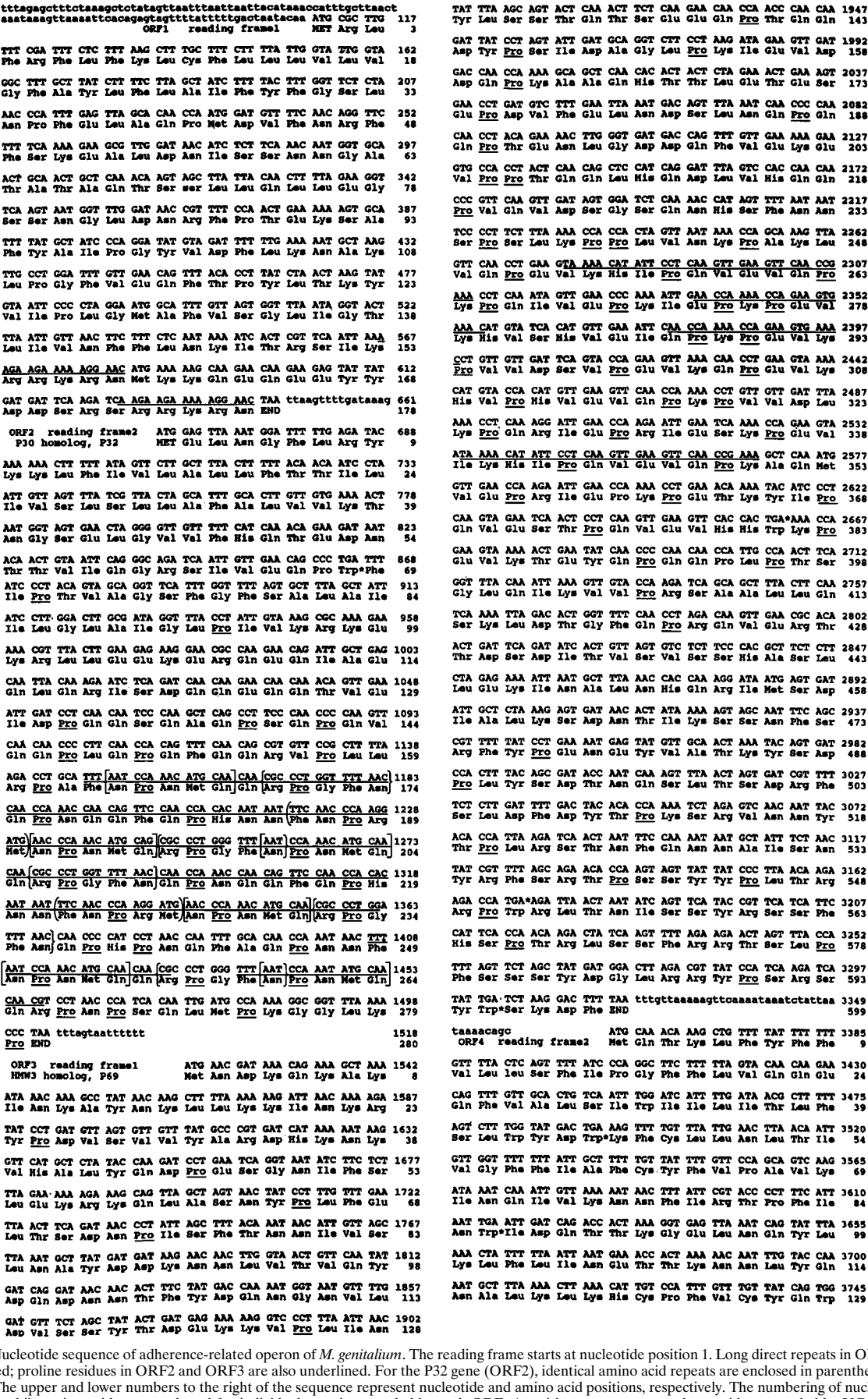

FIG. 3. Nucleotide sequence of adherence-related operon of *M. genitalium*. The reading frame starts at nucleotide position 1. Long direct repeats in ORF1 to ORF3 are underlined; proline residues in ORF2 and ORF3 are also underlined. For the P32 gene (ORF2), identical amino acid repeats are enclosed in parentheses, brackets, and braces. The upper and lower numbers to the right of the sequence represent nucleotide and amino acid positions, respectively. The numbering of nucleotides starts at position 1, while amino acids are numbered for individual proteins encoded by each ORF. Asterisks represent tryptophan residues encoded by UGA.

