Attachment organelle ultrastructure correlates with phylogeny, not gliding motility properties, in *Mycoplasma pneumoniae* relatives

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The *Mycoplasma pneumoniae* cluster is a clade of eight described species which all exhibit cellular polarity. Their polar attachment organelle is a hub of cellular activities including cytadherence and gliding motility, and its duplication in the species *M. pneumoniae* is coordinated with cell division and DNA replication. The attachment organelle houses a detergent-insoluble, electron-dense core whose presence is required for structural integrity. Although mutant analysis has led to the identification of attachment organelle proteins, the mechanistic basis for the activities of the attachment organelle remains poorly understood, with gliding motility attributed alternatively to the core or to the adhesins. In this study we investigated attachment organelle-associated phenotypes, including gliding motility characteristics and ultrastructural details, in seven species of the *M. pneumoniae* cluster under identical conditions, allowing direct comparison. We identified gliding ability in three species in which it has not previously been reported, *Mycoplasma imitans*, *Mycoplasma pirum* and *Mycoplasma testudinis*. Across species, ultrastructural features of attachment organelles and their cores do not correlate with gliding speed, and morphological features of cores are inconsistent with predictions about how these structures are involved in the gliding process, disfavouring a prominent, direct role for the electron-dense core in gliding. In addition, we found *M. pneumoniae* to be an outlier in terms of cell structure with respect to its close relatives, suggesting that it has acquired a special set of adaptations during its evolution.

INTRODUCTION

The bacterial genus *Mycoplasma* includes over 100 species. These organisms lack cell walls and in nature are always associated with vertebrate hosts. Several mycoplasma species exhibit a polarized cell morphology, exemplified by *Mycoplasma pneumoniae*, a significant causative agent of assorted respiratory and non-respiratory human disease conditions (Waites & Talkington, 2004). This surface-adherent organism exhibits the pleomorphy typical of its genus, but is characterized by a prosthecal terminal organelle or attachment organelle at one pole (Balish, 2006). This structure is the site at which adhesins are clustered, enabling the cell to interact productively with host cells (cytadhere). The attachment organelle is the leading end of the cell during gliding motility (Balish & Krause, 2006) and the location of the gliding motor (Hassellbring & Krause, 2007). Gliding appears to be important for spreading from the initial infection site (Jordan et al., 2007). Duplication of the *M. pneumoniae* attachment organelle at a site adjacent to the existing one accompanies initiation of DNA replication (Seto et al., 2001).

The cytoplasm within the *M. pneumoniae* attachment organelle lacks DNA, is clear of large particles, and contains a Triton X-100 (TX)-insoluble structure, the electron-dense core (Biberfeld & Biberfeld, 1970), which appears to be connected to an ill-defined network of cytoskeletal filaments present throughout the cell body (Meng & Pfister, 1980; Göbel et al., 1981). Proper assembly of the core is essential for formation of a functional attachment organelle (Krause & Balish, 2004), making the core essential for virulence. The core consists of several subdomains, including a distal terminal button which is probably in direct contact with the attachment organelle tip, a perpendicularly striated double rod, and a proximal complex that includes a bowl-shaped component at the base (Henderson & Jensen, 2006; Seybert et al., 2006). Although studies of *M. pneumoniae* attachment organelle mutants have led to the identification of many attachment organelle proteins and to the outline of an assembly pathway (Krause & Balish, 2004), how each protein contributes to the structure of the core remains poorly understood.
Mycoplasma gliding is poorly understood compared to other prokaryotic forms of motility. The *M. pneumoniae* motility mechanism has been addressed through characterization of mutants with differences in colony-spread- ing phenotypes, resulting in the identification of a moderate number of genes of both known and unknown function (Hasselbring *et al.*, 2006b). In *Mycoplasma mobile*, a distant relative whose adherence, motility and terminal organelle components are unrelated to those of *M. pneumoniae* (Miyata, 2005), gliding is proposed to involve the cyclical binding and release by the adhesin Gl349 (Uenoymaya *et al.*, 2004) of carbohydrate moieties present on a wide variety of host-cell proteins, including those deposited on the surface of the microscope slide from serum *in vitro* (Nagai & Miyata, 2006). A similar binding-and-release process might occur in *M. pneumoniae*, albeit through the use of an unrelated set of adhesins. In support of this hypothesis, the adhesins P30 (Hasselbring *et al.*, 2005) and P1 (Seto *et al.*, 2005a) have both been implicated in gliding motility of *M. pneumoniae*. Alternative hypotheses have focused on the electron-dense core as the major component of the *M. pneumoniae* motor, citing differences in fine features of individual *M. pneumoniae* cores (Henderson & Jensen, 2006) or postulating that the core is capable of twisting within the attachment organelle (Hegermann *et al.*, 2002). Core-centred models require that the morphology of the core would change during motility, such as by shortening or twisting, and one might further predict that organisms with different motile properties would exhibit corresponding differences in the morphology of the core. The ability to distinguish among these models is predicated on an understanding of the relationship between motile properties and attachment organelle features. One approach to the basis for mycoplasma motility is the characterization of relatives of *M. pneumoniae* that have a similar motility mechanism but differing motility characteristics, thereby constituting a series of 'naturally occurring mutants'.

