Role of *Mycoplasma genitalium* MG218 and MG317 cytoskeletal proteins in terminal organelle organization, gliding motility and cytodherence

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The terminal organelle is a differentiated structure that plays a key role in mycoplasma cytodherence and locomotion. For this reason, the analysis of *Mycoplasma genitalium* mutants displaying anomalous terminal organelles could improve our knowledge regarding the structural elements required for proper locomotion. In this study, we isolated several *M. genitalium* mutants having transposon insertions within the *mg218* or *mg317* genes, which encode the orthologues of *Mycoplasma pneumoniae* HMW2 and HMW3 cytoskeletal proteins, respectively. As expected, *mg218*[−] and *mg317*[−] mutants exhibit a reduced gliding motility, although their ability to attach to solid surfaces was not completely abolished. Interestingly, most of the *mg218*[−] mutants expressed N-terminal MG218 derivatives and showed the presence of short terminal organelles retaining many of the functions displayed by this structure in the wild-type strain, suggesting that the N-terminal region of this protein is an essential element in the architecture of the terminal organelle. Separately, the analysis of *mg317*[−] mutants indicates that MG317 protein is involved in the formation of the terminal button and contributes to anchoring the electron-dense core to the cell membrane. The results presented here clearly show that MG218 and MG317 proteins are implicated in the maintenance of gliding motility and cytodherence in *M. genitalium*.

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INTRODUCTION

Mycoplasmas are genomically streamlined bacteria, characterized by the lack of a peptidoglycan layer and fastidious nutritional requirements. These parasitic micro-organisms have been continuously reemerging as important human and animal pathogens (Baseman & Tully, 1997). In particular, *Mycoplasma genitalium* has been linked to numerous genitourinary as well as extragenitourinary human pathologies (Baseman *et al.*, 1988) and it is a leading cause of Chlamydia-negative, non-gonococcal urethritis (Taylor-Robinson & Horner, 2001; Tully *et al.*, 1981). *M. genitalium* also has a unique and very attractive feature: with a genome size of 580 kb (Fraser *et al.*, 1995), it is the smallest self-replicating cell known to be able to be grown in axenic culture. For this reason, this micro-organism has been considered to be one of the most suitable models for achieving a complete understanding of the biology of a single replicating cell (Roberts, 2004).

M. genitalium cells display a differentiated tip structure, commonly known as the terminal organelle (TO), that

gives them a flask-shaped appearance. The TO plays a key role in mycoplasma cytodherence and further parasitism of host target cells and it is defined by an electron-dense core (EDC) that is a component of the Triton X-100-insoluble fraction (Gobel *et al.*, 1981; Meng & Pfister, 1980). The intriguing complexity and the multifunctional role of the TO have attracted the interest of many researchers. Most of the cytoskeletal proteins present in the complex network that constitutes the TO have been identified primarily as cyadhesins or cytodherence-associated proteins. In addition, the TO is the leading end as mycoplasma cells glide (Radestock & Bredt, 1977) and it constitutes the mycoplasma gliding motor (Hasselbring & Krause, 2007). Motility and cytodherence are closely related in mycoplasmas and many cytodherence-related proteins are also required for cell gliding (Burgos *et al.*, 2007; Hasselbring *et al.*, 2005; Hasselbring & Krause, 2007; Seto *et al.*, 2005). However, the existence of a cytodherence-independent set of motility-related proteins has been disclosed recently for the slow gliding mycoplasmas *M. genitalium* (Pich *et al.*, 2006a) and *Mycoplasma pneumoniae* (Hasselbring *et al.*, 2006).

For several years the isolation and characterization of spontaneous as well as transposon-generated mutants involving different cytodherence-related genes has provided

Abbreviations: EDC, electron-dense core; SAD, surface adherence defects; TB, terminal button; TO, terminal organelle.

A supplementary figure and table are available with the online version of this paper.

important information regarding the spatial and hierarchical development of the attachment organelle in *M. pneumoniae* (Krause & Balish, 2004). These studies have indicated that the cytoskeletal protein HMW2 is a major structural element of the EDC (Balish *et al.*, 2003b). In addition, it has been suggested that polymers of HMW3 protein surround the core and the terminal button (TB) in a linear pattern, possibly serving to stabilize this structure (Krause, 1996). Unfortunately, the specific contribution of the different cytoskeletal proteins from *M. genitalium* to the TO organization has been much less studied (Burgos *et al.*, 2006, 2007). Two independent factors have probably hindered the study of TO formation and the whole cytodherence scenario in the smallest mycoplasma. First, screening for haemadsorption-negative colonies has not allowed the isolation of transposon-generated mutants involving any of the cytodherence-accessory genes from *M. genitalium* (Reddy *et al.*, 1996). Second, the high rates of spontaneous occurrence of class I and class II haemadsorption-negative mutants (Mernaugh *et al.*, 1993), which probably reflect a subjacent cytodherence phase variation mechanism (Burgos *et al.*, 2006), hampers the screening process and jeopardizes the characterization of the mutants isolated.

