Analysis Identifying Common and Distinct Sequences among Texas Clinical Strains of *Mycoplasma genitalium*[▽]

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Mycoplasma genitalium is a human bacterial pathogen linked to urethritis and other sexually transmitted diseases. Here, we assessed the incidence of *M. genitalium* infection in patients attending a sexually transmitted disease clinic in San Antonio, TX, by use of diagnostic real-time PCR. Overall, 16.8% of women and 15.1% of men were found *M. genitalium* positive. Regions of the *mgpB* gene, which encodes the MgPa adhesin, were amplified from positive clinical specimens and evaluated for sequence variability, which demonstrated transmission of the pathogen between sexual partners. Follow-up analysis of a subset of patient specimens revealed reinfection by a different strain of *M. genitalium*, indicating the absence of protective immunity. Eighteen DNA sequence variants were obtained and compared with all other available clinical sequences. Detailed analysis revealed silent mutations of six amino acid residues within the encoded region of the MgPa adhesin in numerous clinical strains. In addition, missense mutations of limited numbers of amino acids were observed. Alignment of putative amino acid sequences revealed the simultaneous occurrence of several mutations and the existence of identical or similar protein variants in strains from different locations.

Mycoplasma genitalium is associated with numerous human genitourinary tract maladies, including nonchlamydial, nongonococcal urethritis in men and cervicitis, endometritis, and pelvic inflammatory disease in women (1, 4, 5, 12, 19, 29, 30, 35, 39). Understanding the epidemiological aspects of infections and developing effective treatment require reliable typing of pathogenic microorganisms. Since *M. genitalium* is very difficult to culture from clinical specimens (13, 21), classical microbiological typing methods are not readily applicable. Furthermore, serological approaches are not widely used because of cross-reactivity with other mycoplasmas, especially with *Mycoplasma pneumoniae* (15, 26). Therefore, typing of *M. genitalium* strains relies on DNA sequence data. Recently, sequence variability of the rRNA operon and tandem repeats in the MG309 locus have been evaluated with clinical specimens (28). However, the majority of *M. genitalium* clinical sequence data available today is based upon the gene *mgpB* (locus MG191 of sequenced reference strain G37 [11]) encoding the *M. genitalium* adhesin MgPa (14, 17, 21). An *M. genitalium*-specific diagnostic PCR assay was designed to target the proximal unique region of this gene (using primers MgPa-1 and MgPa-3 [22] and designated here "MgPa-13"). The MgPa-13 region has exhibited sequence stability in persistently infected patients for up to 21 months (17). Despite this intrastrain stability, high levels of sequence variability between clinical isolates were observed (14, 17). Based on this sequence region, transmission of *M. genitalium* between sexual partners was shown, as was colonization of different anatomical sites of the same patient by identical strains (14).

Here, we present analysis of MgPa-13 sequences generated from clinical specimens collected at the Project S.A.F.E. sexually transmitted disease (STD) clinic in San Antonio, TX (36), from recruited female participants and their male partners. Alignment of MgPa-13 sequences corroborated transmission between partners and colonization of different anatomical sites by the same strain. Comparison of newly recovered sequences with all currently available clinical data (14, 17, 21) revealed not only the presence of several common variants but also distinct sequences. Analyses of encoded amino acids uncovered mutations that determine common features among MgPa-13 region variants.

MATERIALS AND METHODS

Origin and processing of clinical specimens. A group of minority women (Mexican and African American) and their partners were recruited from public health clinics throughout San Antonio, TX, into a controlled randomized trial of behavioral-cognitive interventions to reduce the recurrence of STDs (36). Women were recruited into the study after being diagnosed with a nonviral STD, specifically infection with *Chlamydia trachomatis*, *Neisseria gonorrheae*, *Trichomonas vaginalis*, or *Treponema pallidum*. At each clinic visit, urine specimens were obtained from both female and male subjects, and at the same time, vaginal and cervical swab (VC) specimens were collected from female participants. All urine and VC samples were transported to the University of Texas Health Science Center at San Antonio (UTHSCSA) and were processed immediately using centrifugation and resuspension of pellets in water prior to boiling (2). Specimens examined here were obtained from 500 patient visits during a 13-month period (September 2005 to October 2006).

