Interaction of *Mycoplasma genitalium* with host cells: evidence for nuclear localization

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Mycoplasma genitalium (Mg) is a mollicute that causes a range of human urogenital infections. A hallmark of these bacteria is their ability to establish chronic infections that can persist despite completion of appropriate antibiotic therapies and intact and functional immune systems. Intimate adherence and surface colonization of mycoplasmas to host cells are important pathogenic features. However, their facultative intracellular nature is poorly understood, partly due to difficulties in developing and standardizing cellular interaction model systems. Here, we characterize growth and invasion properties of two Mg strains (G37 and 1019V). Mg G37 is a high-passage laboratory strain, while Mg 1019V is a low-passage isolate recovered from the cervix. The two strains diverge partially in gene sequences for adherence-related proteins and exhibit subtle variations in their axenic growth. However, with both strains and consistent with our previous studies, a subset of adherent Mg organisms invade host cells and exhibit perinuclear targeting. Remarkably, intranuclear localization of Mg proteins is observed, which occurred as early as 30 min after infection. Mg strains deficient in adherence were markedly reduced in their ability to invade and associate with perinuclear and nuclear sites.

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INTRODUCTION

Mycoplasma genitalium (Mg) is an important human mollicute first isolated in 1981 from urethral exudates of men diagnosed with non-gonococcal urethritis (Tully *et al.*, 1981). One of the original isolates, G37, has become the type strain and its genome is fully sequenced. This species possesses the smallest known genome among prokaryotes capable of axenic growth, encoding only 484 proteins (Fraser *et al.*, 1995). The reduced genome permits limited catabolic activities, thereby rendering Mg highly fastidious and dependent on biosynthetic precursors and nutrients supplied *in vitro* or *in vivo*.

Numerous additional studies have determined that Mg is a worldwide cause of urethritis and plays a role in cervicitis, pelvic inflammatory disease and tubal infertility (Stein & Baseman, 2006). Mg-mediated urogenital infection increases the likelihood of symptomatic infection when

compared to colonization by *Chlamydia trachomatis* (Falk *et al.*, 2004). In addition, Mg persists in the urogenital tract following antibiotic usage (Cohen *et al.*, 2007; Taylor-Robinson *et al.*, 2004; Wikstrom & Jensen, 2006).

The initial Mg–host cell adherence event is considered critical to colonization and establishment of human urethritis (Mernaugh *et al.*, 1993). Specific Mg adhesins are surface-exposed, yet linked to an internal cytoskeleton apparatus that constitutes the unique apical tip or terminal organelle. Two major adherence-associated, highly immunogenic proteins, P140 and P110, encoded by the MG191 and MG192 genes, respectively, mediate Mg binding to host determinants (Baseman *et al.*, 2004; Wang *et al.*, 1997). Importantly, these mycoplasma proteins appear to undergo antigenic variation, and their absence markedly reduces cellular colonization (Baseman & Tully, 1997; Stein & Baseman, 2006).

Following adherence of Mg to cultured mammalian cells, internalization of a subset of mycoplasmas occurs (Baseman *et al.*, 1995; Dallo & Baseman, 2000; Jensen *et al.*, 1994; Mernaugh *et al.*, 1993). The biological

Abbreviations: BCA, bicinchoninic acid; CCU, colour-change units; LSCM, laser scanning confocal microscopy; Mg, *Mycoplasma genitalium*; Mp, *Mycoplasma pneumoniae*; Mpen, *Mycoplasma penetrans*.

relevance of this observation is further reinforced by the fact that Mg organisms are detected within cervical and vaginal epithelial cells from infected patients (Blaylock *et al.*, 2004). However, the kinetics of Mg internalization has not been determined, and details of the subcellular locale occupied by these intracellular mycoplasmas are deficient. The possibility that Mg establishes an intracellular niche is especially compelling in the context of the clinical findings of chronic infections and mycoplasma persistence after antibiotic treatments (Stein & Baseman, 2006). However, it is not clear if the current *in vitro* model systems used to examine Mg interactions with host cells are suitable to replicate events occurring during disease. In the past, Mg–host cell studies have exclusively utilized the laboratory high-passage strain Mg G37 with infected tissue culture cells that did not represent the *in vivo* niche where the strains were originally recovered.

To this end, we initiated studies to compare the interaction of type strain Mg G37 with a representative, freshly isolated and low-passage clinical variant designated 1019V (Musatovova *et al.*, 2006) using human cell lines of urogenital origin. This focus provided additional insights into potential cellular compartments in which Mg resides.