In the present study, we aimed to investigate the involvement of *M. genitalium* cytoskeletal proteins in the TO organization and to analyse their contribution to gliding mechanics. To achieve this goal, a methodology recently developed in our laboratory (Pich *et al.*, 2006a) was modified in order to isolate transposon-generated gliding-deficient mutants also displaying surface adherence defects (SAD). Several mutants showing transposon insertions within the *mg218* or *mg317* genes, encoding the orthologues of *M. pneumoniae* HMW2 and HMW3 cytodherence-accessory proteins, respectively, were isolated using this procedure. The analysis of the transposon mutants obtained has revealed that the reduced gliding motility and cytodherence exhibited by these mutants is well correlated with the presence of defects in their respective EDC, revealing the particular roles of MG218 and MG317 proteins in the TO architecture of *M. genitalium*.

METHODS

Culture conditions and transformation. *M. genitalium* strain G37 and the different mutants isolated in this study were grown in SP-4 broth (Tully *et al.*, 1979) at 37 °C under 5 % CO₂ in tissue culture flasks (TPP). For standard colony development, mycoplasma cells were plated in SP-4 medium supplemented with 0.8 % agar (Difco). To check for gliding-deficient strains, cells were attached to cell culture dishes (Corning) and covered with SP-4 broth containing 0.5 % low-melting-point agarose (Iberlabo). SP-4 medium was supplemented with tetracycline (2 µg ml⁻¹; Roche) to select for resistant strains. Transformation of *M. genitalium* by electroporation was performed as described previously (Pich *et al.*, 2006b).

DNA manipulation. Genomic DNA was isolated using the E.Z.N.A. Bacterial DNA kit (Omega Bio-tek). Sequencing of genomic DNA from the different transposon mutants obtained was performed with fluorescent dideoxynucleotides using the Big Dye 3.0 Terminator kit (Applied Biosystems) and Tc upstream and Tc downstream primers (Table 1), according to the recommendations of the manufacturer. Southern blot hybridization was performed using the Dig DNA labelling and detection kit (Roche).

Construction of plasmid pΔmg218. To obtain plasmid pΔmg218, the flanking regions of *mg218* were amplified by PCR. A 1 kb fragment corresponding to the upstream region of *mg218* was amplified using primers URmg218-5' and URmg218-3' (Table 1), cloned into an *EcoRV*-digested pBE plasmid (Pich *et al.*, 2006b) and digested with *PstI* and *EcoRI*. Separately, a 0.8 kb fragment corresponding to the downstream region of *mg218* was amplified using primers DRmg218-5' and DRmg218-3' (Table 1), cloned into an *EcoRV*-digested pBE plasmid, and digested with *BamHI* and *ApaI*. Both flanking regions and an *EcoRI*-*BamHI*-digested *tetM438* marker were finally cloned into a *PstI*-*ApaI*-digested pBSK plasmid to obtain the pΔmg218 construct.

SDS-PAGE and MALDI-MS analysis. The separation of total mycoplasma proteins by SDS-PAGE followed standard procedures. The identification of MG317 protein in the SDS-PAGE was achieved by peptide mass fingerprint analysis, as described previously (Pich *et al.*, 2006a). MG317 protein was identified with a score of 136 and a protein sequence coverage of 38.9 %. Scores greater than 68 were considered significant (Perkins *et al.*, 1999).

Western blotting and preparation of antisera. Western blots were performed as described previously (Burgos *et al.*, 2006), using MG218 monoclonal and polyclonal antibodies developed in this work, or rabbit antiserum to P41 (Krause *et al.*, 1997), HMW1 (Stevens & Krause, 1991) and HMW3 proteins (Stevens & Krause, 1992). Monoclonal antibodies to MG218 protein were obtained by repeated

Table 1. Primers used in this work

Primer	Sequence
Tc upstream	5'-GGTAGTTTTCTCCTGCATCAACATG-3'
Tc downstream	5'-CGTCGTCCAAATAGTCGGATAG-3'
URmg218-5'	5'-CTGCAGCCAGGATTGATCCTAACCAAC-3'
URmg218-3'	5'-GAATTCATAAAATTGGTTGCAGCGAAGG-3'
DRmg218-5'	5'-GGATCCCAACATTAAAAGGTTGATTGGCC-3'
DRmg218-3'	5'-GGGCCCTAACAAATTAACGGTGGTTCTTC-3'
mg218Exp5'	5'-ACTGGTGGATCCCAACAAAAGCAAGATGAT-3'
mg218Exp3'	5'-CAGGTACTCGAGTTTACTTGCTGCTTTTGTG-3'

immunization of BALB/c mice with a *M. genitalium* 0.1 % Triton X-100-insoluble fraction in sterile PBS. Polyclonal antibodies to the MG218 C-terminal region were obtained by repeated immunization of BALB/c mice with a purified, recombinant C-terminal MG218 polypeptide (amino acids 1627–1805). Previously, a 537 bp sequence corresponding to nucleotides 264055–264592 of the *M. genitalium* genome was amplified by PCR using primers mg218Exp5' and mg218Exp3' (Table 1), cloned into a pET21a vector (Novagen), expressed in *Escherichia coli* BL21(DE3) and purified following standard procedures.