Eight species are currently assigned to the *M. pneumoniae* cluster based on 16S rRNA sequence similarity (Johansson & Pettersson, 2002) and the presence of an attachment organelle that contains a core (Balish & Krause, 2005). Gliding has been described for some of these species, including *M. pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma gallisepticum* and *Mycoplasma amphoriforme*, while *Mycoplasma alvi* has been reported to be non-motile (Bredt, 1979; Hatchel *et al.*, 2006). Mean speeds reported in this cluster range from 50 nm s⁻¹ for *M. amphoriforme* to >300 nm s⁻¹ for *M. pneumoniae* (Hatchel *et al.*, 2006). Homologues of the *M. pneumoniae* attachment organelle proteins required for cytadherence and motility are restricted to the *M. pneumoniae* cluster (Tham *et al.*, 1994; Dhandayuthapani *et al.*, 1999; Papazisi *et al.*, 2002), making it likely that a common fundamental mechanism involving this set of proteins underlies attachment organelle-mediated functions in each of these organisms. For most of these species, detailed analysis of the dimensions of the electron-dense cores has not been performed. Correlating molecular and structural aspects of attachment organelle components with cytadherence and motility-related properties in each species will reveal which components are likely participants in the motility process, directing future research on the mechanistic aspects of mycoplasma gliding motility.

We have therefore examined each species of the *M. pneumoniae* cluster with respect to gliding motility and, for motile species, the morphological features of attachment organelles and cores. Time-lapse microcinematographic analysis revealed gliding in all species of the *M. pneumoniae* cluster except *M. alvi*. However, scanning electron microscopy (SEM) revealed substantial differences in cell and attachment organelle dimensions among all species which, while largely associated with phylogenetic relatedness, correlated poorly with gliding motility characteristics, not supporting a direct role for the core in motility. In addition, SEM revealed evidence of DNA association with the proximal end of the core in most species.

**METHODS**

**Growth and culture conditions.** The strains used in this study were: *M. pneumoniae* strain M129; *M. genitalium* strain G37; *M. gallisepticum* strain B50; *Mycoplasma imitans* strain 4229; *M. amphoriforme* strain A39; *Mycoplasma testudinis* strain 01008; *Mycoplasma pireum* strain 70-159; and *M. alvi* strain Ilsley. Cells were grown in plastic tissue-culture flasks from frozen stocks for 2–10 days at 37 °C (30 °C for *M. testudinis*) in SP-4 broth (Tully *et al.*, 1979) to mid-exponential phase (phenol red indicator was orange). *M. alvi* was also grown in a modified SP-4 formulation containing a 1:1 mixture of fetal bovine and porcine sera (HyClone) and grown until slightly turbid. Motility stocks were prepared as previously described (Hatchel *et al.*, 2006). For *M. testudinis* and *M. pireum*, cells were passed three to five times in SP-4 broth to enrich for plastic-attached subpopulations prior to further use and analysis.