METHODS

Growth curve of Mg. In order to establish the growth patterns of multiple-passage reference strain Mg G37 (ATCC 33530) and low-passage (fifth broth transfer) Mg 1019V strain isolated in our laboratory, each strain was grown in 150 cm² tissue culture flasks containing 100 ml SP-4 medium at 37 °C for 72 h and harvested by scraping surface-attached cells into 10 ml fresh SP-4 medium. Cells were divided into ten 1 ml aliquots, and each aliquot was inoculated into 100 ml fresh SP-4 medium in 150 cm² tissue culture flasks and incubated aerobically at 37 °C. The first culture was collected after 24 h incubation, and the remaining cultures were harvested every 12 h for 4 sequential days. At each time interval, Mg surface-attached cells were scraped into 10 ml fresh SP-4 medium and centrifuged at 14 000 g for 30 min at 4 °C. Mycoplasma pellets were washed twice in PBS, suspended in 2 ml PBS, passed repeatedly through 25-gauge needles to disperse large clumps, and analysed by OD₆₀₀, the bicinchoninic acid (BCA) assay for total protein content (Pierce), colour-change units (CCU), which are directly related to the titre of viable, metabolically active mycoplasmas as determined by colony-forming units (Razin & Tully, 1983), and real-time-PCR (Blaylock *et al.*, 2004). All measurements were performed in triplicate and a minimum of two separate times.

DNA isolation and real-time PCR. Genomic DNA was isolated from Mg strains G37 and 1019V using the Easy-DNA kit (Invitrogen). The concentration of genomic DNA was determined by measuring absorbance at 260 and 280 nm. Mg G37 and 1019V genome numbers were quantified using the Mg *gyrA* gene as target (Blaylock *et al.*, 2004). Real-time PCR was performed in triplicate using TaqMan chemistry (Applied Biosystems).

Chromosomal DNA of Mg reference strain G37 was serially diluted (from 10⁷ to 1 genome per reaction) to establish standard curves (Blaylock *et al.*, 2004). All reactions were performed in triplicate using TaqMan Universal PCR Master Mix, a 0.5 µM concentration of primers and a 0.2 µM concentration of probe as suggested by the manufacturer (Applied Biosystems). Amplification conditions con-

sisted of 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min (ABI PRISM HP7900 SDS, Applied Biosystems).

Human HeLa and EM42 cell cultures. HeLa cervical epithelial carcinoma cell line (ATCC CCL-2) and the non-carcinoma endometrial cell line EM42 were used in co-incubation studies with Mg. EM42 cells were established from benign proliferative endometrium (Desai *et al.*, 1994) and exhibit normal chromosomes and epithelial cell morphology, which, combined with their positive staining for cytokeratin and glycodelin, confirm their epithelial phenotype (Poddar *et al.*, 1998). EM42 cells were cultivated in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum and 2 mM L-glutamine (Gibco). HeLa cells were cultivated in Eagle's Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum and 2 mM L-glutamine and Earle's BSS (Gibco). All cell culture working stocks were grown in a 5% CO₂ atmosphere at 37 °C and routinely certified free of mycoplasma contamination using the Mycoprobe Mycoplasma Detection kit (R&D Systems). To prepare Mg-infected samples, EM42 and HeLa cells were grown on glass coverslips to 70% confluence, which was determined by haemocytometer count, and then infected 48 h later.

Mycoplasma–human cell infection protocol. Mg strains G37 and 1019V were grown in 70 ml SP-4 medium at 37 °C until early-to-mid-exponential phase, as determined by individual growth curves. Surface-attached mycoplasmas were harvested by scraping into 5 ml fresh SP-4 medium, dispersed through 25-gauge needles and passed through 0.45 µm Acrodisc filters (Gelman) to minimize mycoplasma clumps. Cell monolayer cultures were inoculated at an m.o.i. of 1–7 mycoplasmas per EM42 or HeLa cell and then incubated for 30 min to 3 h at 37 °C in 5% CO₂. All experiments were repeated four times. For comparative purposes Mg strains deficient in adherence (Dhandayuthapani *et al.*, 1999; Mernaugh *et al.*, 1993; Pich *et al.*, 2006) were grown in SP-4 broth and co-incubated at similar m.o.i. with human cells.

