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SUPPLEMENT ARTICLE

The Unique Microbiology and Molecular Pathogenesis of *Mycoplasma genitalium*

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Mycoplasma genitalium is increasingly appreciated as a common cause of sexually transmitted disease syndromes, including urethritis in men and cervicitis, endometritis, pelvic inflammatory disease, and possibly preterm birth, tubal factor infertility, and ectopic pregnancy in women. Despite these disease associations, which parallel those of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, the mechanisms by which this pathogen elicits inflammation, causes cellular damage, and persists in its only natural host (humans) are unique and are not fully understood. The purpose of this review is to briefly provide a historical background on the discovery, microbiology, and recognition of *M. genitalium* as a pathogen, and then summarize the recent advances in our understanding of the molecular biology and pathogenesis of this unique urogenital organism. Collectively, the basic scientific discussions herein should provide a framework for understanding the clinical and epidemiological outcomes described in the accompanying articles in this supplemental issue.

Keywords. mycoplasma; genitalium; immunity; recombination; regulation.

M. genitalium (MG) is a fastidious and slow growing STD pathogen. This species was first cultured from the urethral exudates of 2 of 13 men with nongonococcal urethritis in 1981 [1], using a broth medium (SP-4) developed for other mycoplasmas. Growth was detected after 50 days of incubation, indicated by a color change in the medium due to the fermentation of glucose. After subsequent subcultures in broth and on solid media, two strains, G37 and M30, were single-colony cloned on soft agar (0.4%) and further characterized. The organism's small size (approximately 0.6 × 0.3 μm), ability to pass through 0.3-μm filters, absence of a cell wall, resistance to penicillin, fried-egg appearance of colonies on soft agar plates, and inability to revert to cell wall–containing bacteria, collectively confirmed their identification as mycoplasmas.

Anti-G37 serum cross-reacted with M30, yet both strains failed to react with antisera against other *Mycoplasma* species, confirming the relatedness between G37 and M30 and their divergence from other, previously described mycoplasmas. These newly described strains were further distinguished from other *Mycoplasma* species by their inability to hydrolyze urea and arginine and their ability to be inhibited by thallium acetate, which is used for selective growth of other mycoplasmas, including the closely related nongenital species, *Mycoplasma pneumoniae*. These distinguishing characteristics, as well as their unique flask shape (Figure 1), led to the official recognition of a novel mycoplasma species, *M. genitalium*, with G37 designated as the type strain [4]. This strain has since been maintained at the American Type Culture Collection and in several laboratories worldwide.

The detection of *M. genitalium* in a subset of men with nongonococcal urethritis in 1981 was intriguing but not sufficient to determine whether *M. genitalium* was associated with, and possibly the cause of, urethritis. Unfortunately the fastidious nature of *M. genitalium* was an impediment to determining the association of the organism with human disease. The characterization of *mgpB* [5], the first *M. genitalium* gene to be sequenced, lead to the identification of *M. genitalium*–specific DNA targets and the development of 2 polymerase chain reaction tests described in 1991 [6, 7]. Shortly thereafter, epidemiological studies confirmed that *M. genitalium* was associated with urethritis [8, 9]. As they say, the rest is history, with the development of several additional laboratory-developed and commercial nucleic acid amplification tests that have since confirmed the role of *M. genitalium* in several reproductive tract disease syndromes in both men and women.

After the isolation of G37 and M30 in 1981, no additional *M. genitalium* strains could be isolated from the genital tract until the 1990s, when Jensen et al [10] discovered that coculture of genital tract specimens with Vero cells (and detection of growth by quantitative polymerase chain reaction) enhanced *M. genitalium* growth in vitro and enabled the recovery of new isolates from male urethral swab and urine specimens. As a result of this technique, collections of geographically distinct *M. genitalium* strains have accumulated in several laboratories, including those of Jensen et al [11] and P. A. T. (unpublished data), which have greatly facilitated the evaluation of genomic