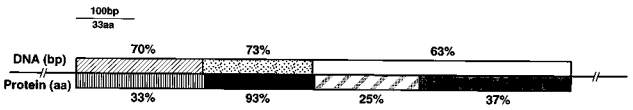

FIG. 4. Comparison of DNA and protein homologies between *M. genitalium* P32 and *M. pneumoniae* P30. The upper bar represents DNA homologies between P32 and P30 genes, while the lower bar shows protein homologies. The numbers above and below indicate percent identities of nucleotides and amino acids, respectively.

of hybridizing fragments were observed when genomic DNAs from five different clinical isolates of *M. genitalium* were probed with the P30 gene (data not shown). Homologous hybridization between *M. pneumoniae* and the P30 gene revealed fragments of different sizes, namely, 10 kb (*Eco*RI), 16 kb (*Xba*I), and 0.95 kb (*Hin*dIII) (Fig. 1).

One of the positive *E. coli* recombinant colonies that carried the 14-kb *Eco*RI fragment (pMG112) of *M. genitalium* genomic DNA was chosen for characterization. After further subcloning, a 2.1-kb *Xba*I fragment (Fig. 1) that contained the homolog P30 adhesin gene of *M. pneumoniae* was cloned into the *Xba*I site of pUC18 (pMG201). Plasmid pMG201 was physically mapped and was found to contain a 1.4-kb *Hin*dIII fragment. Plasmid pMG201 and an adjacent 2-kb region from pMG112 were subjected to DNA sequencing and further analyses.

Sequence analysis of *M. genitalium* adhesin-related genes. Both strands of the *Xba*I fragment were sequenced as shown in Fig. 2. This *Xba*I fragment was 2,019 bp long. Another 2 kb of DNA from the large plasmid pMG112, adjacent to the *Xba*I fragment, was sequenced by an automated sequencer. The resulting 3,745-bp DNA contained four ORFs. Reading frame 1 revealed ORF1 and ORF3 while reading frame 2 (+1) contained ORF2 and ORF4 (Fig. 3). No consensus −10 start or Shine-Dalgarno sequences were detected in any of the four ORFS.

ORF1. ORF1 was 537 bp long and AT rich (67%). Within the 108-bp upstream region of the start of ORF1, no sequence of significance could be detected. A 16-bp direct repeat was found at the 3′ end at nucleotides 567 through 582 and nucleotides 627 through 642. Two inverted repeats with the potential to form hairpin loops and with large amounts of negative free energy as determined by HAIRPIN, a PCGENE program, were found centered around nucleotides 358 and 488 (Fig. 3). ORF1 encoded a basic protein (pI 9.96 at pH 7.0) containing 178 amino acids and with a predicted molecular mass of 20,500 Da (P20). The sequence of the N-terminal amino acids (1 through 33) displayed a transmembrane helical structure with a hydrophobic profile. There were two integral membrane domains, one at amino acids 12 through 28 and the other at amino acids 124 through 140 (Fig. 3).

ORF2. ORF2 started 16 nucleotides downstream of ORF1 (Fig. 3). It was 843 bp long and AT rich (60%). Consistent with mycoplasma gene sequences, ORF2 contained a UGA codon coding for tryptophan at position 68. The 3′ end of ORF2 consisted of two long and two short direct repeats. The 107-bp direct repeats were found at nucleotides 1148 through 1254 and nucleotides 1256 through 1362 and were separated by one nucleotide G at position 1255. The short direct repeats of 23 bp were found 43 bp downstream of the long repeats at nucleotides 1406 through 1428 and nucleotides 1436 through 1458. Two inverted repeats with the potential to form hairpin loop structures were found centered around nucleotides 685 and 732 at the 5′ end of the gene, while one inverted repeat was found centered around nucleotide 1474 at the 3′ end of the gene (Fig. 3). ORF2 encoded a basic integral membrane protein (pI 10.37 at pH 7.0) with an estimated molecular mass of 32,000 Da (P32). P32 contained two transmembrane helices with hydrophobic profiles, comprising amino acids 12 through 28 and 79 through 95. The direct repeats found at the C terminus encoded a proline-rich amino acid sequence containing a variety of repeats of five amino acids; Asn-Pro-Asn-Met-Gln was repeated six times, Arg-Pro-Gly-Phe-Asn was repeated five times, and Phe-Asn-Pro-Arg-Met was repeated twice (Fig. 3).

