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 approx-

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 hemadsorptionnegative 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

imately 25% of individuals with acute nongonococcal, non-

chlamydial urethritis (21, 47, 48). Furthermore, M. genitalium

has been isolated along with M. pneumoniae from nasopharyn-

geal throat swabs of patients with acute respiratory disease (3)

and from synovial fluids from patients with arthritis (43, 45).

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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% CO₂. 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-B-D-thiogalactopyranoside), X-Gal (5-bromo-4-chloro-3-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 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 (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 EcoRI, 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 EcoRI-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 $37^{\circ}C$ (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 Sequences sequencing kit supplied by United States Biochem-



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, *XbaI*, and *Hind*III, respectively; lanes 4 to 6, *M. genitalium* genomic DNA digested with *Eco*RI, *XbaI*, and *Hind*III, respectively. Values on the left represent the molecular sizes (in thousands) of *Hind*III-digested phage lambda genomic DNA.

ical. 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.

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 [$N\alpha$ -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 (*Hind*III) 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, *AluI*; Av, *AvaII*; Dd, *DdeI*; Ev, *Eco*RV; Ha, *HaeII*; Hc, *HincII*; Hd, *HindIII*; Hf, *HinfI*; Hh, *HhaI*; Kn, *KpnI*; Rs, *RsaI*; Sa, *Sau3A*; Sp, *SpeI*; Xb, *XbaI*; Xh, *XhoI*. 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.