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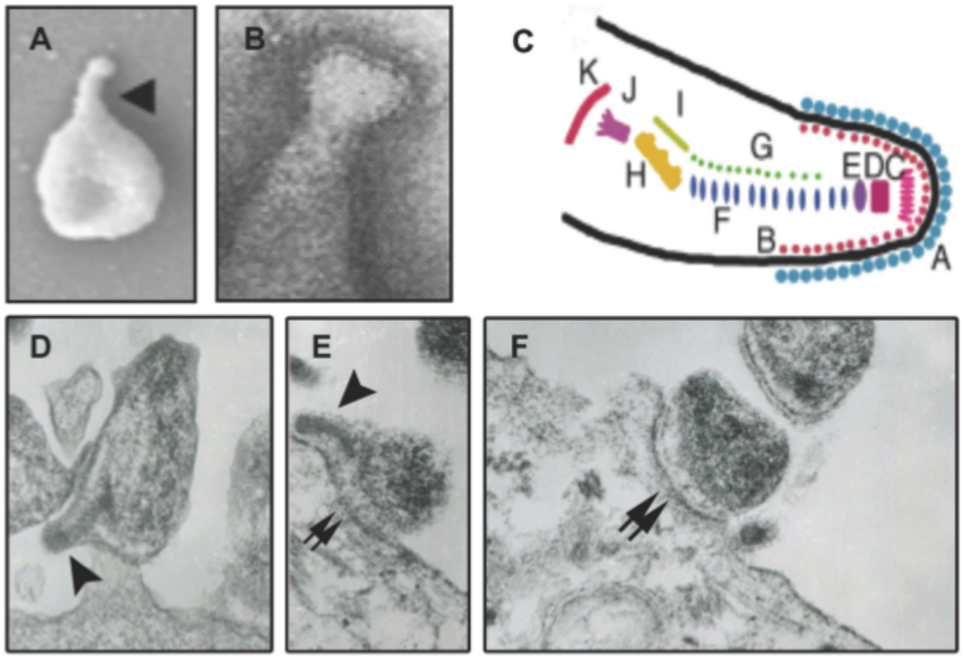

Figure 1. *Mycoplasma genitalium* structure and interactions with human reproductive tract epithelial cells. *A, B,* Curved terminal organelle (*arrowhead in A*) is a dominant feature of the cell body of *M. genitalium* in scanning electron micrographs [2] (*A*) and is covered with a "nap" containing the attachment proteins MgpB and MgpC, as observed by transmission electron microscopy [1] (*B*). Owing to substantial genetic, functional, and morphological homology, the complex structure of the terminal organelle is inferred to be similar to that of *Mycoplasma pneumoniae*. (*C*) *M. pneumoniae* terminal organelle structure, as seen with electron cryotomography [3]; letters indicate different component protein complexes identified in *M. pneumoniae,* which are presumed to correlate with homologous proteins in *M. genitalium*. (D–F) Interaction of *M. genitalium* with cultured human ectocervical epithelial cells. The terminal organelle of *M. genitalium* with its electron dense core is clearly visible in (*D* and *E*), (*arrowheads*) and is involved in attachment to host epithelial cells. The intimate interaction between *M. genitalium* and epithelial cells surface and *M. genitalium* is not limited to the terminal organelle and often involves adherence throughout the cell body (*double arrows in E and F*). Images in *A–C* reproduced with permission from the Microbiology Society [2] (*A*), Elsevier [1] (*B*), and Wiley and Sons [3] (*C*).

diversity, strain differences, and antibiotic susceptibility profiles of this species [12, 13].

Several typing methods have been developed to differentiate *M. genitalium* strains, the most widely used being the detection of single-nucleotide polymorphisms in the semiconserved 5′ region of the *mgpB* gene [14]. This typing system has been used to document the sexual transmission of *M. genitalium* [15], the persistence of strains among longitudinally infected individuals [16–18], and the selection of azithromycin-resistant strains in individuals treated with this antibiotic [12, 13]. These studies have highlighted the genetic heterogeneity of the organism, although many strains maintained at American Type Culture Collection are indistinguishable from G37 by several strain typing systems [14, 19, 20]. Because the laboratory-adapted G37 strain can easily outgrow other less-adapted isolates of *M. genitalium*, the isolation of clinical strains with strain types identical to G37 should be interpreted with caution.