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Mg P32  MELNG---FLRYKKLFIVLALLFTTILIVS-LSLLAFALV----VKTNGS    42
Mp P30  MKLPPRRKLLRF--LLAWMLVLFSALIVLATLILVQHNNTELTEVKSELS    48
        *.*     .**.  *.  . .**..... * *.. . .    **.. *
Mg P32  ELGVVFHQTEDNTTVIQGRSIVEQPWFIPTVAGSFGFSALAIILGLAIGL    92
Mp P30  PLNVVLH-AEEDTVQIQGKPITEQAWFIPTVAGCFGFSALAIILGLAIGL    97
         *.**.* .*..*. ***..*.**.********.****************
Mg P32  PIVKRKEKRLLEEKERQEQIAEQLQRISDQQEQQTVEIDPQQSQAQPSQP   142
Mp P30  PIVKRKEKRLLEEKERQEQLAEQLQRISAQQEEQQA-LEQQAAAEAHAEA   146
        ******************.********.***.* . ...*.........
Mg P32  QVQQPLQPQFQQRVPLLRPAFNPNMQQRPGFNQPNQQFQPHNNFNPRMNP   192
Mp P30  EVEPAPQPVPVPPQPQVQINFGP----RTGF-PPQPGMAPRPGMPPH--P   189
        .*... **    .. * .. .*.*    *.** .*.. ..*. .. *.  *
Mg P32  NMQ-RPGF--NPNMQQRPGFNQPNQQFQPHNNFNPRMNPNMQ-RPGFNQP   238
Mp P30  GMAPRPGFPPQPGMAPRPGM-PPHPGMAPRPGFPPQ--PGMAPRPGM-PP   235
        .*. **** .*.*..***. .*.. ..*. .* *.  *.*. ***. .*
Mg P32  HPNQFAQPNNF--NPNMQQRPGFNPNMQQRPNPSQLMPKGGLKP   280
Mp P30  HPGMAPRPG-FPPQPGMAPRPGMQPPRPGMPPQPGFPPK----R   274
        **.  ..*. *  .*.*..***..* .. .* .. . **    .
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FIG. 5. Amino acid homologies between *M. genitalium* P32 and *M. pneumoniae* P30. The alignment was performed according to the method of Higgins and Sharp (18) with a PCGENE program. Stars indicate identical residues, and dots represent conserved replacements.

The P32 gene of *M. genitalium* is homologous to the P30 adhesin gene of *M. pneumoniae*. P32 of *M. genitalium* shared significant nucleotide and amino acid sequence homologies and other characteristic features of the P30 adhesin of *M. pneumoniae*. Overall, the P32 protein shared 43% identity with P30. The N-terminal region between amino acids 68 and 126 exhibited 93% identity, and this region corresponded to a 73%

	M.genitalium P32 gene	M.pneumoniae P30 gene		M.genitalium P32 gene	M.pneumoniae P30 gene
TTT Phe	17(6.0)	5(1.8)	TAT Tyr	0	0
TTC Phe	4(1.4)	6(2.1)	TAC Tyr	1(0.3)	0(0.0)
TTA Leu	10(3.5)	9(3.2)	TAA ---	1(0.3)	1(0.3)
TTG Leu	2(0.7)	4(1.4)	TAG ---	0	0
CTT Leu	9(3.2)	4(1.4)	CAT His	2(0.7)	1(0.3)
CTC Leu	0	0	CAC His	2(0.7)	5(1.8)
CTA Leu	3(1.0)	3(1.0)	CAA Gln	36(12.0)	20(7.2)
CTG Leu	0	4(1.4)	CAG Gln	9(3.2)	4(1.4)
ATT Ile	7(2.4)	7(2.5)	AAT Asn	13(4.6)	1(0.3)
ATC Ile	4(1.4)	2(0.7)	AAC Asn	17(6.0)	3(1.0)
ATA Ile	2(0.7)	2(0.7)	AAA Lys	7(2.4)	3(1.0)
ATG Met	10(3.5)	14(5.0)	AAG Lys	2(0.7)	7(2.5)
GTT Val	9(3.2)	5(1.8)	GAT Asp	3(1.0)	1(0.3)
GTC Val	0	1(0.3)	GAC Asp	0	0
GTA Val	3(1.0)	4(1.4)	GAA Glu	9(3.2)	15(5.4)
GTG Val	1(0.3)	3(1.0)	GAG Glu	3(1.0)	4(1.4)
TCT ser	0	1(0.3)	TGT Cys	0	0
TCC Ser	2(0.7)	0	TGC Cys	0	1(0.3)
TCA Ser	4(1.4)	0	TGA Trp	1(0.3)	1(0.3)
TCG Ser	1(0.3)	0	TGG Trp	0	1(0.3)
CCT Pro	12(4.2)	15(5.4)	CGT Arg	3(1.0)	6(2.1)
CCC Pro	5(1.7)	13(4.7)	CGC Arg	7(2.4)	5(1.8)
CCA Pro	16(5.6)	25(9.0)	CGA Arg	0(0.0)	1(0.3)
CCG Pro	1(0.3)	4(1.4)	CGG Arg	0(0.0)	0(0.0)
ACT Thr	2(0.7)	2(0.7)	AGT Ser	3(1.0)	3(1.0)
ACC Thr	0(0.0)	2(0.7)	AGC Ser	0(0.0)	1(0.3)
ACA Thr	6(1.7)	3(1.0)	AGA Arg	4(1.4)	4(1.4)
ACG Thr	0(0.0)	0(0.0)	AGG Arg	2(0.7)	0(0.0)
GCT Ala	5(1.7)	9(3.2)	GGT Gly	7(2.4)	17(6.1)
GCC Ala	0(0.0)	5(1.8)	GGC Gly	2(0.7)	4(1.4)
GCA Ala	5(1.7)	5(1.8)	GGA Gly	3(1.0)	2(0.7)
GCG Ala	1(0.3)	7(2.5)	GGG Gly	3(1.0)	0(0.0)

FIG. 6. Compilation of codon usage by *M. genitalium* and *M. pneumoniae* in P32 and P30 genes, respectively. Numbers in parentheses represent percentages of use for the given genes.