**Phylogenetic tree.** The 16S rRNA sequence for each species was entered into BioEdit Sequence Alignment Editor v. 7.0.5.2 (Hall, 1999) for trimming and neighbour-joining alignment by CLUSTAL_X v. 1.8 (Thompson *et al.*, 1997). The phylogenetic tree was generated from these data by NJPlot (Perrière & Gouy, 1996).

**SEM.** SEM was performed as previously described (Hatchel *et al.*, 2006) with minor modifications. Briefly, stocks were grown in 24-well plates containing glass coverslips for 3 h to 1 day before processing. For analysis of TX-insoluble structures, coverslips were incubated for 30 min at 37 °C in a solution containing 2 % TX in TN buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl). For DNase treatment, cells were grown for 1–4 days on coverslips before processing. TX extraction was followed by a 30 min incubation with 5 U bovine pancreas DNase I (Sigma-Aldrich) in 500 μl 1 % TX reaction buffer (10 mM Tris/HCl, pH 7.6, 2.5 mM MgCl₂, 0.5 mM CaCl₂) at room temperature. For RNase A treatment, TX-extracted coverslips were incubated 30 min in 500 μl TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) containing 100 μg RNase A (Qiagen). For whole cells, coverslips were fixed 30 min in fixative A (1.5 % glutaraldehyde/1 % formaldehyde/0.1 M sodium cacodylate, pH 7.2; *M. pneumoniae, M. genitalium, M. gallisepticum, M. imitans* and *M. pireum*) or fixative B (1 % glutaraldehyde/2 % formaldehyde/0.1 M sodium cacodylate, pH 7.2; *M. amphoriforme* and *M. testudinis*). For visualization of TX-insoluble material, fixative A was used for all species. Dehydration, critical-point
Microsystems). The sample was held at 37°C in a heated chamber. After 3 h incubation at 37°C, cells attached to the slide were visualized using a Leica DM IRB inverted microscope (Leica Microsystems). The sample was held at 37°C in a heated chamber. Twenty-seven consecutive phase-contrast images were taken at 5 kV. Dimensions of whole cells, TX-insoluble structures, and structures following DNase treatment were measured individually using SPOT software (Diagnostic Instruments).

**Time-lapse microcinematographic analysis.** Motility sequences were captured as previously described (Hatchel et al., 2006). Briefly, cells were inoculated into chamber slides containing SP-4+3% gelatin. After 3 h incubation at 37°C, cells attached to the slide were visualized using a Leica DM IRB inverted microscope (Leica Microsystems). The sample was held at 37°C in a heated chamber. Twenty-seven consecutive phase-contrast images were taken at appropriate time intervals and later merged using Adobe Photoshop CS version 8.0. The speed of each cell was computed by measuring the distance travelled and dividing it by the duration of movement. Cells moving in fewer than ten frames were scored motile, but were not included in the calculation of mean speed because below this threshold we regarded the speed measurements as unreliable.

**RESULTS**

**Gliding motility**

Of the eight species found in the *M. pneumoniae* cluster based on 16S rRNA sequences (Fig. 1; Pitcher et al., 2005), only *M. alvi* did not attach to glass and exhibit gliding motility, as previously reported (Bredt, 1979). *M. alvi* did not attach under any conditions we tested, and consequently we were unable to investigate it further. For *M. pirum* and *M. testudinis*, only a small minority of the population derived from the initial stocks attached to glass. In these cases, all further studies were performed on plastic-attached subclones. Efforts to enrich for a similar population of *M. alvi* cells were unsuccessful.