Perhaps the most intriguing feature of *M. genitalium* is its predominant slightly curved terminal organelle (Figure 1), a feature shared with, but morphologically distinct from, other pathogenic *Mycoplasma* species [2]. Required for adhesion, motility, and involved in cell division, the terminal organelle is composed of a complex array of proteins that have homologues in *M. pneumoniae*, suggesting similar functions. This structure is remarkably complex, containing surface-exposed proteins visible in electron micrographs as a "nap" at the tip of the organelle [1], a terminal button and 2 parallel rods making up the electron-dense core, and an electron-dense bowl complex proximal to the cell body (Figure 1c.). Adding to the complexity of this terminal organelle is the basal structure thought to be the motor providing energy for motility. The surface-exposed proteins at the tip of the organelle (MgpB and MgpC) mediate attachment to eukaryotic cells, are required for motility, and undergo phase and antigenic variation (see below). The exact mechanism of motility mediated by the terminal organelle is unclear, but has been attributed to the catch, binding, and release activity of the MgpB/MgpC complex in a centipede-like motion [21].

A MINIMAL GENOME

With approximately 580 kb of DNA (the smallest in any organism capable of axenic growth), *M. genitalium* type strain G37 was the second bacterial genome to be fully sequenced. Initially sequenced by the J. Craig Venter Institute in 1995 using shotgun Sanger sequencing [22], this strain was resequenced in 2005 by 454 Life Sciences [23] to demonstrate their novel pyrosequencing technology. These efforts, combined with the 2012

sequencing and annotation of 4 additional strains from Japan, Denmark, and Australia [24], have provided essential data for understanding the genomic structure, energy metabolism, and evolution of *M. genitalium*. For example, the circular, double-stranded DNA genome of *M. genitalium* contains the full genetic complement of the organism, because no plasmids or extrachromosomal DNA have yet been discovered.

Similar to other mycoplasmas and their reduced genomes, the average guanine-cytosine content of the *M. genitalium* genome is low, at approximately 31%. Of the approximately 517 protein-coding open reading frames, roughly 21% are classified as hypothetical genes and lack significant similarity to other bacterial proteins in the National Center for Biotechnology Information database [22]. Similar to other mycoplasmas, *M. genitalium* uses a modified genetic code in which UGA encodes a tryptophan rather than the common translational stop [25]. Through evolutionary reductions in genome size, *M. genitalium* has marked metabolic restrictions as the genome lacks almost all enzymes required for biosynthesis of amino acids, de novo nucleic acid synthesis, and fatty acid biosynthesis. Thus, like many mycoplasmas, *M. genitalium* relies on several metabolic growth factors obtained from the host and exemplifies the highly evolved host cell-pathogen interactions that facilitate persistence in urogenital tissues.

Although complete phylogenetic analyses have yet to be reported, genomic structure seems to be similar among *M. genitalium* strains worldwide [24]. Despite substantial gaps in our understanding of *M. genitalium* pathogenesis, several inferences can be made from its genomic composition. Genomic heterogeneity is primarily contained in hypervariable regions of adjacent genes encoding MgpB and MgpC and in 9 partial, noncoding genomic loci termed MgPar sites. Remarkably, 4.7% of the *M. genitalium* genome is allocated to the *mgpB*/*mgpC* operon and its homologous MgPar sites, highlighting the importance of this system for survival in reproductive tract tissues (see below). Of evolutionary interest, each open reading frame discovered in *M. genitalium* is also encoded in the genome of the human respiratory pathogen *M. pneumoniae* [26]. Despite the genomic similarities, functional studies of putative virulence factors and their interaction with the host are needed to understand the establishment, persistence, and pathogenesis of *M. genitalium* infections in reproductive tract tissues.

HOST IMMUNITY TO INFECTION

In men, urogenital infection is associated with urethral inflammation and discharge (urethritis), which is composed primarily of polymorphonuclear leukocytes. Studies of female reproductive tract syndromes (namely cervicitis) have demonstrated the inflammatory capacity of *M. genitalium* both clinically and with models of the lower reproductive tract epithelium. In short, adhesion of *M. genitalium* to host epithelial cells (Figure 1) elicits acute inflammatory signals via highly expressed innate immune sensors including Toll-like receptors 2 and 6 [27]; the binding of these receptors to *M. genitalium* and its lipoproteins results in NF-κB activation and acute induction of genes involved in host defense [28]. These proinflammatory signals include potent chemokines that ultimately result in leukocyte recruitment to the site of infection [29, 30].