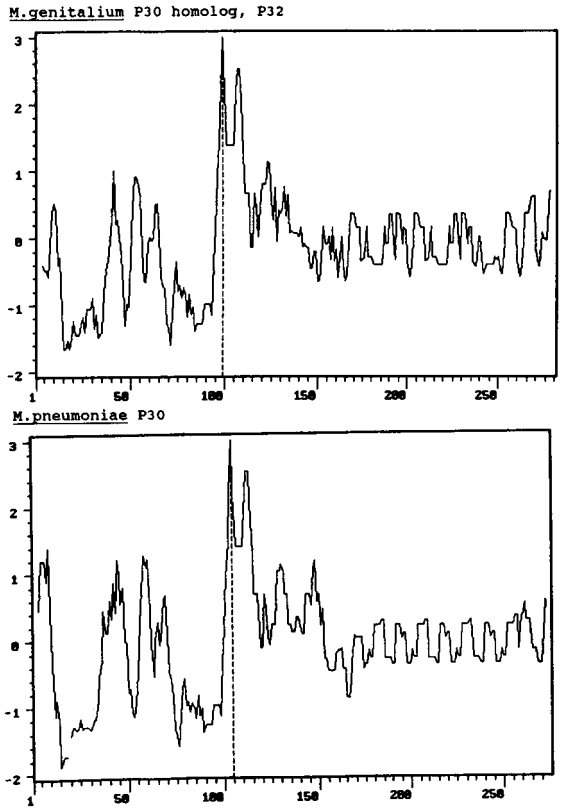

FIG. 7. Hydrophilicity profiles for *M. genitalium* P32 and *M. pneumoniae* P30. Profiles were determined by the method of Hopp and Woods (20) with a PCGENE program. The *y* axis displays hydrophilicity values, and the *x* axis represents the amino acid residue numbers. Vertical dashed lines indicate maximum hydrophilicities.

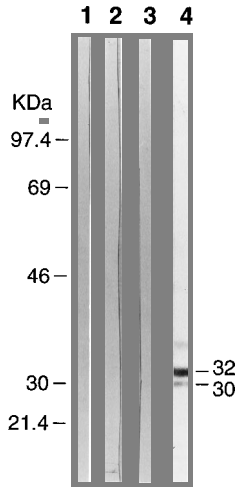

FIG. 8. Immunoblots of *M. pneumoniae* and *M. genitalium* total cell proteins. Rabbit antiserum generated against a 12-amino-acid synthetic peptide of the deduced P32 adhesin of *M. genitalium* was used for immunoblots. Lanes 1 and 2, *M. pneumoniae* proteins; lanes 3 and 4, *M. genitalium* proteins. Lanes 1 and 3 were treated with preimmune serum, while lanes 2 and 4 were treated with immune serum. Serum generated against KLH coupled to an irrelevant peptide showed no cross-reactions to mycoplasma proteins. All sera were diluted to 1:50,000. Values on the left represent the molecular masses of proteins used as standards.

DNA sequence identity with the P30 gene. Overall, the DNA sequence identity between P32 and P30 genes was 68% (Fig. 4). The C termini of both proteins were rich in proline residues and contained similar, but not identical, novel repeats consisting of five to six amino acids (Fig. 3). However, the P32 deduced amino acid sequence contained 23 fewer prolines, 15 fewer alanines, and 8 fewer glycines compared with P30 of *M. pneumoniae* (Fig. 5 and 6). Reflecting a similar trend in codon usage, P32 contained 26 more asparagines, 21 more glutamines, and 10 more phenylalanines. Both proteins contained one tryptophan residue encoded by UGA, and only P30 contained a cysteine residue (Fig. 6). Interestingly, the hydrophilicity profiles of these proteins were almost identical (Fig. 7).

In order to establish that ORF2 encoded an *M. genitalium* protein, a 12-amino-acid synthetic peptide from the C-terminal end of P32 was constructed. This peptide contained the amino acids FNPNMQQRPGFN, which were repeated three times (at amino acids 163 to 174, 199 to 210, and 249 to 260) (Fig. 3 and 5) and exhibited no correlative sequence in P30 of *M. pneumoniae*. Antibodies raised against this peptide coupled to KLH cross-reacted strongly with a 32-kDa protein in *M. genitalium* and failed to react with *M. pneumoniae* proteins (Fig. 8). Weaker reactions to 37- and 30-kDa *M. genitalium* proteins were also detected. The location of P32 on *M. genitalium* cells was examined with various proteases and P32 gel migration patterns. Protease-treated intact mycoplasmas were subjected to SDS-PAGE, and gels were immunoblotted with antipeptide antiserum. Papainase completely digested P32, trypsin and *S. griseus* protease partially cleaved P32 into a 30-kDa peptide, and chymotrypsin was without effect (Fig. 9).