Immunofluorescence and confocal microscopy. Coverslips with infected cell monolayers were washed twice with PHEM buffer (60 mM PIPES, 25 mM MgCl₂, 10 mM EGTA, pH 7.2) and fixed with 2% paraformaldehyde dissolved in PHEM buffer for 30 min at room temperature. Then, cells were washed twice with PHEM buffer and permeabilized with 0.1% saponin for 5 min (Boleti *et al.*, 2000). After washing cells three times with PHEM buffer containing normal goat serum, cell preparations were incubated for 40 min with 0.1 ml antibody directed to the C-terminal portion of adhesin P140 (rabbit anti-MgPa-III) (Svenstrup *et al.*, 2002) at a 1/1000 dilution in PHEM with 10% normal goat serum. Rabbit antisera reactive against Mg proteins P110 (Mg 192) and elongation factor Tu (Mg 451) at a 1/1000 dilution were also used to detect intranuclear Mg protein localization. Coverslips were rinsed with PHEM and blocked with 1% normal goat serum (Gibco) for 20 min. Then, cells were rinsed with PHEM and incubated for 40 min with 0.1 ml goat anti-rabbit IgG-Alexa 488 (Molecular Probes) at 1/2000 dilution in PHEM with 10% normal goat serum (Gibco). Each incubation step was performed with gentle shaking at room temperature. After the final incubation, coverslips were washed three times with PHEM buffer and mounted on slides with 25 µl Vectashield Hardset mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). For negative controls, uninfected cells were treated identically, and infected cells were incubated with normal goat serum in the absence of anti-Mg antibodies.

Images of stained cells were acquired by laser scanning confocal microscopy (LSCM) with an Olympus FV500 imaging system and IX-81 microscope. The UPLANAPO 60× objective (1.4 numerical aperture) was used for all datasets, and an additional electronic zoom

of 2 was applied. The DAPI signal was excited with a 405 Blue Diode laser and collected through a 430–460 nm emission filter. The Alexa 488 signal was excited with the 488 nm line of an argon laser and collected through a 505–525 nm emission filter. Signals from each fluorophore were collected in sequence to minimize cross-talk. Z-series datasets were generated at 0.5 μm intervals from the basal to apical side of the cell monolayer using samples prepared 30 min, 1 h and 3 h post-infection. The percentage of Mg nuclear-positive cells was determined by counting the number of human cells with Mg-related punctate immunofluorescence inside individual nuclei in each field and dividing by the total number of cells in that same field. Five fields were counted for each experiment, and each experiment was repeated four times. The presence of Mg immunofluorescence within the volume of individual nuclei was determined by evaluating Z-series datastacks using the multiplane form function in the Fluoview software. This function enables the datastack to be viewed in the XY, XZ and YZ dimensions. Inclusion of Mg-associated immunofluorescence within the nucleus was considered positive if the DAPI signal was shown to surround the Alexa 488 signal in the X, Y and Z directions. Further, to determine whether the Alexa 488 signal was within the DAPI signal, a line trace was made through orthogonal sections to obtain a profile of the fluorescence distribution in both the DAPI and the Alexa 488 detection channels.

Statistical analysis. All results were expressed as the mean $\pm$ SD. Statistical differences were determined using a non-parametric one-way ANOVA (Kruskal–Wallis) with GraphPad In-Stat3 (GraphPad Software). A value for $P < 0.05$ was considered significant.

RESULTS

Growth characteristics of Mg G37 and 1019V

Mg type strain G37 has been subcultured in the laboratory for many years, and its axenic growth characteristics have been determined. In contrast, strain 1019V is a recent, low-passage isolate from a female patient, and its growth characteristics have not been examined. Therefore, growth curves were performed on both strains in parallel, so that comparable numbers of each strain at similar growth phases could be obtained for infection studies with human cell lines. The G37 strain was used at its fortieth passage while the 1019V strain was at its fifth passage. Mg is a highly fastidious organism that grows as adherent microcolonies on the plastic surface of tissue culture flasks in liquid culture. Therefore, harvesting of bacteria requires scraping from the vessel surface of each culture and disaggregation of mycoplasmal clumps by needle passage and filtration. To evaluate bacterial growth rate as a function of time, multiple criteria were measured including spectrophotometric measurement of optical density, determination of total protein by BCA assay, quantification of mycoplasma chromosome number by real-time PCR, and use of CCU as a mycoplasma growth-associated metric.