Detection of *M. genitalium*. Detection of *M. genitalium* was performed by quantitative duplex real-time PCR assay with inhibition control (20). Serial dilutions representing from 1 to 10^8 genome copies of *M. genitalium* reference strain G37 (14th passage) were used to determine the efficiency and detection limit of the assay. We consistently detected five or more genomes per reaction and one genome per reaction with 60% probability. Five dilutions ranging from 1 to 10^7 genomes were included as standard concentrations on each test plate for quantification. Duplex TaqMan assays were performed on 5-μl aliquots of DNA preparation in 50-μl reaction mixtures with universal master mix, as suggested by the manufacturer (Applied Biosystems). Individual aliquots represented 400 μl of urine specimens or ~1% of collected VC specimens. Assays were performed using the ABI Prism 7900HT sequence detection system.

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M2341|2|
TX195
H12202o1|37|
MPN141
111-0|51|
TX443
H13051f1|52|
TX229
TX414
TX260
TX277
DK15776|31|
TX167
M2321|11|
M6271 E17-aus|7|
DK11844|49|
168-0|44|
H12755v1|42|
H-p7791B11|3|
123-0|43|
399F rus|45|
RYH181|46|
18-0R|47|
10089
10177
LF-p7523 O11|13|
Taqman3672|48|
H8692|55|
N4219|38|
LF-p6486 L11|4|
RYH115|41|
TX140
H97050g2|54|
129-0|39|
RYH23|40|
M6286 R67|14|
TX124
LF-p7551 S11|50|
TX84
10114
DK pr9|6|
H346j1|30|
2094 Ume?21|
TX27
H9504k1|12|
19-0|34|
TX468
76-0|18|
10163
LF-p6257 J11|5|
M6285 R65|9|
RYH214|17|
LF-p6601 M11|16|
314F rus|22|
LF-p7262 P11|19|
1-0|29|
66-0|8|
5336 D|24|
10081
RYH70|35|
TX403
DK523|15|
M2300|20|
35M rus|27|
RYH117|26|
MgPa|1|
1182 Ume?36|
M6328 R66|33|
DK16221|9|
M6270 D24-aus|28|
M2288|32|
H-p9300A191|53|
H7755g1|10|
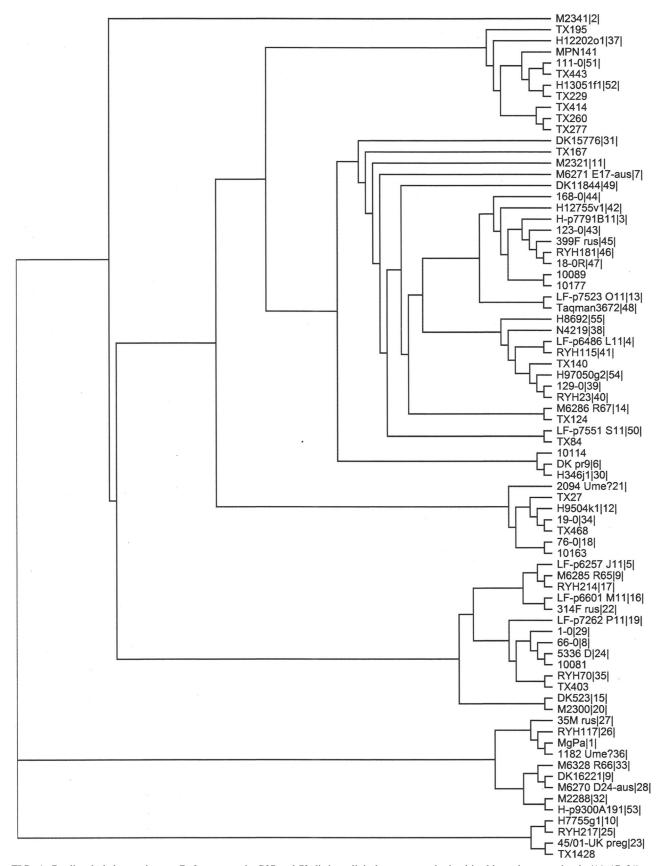RYH217|25|
45/01-UK preg|23|
TX1428