ORF3. ORF3 was located 14 bp downstream of ORF2 and was transcribed from reading frame 1 (Fig. 3). ORF3 was 1,800 bp long and AT rich (65.7%). The DNA sequence of ORF3 was highly repetitious, containing several direct repeats of various lengths, from 10 to 35 bp. Among the large repeats, a 35-bp direct repeat was located at nucleotides 2276 through 2310 and 2534 through 2568. A 21-bp direct repeat was found at nucleotides 2336 through 2356 and 2378 through 2398. Numerous inverted repeats were found scattered across the ORF3 sequence. Several repeats showed the potential to form hairpin loops with large amounts of negative free energy (determined by HAIRPIN, a PCGENE program) and were centered around nucleotides at positions 1850, 2363, 2450, 2641, 2849, 3300, and 3432. ORF3 encoded a hydrophilic protein with a predicted molecular mass of 68,720 Da (P69). P69 did not contain transmembrane or membrane-associated helices. All three tryptophan residues located at positions 381, 551, and 595 were encoded by UGA codons (Fig. 3).

P69 of *M. genitalium* is homologous to HMW3 protein of *M. pneumoniae*. ORF3 and its protein product P69 shared significant DNA and amino acid sequence homologies with the HMW3 gene and corresponding protein of *M. pneumoniae* (33). The ORF3 DNA sequence exhibited 74% identity compared with that of the HMW3 gene of *M. pneumoniae* (Fig. 3).

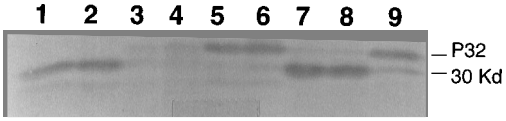

FIG. 9. Protease sensitivity of P32 protein in intact *M. genitalium* cells. Lanes 1 and 2, *S. griseus* protease (50 and 5 μg of enzyme, respectively); lanes 3 and 4, papainase (50 and 5 μg, respectively); lanes 5 and 6, chymotrypsin (50 and 5 μg, respectively); lanes 7 and 8, trypsin (50 and 5 μg, respectively); lane 9, untreated control.

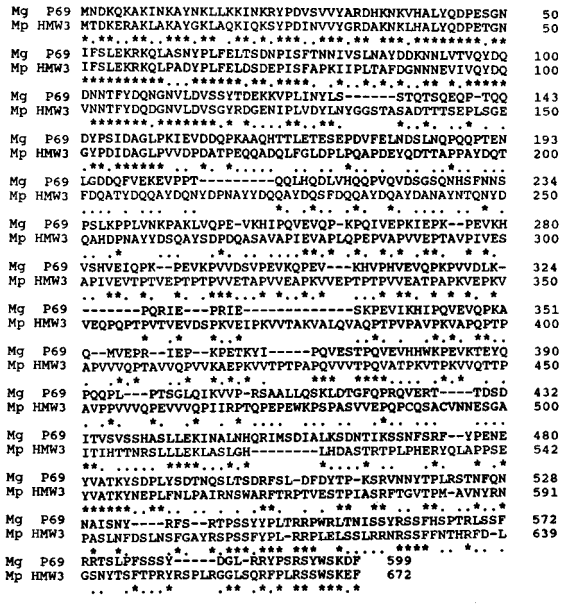

FIG. 10. Amino acid homologies between P69 of *M. genitalium* and HMW3 of *M. pneumoniae*. The alignment was performed with a PCGENE program by the method of Higgins and Sharp (18). Stars represent amino acid residue identities, and dots indicate conservative replacements.

The first 120 amino acids in the N terminus and the last 60 amino acids in the C terminus of P69 shared 70 and 45% identity with the N and C termini of the HMW3 protein, respectively (Fig. 10). The hydrophilicity plots of P69 and HMW3 protein were similar, with three amino acid regions (between amino acid residues 3 and 8, 33 and 38, and 53 and 58) exhibiting the highest hydrophilicity (Fig. 11). However, HMW3 was acidic with a pI of 4.39 while P69 was slightly basic with a pI of 7.2. This can be explained by the fact that the HMW3 protein of *M. pneumoniae* contained a total of 80 negatively charged residues (Asp plus Glu) and 51 positively charged residues (Arg plus Lys), while P69 of *M. genitalium* contained 74 negatively charged residues and 73 positively charged residues, including 21 more lysine residues (Fig. 12). The codon usage for ORF3 of *M. genitalium* and HMW3 of *M. pneumoniae* differed in that ORF3 was more AT rich (65.7%) than was HMW3 (52.6%). Accordingly, P69 contained 45 fewer alanines, 31 fewer prolines, 20 fewer valines, and 18 fewer threonines. Like P32, P69 did not contain any cysteine residues, while HMW3 contained two cysteines (Fig. 12). Because of the apparent amino acid homologies between P69 and HMW3 proteins, a polyclonal antiserum against the HMW3 protein of *M. pneumoniae* (provided by D. Krause, University of Georgia) was used to detect cross-reacting proteins in *M. genitalium* by immunoblot. A major protein band with a molecular mass ranging from 105 to 109 kDa and three proteins with molecular masses of 97, 92, and 38 kDa were detected (Fig. 13).