The growth data obtained by measuring optical density gave rise to classical-appearing growth curves (Fig. 1A) that seemed nearly identical for both the G37 and 1019V isolates. The lag phase for both strains lasted approximately 2 days, followed by an exponential growth phase that spanned the period from 48 to 96 h. Then the mycoplasmas entered into

Fig. 1. Growth of Mg G37 (■) and 1019V (●) measured by optical density (A), total protein (B), and real-time PCR (C). Samples of both strains grown axenically in SP-4 medium were analysed as detailed in Methods.

stationary phase, which ended after 4–5 days, followed by an apparent decrease in culture optical density, consistent with bacterial lysis associated with death.

The evaluation of growth as a function of total bacterial protein for strain G37 (Fig. 1B) demonstrated that initial increases in bacterial protein occurred between 24 and 48 h, which preceded the start of the OD₆₀₀-based exponential growth phase and ended shortly after the OD₆₀₀ exponential growth declined, as is common for typical bacterial growth. Early growth anomalies observed with 1019V (Fig. 1B) could represent unbalanced growth caused by the adjustment of this recently isolated, low-passage clinical strain to axenic culture conditions. However, at later points total 1019V protein calculations were consistent with G37.

The results of quantitative PCR to enumerate Mg chromosomal copies are shown in Fig. 1(C). The culture conditions utilized for these assays reveal that G37 chromosome numbers increased in a linear manner until 72 h. However, a net increase for 1019V genome numbers was delayed until 48 h, after which mycoplasma cell replication occurred until 96 h. Nevertheless, the genome copies for both organisms were comparable when stationary phase was reached.

The most suggestive evidence for differences in growth behaviour by the two strains was obtained when their growth dynamics were examined by CCU enumeration. As might be expected, the high-passage G37 strain appeared more adapted to *in vitro* conditions than the low-passage 1019V strain, as judged by faster growth rates between starting inocula of 10⁶ and 10³. Surprisingly, we observed that G37 growth dropped off rather abruptly when lower starting densities were used. For example, G37 appeared to require at least 100 starting organisms to establish growth in broth culture (Table 1). Unexpectedly, strain 1019V grew in broth culture when only 1–10 bacteria were inoculated as determined by limiting dilution assay (Table 2) consistent with colony-forming units (data not shown).

Invasion by Mg of HeLa and EM42 cells

Based upon quantitative growth curve determinations, cultures of each strain harvested at mid-exponential phase were used to infect host cells at low m.o.i. (1–7 mycoplasmas per human cell). Both EM42 and HeLa cells were co-incubated with Mg and, at differing post-infection times, were prepared as described in Methods. Mg-associated immunofluorescence was identified with specific antibodies directed to mycoplasma proteins P140, P110 and EF-Tu. Nuclei of human cultured cells were delineated by staining with DAPI so that the intracellular plane could be identified during optical sectioning via LSCM.

Remarkably, we observed a subset of Mg-associated immunofluorescence exhibiting an unusual subcellular localization pattern that could be clearly enumerated by LSCM. Specifically, image analysis revealed evidence of mycoplasma protein distribution within host cell nuclei (Figs 2 and 3) in a rapid and efficient manner (Fig. 4). Similar results were obtained when either G37 or 1019V isolates were examined, so while the micrographs shown here depict G37, the images obtained with 1019V were indistinguishable. As shown in Fig. 2, images taken at the apical, central, and basal planes of nuclei in infected EM42 (A–C) or HeLa (D–F) cells demonstrate distinct immunofluorescence clearly included within the internal volumes. Furthermore, digital orthogonal sections through Z-series datasets (Figs 2 and 3) confirm that a portion of internalized Mg-associated immunofluorescence is within the volume of nuclei in both EM42 (Fig. 3A–C) and HeLa (Fig. 3D–F) cells 30 min after infection. To confirm that the Alexa 488 signal from Mg was within the DAPI signal delineating host nuclei, line traces measuring pixel intensity in both channels were performed through orthogonal sections to obtain a profile of the fluorescence distribution in both DAPI and Alexa 488 detection channels (Fig. 3D–F). As demonstrated by the trace of fluorescence intensities within a selected region (Fig. 3; white box) of an infected host nucleus (Fig. 3; red box), the fluorescence distribution of Alexa 488-stained Mg, depicted as a green line in the intensity profile graph, is fully contained within the area occupied by the DAPI-stained host cell nucleus represented by the blue line in the intensity profile graph (Fig. 3). Therefore, Mg immunofluorescence in this micrograph is definitively located within the host cell nucleus. Importantly, this staining pattern is specific, as negative controls (see Methods) exhibited no evidence of Mg-related immunofluorescence.