Microcinematography. Gliding motility data for the wild-type strain and the different mutants was obtained from microcinematographic studies (Pich *et al.*, 2006a). The motility of 25 individual cells from each particular clone of mg317[−] or mg218[−] mutants was analysed. Data obtained from the different mg317[−] mutant clones or, separately, from the different mg218[−] mutant clones, were combined to obtain a single value. Similarly, a total of 300 cells from mg317[−] or mg218[−] mutants, including cells from different clones, were examined to calculate the respective gliding frequencies.

Haemadsorption activity. For quantitative assessment of the haemadsorption activity, aliquots of 25 µl containing ~10⁷ c.f.u. ml^{−1} of the wild-type strain, mutants c9, c5, c12, c6, c4 and c16, and a representative clone of Δmg218 and Δmg191 mutants (Burgos *et al.*, 2006), were incubated at 37 °C for 2 h with 475 µl diluted (1:50) human erythrocytes. The mixture was shaken gently throughout the incubation. Erythrocytes and bound mycoplasmas were pelleted by centrifugation at 300 g for 2 min. Different dilutions of the supernatant were plated onto solid SP-4 medium in order to estimate the mycoplasma concentration. Prior to the incubation with erythrocytes, different dilutions of the original samples were also plated onto solid SP-4 medium to determine the initial mycoplasma concentration. The haemadsorption activity values are derived from two independent experiments. In each experiment, data obtained from the two clones representing the same group of mutants were combined.

Scanning electron microscopy. Scanning electron microscopy analyses were performed using Permax chamber slides (Nunc). For this purpose, cells from the wild-type strain, all mg317[−] mutants, and mg218[−] mutants c12, c13, c11, c3 and c6 were grown for 24 h, while cells from Δmg218 mutants and mg218[−] mutants c4 and c16 were grown for 48 h. SP-4 medium was removed and cells were washed gently with PBS and fixed with 1 % glutaraldehyde for 1 h. Samples were washed again with PBS and dehydrated sequentially with 25, 50, 75 and 100 % ethanol concentrations for periods of 10 min each. Immediately, samples were critical-point dried (K850 Critical Point Drier, Emitech) and sputter coated with gold. Samples were observed using a Hitachi S-570 scanning electron microscope.

Transmission electron microscopy. Cells from the wild-type strain, mg218[−] mutants c4, c16 and c6, mg317[−] mutant c5 and Δmg218 mutant c8 were grown to late-exponential phase, scraped, pelleted by centrifugation, and washed with PBS. Pellets were fixed with 2.5 % (v/v) glutaraldehyde and 2 % (v/v) paraformaldehyde (EM grade, TAAB) in 100 mM phosphate buffer (PB, pH 7.4) for 2 h and rinsed with 100 mM PB. Pellets were then post-fixed in 1 % (w/v) osmium tetroxide (TAAB) containing 0.8 % (w/v) potassium hexacyanoferrate (III) (Sigma) for 2 h and washed with 100 mM PB. All these steps were done at 4 °C. Samples were dehydrated through a graded ethanol series, infiltrated in Spurr's resin and polymerized for 48 h at 60 °C. Ultrathin sections were mounted on copper grids, contrasted with standard uranyl acetate and lead citrate double-staining, and observed in a Hitachi H-7000 transmission electron microscope at 75 kV. The length of the EDC in a particular strain was determined from measurements of 18–25 TO.

RESULTS

Isolation of *M. genitalium* gliding-deficient mutants with SAD

A random transposon library was obtained by electroporating wild-type cells in the presence of MTnTetM438, a modified version of Tn4001 that confers tetracycline resistance and gives rise to random and stable insertions in the *M. genitalium* genome (Pich *et al.*, 2006b). Transformed cells were dispensed into cell culture dishes, left to adsorb onto the plastic surface and covered with 0.5 % low-melting-point agarose SP-4 medium. In contrast to our previous report (Pich *et al.*, 2006a), the adsorbed cells were not washed with PBS, in order to retain those mutants not strongly adhered to the plastic surface. The morphology of ~12 000 colonies was examined and 40 colonies exhibiting a compact morphology, compatible with the existence of gliding deficiencies, were identified and isolated. Since gliding-deficient mutants without SAD are also isolated by this procedure, we first discarded those mutants showing transposon insertions in the *mg200* and *mg386* genes, analysed extensively in our previous report. Twenty-two transposon mutants lacking MG386 or MG200 polypeptides were identified by SDS-PAGE analysis and consequently were not examined. Separately, seven clones containing mixed populations were also discarded.