ORF4. ORF4 was located 31 bp downstream of ORF3 and was transcribed from reading frame 2. The partial sequence of ORF4 revealed a phenylalanine-rich protein that shared significant identity (46%) with an NCBI bank *M. pneumoniae* sequence whose function is not known.

DISCUSSION

The biological, morphological, and serological similarities shared by *M. genitalium* and *M. pneumoniae* suggest common mechanisms of host-cell interaction. Earlier, we identified and characterized the P140 adhesin of *M. genitalium* and showed that high degrees of DNA and amino acid sequence homologies between P140 and the P1 adhesin of *M. pneumoniae* (9, 32) existed. These similarities occurred despite the limited total genomic DNA homology (8%) detected between *M. genitalium* and *M. pneumoniae* and the substantial differences in codon usage (2, 9). Because of the apparent sequence conservation among the adhesin-related genes and proteins associated with mycoplasma cytadherence, we attempted to identify and clone additional *M. genitalium* genes by using the P30 adhesin gene from *M. pneumoniae*, which has been shown to encode a 30-kDa protein essential for *M. pneumoniae* cytadherence and virulence (2, 4, 8). A unique 2.1-kb *Xba*I restriction fragment of genomic DNA of *M. genitalium*, which hybridized to the P30 probe, was sequenced along with a 1.5-kb fragment downstream. Four ORFs on a single sense strand, transcribed from different and nonoverlapping reading frames, were identified. ORF1 was 567 bp long, encoding a 20-kDa basic protein (P20) whose function is not known. Its N-terminal sequence (5 through 14) shares homology with cell surface receptor proteins, particularly, human leukocyte adhesin glycoprotein P150 and viral coat proteins, such as simian virus 40 large T antigen and hemagglutinin precursor of influenza A virus, while its C terminus amino acids (160 through 176) share homology with a consensus sequence for bipartite nuclear transport proteins (11, 37). This makes ORF1 an interesting candidate to uncover functions associated with mycoplasma adherence and the subsequent invasion of host cells. ORF2

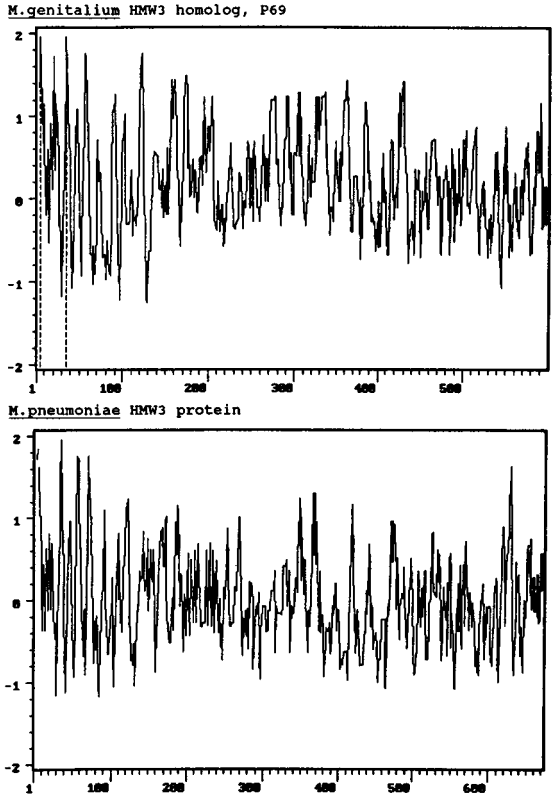

FIG. 11. Hydrophilicity profiles for *M. genitalium* P69 and *M. pneumoniae* HMW3. Profiles were determined by the method of Hopp and Woods (20) with a PCGENE program. The hydrophilicity values are shown on the *y* axis, and the amino acid residue numbers are shown on the *x* axis. Vertical dashed lines indicate maximum hydrophilicities.