Gliding characteristics, which were previously determined by our method for *M. pneumoniae*, *M. amphoriforme* and *M. gallisepticum*, were assayed for the remaining species (Hatchel et al., 2006). Mean speeds ranged from 28 nm s⁻¹ for *M. pirum* to 2970 nm s⁻¹ for *M. testudinis* (Table 1; see also Supplementary Fig. S1, available with the online version of this paper). *M. testudinis* was the fastest, though with the lowest percentage of motile cells. Although cells glided in the direction of the attachment organelle, large-scale cellular movement appeared generally random for most species. However, ~80% of *M. genitalium* and *M. testudinis* cells moved in nearly circular patterns (not shown). There was no relationship between phylogeny and gliding speed.

**Attachment organelle and cell morphology**

To test whether gliding characteristics correlated with ultrastructural features of the attachment organelle, we analysed both whole cells and TX-insoluble electron-dense cores in each of the seven adherent, motile species by SEM. Attachment organelles were divided into substructures for common reference: the distal moiety was termed the knob, and the proximal portion, the shaft (see Fig. 2h). Dimensions of individual attachment organelles and cells (Table 2) were measured.

*M. gallisepticum* (Fig. 2a), *M. imitans* (Fig. 2b), *M. amphoriforme* (Fig. 2c), and *M. testudinis* (Fig. 2d) exhibited short, wide attachment organelles, with mean lengths from 120 to 150 nm, and widths from 130 to 150 nm (Table 2). The knob was distinct from the shaft in *M. gallisepticum* and *M. amphoriforme*, sometimes in *M.

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**Table 1. Gliding motility parameters at 37°C**

<table>
<thead>
<tr>
<th>Mycoplasma species</th>
<th>Mean speed (nm s⁻¹) ± sd</th>
<th>Range (nm s⁻¹)</th>
<th>Percentage of cells moving per frame (total cells analysed)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. pneumoniae</em> str. M129*</td>
<td>336 ± 59</td>
<td>197–538</td>
<td>51 (149)</td>
</tr>
<tr>
<td><em>M. genitalium</em> str. G37</td>
<td>111 ± 22</td>
<td>62–172</td>
<td>72 (126)</td>
</tr>
<tr>
<td><em>M. gallisepticum</em> str. R0oa*</td>
<td>131 ± 38</td>
<td>50–286</td>
<td>64 (421)</td>
</tr>
<tr>
<td><em>M. imitans</em> str. 4229</td>
<td>107 ± 31</td>
<td>41–194</td>
<td>65 (173)</td>
</tr>
<tr>
<td><em>M. testudinis</em> str. 01008</td>
<td>2970 ± 570</td>
<td>2180–4740</td>
<td>37 (100)</td>
</tr>
<tr>
<td><em>M. amphoriforme</em> str. A39*</td>
<td>49 ± 19</td>
<td>15–133</td>
<td>53 (1034)</td>
</tr>
<tr>
<td><em>M. pirum</em> str. 70-159</td>
<td>28 ± 8</td>
<td>12–58</td>
<td>45 (289)</td>
</tr>
</tbody>
</table>

*Data from Hatchel et al. (2006).
Fig. 2. Scanning electron micrographs of mycoplasma cells grown on glass coverslips. Three representative cells are shown for each species. Images are aligned so that the attachment organelle is at the top. (a) *M. gallisepticum*; (b) *M. imitans*; (c) *M. amphoriforme*; (d) *M. testudinis*; (e) *M. pireum*; (f) *M. genitalium*; (g) *M. pneumoniae*; (h) schematic of mycoplasma cell. Open and black arrows indicate a ridge-like feature at the base of the attachment organelle shaft in *M. gallisepticum* and *M. amphoriforme* respectively; white arrows indicate striations on the *M. pireum* attachment organelle; black arrowheads indicate the curvature of *M. testudinis* and *M. genitalium* attachment organelles. Scale bar, 250 nm.
attachment organelles were curved an average of 10° ± 10° from the long axis of the cell (Fig. 2f), and the knob, prominent in this species, was off-centre in the direction of this curvature.