Identification of the transposon insertion points

Insertion points of MTnTetM438 in the eleven gliding-deficient mutants selected for further analysis (Table 2) were determined by sequencing the corresponding genomic DNA using the Tc upstream and Tc downstream primers (Table 1) present in the MTnTetM438 transposon. Seven transposon mutants showed MTnTetM438 insertions within the *mg218* coding region (mg218[−] mutants; Fig. 1a). The remaining transposon mutants showed minitransposon insertions within the *mg317* coding region (mg317[−] mutants; Fig. 1b).

Colony morphology of the mutants

Cells from mg218[−] mutants c4 and c16 covered with 0.5 % low-melting-point agarose SP-4 medium developed flat and shiny colonies, with several small colonies surrounding the main one (Fig. 2a). Interestingly, cells from both clones did not attach avidly to the plastic surface of the cell culture flask. In contrast, under similar culture conditions, cells from the remaining mg218[−] mutant clones (Fig. 2b) and all the mg317[−] mutants (Fig. 2c) developed colonies with rough surfaces and a granulate appearance in the centre. The presence of some satellite microcolonies in these clones indicated that a certain degree of locomotion was still preserved. Cells from these nine transposon mutants attached quite well to the plastic surface of cell culture flasks. Hence, the two different classes of colony morphologies described here reflect the effect of the gene disruption

Table 2. MTn*TetM438* insertion point in the 11 gliding deficient mutants

Disrupted gene	Clone	Insertion point*	Amino acids in the N-terminal truncated fragments	Expected mass (kDa)	Cell adhesion†
<i>mg218</i>	c4	259188 (–)	3	–	–
	c16	259895 (+)	239	28.5	–
	c12	260913 (+)	577	69.0	±
	c13	261124 (+)	648	77.7	±
	c11	261142 (+)	655	78.5	±
	c3	261568 (+)	797	95.0	±
	c6	261575 (+)	799	95.3	±
<i>mg317</i>	c1	396783 (–)	213	24.4	±
	c9	396712 (+)	237	26.9	±
	c5	396582 (–)	280	31.8	±
	c15	396348 (–)	358	40.7	±

*The insertion point refers to the NC 000908 GenBank genome sequence from *M. genitalium*. Symbols (+) [plus strand] and (–) [minus strand] indicate the sense of the MTn*TetM438* insertions in the genome.

† ±, Presence of moderate cell adherence defects; –, presence of severe cell adherence defects.

on the surface-adherence capability of the mutant strain. This important divergence prompted us to consider the separation of the *mg218*[–] mutants into two groups: *mg218*[–] mutants with severe SAD (c4 and c16), and *mg218*[–] mutants with moderate SAD (c3, c6, c11, c12 and c13).

Construction of Δ *mg218* mutants

In the light of the two different phenotypes observed after transposon disruption of *mg218*, we decided to analyse the effect of the complete loss of MG218 protein in *M. genitalium* by deleting the *mg218* coding region completely by homologous recombination. For this purpose, the suicide plasmid p Δ *mg218* was engineered to contain the *tetM438* selectable marker (Pich *et al.*, 2006b) and the flanking regions of *mg218* (Fig. 3a). A double recombination event between plasmid p Δ *mg218* and the mycoplasma genome promotes the substitution of bases 43 to 5376 of

mg218 (98.5 % of the *mg218* coding region) by the *tetM438* marker. Several tetracycline-resistant clones were isolated after electroporation of *M. genitalium* in the presence of p Δ *mg218*. The presence of the expected deletion within *mg218* in these strains was assessed by Southern blotting. Genomic DNA from the wild-type strain and eight p Δ *mg218* transformants was digested with *Acc65I* and probed with a 0.8 kb *Bam*HI–*Apa*I fragment obtained from plasmid p Δ *mg218*. A single band of 2.2 kb, clearly different from the 10.9 kb band expected for the wild-type strain, was detected for the eight p Δ *mg218* transformants analysed (Fig. 3b). This single band of 2.2 kb is compatible only with a double recombination event and confirms the expected deletion of *mg218* in these strains. Hereinafter, we will refer to these strains as Δ *mg218* mutants.