Lacking the discovery of any specific toxins or secreted virulence factors, it is primarily via the large number of surface-exposed lipoproteins that *M. genitalium* stimulates the cells lining the reproductive tract epithelium. Metabolic byproducts of *M. genitalium* and of the local host response include reactive oxygen species and nitric oxide that may have a cytopathological impact on the epithelium. Importantly, recruited leukocytes are robust potentiators of the local inflammatory response; in fact, *M. genitalium* induces very potent proinflammatory responses from monocytes/macrophages that are common to female reproductive tract tissues [31]. In persistently infected women, neutrophils are a prominent component of the proinflammatory response to *M. genitalium*, which seems to be chronic, varies in severity over time, and is ablated with microbiological cure [29].

M. genitalium infection elicits systemic [32] and mucosal [33] antibody responses in humans and in experimentally infected primates [34, 35]. Unfortunately, development of serologic tests has been challenging due to the difficulty in distinguishing between past and current infection, discerning potential cross-reactions with other mycoplasmas, and identifying the optimal antigens (purified lipid-associated membrane proteins, whole-cell proteins, and/or purified peptides) and immunoassay format (enzyme immunoassays, Western blot analyses) [32, 36]. For a primary *M. genitalium* infection, the local immune response without doubt precedes the generation of specific antibodies, but the relative importance of epithelial cell responses and cell-mediated immunity to generation of a robust antibody response are unknown. Nonetheless, both men and women infected with *M. genitalium* develop specific antibodies, primarily against 2 prominent outer membrane proteins, MgpB and MgpC.

Seroconversion to these antigens has been corroborated in several small animal models of infection and in nonhuman primates [33–35, 37] and mice [38]. Importantly, specific antibodies are measurable in lower genital tract secretions from women, implying that they are present at the site of infection and that *M. genitalium* must actively circumvent this response to survive. Although antigenic variation may facilitate survival (discussed below), the role of antibodies and cell-mediated immunity in clearance requires further study. Clearance without documented antibiotic treatment has been observed in several studies [39–41]; evaluation of the processes mediating clearance, including the host immune response, is critical to understanding the natural history of *M. genitalium* infections. In addition, we still lack unequivocal evidence as to whether or

not antibody responses protect from subsequent exposures to *M. genitalium*.

PERSISTENCE AND IMMUNE EVASION

Despite the infiltration of neutrophils, production of inflammatory mediators, and induction of local and systemic antibodies, *M. genitalium* has a remarkable ability to evade the host immune responses leading to chronic urogenital infections. For example, among Kenyan female sex workers, *M. genitalium* persisted for ≥7 months in 21% of these women, and up to 2 years in 2 women [41]. Combined with the observed persistence of *M. genitalium* in men [18], chimpanzee [34], macaque [37], and mouse models of infection [42], these studies indicate that urogenital persistence is common in both male and female reproductive tract tissues, possibly by antigenic and phase variation of their surface-exposed proteins as detailed below.

The discovery of the MgPar sequences containing multiple partial copies of the *mgpB* and *mgpC* genes in the *M. genitalium* genome suggested that gene variation of the surface-exposed proteins, MgpB (also known as MgPa and P140) and MgpC (also known as P110) [22, 43], may occur. Recent studies have confirmed 2 types of gene variation in *M. genitalium*: antigenic variation, which results in the expression of MgpB and MgpC variants with differing amino acid sequences, and phase variation, during which cells lose the ability to adhere to cultured cells and bind to red blood cells (hemadsorption). The experimental demonstration that both types of gene variation occur by recombination in vitro and in vivo, as well as the mechanism, regulation, and repercussions of this process, are the focus of the following discussion.