Measurements of cell bodies revealed a positive correlation between cell width and attachment organelle width (Table 2). M. pneumoniae and M. pirum cells were distinctly narrow in comparison to the others (Fig. 2e, g; Table 2). In contrast, cell length appeared unrelated to other features (Table 2). M. pneumoniae cells were unique in the prominence of the trailing filament found at the pole opposite the attachment organelle (Fig. 2g). This structure, whose length varied greatly, drove the mean length of the M. pneumoniae cell to 1500 nm (Table 2). M. gallisepticum and M. amphoriforme cell bodies often had a ridge-like feature in the vicinity of the base of the attachment organelle shaft (Fig. 2a, c).

### Cell division

We observed SEM fields of cells from each of the seven adherent species of this cluster (Fig. 3) to characterize cells that appeared to be in the process of dividing. Either the appearance of two attachment organelles at the same pole on a single cell or two attachment organelles pointing in different directions was taken to indicate that cell division was in progress. All species except M. imitans (Fig. 3b) were observed to have cells exhibiting one or both of these phenotypes. Dividing M. pirum cells were only found to have attachment organelles at opposite poles (Fig. 3e), and in M. gallisepticum, adjacently paired attachment organelles were not observed (Fig. 3a), suggesting that in these species the new attachment organelle might not form immediately adjacent to the old one as in M. pneumoniae.

### TX-insoluble structures

Substructures of the electron-dense core of the attachment organelle, namely the terminal button, the rod, the base and a tuft of fibrous material (Fig. 4h; Hatchel et al., 2006), have been described. Although we previously had difficulty resolving structures in M. gallisepticum (Hatchel et al., 2006), the use of a fresh preparation of TX resulted in consistent observation of core-like structures in all seven species (Fig. 4).

Rod length was conserved across species over a narrow range from 130 to 170 nm; inclusion of the terminal button raised the values to 180–240 nm (Table 3). However, rod width varied substantially among species, from 30 to 80 nm (Table 3), and the variation correlated well with phylogenetic relatedness. The length of the M. imitans rod (Table 3) is underestimated on account of being variably obscured by the unusual base (see below). The slight difference between the current results and those previously reported for rod width in M. amphoriforme might be due to the use of a different fixative preparation (Hatchel et al., 2006). Terminal button length and width varied somewhat across species, and although the variation appeared unrelated to gliding speed, width appeared to have a phylogenetic component, with M. pneumoniae and M. genitalium exhibiting the narrowest terminal buttons (Table 3). The M. pneumoniae terminal button was frequently difficult to distinguish from the rod (Fig. 4g), as previously described (Hatchel et al., 2006). The M. genitalium rod exhibited a pronounced lateral curvature, and the terminal button was consistently offset in the direction of this curvature (Fig. 4f). Curvature of the electron-dense core was also visible in ~25% of M.

**Table 2. Whole-cell dimensions (nm) ± SD, mean of 30 cells**

<table>
<thead>
<tr>
<th>Whole-cell length</th>
<th>Cell body</th>
<th>Attachment organelle</th>
<th>Knob</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
<td></td>
</tr>
<tr>
<td>M. pneumoniae</td>
<td>1500 ± 420</td>
<td>520 ± 90</td>
<td>220 ± 30</td>
</tr>
<tr>
<td>M. genitalium</td>
<td>590 ± 50</td>
<td>420 ± 50</td>
<td>280 ± 50</td>
</tr>
<tr>
<td>M. gallisepticum</td>
<td>830 ± 120</td>
<td>710 ± 110</td>
<td>330 ± 70</td>
</tr>
<tr>
<td>M. imitans</td>
<td>620 ± 50</td>
<td>500 ± 50</td>
<td>350 ± 30</td>
</tr>
<tr>
<td>M. amphoriforme</td>
<td>770 ± 70</td>
<td>620 ± 70</td>
<td>360 ± 60</td>
</tr>
<tr>
<td>M. testudinis</td>
<td>680 ± 100</td>
<td>560 ± 100</td>
<td>280 ± 20</td>
</tr>
<tr>
<td>M. pirum</td>
<td>720 ± 110</td>
<td>480 ± 90</td>
<td>230 ± 20</td>
</tr>
</tbody>
</table>

**ND, Not determined because knob and shaft were not uniformly distinguishable.**
testudinis cells, though in this species the curvature was more abrupt and located close to the terminal button (Fig. 4d). Although we could not image the non-adherent M. alvi, it is known to have an electron-dense core (Gourlay et al., 1977).