In order to determine the kinetics and frequency of Mg G37 or 1019V nuclear localization within EM42 and HeLa, a time-course infection (30, 60 and 180 min) was

Table 1. Determination of growth of Mg G37 using colour-change units (CCU)

Mycoplasma CCU were determined over a period of 360 h, from cultures inoculated with the number of bacteria shown. + indicates a change in the colour of the medium from red to yellow; – indicates no change in medium colour.

No. of bac- teria	Time (h)														
	24	48	72	96	120	144	168	192	216	240	264	288	312	336	360
10 ⁶	–	+*													
10 ⁵	–	–	+												
10 ⁴	–	–	–	+											
10 ³	–	–	–	–	–	+									
10 ²	–	–	–	–	–	–	–	+							
10 ¹	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10 ⁰ †	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*The + sign indicates the time of incubation at which initial growth occurred.
†Equivalent to 1 or less.

Table 2. Determination of growth of Mg 1019V using colour-change units (CCU)

Details as for Table 1.

No. of bacteria	Time (h)														
	24	48	72	96	120	144	168	192	216	240	264	288	312	336	360
10 ⁶	—	—	+	*											
10 ⁵	—	—	—	+											
10 ⁴	—	—	—	—	+										
10 ³	—	—	—	—	—	—	+								
10 ²	—	—	—	—	—	—	—	+							
10 ¹	—	—	—	—	—	—	—	—	—	+					
10 ^{0†}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+

*The + sign indicates the time of incubation at which initial growth occurred.

†Equivalent to 1 or less.

Fig. 2. LSCM of the nuclear region (blue) of Mg G37 (green)-infected uroepithelial cells at 30 min post-infection. Panels A–C represent different areas (A, apical plane; B, central plane; C, basal plane) within the nuclei of the same group of Mg G37-infected EM42 endometrial cells. Panels D–F represent different areas (D, apical plane; E, central plane; F, basal plane) within the nuclei of the same group of Mg G37-infected HeLa cervical epithelioid cells. The DAPI-stained host nuclei appear blue while the green-appearing Mg G37 were visualized using primary rabbit antibody directed to the C-terminal region of P140 adhesin and Alexa 488-conjugated secondary antibody.

Fig. 3. LSCM of nuclear region (blue) of Mg G37 (green)-infected uroepithelial cells at 30 min post-infection. Digital orthogonal sections through Z-series datasets confirm that Mg G37-associated immunofluorescence is within the volume of EM42 (A–C) and HeLa (D–F) cell nuclei. Images show the central section of individual nuclei of EM42 (A) and HeLa (D) cells and extended views of the horizontal (B and E) and vertical (C and F) Z-projections of EM42 and HeLa cells, respectively. Colours of arrows (red, yellow and white) indicate the localization of Mg immunofluorescence in the horizontal and vertical Z-projections. The small red-boxed portion of (E) is enlarged (large red box) to better delineate DAPI and Alexa 488. The white box defines the nuclear area through which the trace of relative intensities of green (Mg) and blue (host nucleus) pixels was determined. Data are graphically depicted in the Intensity Profile, where all green pixels representing Mg are contained within the blue pixels defining the host nucleus.

performed and samples fixed and fluorescently stained (Fig. 4). For example, the percentage of EM42 cells containing Mg G37-associated immunofluorescence within their nuclei increased from approximately 15 % by 30 min post-infection to 40 % by 1 h and 60 % by 3 h post-infection. Mg strain 1019V was detected in approximately 30 % of EM42 cell nuclei at 30 min and 1 h post-infection, and in 50 % at 3 h post-infection. This rapid intranuclear localization event was observed in all four

independent experiments conducted, with at least half of all infected cells containing evidence of intranuclear mycoplasma proteins (P140, P110 and EF-Tu) within 3 h after invasion. Similar intranuclear targeting occurred when HeLa cells were used (data not shown). Importantly, Mg mutants deficient in adherence revealed less than 1 % nuclear-associated immunofluorescence using antisera reactive against P140, P110 and EF-Tu (data not shown).