titagagottiotaaagototatagitaattaattaattaataaacoattigottaact aaataaagttaaaattoacagagtagtittäättitgaotaatacaa AVG CGC TG 117 ORP1 raadiug framal MET AVG Tau 1	TAT TTA AGC AGT ACT CAA ACT TCT CAA GAA CAA CCA ACC CAA CAA 1947 Tyr Leu Ser Ser Thr Gin Thr Ser Glu Glu Gin <u>Pro</u> Thr Gin Gin 143
TTT CGA TTT CTC TTT AM CTT TGC TTT CTT TTA TTG GTA TTG GTA 162	GAT TAT CCT AGT ATT GAT GCA GGT CTT CCT ANG ATA GAA GTT GAT 1992 Asp Tyr <u>Pro</u> Ser Ile Asp Ale Gly Leu <u>Pro</u> Lys Ile Glu Val Amp 158
GOC THT GCT TAT CTT TTC TTA GCT ATC TTT TAC TTT GGT TCT CTA 207	GAC CAA CCA AAA GCA GCT CAA CAC ACT ACT CTA GAA ACT GAA AGT 2037 Asp Gin <u>Pro</u> Lys Als Als Gin His Thr Thr Leu Giu Thr Giu Ser 173
ANC CCA THE GAG TEA GCA CAA CCA ATG GAT GTT THE AAC AGG TEA CO	GAA CCT GAT GTC TTT GAA TTA AAT GAC AGT TTA AAT CAA CCC CAA 2082 Glu <u>Pro</u> Asp Val Phe Glu Leu Asn Asp Ser Leu Asn Gln <u>Pro</u> Gln 188
TIT TCA ANA GAA GCG TTG GAT AAC ATC TCT TCA AAC AAT GGT GCA 297	CAA CCT ACA GAA AAC TTG GGT GAT GAC CAG TTT GTT GAA AAA GAA 2127 Gin <u>Pro</u> Thr Giu Asn Leu Giy Asp Asp Gin Phe Val Giu Lys Giu 203
ACT GCA ACT GCT CAA ACA AGT AGC TTA TTA CAA CTT TTA GAA GGT 342	GTG CCA CCT ACT CAA CAG CTC CAT CAG GAT TTA GTC CAC CAA CAA 2172 Val <u>Pro</u> Pro Thr Gln Gln Leu His Gln Asp Leu Val His Gln Gln 218
The Ala the Ala Gin the see see law and cin law ciu ciu ciy /s	CCC GTT CAA GTT GAT AGT GGA TCT CAA AAC CAT AGT TTT AAT AAT 2217
TCA AGT AAT GGT TTG GAT AAC CGT TTT CCA ACT GAA AAA AGT GCA 387	<u>Pro</u> Val Gln Val Amp Ser Gly Ser Gln Amn Him Ser Phe Amn Amn 233
Ser Ser Ann Gly Leu And Ann Arg phe pro the glu Lyn Ser Ala 93	TCC CCT TCT TTA ANA CCA CCA CTA GTT ANT ANA CCA GCA ANG TTA 2262
The tat get ate cea gga tat get gat tit its gaa aat get ang 432	Ser <u>Pro</u> Ser Leu Lys <u>Pro Pro</u> Leu Vel Asn Lys <u>Pro</u> Ale Lys Leu 248
THE COT GGA TIT GIT GAA CAG TIT ACA COT TAT CTA ACT ANG TAT 477	GTT CAA CCT GAA G <u>TA AAA CAT ATT CCT CAA GTT GAA GTT CAA CCG</u> 2307 Val Gin <u>Pro</u> Glu Val Lys His Ile <u>Pro</u> Gin Val Giu Val Gin <u>Pro</u> 263
Leu Pro Gly Phe Vel Glu Gln Phe Thr Pro Tyr Leu Thr Lys Tyr 123	ANA CCT CAN ATA GTT GAN CCC ANA ATT GAN CCA ANA CCA GAN GTG 2352
GTA ATT CCC CTA GGA ATG GCA TTT GTT AGT GGT TTA ATA GGT ACT 522	Lys pro Gin ile Val Giu pro Lys ile Giu pro Lys Pro Giu Val 278
Val 11e Pro Leu Gly Met Ale Phe Val Ber Gly Leu 11e Gly Thr 138	ANA CAT GTA TCA CAT GTT GAA ATT C <u>AA CCA ANA CCA GAA GTG ANA</u> 2397
TTA ATT GTT AAC TTC TTT CTC AAT AAA ATC ACT CGT TCA ATT AAA 567	Lys His Val Ser Bis Val Glu 11e Glu <u>Pro</u> Lys <u>Pro</u> Glu Val Lys 293