Except in M. pneumoniae, the base of the electron-dense core was obscured by irregular material. Treatment of adherent TX-insoluble fractions with DNase I resulted in highly consistent loss of this material, exposing the base of the core (Fig. 4). Incubation in buffer alone had no effect,
nor did treatment with RNase A (Fig. 4i). Pronase caused loss of all visible structures (data not shown). In *M. genitalium* (Fig. 4f) and *M. amphoriforme* (Fig. 4c) the DNase-sensitive material was predominantly filamentous, with some clumpier regions; in *M. gallisepticum* (Fig. 4a), *M. imitans* (Fig. 4b), and *M. testudinis* (Fig. 4d) this mass was mostly nodular, with occasional filaments; and in *M. pirum* (Fig. 4e) the material had a smooth appearance with some short filaments extending from it. These filaments are consistent with the appearance of loops of DNA that have been locally denuded of protein (Cunha et al., 2001). Taken together, these data are consistent with the identification of the core-associated material as DNA, rendered condensed by treatment with detergent and salt (Cunha et al., 2001), although whether the interaction between the DNA and the core is physiologically relevant is unclear. Treatment with DNase had no significant effects on measurements of other core substructures, except for occasional lengthening of the rod due to its being less obscured.

The dimensions of the exposed bases were highly variable across species (Table 3; Fig. 4). The base of *M. pneumoniae* was by far the smallest in both length and width. At the other extreme was *M. imitans*, which had a very large and highly irregularly shaped base (Fig. 4b). The DNase-treated bases of *M. gallisepticum* and *M. imitans* consistently had a notch-like feature of irregular orientation (Fig. 4a, b), suggesting that the removal of DNA was equally complete in each species. No relationship between base dimensions and gliding speed or phylogeny was apparent across species. In fact, although attachment organelle morphology generally correlated well with phylogeny, no relationship was detected between gliding speed and any element of attachment organelle morphology.

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**Fig. 4.** Scanning electron micrographs of mycoplasma electron-dense cores. Under ‘TX only’, cells were grown on glass coverslips and extracted with 2 % TX for 30 min at 37 ºC (see Methods). Under ‘TX+DNase I’, cells were grown on glass coverslips, extracted with TX as above, and then treated with DNase I for 30 min at room temperature (see Methods). Two representative cores are shown for each species with and without DNase treatment, with terminal buttons aligned at the tops of images. (a) *M. gallisepticum*; (b) *M. imitans*; (c) *M. amphoriforme*; (d) *M. testudinis*; (e) *M. pirum*; (f) *M. genitalium*; (g) *M. pneumoniae*; (h) schematic of the mycoplasma electron-dense core substructures; (i) *M. gallisepticum* treated with TX and then RNase A. Arrows point to notches in *M. gallisepticum* and *M. imitans* bases. Scale bar, 250 nm.
M. imitans, the addition of total number of motile mycoplasma species to nine with M. testudinis among mycoplasmas (Miyata, 2005). Its ultrastructure and mobile but interestingly, the motile cells glided as rapidly as testudinis but that it and M. mobile have evolved, perhaps under similar unidentified pressures, to glide rapidly. Alternatively, despite its close relatedness to M. mobile, whose gliding speed was previously unchallenged among mycoplasmas (Miyata, 2005). Its ultrastructure and phylogenetic position suggest that M. testudinis has a mechanistically similar motor to that of its close relatives, but that it and M. mobile have evolved, perhaps under similar unidentified pressures, to glide rapidly. Alternatively, despite its close relatedness to M. pneumoniae and its similar appearance to its relative, M. testudinis might have acquired a different mechanism for motility, perhaps through horizontal gene transfer. However, the absence of a relationship between the motor mechanism and gliding speed is observed in Mycoplasma pulmonis, which appears to have the same gliding machinery as M. mobile (Seto et al., 2005b), despite its mean speed being much slower (Bredt & Radestock, 1977).