Fig. 4. Percentage of human EM42 cells with nuclear-associated Mg G37 and 1019V immunofluorescence at 30 min, 1 h or 3 h post-infection. Black bars, Mg G37; white bars, Mg 1019V; * $P < 0.05$; *** $P < 0.001$.

DISCUSSION

In the present study, we monitored Mg–host cell interactions comparing two distinct mycoplasma strains that differ in terms of both the amount of axenic passage they have experienced since initial isolation and sequence heterogeneity within adherence-associated proteins P140 and P110. Care was taken to evaluate their growth features, such that comparable input numbers of similarly grown mycoplasmas (Fig. 1) were co-incubated with human non-phagocytic cultured cells of urogenital origin. The low-passage isolate 1019V grew more slowly in broth compared to G37, when mycoplasma cultures were started with a high inoculum density (Tables 1 and 2). However, both isolates reached comparable numbers by stationary phase (Fig. 1). Surprisingly, the multiple-passage and laboratory-adapted strain G37 required a starting inoculum at least two orders of magnitude higher than strain 1019V (Tables 1 and 2). These starting density-related features of G37 are probably the result of inadvertent adaptation to high starting densities commonly used in the laboratory in order to obtain large numbers of bacteria for investigative purposes. In contrast, strain 1019V may benefit in the clinical setting from being able to efficiently outgrow from a single cell while being transmitted to a new host or colonizing the urogenital tract as an ascending urogenital infection. Despite the subtle axenic growth differences observed between the two strains, time-course analysis using LSCM to identify intracellular mycoplasmas revealed the striking phenotype of intranuclear localization of Mg proteins within 30 min, and in at least half of host cells 3 h post-infection (Figs 2, 3 and 4).

Our results indicate that both high- and low-passage Mg strains behaved similarly in terms of the host–mycoplasma interaction, although studies with other mycoplasmas have revealed pronounced strain-dependent differences. For example, distinct *Mycoplasma fermentans* strains exhibited different rates of infection in HeLa cells, severity of tissue damage, and localization (Stadtlander *et al.*, 1993). *Mycoplasma gallisepticum* high- or low-passaged strains

differed significantly in their entry into HeLa cells (Winner *et al.*, 2000). Extension of these findings to the *M. gallisepticum* chicken infection model demonstrated a correlation between the *in vitro* invasive potential of specific mycoplasma strains and their mucosal penetration and virulence *in vivo* (Much *et al.*, 2002). Thomas *et al.* (2003) described varied adherence rates when *Mycoplasma bovis* isolates that differed in the number of *in vitro* passages were used to infect a variety of cultured host cells. Despite past precedent for altered host function as a consequence of laboratory passage, we observed little difference between the high-passage G37 and low-passage 1019V strains using the cellular interaction phenotype of nuclear localization. However, this is only one cell-association-related phenotype, and it is possible that G37 and 1019V strains will display differences in host interaction when additional criteria are examined. Interestingly, Mg mutants derived from G37 and deficient in host-cell interactions demonstrated markedly reduced internalization and perinuclear/intranuclear localization capabilities, reinforcing the invasive properties of clinically relevant wild-type Mg strains and their relationship to disease pathogenesis.

The invasive potential of Mg described herein has been reported previously for this and other human mycoplasma pathogens. Jensen *et al.* (1994) observed internalization of Mg G37 in Vero monkey kidney cells, while Mernaugh *et al.* (1993) described Mg G37 entry into human lung fibroblasts. In addition, the highly invasive *Mycoplasma penetrans* (Mpen) was observed to attain cytoplasmic and perinuclear localization in WI-38 cells (Baseman *et al.*, 1995). Furthermore, *Mycoplasma pneumoniae* (Mp), which is the closest relative to Mg, achieves cytoplasmic and perinuclear localization in WI-38 (Baseman *et al.*, 1995) by 2 h, and a similar localization was reported for this pathogen within the human hepatocyte cell line Hep-G2 (Meseguer *et al.*, 2003). Yavlovich *et al.* (2004) observed Mp internalization in human lung carcinoma A549 cells but not in HeLa epithelial cells. Finally, evidence for a nuclear locale for some mycoplasma species has been suggested. For example, examination of tissue from non-human primates fatally infected with *M. fermentans* identified bacteria inside Kupffer cells via electron microscopy (Lo *et al.*, 1993). Subcellular fractionation of HEp-2 cells persistently infected with Mp or Mpen demonstrated that viable mycoplasmas could be grown from nuclear fractions (Dallo & Baseman, 2000). The observation that a subfraction of intracellular Mpen associated with the nucleus, in the context of reports that host DNA damage occurs within Mpen-persistently infected cells, may imply that mycoplasma nuclear association could have virulence consequences (Feng *et al.*, 1999).