The complete *mgpB* and *mgpC* genes are present in a single expression site and consist of conserved regions interspersed with variable regions B, EF, G, and KLM. Homologous copies of the variable regions are present in multiple MgPar sites distributed around the chromosome (Figure 2 and [16, 17, 44, 45]). Antigenic variants arise when the variable regions of the expression site exchange sequences through segmental recombination with ≥1 of the MgPar sites. These recombination events are reciprocal; changes in the *mgpBC* sequence are accompanied by corresponding changes in the MgPar sites [16, 17], distinguishing *mgpBC*/MgPar recombination from antigenic variation systems in other bacteria, in which recombination is typically unidirectional (termed *gene conversion*) [46]. Furthermore, genetic diversity is achieved by recombination between different MgPar sites followed by subsequent *mgpBC*/MgPar recombination [17]. Phase variants, which do not express the MgpB and/or MgpC proteins and are therefore nonadherent, arise by multiple recombination mechanisms. For example, recombination between the expression site and the MgPar sites can involve 2 variable regions and intervening conserved sequences, resulting in the translocation of the conserved *mgpBC* sequences to the participating MgPar site, leaving an incomplete *mgpBC* operon ([47, 48]; P.A.T., unpublished data).

Experimental primate models of infection have provided opportunities to study the immune response, *mgpBC*/MgPar recombination, selection of MgpB and MgpC variants, and *M. genitalium* persistence. In a female pig-tailed macaque inoculated cervically, serum and cervical antibodies induced by the G37 inoculum were less reactive with an MgpB variant that appeared and became predominant after 8 weeks of infection,

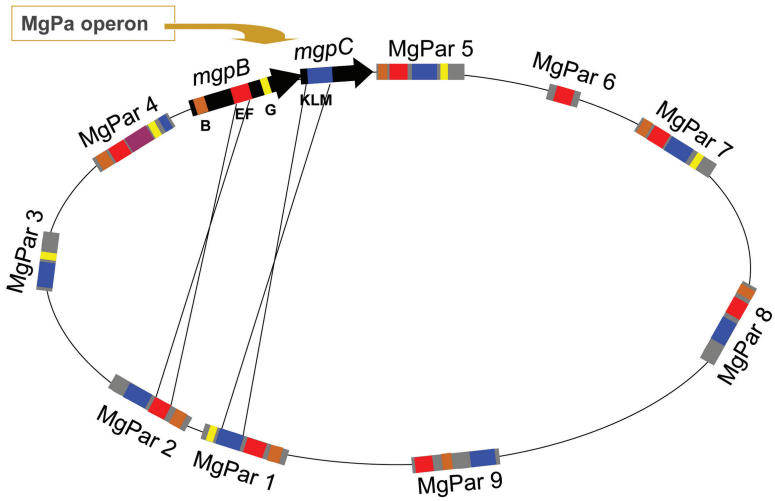

Figure 2. Schematic of the *Mycoplasma genitalium* genome showing the MgPa operon containing the adjacent *mgpB* and *mgpC* genes, the 9 homologous MgPar sequences and the mechanism of segmental reciprocal exchange between *mgpBC* and the MgPar sites. Variable regions B (*brown*), EF (*red*), and G (*yellow*) in *mgpB* and variable region KLM in *mgpC* (*blue*) are separated by conserved regions (*black*) found only in the *mgpB* and *mgpC* genes. Truncated but divergent copies of these variable regions are also found in the MgPar sites, separated by adenine-thymine-rich sequences found only in the MgPar sites (*gray*). Reciprocal recombination between short stretches of the *mgpBC* genes and the MgPar sites (*thin black lines*) results in antigenic and phase variation of the MgpB and MgpC proteins and replacement of sequences in the MgPar sites with those previously in the *mgpBC* expression sites, in a process termed *segmental reciprocal recombination*.

consistent with a role for antigenic variation in immune avoidance in vivo [35]. The selection of *mgpC* variants is also supported by the accumulation of *mgpC* sequence variants in urethral cultures over 11 weeks of infection in chimpanzees, coincident with development of antibodies [49]. In both primate models, the emerging variant sequences were consistent with replacement of the *mgpBC* sequences in the inoculating strain with an MgPar-specific sequence. Unfortunately, current studies on chimpanzees must be performed with archived specimens from these animals [49], which are no longer available for *M. genitalium* research. However, the pig-tailed macaque model is a promising alternative for studying the persistence, clearance, and specific antibody responses that could be exploited in future experiments to examine bacterial-host interactions, pathogenesis, and the dynamics of antigenic variation and immune evasion.