Aside from the curvature of the rod in relation to large-scale near-circular movement in M. genitalium and M. testudinis, no measured dimension of the attachment organelle appeared to correlate with gliding characteristics. Curved paths were previously indicated for M. genitalium (Pich et al., 2006). The curvature of these structures is quite different in the two species, with the rod of the M. genitalium core exhibiting a shallow curvature throughout (Fig. 4f), and that of the M. testudinis core, when curved, being sharply bent at a single location (Fig. 4d). Importantly, this curvature is distinct from the bend observed in M. pneumoniae cores in cells that are not attached to the substrate (Henderson & Jensen, 2006). We do not observe this bend under our conditions, as confirmed by high-angle tilt SEM (not shown), perhaps because when attached to the substrate the attachment organelle is under tension.

In no case did we observe cores that were twisted, as predicted by one model (Hegermann et al., 2002). Although the M. pneumoniae core is clearly suggested to have a broad and a narrow side (Regula et al., 2001; Hegermann et al., 2002; Henderson & Jensen, 2006), there was little variation in rod width within any species. The inchworm model, an alternative model for core function during gliding (Henderson & Jensen, 2006), invokes repeated contraction and expansion of the core along its length, which might have been borne out by substantial differences in rod length of cells within a single species. However, except for M. imitans, whose rod was difficult to measure because of obscuration by the large, irregular base, distributions of the length of the terminal button plus the rod were not dramatic. It is possible that the degree of contraction is too small to be measured by our techniques, but also possible that contraction does not occur. Also, observations of gliding cells did not suggest any quantized movement, though the step size could be smaller, or the steps faster, than our ability to detect. The consistency in the dimensions of cores within each species leads us to propose that the electron-dense core, though an important determinant in attachment organelle biogenesis, is not especially dynamic, and not directly involved in motility.

### Cell division

Whether or not the electron-dense core has direct involvement in gliding motility, it might well play a direct role in some other process. Its roles in normal attachment organelle formation and adhesin localization in M. pneumoniae are well documented (Krause & Balish, 2004), though other core-lacking mycoplasmas such as M. mobile (Shimizu & Miyata, 2002) form head-like structures that appear to function analogously to attachment organelles. The special relationship between attachment organelle formation and DNA replication in M. pneumoniae might highlight a role for the electron-dense

### Table 3. Electron-dense core dimensions (nm) ± SD, mean of 30 cells

<table>
<thead>
<tr>
<th></th>
<th>Terminal button</th>
<th>Rod</th>
<th>Terminal button + rod</th>
<th>Base</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length Width</td>
<td>Length Width</td>
<td>Length Width</td>
<td></td>
</tr>
<tr>
<td>M. pneumoniae</td>
<td>ND 60 ± 10</td>
<td>ND 50 ± 10</td>
<td>240 ± 20</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>M. genitalium</td>
<td>50 ± 10 60 ± 20</td>
<td>160 ± 20 50 ± 10</td>
<td>220 ± 20</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>M. gallisepticum</td>
<td>90 ± 10 90 ± 20</td>
<td>140 ± 30 30 ± 0</td>
<td>230 ± 30</td>
<td>120 ± 40</td>
</tr>
<tr>
<td>M. imitans</td>
<td>60 ± 20 60 ± 20</td>
<td>130 ± 60 40 ± 10</td>
<td>180 ± 70</td>
<td>240 ± 60</td>
</tr>
<tr>
<td>M. amphoriforme</td>
<td>70 ± 10 90 ± 20</td>
<td>160 ± 20 60 ± 10</td>
<td>220 ± 20</td>
<td>90 ± 20</td>
</tr>
<tr>
<td>M. testudinis</td>
<td>80 ± 10 80 ± 10</td>
<td>170 ± 20 80 ± 10</td>
<td>240 ± 20</td>
<td>150 ± 40</td>
</tr>
<tr>
<td>M. pirum</td>
<td>90 ± 10 90 ± 10</td>
<td>130 ± 10 80 ± 10</td>
<td>210 ± 10</td>
<td>100 ± 20</td>
</tr>
</tbody>
</table>