FIG. 1. Predicted phylogenetic tree. Reference strain G37 and 73 distinct clinical sequences obtained in this study or previously (14, 17, 21) were aligned by CLUSTALW. The sequence of the corresponding region of the *M. pneumoniae* adhesin P1 gene (MPN141) was included as an outlier.
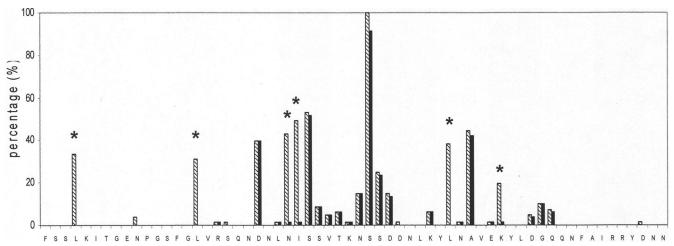
FIG. 2. Comparison of MgPa-13 nucleotide and deduced amino acid sequences. The region of F^{75} (extreme left) to N^{136} (extreme right) of the MgPa protein in reference strain G37 is presented. Percentages of mutated codons (striped bars) and of detected missense mutations (black bars) are shown. Percentages represent the fraction of the 73 aligned clinical sequences that contained a codon/amino acid different from that of the G37 sequence. Recurrent silent mutations of six amino acid residues are indicated (asterisks).

PCR amplifications of MgPa-13 regions. With *M. genitalium*-positive speci-mens, amplification of MgPa-13 regions was performed using primers MgPa-1 (5′-AGTTGATGAAACCTTAACCCCTTGG-3′) and MgPa-3 (5′-CCGTTGA GGGGTTTTCCATTTTTGC-3′) (22) and Platinum *Taq* DNA high-fidelity polymerase system, as suggested by the manufacturer (Invitrogen). Chromo-somal DNA isolated from reference strain G37 (100 ng) was used as a positive control alongside a control for contamination of reagents. All amplified products were visualized on 2% agarose and purified (QIAquick PCR purification kit; Qiagen) prior to sequencing.

Sequence analysis. Sequencing of both strands from *M. genitalium*-positive specimens was accomplished by the Department of Microbiology and Immunol-ogy Nucleic Acids Facility, UTHSCSA. DNA and amino acid sequence align-ments and phylogenetic tree predictions were performed by CLUSTALW (Fig. 1). The sequence of the corresponding region of the *M. pneumoniae* adhesin P1 gene (MPN141) was included as an outlier. Positions of amino acid residues (Fig. 2 and 3) correspond to the protein sequence in G37.

Sequence designation. Sequences are identified by numbers assigned to the specimen upon arrival to the laboratory. If identical sequences were generated from both partners, only the sequence from the woman is presented. If identical sequences were obtained from unrelated participants (i.e., different participants with no known sexual relationship), only the sequence with the earliest collection date is presented.

Nomenclature. The major *M. genitalium* adhesin MgPa (16) (also known as protein P140 [33]) is encoded by locus MG191 of the sequenced reference strain G37 (gene *mgpB*, nucleotides 221570 to 225904). In this paper we refer to the analyzed region as MgPa-13, since it is amplified using primers MgPa-1 and MgPa-3 (22).

Nucleotide sequence accession numbers. Sequences of MgPa-13 regions ob-tained for clinical specimens collected in the San Antonio, TX, area have been submitted to the GenBank database and assigned accession numbers FJ750827 to FJ750844.

RESULTS

Incidence of *M. genitalium* in patient population. Among specimens collected from 268 females, 11.5% of urine speci-mens and 10.9% of VC specimens were positive for *M. geni-talium*. Overall, 16.8% women were *M. genitalium* positive (based upon at least one of the analyzed specimens being positive) and of those, 15 were diagnosed positive by both urine and VC. Of 232 male participants, 15.1% were *M. geni-talium* positive. Using a quantitative real-time PCR assay, the number of *M. genitalium* genomes per reaction in positive specimens ranged from 1 to ~10,000.

All *M. genitalium*-positive specimens were used to amplify MgPa-13 regions. Limited volumes of specimens and low ge-nome numbers of *M. genitalium* in many positive samples (only 30% of specimens contained 10 or more *M. genitalium* ge-nomes per reaction) resulted in the generation of 31 MgPa-13 amplicons. Only one sequence variant was obtained from each specimen. Sequences generated from related specimens (i.e., same patients or sexual partners) were excluded from further examination, and DNA sequence analyses revealed 18 differ-ent sequences. The highest *M. genitalium* load was found in specimen TX135 during the patient's initial visit. On her sec-ond visit (after 6 months), she was *M. genitalium* negative. On her subsequent two visits (after 18 and 20 months), she was again *M. genitalium* positive and the generated MgPa-13 se-quences were identical (TX1428). However, alignment with TX135 showed that she was reinfected by a different strain of *M. genitalium* (differences in seven nucleotides).