Gliding motility data

Gliding motility of the wild-type strain and *mg218*[–], *mg317*[–] and Δ *mg218* mutants was analysed quantitatively by microcinematography (Table 3). Motile cells from the wild-type strain represented 93.8 % of the cell population and exhibited a mean gliding speed of 0.152 μ m s^{–1}. However, attached cells from *mg317*[–] and *mg218*[–] mutants with moderate SAD displayed a mean gliding speed between four and fivefold below that of the wild-type strain. Motile cells from *mg317*[–] mutants represented 48.3 % of the cell population while the percentage of motile cells from *mg218*[–] mutants with moderate SAD was slightly lower (43.9 %). In contrast, Δ *mg218* and *mg218*[–] mutants with severe SAD were non-motile.

In addition, numerous cells from *mg218*[–] and *mg317*[–] mutants displayed filaments (see below: Cell morphology) similar to those documented previously for *mg386* gliding-deficient mutants (Pich *et al.*, 2006a). These filaments were often found connecting two different cells but were also

Fig. 1. Schematic representation showing the respective locations of MTn*TetM438* insertions within the *mg218* (a) and *mg317* (b) genes. Full arrows represent the genes for which the locus_tag is shown. Gene names in parentheses correspond to the respective *M. pneumoniae* homologues. *mg218.1* has also been annotated as *mg491*.

Fig. 2. Colony morphology of mycoplasma cells attached to cell culture dishes and covered with SP-4 medium containing 0.5% low-melting-point agarose. (a) *mg218*[−] mutant c4, (b) *mg218*[−] mutant c6, (c) *mg317*[−] mutant c5, (d) wild-type strain.

present in isolated mycoplasmas. We observed that the filament linking two cells was broken as the cells became further apart (data not shown). When the resulting individual cells resumed gliding, the filament was clearly detected at the pole opposite to the direction of movement.

Protein profile

The protein profiles of the different mutants isolated in this study were analysed by SDS-PAGE (Fig. 4a) and Western blotting (Fig. 4b–f). As expected, a 220 kDa band corresponding to full-length (1805 amino acids) MG218 protein (Dhandayuthapani *et al.*, 1999) was absent in both Δ *mg218* and *mg218*[−] mutants (Fig. 4a, b). Nevertheless, the use of MG218 monoclonal antibodies revealed the presence of N-terminal MG218 derivatives in all *mg218*[−] mutants with moderate SAD (Fig. 4b). Polyclonal antibodies raised against amino acids 1627 to 1805 of MG218 protein revealed also the presence of several C-terminal

MG218 derivatives (bands of 118, 115 and 25 kDa, Fig. 4d), most of them visible both in the wild-type strain and in all the *mg218*[−] mutants isolated. As expected, no N-terminal and C-terminal MG218 derivatives were observed among the Δ *mg218* mutants.

All *mg317*[−] mutants lacked the band corresponding to full-length (599 amino acids) MG317 protein (Svenstrup *et al.*, 2006). However, the presence of truncated MG317 fragments was not detected in this case. Δ *mg218* and *mg218*[−] mutants c16 and c12 (Fig. 4a, c) as well as mutant c4 (data not shown) also exhibited reduced levels of MG317. Likewise, levels of MG312 protein, the orthologue of *M. pneumoniae* HMW1, were also reduced in Δ *mg218* and *mg218*[−] mutants c4, c16 and c12 (Fig. 4e). These observations strongly suggest that the stability of MG312 and MG317 depends on the expression of the MG218 N-terminal region, in particular of the short stretch located between amino acids 577 and 648 (the length of the N-terminal MG218 derivatives in mutants c12 and c13). In addition, all the mutants isolated in this study showed reduced levels of MG386, P140 and P110 proteins. The stability of MG386 protein seems to be directly linked to the expression of P140 and P110 proteins at wild-type levels (Burgos *et al.*, 2006), suggesting a cooperative interaction between these proteins. Finally, the presence of MG218.1 protein, the orthologue of *M. pneumoniae* P41, was detected in all Δ *mg218* and *mg218*[−] mutants (Fig. 4f). This result rules out the existence of polar effects on the *mg218.1* gene derived from disruption or deletion of *mg218*. Polar effects derived from the transposon insertion in *mg317*[−] mutants are not expected because the distance between *mg316* and *mg317* (40 bp) is too long to allow translational coupling of the corresponding polypeptides (Lluch-Senar *et al.*, 2007).

Haemadsorption activity

Haemadsorption activity of the different mutants isolated in this study, the wild-type strain and the non-adherent mutant Δ *mg191* was analysed quantitatively. As expected, *mg317*[−] and *mg218*[−] mutants with moderate SAD retained around 50% of the wild-type haemadsorption

Fig. 3. (a) Schematic representation showing the genomic rearrangements when obtaining Δ *mg218* mutants and (b) Southern blot analysis of these mutants. UR, upstream region; DR, downstream region; WT, wild-type.

Table 3. Motility and cytodherence data

ND, Not determined; HA, haemadsorption; WT, wild-type.