Lou Ile Val Asn Phe Phe Leu Asn Lys Ile Thr Arg Ser Ile Lys 153	CCT GTT GTT GAT TCA GTA CCA GAA GTT ANA CAA CCT GAA GTA AAA 2442
<u>Aga aga ama agg ama</u> atg ama ang caa gaa gaa gag tat tat 612	Pro Val Val Asp Ser Val Pro Glu Val Lys Gln Pro Glu Val Lys 308
Arg Arg Lys Arg Asn Met Lys Lys Gin Glu Gin Glu Glu Hyr Tyr 168	CAT GTA CCA CAT GTT GAA GTT CAA CCA AAA CCT GTT GTT GAT TTA 2487
GAT GAT TCA AGA T <u>CA AGA AGA AAA AGG AAC</u> TAA ttaagttttgataaag 661	His Val <u>Pro</u> His Val Glu Val Gln <u>Pro</u> Lys <u>Pro</u> Val Val Asp Leu 323
Asp Asp Ser Arg Ser Arg Arg Lys Arg Asn END 178	AAA CCT CAA AGG ATT GAA CCA AGA ATT GAA TCA AAA CCA GAA GTA 2532
ORF2 reading frame2 ATG GAG TTA AAT GGA TIT TTG AGA TAC 688	Lys <u>Pro</u> Gin Arg Ile Giu <u>Pro</u> Arg Ile Giu Ser Lys <u>Pro</u> Giu Val 338
P30 homolog, P32 HET Glu Leu Ash Gly Phe Leu Arg Tyr 9	ATA AAA CAT ATT CCT CAA GTT GAA GTT CAA CCG AAA GCT CAA ATG 2577
Ana ana cit itt ata git cit gct ita cit itt aca aca arg cit 733	Ile Lys His Ile <u>Pro</u> Gin Val Glu Val Gin <u>Pro</u> Lys Ale Gin Met 353
Lys Lys Leu Pho IIe Val Leu Ala Leu Leu Pho Thr Thr IIe Leu 24	GTT GAA CCA AGA ATT GAA CCA AAA CCT GAA ACA AAA TAC ATC CCT 2622
ATT GTT AGT TTA TCG TTA CTA GCA TTT GCA CTT GTG ANA ACT 778	Val Glu <u>Pro</u> Ary Ile Glu Pro Lys <u>Pro</u> Glu Thr Lys Tyr Ile <u>Pro</u> 368
Ile Val Ser Leu Ber Leu Leu Ala Phe Ala Leu Val Val Lys Thr 39	CAA GTA GAA TCA ACT CCT CAA GTT GAA GTT CAC CAC TGA*AAA CCA 2667
Ant get Agt gaa eta gog git git tit cat caa aca gaa gat ant 823	Gln Val Glu Ser Thr Pro Gln Val Glu Val His His Trp Lys Pro 383
And Gly Ser Glu Leu Gly Val Val Phe Bib Gin Thr Glu Asp Ash 54	GAA GTA AAA ACT GAA TAT CAA CCC CAA CAA CCA TTG CCA ACT TCA 2712
ACA ACT GTA ATT CAG GGC AGA TCA ATT GTT GAA CAG CCC TGA TTT 868	Glu Val Lys Thr Glu Tyr Gln <u>Pro</u> Gln Gln Pro Law Pro Thr Ser 398
Thr Thr Val Ile Gin Gly Ary Ser Ile Val Glu Gin Pro TrpPPhe 69 ATC CCT ACA GTA GCA GGT TCA TTT GGT TTT AGT GCT TTA GGT ATT 913 Ile Pro Thr Val Ala Giv Ser Phe Giv Phe Ser Ala Leu Ala Ile 84	GGT TTA CAA ATT AAA GTT GTA CCA AGA TCA GCA GCT TTA CTT CAA 2757 Gly Leu Gln ile Lys Val Val Pro Arg Ser Ala Ala Leu Leu Gln 413
ATC CTT GGA CTT GGG ATA GGT TTA CCT ATT GTA ANG CGC AAA GAA 958	TCA ANA TTA GAC ACT GGT TTT CAA CCT AGA CAA GTT GAA CGC ACA 2802
Tie Leu Giv Leu Ais Tie Giv Leu Pro Ile Val Lys Arg Lys Glu 99	Ser Lys Leu Asp Thr Gly Phe Gln <u>Pro</u> Arg Gln Val Glu Arg Thr 428
AAA COT TTA CTT GAA GAG AAG GAA CGC CAA GAA CAG ATT GCT GAG 1003	ACT GAT TCA GAT ATC ACT GTT AGT GTC TCT TCC CAC GCT TCT CTT 2847
Lys Arg Leu Leu Glu Glu Glu Ays Glu Arg Gin Glu Gin Ile Als Glu 114	Thr Asp Ser Asp Ile Thr Val Ser Val Ser Ser His Ala Ser Leu 443