Sequences generated from different anatomical sites of fe-male participants were identical. As mentioned earlier, both urine and VC specimens collected from 15 female patients were *M. genitalium* positive, and we examined MgPa-13 regions from both specimens of 4 women. Comparison of these se-quences showed that each woman was infected by a different *M. genitalium* strain. Additionally, identical sequences were obtained from both anatomical sites of the patient. This dem-onstrated colonization of each patient by a single *M. genitalium* strain, as previously observed (14).

Partners were infected by the same *M. genitalium* strain. The majority of the analyzed specimens were collected from sexual partners (172 dyads). We detected *M. genitalium* in both part-ners in 19 couples (11% of participating dyads). All positive dyad specimens were further used for MgPa-13 region ampli-fication, and we generated paired products for four dyads. Analysis of these MgPa-13 regions revealed that sequences were identical between partners, which corroborated sexual transmission of *M. genitalium* among partners (14).

Both common and unique MgPa-13 variants identified among Texas strains. Comparison of DNA sequences gener-
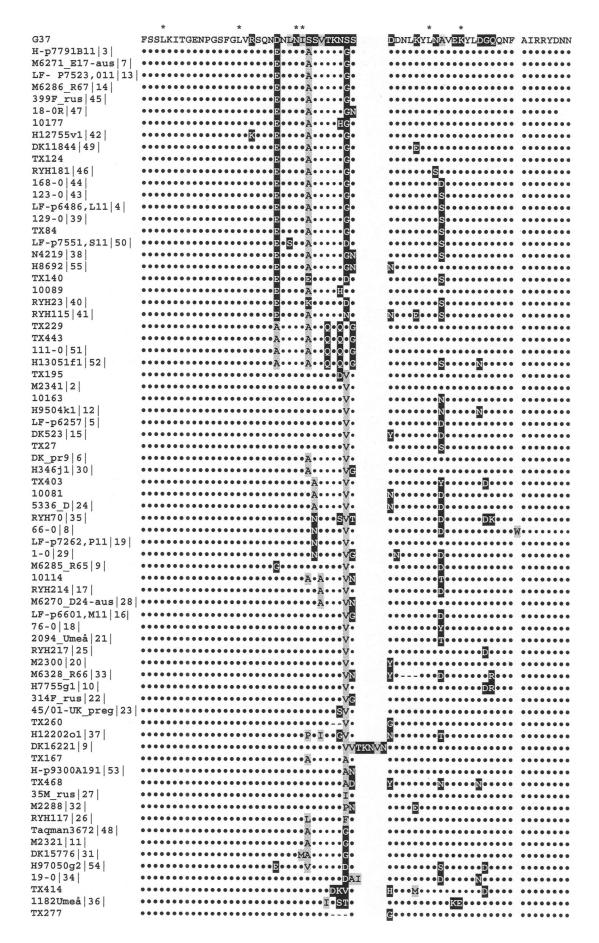
G37   FSSLKITGENPGSFGLVRSQNDNLNISSVTKNSS DDNLKYLNAVEKYLDGQQNF AIRRYDNN

H-p7791B11|3|
M6271_E17-aus|7|
LF- P7523,011|13|
M6286_R67|14|
399F_rus|45|
18-0R|47|
10177
H12755v1|42|
DK11844|49|
TX124
RYH181|46|
168-0|44|
123-0|43|
LF-p6486,L11|4|
129-0|39|
TX84
LF-p7551,S11|50|
N4219|38|
H8692|55|
TX140
10089
RYH23|40|
RYH115|41|
TX229
TX443
111-0|51|
H13051f1|52|
TX195
M2341|2|
10163
H9504k1|12|
LF-p6257|5|
DK523|15|
TX27
DK_pr9|6|
H346j1|30|
TX403
10081
5336_D|24|
RYH70|35|
66-0|8|
LF-p7262,P11|19|
1-0|29|
M6285_R65|9|
10114
RYH214|17|
M6270_D24-aus|28|
LF-p6601,M11|16|
76-0|18|
2094_Umeå|21|
RYH217|25|
M2300|20|
M6328_R66|33|
H7755g1|10|
314F_rus|22|
45/01-UK_preg|23|
TX260
H12202o1|37|
DK16221|9|
TX167
H-p9300A191|53|
TX468
35M_rus|27|
M2288|32|
RYH117|26|
Taqman3672|48|
M2321|11|
DK15776|31|
H97050g2|54|
19-0|34|
TX414
1182Umeå|36|
TX277

1472

TABLE 1. Typical silent mutations observed with MgPa-13 region
of *M. genitalium* clinical strains[a]