Strain	Mean gliding speed ($\mu\text{m s}^{-1}$) $\pm$ SEM	Mean gliding speed ranges* ($\mu\text{m s}^{-1}$)	Motile cells	HA activity (% of WT)
WT	0.152 ± 0.007	—	93.8 %	100
mg218 ⁻ mutants†	0.034 ± 0.004	0.033–0.037	43.9 %	46.5
mg317 ⁻ mutants	0.032 ± 0.004	0.031–0.034	48.3 %	62.4
Δ mg218 mutants	0	—	0 %	19.3
Δ mg191 mutants	ND	—	ND	10.8

*Maximum and minimum mean gliding speed values obtained for the different clones.

†Clones with moderate SAD only.

activity (Table 3). Accordingly, cells from these mutants grow mainly attached to the plastic surface of the cell culture flask in liquid cultures. In contrast, Δ mg218 mutants

displayed haemadsorption activities 20% of that of the wild-type strain. Correspondingly, cells from Δ mg218 mutants grow mainly in suspension in liquid cultures. The

Fig. 4. Protein profile of the wild-type strain (WT) and different mutant strains analysed by 8% SDS-PAGE (a) or Western blotting using antiserum to MG218 N-terminal region (b), HMW3 (c), MG218 C-terminal region (d), HMW1 (e) or P41 proteins (f). MW, size markers.

haemadsorption activity displayed by the two $mg218^-$ mutants with severe SAD was similar to the activity observed for $\Delta mg218$ mutants (data not shown). All data regarding $mg218^-$ mutants with severe SAD obtained in this study are similar to the data obtained for $\Delta mg218$ mutants and will not be addressed further in this manuscript.

Cell morphology

Scanning electron microscopy analyses revealed that cells from $mg218^-$ mutants with moderate SAD (Fig. 5b) and $mg317^-$ mutants (Fig. 5c) exhibit a similar morphology, characterized by the presence of a short TO. The thin neck that joins the cell body and the TB in the wild-type cells (Fig. 5a, arrows) is less apparent in these mutants. Interestingly, most of the cells from these mutants show long tails at the pole opposite to the TO. These long tails often join cells oriented in opposite directions. Among the population of $\Delta mg218$ mutants (Fig. 5d, e), we observed rounded cells, reminiscent of those described previously for $\Delta mg191$ and $\Delta mg192$ mutants (Burgos *et al.*, 2006), as well as elongated cells. However, the most remarkable feature of $\Delta mg218$ mutants is the presence of rounded bodies, two or threefold larger than a single wild-type cell, suggesting the existence of strong deficiencies in cell division.

Ultrastructural analyses

TOs were easily recognized in cells from the wild-type strain and they were defined by a long EDC (214 $\pm$ 7 nm)

with a characteristic curvature at the proximal end (Fig. 6a). In agreement with previous work (Henderson & Jensen, 2006), some views revealed the origin of two independent rods at the proximal end of the EDC (Fig. 6a, numbers 1 and 2). In addition, a slightly electron-dense material that separates the cytoplasm from the proximal end of the EDC was observed (Fig. 6a, arrows). The distal end of the EDC showed the thicker region currently known as the TB. Notably, some transverse sections of the TB revealed the presence of several discrete highly electron-dense rounded particles (Fig. 6a, asterisk; see also Supplementary Fig. S1, available with the online version of this paper). Since the overall structure found in the *M. genitalium* TO seems similar to that observed in the *M. pneumoniae* TO, we hypothesize that these rounded particles correspond to the arched path of discrete globular proteins (Fig. 7b, component C) recently identified in *M. pneumoniae* by cryo-electron tomography (Henderson & Jensen, 2006).

Cells from $mg218^-$ mutants with moderate SAD showed TOs with EDCs that were shorter (138 $\pm$ 10 nm) than those of the wild-type strain (Fig. 6b). TBs were still present and some sections retained the highly electron-dense rounded particles described for the wild-type strain. In contrast, these electron-dense particles were not observed in preparations from $mg317^-$ mutants. Most of the EDCs from $mg317^-$ mutants were devoid of a clear TB, and the distal end of the EDC often appeared unconnected to the cell membrane (Fig. 6c). Interestingly, we also observed electron-dense structures embedded in the cytoplasm, not

Fig. 5. Scanning electron microscopy of the wild-type strain (a), $mg218^-$ mutant c6 (b), $mg317^-$ mutant c5 (c) and a $\Delta mg218$ mutant (d and e). Arrows show the neck that joins the cell body and the TB in the wild-type strain.

Fig. 6. Transmission electron microscopy of the wild-type strain (a), *mg218*⁻ mutant c6 (b), *mg317*⁻ mutant c5 (c) and a Δ *mg218* mutant (d). The presence of two independent rods in the EDC is indicated by the numbers 1 and 2. Arrows indicate the slightly electron-dense material that separates the cytoplasm from the EDC. The asterisk highlights the presence of rounded particles in transverse sections of the TB. The circle in the right panel of (c) draws attention to an EDC free in the cytoplasm and not associated with any membrane protrusion.