CAN TTA CAN AGA ATC TCA GAT CAN GAN GAN CAN CAN ACA GTT GAN 1048	CTA GAG ANA ATT AAT GCT TTA AAC CAC CAA AGG ATA ATG AGT GAT 2892
Gin Leu Gin Arg Ile Ser Amp Gin Gin Giu Gin Gin Thr Val Giu 129	Leu Glu Lys Ile Asn ala Leu Asn His Gln Arg Ile Het Ser Asp 458
ATT GAT CCT CAA CAA TCC CAA GCT CAG CCT TCC CAA CCC CAA GTT 1093	ATT GCT CTA AAG AGT GAT AAC ACT ATA AAA AGT AGC AAT TTC AGC 2937
Ile Asp <u>Pro</u> Gin Gin Ser Gin Ale Gin <u>Pro</u> Ser Gin <u>Pro</u> Gin Val 144	Ile Ala Leu Lys Ser Asp Asn Thr Ile Lys Ser Ser Asn Phe Ser 473
CAN CAN CCC CTT CAN CCA CAG TTT CAN CAG CGT GTT CCG CTT TTA 1138	CGT TTT TAT CCT GAA AAT GAG TAT GTT GCA ACT AAA TAC AGT GAT 2982
Gin Gin <u>Pro</u> Lau Gin <u>Pro</u> Gin Phe Gin Gin Arg Val <u>Pro</u> Lau Lau 159	Arg Phe Tyr <u>Pro</u> Glu Asn Glu Tyr Val Ale Thr Lys Tyr Ser Asp 488
AGA CCT GCA TTT AAT CCA AAC ATG CAA CAA CCAC CCT GGT TTT AAC 1183	CCA CTT TAC AGC GAT ACC AAT CAA AGT TTA ACT AGT GAT CGT TTT 3027
Arg Pro Ala Phe Ass Pro Ass Not Gin Gin Arg Pro Gly Phe Ass 174	<u>Pro</u> Leu Tyr Ser Asp Thr Asn Gln Ser Leu Thr Ser Asp Arg Phe 503
CAN CCA ANC CAN CAG TTC CAN CCA CAC ANT ANT TTC ANC CCA AGG 1228 Gin Pro Asn Gin Gin Fhe Gin Pro His Asn Asn Phe Asn Pro Arg 189	TCT CTT GAT TIT GAC TAC ACA CCA AAA TCT AGA GTC AAC AAT TAC 3072 Ser Leu Asp Phe Asp Tyr Thr \underline{Pro} Lys Ser Arg Val Asn Asn Tyr 518
ATGAAC CCA AAC ATG CAG CGC CCT GGG TTT AAT CCA AAC ATG CAA 1273	ACA CCA TTA AGA TCA ACT AAT TTC CAA AAT AAT GCT ATT TCT AAC 3117
Het Jasn Pro Asn Net Gin Arg Pro Gly Phe Asn Pro Asn Net Gin 204	Thr <u>Pro</u> Leu Arg Ser Thr Asn Phe Gln Asn Asn Ala Ile Ser Asn 533
CAA CGC CCT GGT TTT AAC CAA CCA AAC CAA CAG TTC CAA CCA CAC 1318	TAT CGT TTT AGC AGA ACA CCA AGT AGT TAT TAT CCC TTA ACA AGA 3162
Gln Arg Pro Gly Phe Asn Gln Pro Asn Gln Gln Phe Gln Pro His 219	Tyr Arg Phe Ser Arg Thr <u>Pro</u> Ser Ser Tyr Tyr <u>Pro</u> Leu Thr Arg 548
ANT ANT TTC ANC CCA AGG ATG ANC CCA ANC ATG CAA CCC CCT GGA 1363	AGA CCA TGA*AGA TTA ACT AAT ATC AGT TCA TAC CGT TCA TCA TTC 3207
Asn Asn Phe Asn Pro Arg Met Aan Pro Asn Met Gin Arg Pro Giy 234	Arg <u>Pro</u> Trp Arg Leu Thr Asn Ile Ser Ser Tyr Arg Ser Ser Phe 563
TTT AAC CAA CCC CAT CCT AAC CAA TTT GCA CAA CCA AAT AAC <u>TTT</u> 1408	CAT TCA CCA ACA AGA CTA TCA AGT TTT AGA AGA ACT AGT TTA CCA 3252
Phe AsnjGln <u>Pro</u> His <u>Pro</u> Asn Gln Phe Ale Gln <u>Pro</u> Asn Asn Phe 249	His Ser <u>Pro</u> Thr Arg Leu Ser Ser Phe Arg Arg Thr Ser Leu <u>Pro</u> 578
ANT CCA AAC ATG CAA CGC CCT GGG TTT AAT CCA AAT ATG CAA 1453	TTT AGT TCT AGC TAT GAT GGA CTT AGA CGT TAT CCA TCA AGA TCA 3297