The frequency of *mgpBC*/MgPar variation, estimated to be $>1.25 \times 10^{-4}$ events per genome per generation, is similar to that observed in the notoriously antigenically variable *Neisseria gonorrhoeae* [50]. This finding begs the question of how such variation is achieved in *M. genitalium,* which lacks many of the recombination enzymes found in other bacteria. Homologues of only a few recombination genes, including those that encode RecA (a central recombination enzyme), RuvA and RuvB (required for DNA strand exchange), and RecU (required for resolution of DNA recombination intermediates) were identified in the G37 genome and were subsequently shown to be essential for *mgpBC*/MgPar recombination [51, 52]. Significantly, both antigenic and phase variation were decreased in a *recA* mutant, adding further support for the evidence that both variant types derive predominantly from recombination events, rather than from point mutations.

GENE REGULATION IN *M. GENITALIUM*

Sequencing of the *M. genitalium* genome has revealed few potential regulatory genes that are important for responding to the different metabolic environments and niches encountered in its human host. Although recombination is one of the most highly regulated systems in bacteria, proteins controlling this system in *M. genitalium* were not identified until 2014, when Burgos and Totten [53] revealed that MG428 is a positive regulator of *mgpBC*/MgPar recombination. MG428 coordinates the expression of key recombination genes, including *recA*, *ruvA*, and *ruvB*, as well as other novel proteins required for recombination [53, 54]. The homology of MG428 to alternative sigma factors, the binding of purified MG428 protein to core RNA polymerase, and the presence of the unique promoter sequences upstream of MG428-activated genes, collectively confirmed that MG428 is an alternative sigma factor [53, 54].

The identification of MG428 as a sigma factor was surprising because, until this discovery, mycoplasmas were presumed to lack alternative sigma factors and other means of globally controlling gene expression. Alternative sigma factors, as identified in other organisms, coordinately control the expression of genes involved in a defined physiological process, usually in response to an external stimulus, by binding to and directing RNA polymerase to a subset of genes containing promoter sequences targeted by the sigma factor. Identification of environmental signals that affect MG428 activity and *mgpBC*/MgPar recombination is needed to understand the activation of phase and antigenic variation in vivo.

The *M. genitalium* RecA protein is expressed as 3 distinct isoforms, which may represent a novel mechanism of regulation of recombination not found in other bacteria [53]. Although only the full-length isoform is active in recombination, the 2 N-terminally truncated isoforms, produced by alternative translation initiation, are hypothesized to posttranscriptionally regulate RecA activity, thereby fine-tuning the regulation of antigenic and phase variation in *M. genitalium*. Taken together, these results indicate that recombination leading to antigenic and phase variation in *M. genitalium* is complex and tightly regulated. The identification of environmental factors that modulate the activity of MG428, the full extent of the Mg428 regulon, and the function of the RecA isoforms in *mgpBC*/MgPar recombination are crucial for understanding the pathogenesis of this human pathogen.

SUMMARY AND PRIORITIES FOR THE FUTURE

Clearly much remains to be learned about the molecular pathogenesis of *M. genitalium* and the factors that enable the intimate interactions with host cells of the urogenital tract, long-term survival in vivo, evasion of the host immune response, and transmission. The recombination enzymes required for *mgpBC*/MgPar recombination, the extent of the regulon controlling this process, and environmental factors enhancing recombination are essential for understanding the pathogenesis and unique molecular strategies used by *M. genitalium* to persist at reproductive tract sites despite robust immune responses. Although the primary rationale for selection of antigenic and phase variants is believed to be the evasion of host antibodies resulting in persistence, other possibilities exist, such as differential adherence to host cells or regulation of host cell invasion and egress.

Additional studies are needed to define other factors that facilitate survival in reproductive tract tissues. For example, the life cycle of *M. genitalium* within reproductive tract tissues is essentially unknown, as are the mechanisms of invasion and the importance of intraepithelial cell residence for persistence and immune evasion. In addition, characterization of the surface-exposed antigenic epitopes important for antibody-mediated killing should be a priority for the future development of effective vaccines. Finally, the use of well-defined animal

models, the continued exploration of enzymes required for recombination, and the regulation of recombination and other critical processes in this unique bacterium are critical to our understanding of the pathobiology of this emerging pathogen and its associated disease processes.

Notes

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