ND, Not determined because rod and terminal button were difficult to distinguish.
core in coordination between the two events. An increase in the amount of cellular DNA is accompanied by appearance of a second attachment organelle adjacent to the first one in *M. pneumoniae*, and the distance between the two organelles increases with continually increasing DNA levels (Seto et al., 2001). Furthermore, time-lapse microcinematography of dividing *M. pneumoniae* cells reveals that a new attachment organelle generally appears adjacent to the old one, and the new one anchors the cell in place while the old one glides away from it (Hasselbring et al., 2006a), resulting in the two structures being present at opposite poles. Finally, in *M. gallisepticum*, newly synthesized DNA is specifically enriched in a biochemical fraction itself enriched for attachment organelles (Maniloff & Quinlan, 1974). Although it could be artefactual, the physical interaction observed in this study between the cellular DNA and the electron-dense core is intriguing and will be a subject of future studies.

**Evolution**

Clearly, the electron-dense core and the attachment organelle, as well as gliding motility, were present in the last common ancestor of the *M. pneumoniae* cluster; these features were presumably conferred by acquisition of the set of cytadherence-associated genes present in *M. pneumoniae* and its relatives from an unidentified source. The considerable lengths of the *M. pneumoniae* and *M. pirum* structures appear to have arisen independently, since the *M. genitalium* attachment organelle is not much longer than that of *M. amorphiforme*. Curvature in *M. genitalium* and *M. testudinis* also appears to have developed independently, as discussed above. Thus, the progenitor of these species most likely harboured a short, wide attachment organelle like that of *M. gallisepticum* and *M. amorphiforme*. As the knob is markedly reduced only in *M. pneumoniae*, it was probably prominent in the common ancestor. In addition, the trailing filament appears to be unique to *M. pneumoniae* and therefore unlikely to have been present in the parent species. Finally, the presence of faster gliding only in *M. pneumoniae* and especially *M. testudinis* suggests that slower motility was probably ancestral.

*M. pneumoniae* appears to be the most derived member of its cluster with respect to attachment organelle and other ultrastructural phenotypes. Its speed is not close to that of any of its relatives, and the reduction in the size of the distal elements of the structure is likely an adaptation to an unknown pressure. Numerous proteins involved in gliding are apparently unique to the clade containing *M. pneumoniae* and *M. genitalium* (Hasselbring et al., 2006b), and it is conceivable that some of these proteins are involved in processes special to one or both of these organisms. Thus, extrapolation of *M. pneumoniae* attachment organelle-related data to other species ought to be applied cautiously. In contrast, *M. amorphiforme* might be the most primitive, with its slow speed and average dimensions; as such, it might constitute a more general model for attachment organelle function in species of the *M. pneumoniae* cluster.

**ACKNOWLEDGEMENTS**

This work was supported by funds from Miami University. SEM was performed at the Miami University Electron Microscopy Facility. We thank the following for strains: L. Duffy and K. Waites, University of Alabama-Birmingham (*M. genitalium*); S. Kleven, University of Georgia (*M. imitans*); M. Brown, University of Florida (*M. testudinis*); M. Davidson, Mollicutes Culture Collection, Purdue University (*M. alvi*, *M. pirum*). Thanks also to R. Balish and E. Bridge for suggestions about the manuscript.

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Edited by: C. Citti