Therefore, the observed perinuclear localization and co-fractionation results reported previously suggest that mycoplasmas associate with the host nucleus. However, our study distinguishes itself from earlier work by the finding of internalization in uroepithelial-derived cells as

early as 30 min, with a high percentage of host cells infected by 3 h (>50%) as adjudged by nuclear localization immunofluorescence. Furthermore, the nature of the nuclear association reported herein based upon the intranuclear distribution of three distinct Mg proteins is fully consistent with Mg and its products breaching the nuclear membrane.

The disease-related biological consequences of mycoplasma internalization have not been established. However, there is an association between mycoplasma internalization and the traits of persistence and drug resistance observed in cultured host cells (Dallo & Baseman, 2000). As stated above, Mg, Mp and Mpen survived in cultured cells for at least 6 months, with replication observed in the presence of antibiotic concentrations that should kill extracellular bacteria. Therefore, persistence correlated with invasion. The intracellular phenotype of Mg is also clinically relevant, as host cells derived directly from patients were found to be heavily parasitized intracellularly based upon confocal immunoanalysis and transmission immunoelectron microscopy of clinical samples from infected women (Blaylock *et al.*, 2004). Therefore, we speculate that the invasive potential of Mg is substantial, especially given our finding that intranuclear evidence of mycoplasmas suggests an ability to transit from the extracellular environ through two host membranes (cytosolic and nuclear), further reinforcing the clinically observed persistence of this pathogen.

The nuclear localization observed for Mg is apparently without precedent for other bacterial pathogens. This is not to imply that other bacterial pathogens do not exploit the host nucleus by the targeting of protein effectors and toxins. For example, *Mycobacterium tuberculosis* delivers nucleoside-diphosphate kinase to the host cell nucleus, where this enzyme mediates *in situ* DNA damage that contributes to host cell death (Saini *et al.*, 2004). *Shigella flexneri* translocates two type III effectors into the host cell nucleus, and they alter cellular function by modulating select signal transduction pathways (Zurawski *et al.*, 2006). The cytolethal distending toxin of specific pathogenic bacteria exhibits nuclear translocation and leads to host cell DNA damage and cell cycle arrest (McSweeney & Dreyfus, 2004). Here, we describe the rapid delivery of three distinct Mg proteins and possibly the entire bacterium into the nucleus, which seems to present novel challenges. The exact mechanisms for mycoplasma nuclear-associated entry and the potential signal transduction and genetic and pathogenetic consequences associated with this biological manoeuvre remain to be elucidated. However, initial events in the Mg-human cell invasive process could lead to alterations in host cell membrane function, evidence of early-stage loss of cell viability and nuclear membrane vulnerability leading to Mg intranuclear penetration. Also, the intense perinuclear association of Mg reported earlier (Baseman *et al.* 1995; Dallo & Baseman, 2000) could result in intranuclear entrapment of Mg during host cell division and nuclear membrane disruption and re-formation.

With the establishment of the mycoplasma-host cell interaction model system utilized in this study, future investigations are readily feasible, which should demystify these biological associations and provide unique insights as to how this smallest of self-replicating pathogens can successfully circumvent the immune response and navigate through host cell structures. Specifically, how do this bacterium and its products exploit host cell function to traffic to the nucleus? Virus trafficking to the nucleus seems the most related pathogenic system, and the extensive body of work on viral nuclear trafficking should serve as a guide for future Mg studies. Our future studies will focus on the identification of host cytoskeletal elements that are needed to impart the observed Mg nuclear tropism and the characterization of disrupters of normal cellular architecture trafficking that alter Mg nuclear association. The association of host nuclear protein complexes and domains in relation to the location of intranuclear mycoplasma may also provide insights into potential entry mechanisms exploited by Mg. While previous studies support the viability and replicative potential of nuclear-associated mycoplasmas (Dallo & Baseman, 2000), it still remains to be determined if nuclear-associated Mg replicate in this locale and how nuclear-associated mycoplasmas might segregate within dividing host cells. Both transcriptional analysis of nuclear fractions and live imaging analysis of infected cells should help to address the intranuclear viability and replication issues associated with Mg parasitism.

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