	M.genitalium HMW3 homolog	M.pneumoniae HMW3 gene		M.genitalium HMW3 homolog	M.pneumoniae HMW3 gene
TTT	Phe 13(2.1)	15(2.2)	TAT Tyr	19(3.1)	17(2.5)
TTC	Phe 5(0.8)	5(0.7)	TAC Tyr	7(1.1)	17(2.5)
TTA	Leu 22(3.6)	7(1.0)	TAA ---	1(0.1)	1(0.1)
TTG	Leu 5(0.8)	11(1.6)	TAG ---	0	0
CTT	Leu 7(1.1)	4(0.5)	CAT His	11(1.8)	0
CTC	Leu 1(0.1)	7(1.0)	CAC His	6(1.0)	7(1.0)
CTA	Leu 6(1.0)	6(0.8)	CAA Gln	41(6.8)	32(4.7)
CTG	Leu 0	4(0.5)	CAG Gln	6(1.0)	13(1.9)
ATT	Ile 16(2.6)	11(1.6)	AAT Asn	20(3.3)	10(1.4)
ATC	Ile 4(0.6)	4(0.5)	AAC Asn	18(0.3)	20(2.9)
ATA	Ile 6(1.0)	4(0.5)	AAA Lys	37(6.1)	12(1.7)
ATG	Met 3(0.5)	2(0.2)	AAG Lys	10(1.6)	14(2.0)
GTT	Val 34(5.6)	22(3.2)	GAT Asp	29(4.8)	30(4.4)
GTC	Val 5(0.8)	5(0.7)	GAC Asp	7(1.1)	12(1.7)
GTA	Val 10(1.6)	30(4.4)	GAA Glu	35(5.8)	27(4.0)
GTG	Val 3(0.5)	15(2.2)	GAG Glu	3(0.5)	11(1.6)
TCT	Ser 12(2.0)	4(0.5)	TGT Cys	0	2(0.2)
TCC	Ser 2(0.3)	6(0.8)	TGC Cys	0	0
TCA	Ser 18(3.0)	4(0.5)	TGA Trp	3(0.5)	2(0.2)
TCG	Ser 0	2(0.2)	TGG Trp	0	1(0.1)
CCT	Pro 24(4.0)	22(3.2)	CGT Arg	6(1.0)	10(1.4)
CCC	Pro 5(0.3)	6(0.8)	CGC Arg	1(0.1)	8(1.1)
CCA	Pro 29(4.8)	49(7.2)	CGA Arg	0	0
CCG	Pro 2(0.3)	14(2.0)	CGG Arg	0	2(0.2)
ACT	Thr 22(3.6)	11(1.6)	AGT Ser	21(3.5)	17(2.5)
ACC	Thr 2(0.3)	17(2.5)	AGC Ser	9(1.5)	10(1.4)
ACA	Thr 9(1.5)	20(2.9)	AGA Arg	17(2.8)	4(0.5)
ACG	Thr 0	3(0.4)	AGG Arg	2(0.3)	1(0.1)
GCT	Ala 11(1.8)	28(4.1)	GGT Gly	6(1.0)	12(1.7)
GCC	Ala 2(0.3)	7(1.0)	GGC Gly	0	4(0.5)
GCA	Ala 5(0.8)	20(2.9)	GGA Gly	2(0.3)	1(0.1)
GCG	Ala 0	8(1.1)	GGG Gly	0	3(0.4)

FIG. 12. Compilation of codon usage by *M. genitalium* and *M. pneumoniae* in P69 and HMW3 genes, respectively. Numbers in parentheses represent percentages of use for the given genes.

was 843 bp long, encoding a 32-kDa basic integral and surface exposed membrane protein (P32). Importantly, P32 shared substantial DNA and amino acid sequence homologies with the P30 adhesin of *M. pneumoniae*. Amino acid sequences at the N terminus of P32 exhibited 93% identity with P30, and like the P30 adhesin of *M. pneumoniae*, P32 contained proline-rich repeats of five to six amino acids at the C terminus. Characterization of spontaneous hemadsorption-negative *M. pneumoniae* mutants lacking P30 showed that P30 was essential for *M. pneumoniae* cytadherence (2, 4, 25, 26). The study of mutants of *M. genitalium* defective in P32, generated either spontaneously or by transposon Tn*4001*, should define the role of P32 in mycoplasma pathogenicity (36). The third ORF, located downstream of P32, encoded a 69-kDa protein (P69) with significant identity to the HMW3 adherence-related accessory protein of *M. pneumoniae*. Mutants that lacked HMW3 failed to achieve cytadherence and were avirulent in the hamster model (25). In *M. genitalium*, P69 is the first putative adherence-accessory homolog protein identified whose role in cytadherence and other biological functions must be established. The difference between the calculated molecular mass of P69 (69 kDa) and those of the higher-molecular-mass protein bands detected immunologically by anti-HMW3 antisera (105 to 109 kDa and 90 to 95 kDa, Fig. 13) could be attributed to the acidic amino acid residues present in P69 (Fig. 3 and 12). A similar anomaly in protein sizes was seen with the HMW3 protein of *M. pneumoniae* (33) and the MB antigen of *Ureaplasma urealyticum* (50).