Ann Pro Ann Net Cin Gin Jarg Pro Ciy Phe Ann Pro Ann Net Cin 264	Phe Ser Ser Tyr Asp Gly Leu Arg Arg Tyr <u>Pro</u> Ser Arg Ser 593
CAA COT CCT AAC CCA TCA CAA TTG ATG CCA AAA GGC GGT TTA AAA 1498	TAT TGA-TCT AAG GAC TTT TAA tttgttaaaaagttoaaaataaatotattaa 3349
Gin Arg Pro Asn Pro Ser Gin Lau Met Pro Lys Gly Gly Leu Lys 279	Tyr Trp*Ser Lys Asp Phe END 599
CCC TAA tttagtaattttt 1518	tasaacagc ATG CAA ACA AAG CTG TTT TAT TTT 3385
Pro END 280	ORF4 reading frame2 Het Gin Thr Lys Leu Phe Tyr Phe Phe 9
ORF3 reading frame1 ATG AAC GAT AAA CAG AAA GCT AAA 1542	GTT TTA CTC AGT TTT ATC CCA GGC TTC TTT TTA GTA CAA CAA GAA 3430
RMM3 homolog, P69 Net Am Amp Lym Gin Lym Ala Lym 8	Val Leu leu Ser Fhe Ile Pro Gly Fhe Phe Leu Val Gln Gln Glu 24
ATA ARC ARA GCC TAT ARC ARG CTT TTA ARA RAG ATT ARC ARA AGA 1587	CAG TTT GTT GCA CTG TCA ATT TGG ATC ATT TTG ATA ACG CTT TTT 3475
Ile Asm Lys Ale Tyr Asm Lys Leu Leu Lys Lys Ile Asm Lys Arg 23	Gin Phe Val Ala Leu Ser Ile Trp Ile Ile Leu Ile Thr Leu Phe 39
TAT COT GAT GTT AGT GTT GTT TAT GCC CGT GAT CAT AAA AAT AAG 1632	AGT CTT TGG TAT GAC TGA AAG TTT TGT TTA TTG AAC TTA ACA ATT 3520
Tyr <u>Pro</u> Asp val ser val val tyr ala Arg Asp His Lys Asn Lys 38	Ser Leu Trp Tyr Asp Trp*Lys Fhe Cys Leu Leu Asn Leu Thr Ile 54
GTT CAT GCT CTA TAC CAA GAT CCT GAA TCA GGT AAT ATC TTC TCT 1677	GIT GGT TIT TIT AIT GCT TIT TGT TAT TIT GTT CCA GCA GTC AAG 3565
Val His Ala Leu Tyr Gln Asp <u>Pro</u> Glu Ser Gly Asn Ile Phe Ser 53	Val Gly Phe Phe Ile Ale Phe Cys Tyr Phe Val Pro Ale Val Lys 69
TTA GAN ARA AGA ANG CAG TTA GOT AGT ANC TAT COT TTG FIT GAN 1722	ATA AAT CAA ATT GTT AAA AAT AAC TTT ATT CGT ACC CCT TTC ATT 3610
Leu Glu Lys Ary Lys Gin Leu Ala Ser Asn Tyr <u>Pro</u> Leu Phe Glu 68	Ile Asm Gin Ile Val Lys Asm Asm Phe Ile Arg Thr Pro Phe Ile 84
TTA ACT TCA GAT AAC CCT ATT AGC TTT ACA AAT AAC ATT GTT AGC 1767	AAT TGA ATT GAT CAG ACC ACT AAA GGT GAG TTA AAT CAG TAT TTA 3655
Leu Thr Ser Aep Asn <u>Pro</u> Ile Ser Phe Thr Asn Asn Ile Vel Ser 83	Asn Trp+Ile Asp Gin Thr Thr Lys Gly Giu Leu Asn Gin Tyr Leu 99
TTA AAT GOT TAT GAT GAT AAG AAC AAC TTG GTA ACT GTT CAA TAT 1812	AAA CTA TIT ITA ATT AAT GAA ACC ACT AAA AAC AAT TIG TAC CAA 3700
Leu aan ala tyr asp asp Lys asn asn Leu Val Thr Val Gin Tyr 98	Lys Leu Phe Leu Ile Asn Glu Thr Thr Lys Asn Asn Leu Tyr Gln 114
GAT CAG GAT AAC AAC ACT TIC TAI GAC CAA AAI GGT AAI GII TIG 1857	AAT GCT TTA AAA CTT AAA CAT TGT CCA TTT GTT TGT TAT CAG TGG 3745
Asp Gin Asp Asn Asn Thr Phe Tyr Asp Gin Asn Giy Asn Vel Leu 113	Asn Ala Leu Lys Leu Lys His Cys Pro Phe Val Cys Tyr Gin Trp 129
GAT GTT TCT AGC TAT ACT GAT GAG AAA AAG GTC CCT TTA ATT AAC 1902 Asp Val Ser Ser Tyr Thr Asp Glu Lys Lys Val <u>Pro</u> Leu Ile Asn 128	