Fig. 7. Overall structure of *M. genitalium* TO (a) and schematic representation of the attachment organelle of *M. pneumoniae* with components labelled (b); modified from Henderson & Jensen (2006).

associated with any membrane protrusion (Fig. 6c). These structures resemble bona fide EDCs that appear to be free in the cytoplasm, since the tip structure could not be identified in such cells. These observations suggest that the distal end of the EDC is not attached tightly to the cell membrane in these mutants. In contrast, EDCs could not be identified in cells from Δ *mg218* mutants (Fig. 6d). In accordance with the images obtained by scanning electron microscopy, cell bodies from Δ *mg218* mutants were larger than those observed for the wild-type strain. Finally, many cells from all the mutants analysed in this study displayed long tails with alternating electron-dense and electron-

transparent regions, as could be seen for Δ mg218 mutants (Fig. 6d).

DISCUSSION

In the current study, we have found that transposon disruption of *mg218* or *mg317* genes does not completely eliminate the ability of the respective mutant cells to attach to solid surfaces, a feature that is thought to be essential for mycoplasma locomotion *in vivo*. The examination of *mg218*[−] and *mg317*[−] mutants reveals a significant reduction in their number of motile cells and their gliding speed, in addition to the presence of anomalous TOs. This fact suggests that the involvement of MG218 and MG317 proteins in the gliding mechanics is a direct consequence of their respective roles in TO organization. However, we cannot discard the possibility that the reduced levels of MG386, P140 and P110 proteins, previously linked to mycoplasma gliding motility (Hasselbring *et al.*, 2006; Pich *et al.*, 2006a; Seto *et al.*, 2005), could also contribute to the reduced locomotion observed in the *mg218*[−] and *mg317*[−] mutant cells.

The two different phenotypes observed among *mg218*[−] mutants are well correlated with the presence of N-terminal MG218 truncated fragments. Supporting this information, we identified the presence of N-terminal MG218 derivatives in all *mg218*[−] mutants with moderate SAD. These MG218 truncated forms seem to be responsible for the EDC formation and the intermediate cytodherence activity displayed by this group of *mg218*[−] mutants. The stability of the N-terminal MG218 derivatives with lengths ranging from 577 to 799 amino acids is interesting, because it has been reported that N-terminal HMW2 derivatives smaller than 1040 amino acids are unstable in *M. pneumoniae* (Balish *et al.*, 2003a). Moreover, in contrast to *M. pneumoniae* *hmw2* transposon mutants (Hedreyda & Krause, 1995), N-terminal MG218 derivatives are not only stable but they are still functional in *M. genitalium*. Accordingly, the shorter EDCs observed in *mg218*[−] mutants with moderate SAD probably reflect the truncated nature of the N-terminal MG218 derivatives. Thereby, our results provide experimental evidence that the length of the EDC corresponds to the length of MG218 modelled as a fully extended α -helix, as has been addressed previously for *M. pneumoniae* HMW2 (Balish *et al.*, 2003b). Interestingly, we also identified the existence of multiple C-terminal MG218 derivatives in all *mg218*[−] mutants as well as in the wild-type strain. The presence of these internal products in *mg218*[−] mutants rules out the possibility that they could arise from the proteolytic processing of full-length MG218 protein and suggests the existence of additional transcriptional and translational start sites in the *mg218* coding region. Two different ATG codons, encoding methionines 804 and 822 of MG218 protein, could also function as internal start codons, giving rise to polypeptides with a size compatible with the 118 and 115 kDa C-terminal MG218 derivatives identified in this work (see Fig. 4d and Supplementary Table S1, available with the online version

of this paper). The function of these MG218 derivatives, not identified in *M. pneumoniae*, is still unknown. In contrast, the 25 kDa C-terminal MG218 derivative, which could arise from the translation of MG218 starting at either methionine 1574 or 1615 (Supplementary Table S1), is probably the orthologue of *M. pneumoniae* P28 protein (Krause *et al.*, 1997). The presence of this small derivative in both mycoplasma species suggests that it plays a significant biological function that still remains unknown. Thus, further work is needed to unravel the significance of the different C-terminal derivatives found in *M. genitalium*. On the other hand, it is noteworthy that Δ mg218 mutants, lacking such C-terminal MG218 derivatives, exhibit a phenotype similar to that of *mg218*[−] mutants with severe SAD. In addition, these C-terminal derivatives do not seem to be involved in the stabilization of MG312 and MG317 proteins, because MG312, and to a lesser extent MG317, are unstable in *mg218*[−] mutants with severe SAD. Thus, it seems that the stability of MG312 and MG317 depends on the expression of the MG218 N-terminal region, in particular of the short stretch located between amino acids 577 and 648 (the length of the N-terminal MG218 derivatives in mutants c12 and c13). Altogether, these observations indicate that the MG218 N-terminal region can fulfil many of the roles currently associated with the expression of a full-length MG218 protein, a result that is in acute contrast to that observed in *M. pneumoniae* (Balish *et al.*, 2003a).