Amino acid	Codon change[b]	No. of strains with mutated codon
L^{78}	CTA (1.26) → CTG (0.43)	26
L^{90}	TTA (5.03) → CTA (1.26)	23
L^{115}	CTC (0.49) → CTT (1.99)	28
N^{99}	AAT (4.61) → AAC (2.89)	30
I^{100}	ATT (5.18) → ATC (1.78)	34
K^{120}	AAA (7.03) → AAG (2.43)	15

[a] Seventy-three distinct clinical sequence variants were obtained previously (14, 17, 21) and in this study.
[b] Percentage of codon usage (abundance) within *M. genitalium* reference strain G37 genome is provided in parentheses.

ated from this study with all currently available clinical sequences (14, 17, 21) revealed identical sequences. For example, sequences indistinguishable from TX135, TX302, and TX1428 were found among previously published sequences (14, 17). However, 13 DNA sequence variants specific to the San Antonio area were identified.

For phylogenetic tree prediction, 73 distinct clinical sequences of the MgPa-13 region were compared with reference strain G37. Identified common sequences were represented by their earliest published types, and consequently, the San Antonio area sequences and the Kenya collection (17) were represented only by their unique sequences. Results suggested that site-specific *M. genitalium* strains were endemic in different areas and are presented in Fig. 1.

Alignment of putative amino acid sequences of the MgPa-13 region reveals high occurrence of silent mutations. When putative amino acid sequences encoded by all unique clinical variants were examined, strains had uninterrupted reading frames (amino acids 61 to 153 of G37-MgPa). Upon comparison of the deduced amino acid sequences in clinical strains with the G37-MgPa sequence, a high occurrence of silent mutations of six codons was observed (Fig. 2 and Table 1). They represented 38% of all detected codon mutations. It also appears that several of the silent mutations were found together in the same sequence variant. For example, among 26 strains with the L^{78} silent mutation, 23 strains also carry the I^{100} silent mutation (out of 34); 24 strains carry the L^{115} silent mutation (out of 28); 21 strains carry the L^{90} silent mutation (out of 23); 21 strains carry the N^{99} silent mutation (out of 30); and 12 strains carry the K^{120} silent mutation (out of 15). Despite the DNA sequence variability, amino acid residues were identical.

Missense mutations lead to distinct sequence variants in MgPa. The majority of missense mutations were limited to a restricted number of amino acids (Fig. 2 and 3) with noticeably favored codon mutations. For example, 23 D^{96} mutations (out of 28 detected) were due to replacement of GAC by GAG (encoding E) and four were GAC-to-GCG (encoding A) mu-

tations. Interestingly, simultaneous occurrence of several missense mutations was also detected with strains of different geographic origin, resulting in identical variants of the protein (Fig. 3). For example, simultaneous substitutions of D^{96} by E, S^{101} by A, and S^{107} by D/G were found in 20 strains. Another recognized variant contained simultaneous D^{96}-to-A, S^{101}-to-A, T^{104}-to-Q, N^{106}-to-Q, and S^{108}-to-G mutations.

DISCUSSION

The *M. genitalium mgpB* gene consists of regions that share homologies with nine repetitive sequences (MgPar) spread throughout the chromosome (7, 17). Extensive sequence variability resulting from recombination between repetitive elements of *mgpB* and MgPars was recently confirmed with reference strain G37 as well as with clinical strains (17, 18, 27). However, the MgPa-13 region (nucleotides 181 to 460) does not share homology with other regions in the G37 genome, and this region was chosen for development of a conventional diagnostic PCR assay (22). Later, longitudinal sequence stability within a strain was demonstrated (14, 17), and the use of this region for typing purposes was proposed. Analysis of a growing number of clinical data uncovered significant interstrain sequence variability. Sequences of the MgPa-13 region represent the largest set of data generated from different clinical strains of *M. genitalium* (13, 14, 17).

During a 13-month period (2005 to 2006), we evaluated *M. genitalium* incidence with patients recruited under Project S.A.F.E. in San Antonio, TX (25, 36). Specimens (urine and VC swab) were collected during 500 visits and assessed using a diagnostic *M. genitalium*-specific real-time PCR assay (20). Overall, 16.8% of women and 15.1% of men were found *M. genitalium* positive. These numbers are higher than results of similar studies of STD clinic attendees (1, 3, 10, 29, 38), which could be explained by the facts that our specimen collection was conducted over a rather short time period and the patient population consisted of women (and their partners) who were recruited after being diagnosed with nonviral STDs (36).