The organization of genes encoding P32 and P69 of *M. genitalium* was similar to the organization of their counterpart P30 and HMW3 genes of *M. pneumoniae*. The HMW3 gene was located 13 bp downstream of the P30 adhesin gene and was read from a different nonoverlapping reading frame (35). This similarity in organization of adhesins and adherence-accessory genes between *M. genitalium* and *M. pneumoniae* suggests that these two mycoplasmas share related and complex operons that encode cytadherence-related functions. Each of the three *M. genitalium* proteins P20, P32, and P69 could be phosphorylated and glycosylated, as revealed by the PCGENE program. Recently, the phosphorylation of cytadherence-accessory proteins and the P1 adhesin of *M. pneumoniae* was demonstrated (12), although the role of phosphorylation in cytadherence and virulence remains unknown.

The roles of long and short direct repeats present at the 3′ ends of ORF1, ORF2, and ORF3 are unclear, although direct repeats have been observed by other investigators (6, 13, 22, 49, 50). The involvement of short repeats in phase variation has been demonstrated for pathogenic bacteria (14, 23, 30, 34). Variations in the antigenic (hydrophilic) domains of proteins associated with pathogenicity have been related to the involvement of repetitive sequences, as in the cases of the streptococci (19), *U. urealyticum* (50), and *Mycoplasma hyorhinis* (49). We have described the single- and multiple-copy nature of the P1 gene of *M. pneumoniae* and its homologous P140 gene in *M. genitalium*, which may represent mechanisms for generating antigenically different adhesins by genetic recombination (7, 10, 41, 42). Although the genes encoding P30 and P32 are present as single copies (Fig. 1), the repeat structures detected at the C termini may contribute to possible antigenic variation events by deletion or duplication of the repeats, as demonstrated in *U. urealyticum* (50). Also, the roles of the inverted repeats, some of which have the potential to form hairpin loops, need to be determined.

We observed reading frame shifts in the expression of three ORFs in *M. genitalium*. Reading frame shifts during translation have been observed for procaryotes and eucaryotes as a means of utilizing limited genomes effectively with the capacity to produce more proteins or as a regulatory mechanism in the production of different amounts of gene products (1, 5, 15, 16). The inverted repeats with the potential to form secondary structures may participate in ribosomal bypassing of nucleotides (5, 15), resulting in the expression of adhesin genes from different reading frames as well as in the regulation of adhesin synthesis. In bacteria such as *Bordetella* spp. (17, 38) and *Neisseria* spp. (30) and in the malarial parasite (23), repeat structures are used to bring genes in frame for transcription by deletion or duplication of repeats. With *M. genitalium*, two

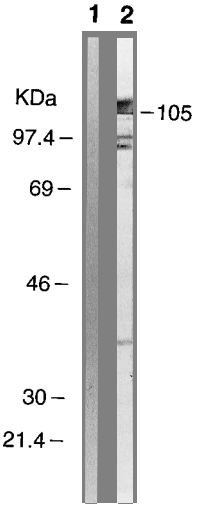

FIG. 13. Immunoblots of *M. genitalium* total cell proteins. *M. pneumoniae* anti-HMW3 antiserum was used for immunoblots. Lane 1, preimmune serum; lane 2, immune serum. Both sera were diluted 1:50,000.

interesting possibilities may result if the hairpin loops with large amounts negative free energy ($-dG°$) are introduced into the sense strand. The hairpin loops from individual ORFs could generate smaller proteins with different amino acid sequences without affecting the reading frame of the downstream neighbor. Some of these loops could restore a single reading frame for different ORFs. As an example, the introduction of a hairpin loop at the 3′ end of the repeat structure in ORF2 (an inverted repeat between nucleotides 1467 and 1480 that could form a hairpin loop with a $dG°$ of -2 Kcal [-8.368 KJ/mol) could generate a 30-kDa protein and align ORF3 into the same reading frame as ORF2 (Fig. 3). This may explain why antipeptide antiserum detected a 30-kDa protein in addition to P32 (Fig. 8). Similarly, the presence of several repeats with the potential to form hairpin loops spontaneously in ORF3 could generate several peptides (37, 92, and 97 kDa), as detected by anti-HMW3 antiserum (Fig. 13). However, this explanation does not exclude the possibility that these monospecific antisera detected antigenically similar, yet unrelated, proteins. The use of monoclonal antibodies directed against specific regions of P32 or P69 would address this issue. It is not known how these secondary structures may be involved in gene expression and/or regulation. However, a possible role of secondary structures in the translation of the P140 operon of *M. genitalium* was proposed (22). The identification and characterization of similar adhesins and adherence-related accessory genes and proteins among the pathogenic human mycoplasmas reinforce the superfamily nature of this group of molecules and provide new opportunities to uncover and elucidate the mechanisms by which mycoplasmas parasitize and infect human hosts.

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