Electron microscopy analyses reveal that MG317 protein is required to build up a standard TB. Since many cells from *mg317* transposon mutants show EDC free in the cytoplasm, the TB seems to be involved in the attachment of the distal end of the EDC to the inner surface of the plasma membrane. It is tempting to speculate that MG317 is involved in the attachment of the EDC to the intracellular domains of P140/P110 complexes present at the terminal tip membrane. To fulfil such a possible function, MG317 should be localized in the vicinity of the TB in *M. genitalium*, which is in agreement with the localization of HMW3 in *M. pneumoniae* revealed by immunoelectron microscopy (Stevens & Krause, 1992). In our micrographs, the chevron-like structures previously observed in a *M. pneumoniae* *hmw3* mutant (Willby & Krause, 2002) could not be identified. Regardless of this, the intermediate cytodherence displayed by our *mg317*[−] mutants is in accordance with the phenotype described for the *M. pneumoniae* *hmw3* mutant (Willby & Krause, 2002). Thus, the partial cytodherence activity displayed by *mg317*[−] and *mg218*[−] mutants provides an explanation for the previous failure to isolate *M. genitalium* haemadsorption-negative mutants showing transposon insertions in these two cytodherence-related genes (Reddy *et al.*, 1996). Interestingly, a similar situation could be hampering the isolation of *Mycoplasma gallisepticum* haemadsorption-negative mutants bearing transposon insertion in genes different from *gapA* and *crmA* (Mudahi-Orenstein *et al.*, 2003). Therefore, screening for colonies exhibiting a compact morphology could provide a direct way to isolate these mutants.

As stated previously, all the mutants isolated in this study show reduced levels of P140 and P110 proteins, which are involved in the maintenance of *M. genitalium* cytodherence (Burgos *et al.*, 2006). This result indicates that beyond the mutual interdependence of P140 and P110, demonstrated previously in Δ mg191 and Δ mg192 mutants (Burgos *et al.*, 2006), the levels of these two proteins seem to be also coordinately regulated in *M. genitalium*. In addition, the reduced levels of P140 and P110 also provide an explanation for the reduced haemadsorption activity exhibited by mg218⁻ and mg317⁻ mutants. However, additional factors are needed to explain the reduced cytodherence observed in mg218⁻ mutants with severe SAD. Probably, the absence of a TO in these mutants is also hampering the correct localization of these two adhesins in the outer side of the cell membrane. Notably, a previous study in which *M. genitalium* mg218 was disrupted through homologous recombination allowed the isolation of some mutants (designated JB1 mutants) displaying a cytodherence-negative phenotype (Dhandayuthapani *et al.*, 1999). JB1 mutants exhibited a dramatic instability of P140 and P110 that might also explain the complete loss of cytodherence of these mutants. In contrast, P140 and P110 are still found in all the Δ mg218 mutants isolated in our study, although at reduced levels. At the present time, the origin of the different behaviour of P140 and P110 in JB1 and Δ mg218 mutants is unclear.

Finally, most of the cells from the mg218⁻ and mg317⁻ mutants showed thin filaments similar to those described previously for mg386 and mg200 gliding-deficient mutants (Pich *et al.*, 2006a). Scanning electron microscopy images from mg218⁻ and mg317⁻ mutants also revealed that cells coupled by these filaments are always oriented in opposite directions, a configuration characteristic of dividing mycoplasma cells. Altogether, these observations suggest that cytokinesis is impaired in *M. genitalium* mutants with gliding motility defects. In addition, locomotion clearly contributes to cell dispersion in adherent mycoplasmas, a fact that is supported by the formation of large cell aggregates in liquid cultures of mutants with gliding deficiencies (Pich *et al.*, 2006a). The dramatic consequences for cell division, probably magnified by the complete loss of the TO, are particularly evident in Δ mg218 mutants. However, the large cell bodies observed in Δ mg218 mutants may reveal an additional role for MG218. Interestingly, further investigation of conserved domains within MG218 (Marchler-Bauer & Bryant, 2004) shows the presence of two independent but overlapping SMC domains (Structural Maintenance of Chromosomes; COG1196). Proteins that contain SMC-domains have been shown to participate in many aspects of higher-order chromosome organization. Like SMC proteins, MG218 is predicted to form large coiled-coils that are often involved in protein–DNA interactions (Akhmedov *et al.*, 1999). Additional analyses are needed to determine whether DNA is present in the TO and whether MG218 protein is also involved in chromosome dynamics of *M. genitalium*.

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