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 (*Hind*III) (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 *XbaI* fragment (Fig. 1) that contained the homolog P30 adhesin gene of *M. pneumoniae* was cloned into the *XbaI* site of pUC18 (pMG201). Plasmid pMG201 was physically mapped and was found to contain a 1.4-kb *Hind*III 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 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

42	MELNGFLRYKKLFIVLALLFTTILIVS-LSLLAFALVVKTNGS	P32	Mg
48	MKLPPRRKLLRFLLAWMLVLFSALIVLATLILVQHNNTELTEVKSELS	P30	Mp
	. .**. *** * * ** *		-
92	ELGVVFHQTEDNTTVIQGRSIVEQPWFIPTVAGSFGFSALAIILGLAIGL	P32	Mg
97	PLNVVLH-AEEDTVQIQGKPITEQAWFIPTVAGCFGFSALAIILGLAIGL	P30	Mp
	*.**.* .**. ****.******************		-
142	PIVKRKEKRLLEEKERQEQIAEQLQRISDQQEQQTVEIDPQQSQAQPSQP	P32	Mq
146	PIVKRKEKRLLEEKERQEQLAEQLQRISAQQEEQQA-LEQQAAAEAHAEA	P30	Mp
	******************		-
192	QVQQPLQPQFQQRVPLLRPAFNPNMQQRPGFNQPNQQFQPHNNFNPRMNP	P32	Mg
189	EVEPAPQPVPVPPQPQVQINFGPRTGF-PPQPGMAPRPGMPPHP	P30	Mp
	.* ** **.* *.** .** *. *		-
238	NMQ-RPGFNPNMQQRPGFNQPNQQFQPHNNFNPRMNPNMQ-RPGFNQP	P32	Mq
235	GMAPRPGFPPQPGMAPRPGM-PPHPGMAPRPGFPPQPGMAPRPGM-PP	P30	Mp
	.*. **** .*.******* *. *.*. ****		-
	HPNQFAQPNNFNPNMQQRPGFNPNMQQRPNPSQLMPKGGLKP 280	P32	Mq
	HPGMAPRPG-FPPQPGMAPRPGMQPPRPGMPPQPGFPPKR 274	P30	Mp
	. * .*.** **		-