The MgPa-13 region was further amplified from *M. genitalium*-positive specimens, and sequence analysis revealed a high degree of variability among local strains. From the originally amplified 31 sequences, we eliminated related (i.e., originating from the same patient or sexual partners) sequences and identified 18 different MgPa-13 region variants through comparative sequence analysis. Identical strains were detected at different anatomical sites of four female patients, which demonstrated colonization with the same strain. Further, identical strains were observed among partners (four dyads), corroborating previously observed transmission of *M. genitalium* between sexual partners (14, 17, 27).

We compared San Antonio area-originating MgPa-13 nucleotide sequences with all available clinical sequences collected

FIG. 3. Alignment of deduced amino acid sequence from F^{75} to N^{136} of reference strain G37-MgPa with sequences of clinical strains. Amino acids identical to those of G37 are presented as dots (●); missense mutations are highlighted with gray (hydrophobic amino acid) or black (hydrophilic amino acid). Amino acid deletions compared with those of G37-MgPa are indicated (−). Positions of the majority of silent mutations are indicated (asterisks). Sequences were grouped to demonstrate existence of observed protein variants. Seventy-three distinct clinical sequence variants were obtained previously (14, 17, 21) and in this study.

from different locations. Fifty-six different MgPa-13 variants were recently recognized among 267 sequences found in positive specimens from nine countries (14). Eight different strains were identified among female commercial sex workers in Nairobi, Kenya (17). Alignment of DNA sequences revealed identical MgPa-13 variants in different groups of patients, implying the existence of several common sequence types worldwide. Alongside these common sequence variants, location-specific sequences were represented.

Although MgPa-13 regions demonstrated extensive DNA sequence variability, amino acid substitutions seemed to involve only a limited number of amino acids (Fig. 2 and 3). In addition, numerous silent mutations were observed (38% of observed codon mutations). For example, comparison of deduced amino acid sequences indicated that all detected leucine codon changes (L^{78}, L^{90}, and L^{115}) were identical and silent. Furthermore, conservation of N^{99}, I^{100}, and K^{120} in their positions was observed (i.e., in each case, except for a single missense mutation, all substitutions were identical and silent) (Fig. 2 and 3). Additionally, it appears that several of the silent mutations were found together in the same sequence variant.

As mentioned above, missense mutations involved a limited number of amino acids (Fig. 2 and 3), and simultaneous occurrence of several missense mutations was also detected in strains of different geographic origin. As a result, several distinct sequence variants of adhesin MgPa were identified (Fig. 3).

In the majority of synonymous mutations, except for L^{115}, codons (as found in G37) were replaced by less abundant codons (Table 1). Thus, even though the amino acid sequence of MgPa remains unchanged, codon mutations could affect expression of the MgPa protein. Effects of silent mutations on protein folding have been reported (6, 24). The function of MgPa as a major adhesin and its antigenicity have been demonstrated, but the exact positioning of the protein in the mycoplasma membrane, as well as the role of individual domains or regions, has not been detailed. The impact of detected mutations on structural or functional properties of MgPa necessitates additional investigation.

Interestingly, the *mgpB* gene of *M. genitalium* and the adhesin P1 coding gene MPN141 of *M. pneumoniae* share much in common. Both genes contain repetitive sequences with multiple copies spread throughout the chromosome, and both genes contain a unique region within the proximal ~500 nucleotides (7, 31, 32). Still, no sequence variability within this region has been observed with *M. pneumoniae* (37). In contrast to the recent findings of extensive sequence variability in *M. genitalium* (17, 18, 27, 34), the existence of two highly conserved types of *M. pneumoniae* P1 gene sequences has been demonstrated repeatedly (9). The existence of these two types of the P1 gene (type 1 and type 2) was shown to be an excellent marker for numerous other variations within their genomes, some of which likely lead to virulence differences observed among strains (8). It is possible that well-documented shifts of clinical *M. pneumoniae* isolates in different locations over time could be a result of type-associated differences (23). One hypothesis speculates that type-specific antibodies render protection against the causative type strain, leading to replacement by the other strain type. It is unclear whether any correlation exists between the particularly expressed MgPa region and *M. genitalium* virulence. As increased numbers of sequence vari-

ants arise, possible connections between MgPa-13 sequence variants and clinical symptoms, host immunoresponsiveness, and/or persistence of infection need to be evaluated. Accumulation and analysis of additional MgPa-13 region sequence data should further enhance studies of *M. genitalium* infections, evaluation of treatment effectiveness, and understanding of its epidemiology, transmission, pathogenicity, and phylogeny.

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