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.

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).

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%

	<u>M.genitalium</u>		M.pneumoniae		M.genitalium		M.pneumoniae	
	P32 gene		P30 gene		P32 gene		P30 gene	
TTT TTC TTA TTG	Phe Phe Leu Leu	17(6.0) 4(1.4) 10(3.5) 2(0.7)	5(1.8) 6(2.1) 9(3.2) 4(1.4)	TAT TAC TAA TAG	Tyr Tyr 	0 1(0.3) 1(0.3) 0	0 0(0.0) 1(0.3) 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 PC-GENE program. The *y* axis displays hydrophilicity values, and the *x* axis represents the amino acid residue numbers. Vertical dashed lines indicate maximum hydrophilicities.

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* 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.*



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.

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 μ g of enzyme, respectively); lanes 3 and 4, papainase (50 and 5 μ g, respectively); lanes 5 and 6, chymotrypsin (50 and 5 μ g, respectively); lanes 7 and 8, trypsin (50 and 5 μ g, respectively); lane 9, untreated control.

Mg	P69	MNDKQKAKINKAYNKLLKKINKRYPDVSVVYARDHKNKVHALYODPESGN	50
Mp	HMW3	MTDKERAKLAKAYGKLAQKIQKSYPDINVVYGRDAKNKLHALYODPETGN	50
-		*.*******.** .**.*.***.*** *** ***	
Mo	P69	IFSLEKRKQLASNYPLFELTSDNPISFTNNIVSLNAYDDKNNLVTVOYDO	100
Mp	HMW3	IFSLEKRKQLPADYPLFELDSDEPISFAPKIIPLTAFDGNNNEVTVOYDO	100
		***************************************	100
Ma	P69	DNNTFYDONGNVLDVSSYTDEKKVPLINYLSSTOTSOFOP-TOO	143
Mrc	HMW3	VNNTFYDODGNVLDVSGYRDGENIPLVDVLNVGGSTASADTTTSEPLSGE	150
		******* ******* * * ** ** . **	100
Ma	D60	DYPSTDAGLPKTEVDDOPKAAOHTTLETESEDDUEETNDSLNOBODTEN	107
Mn	HMWD	GVPDTDAGL PVVDPDATDF00AD01 FCI DDI D0ADDFV0DTTADDAVD0T	200
np	10103	tt ttttt	200
Ma	DCO		224
Mo	109		2.54
мp	utum 2	PDQATEDQQATDQATDPAATEDQQATDQATDQATDQATDQATAQAT	200
Mg	P69	PSLKPPLVNKPAKLVQPE-VKHIPQVEVQP-KPQIVEPKIEPKPEVKH	280
мp	HMW3	QAHDPNAYYDSQAYSDPDQASAVAPIEVAPLQPEPVAPVVEPTAVPIVES	300
Mg	P69	VSHVEIQPKPEVKPVVDSVPEVKQPEVKHVPHVEVQPKPVVDLK-	324
Мp	HMW 3	APIVEVTPTVEPTPTPVVETAPVVEAPKVVEPTPTPVVEATPAPKVEPKV	350
		. *. *** *** **. * **. * *	
Mg	P69	SKPEVIKHIPQVEVQPKA	351
Мp	HMW 3	VEQPOPTPVTVEVDSPKVEIPKVVTAKVALQVAQPTPVPAVPKVAPQPTP	400
		* .* ****.*.	
Mg	P69	QMVEPRIEPKPETKYIPQVESTPQVEVHHWKPEVKTEYQ	390
Мp	HMW 3	APVVVQPTAVVQPVVKAEPKVVTPTPAPQVVVTPQVATPKVTPKVVQTTP	450
		*.** *.*.* . *** *****.*	
Mg	P69	PQQPLPTSGLQIKVVP-RSAALLQSKLDTGFQPRQVERTTDSD	432
Mp	HMW 3	AVPPVVVQPEVVVQPIIRPTQPEPEWKPSPASVVEPQPCQSACVNNESGA	500
		*. `** . ***	
Mg	P69	TTVSVSSHASLLEKINALNHORIMSDIALKSDNTIKSSNFSRFYPENE	480
Mp	HMW3	ITTHTTNRSLLLEKLASIGHLHDASTRTPLPHERYQLAPPSE	542
		** *****.* **.**. * .*	
Mg	P69	VUATKYSDPLYSDTNOSLTSDRESL-DEDYTP-KSRVNNYTPLRSTNFON	528
Mp	HMW3	VUATEVNEDI ENI DA TENSWARETRETRETRESTETASRETGVTPM-AVNYRN	591
-		****** ** ** ** ** ** **	
Ma	P69	NATENY PEC PECSYVEL TERPWELTNISSYRSSFHSPTRLSSF	572
Mp	HMW3	DAGINEDICINGECANDEDESEVDI-BEDIELSSI.RENRSSFENTHRED-L	639
Mg	P69	RRTSLPFSSSYDGL-RRYPSRSYWSKDF 599	
Mp	HMW3	GSNYTSFTPRYRSPLRGGLSQRFPLRSSWSKEF 672	
		** .** .*.* ** ***.*	

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 XbaI 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.ge	enitalium	M.pneumon	iae		M.genitalium	M.pneumoniae
	HMW3	3 homolog	HMW3 gen	e		HMW3 homolog	HMW3 gene
ጥጥጥ	Phe	13/2.11	15(2 2)	ጥልጥ	TVr	19(3.1)	17(2 5)
ጥጥሮ	Phe	5/0 8)	5(0.7)	TAC	TUT	7/1 1	17/2 5
TTA	Ten	22/3.6)	7(1.0)	TAA	111	1/0 1)	1(0,1)
TTG	Len	5(0.8)	11/1 6	TAG		1(0.1)	1(0.1)
113	Deu	5(0.8)	11(1.0)	ING		0	U
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)
2.000	т1.	1612 61	11/1 6	***		20/2 21	30/3 43
ATT	110	10(2.0)	11(1.0)	AAI	ASI	20(3.3)	10(1.4)
ATC ATA	TIC	4(0.0)	4(0.5)	AAC	ASI	10(0.3)	20(2.9)
ATA	TTE	2(0.5)	4(0.5)	AAA	Lys	10(1 ()	12(1.7)
AIG	met	3(0.5)	2(0.2)	AAG	гуз	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)
	• • • •		440 5				
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	ō	2(0.2)
		-,,				•	2(012)
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)
CCT	۸la	11/1 8)	28(4.1)	COT	61v	6(1.0)	12(1 7)
acc	Ala	2(0.3)	7/2 0	GGC	610	0,1.0)	4(0.5)
CCN	Ala	5(0.8)	20(2.9)	CCA	civ	2(0.3)	1(0.5)
aca	Ala	0,0.07	8(1 1)	GGG	GIV	0	3(0,4)
929	n1a	v	0(111)	0.50	OL Y	v	5(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 prolinerich 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 Tn4001, 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 $(-dG^{\circ})$ 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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