Protein P200 Is Dispensable for *Mycoplasma pneumoniae* Hemadsorption but Not Gliding Motility or Colonization of Differentiated Bronchial Epithelium


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*Mycoplasma pneumoniae* protein P200 was localized to the terminal organelle, which functions in cytadherence and gliding motility. The loss of P200 had no impact on binding to erythrocytes and A549 cells but resulted in impaired gliding motility and colonization of differentiated bronchial epithelium. Thus, gliding may be necessary to overcome mucociliary clearance.

*M. pneumoniae* causes tracheobronchitis, bronchopneumonia, and a variety of extrapulmonary manifestations in humans (35). A distinct polar structure (5, 18) mediates host cell attachment (cytadherence) by this novel cell wall-less prokaryote. Electron micrographs of experimentally infected human and animal cells, as well as cells collected from natural *M. pneumoniae* infections, reveal an intimate relationship between this terminal organelle and the epithelial cell surface (7). The terminal organelle also mediates gliding motility (16, 29), which may facilitate mycoplasma traversal of the airway surface liquid to allow access to host receptors on the apical surface of respiratory epithelium and refuge from the turbulent mucociliary escalator.

*M. pneumoniae* binds erythrocytes (hemadsorption [HA]), and while not thought to encounter erythrocytes typically during infection, a limited correlation exists between HA and virulence (14, 25, 32). Characterization of HA mutants (Table 1) has identified a number of proteins associated with cytadherence, including HMW1, HMW2, HMW3, P65, and adhesins P1 and P30 (13, 14, 22, 24). Proteins HMW1, HMW2, HMW3, and P65 are mostly or entirely insoluble in the detergent Triton X-100 (TX) (3), a parameter that serves as a working definition for cytoskeletal elements of eukaryotic cells (3, 21). HMW1, HMW3, and P65 also contain acidic proline-rich (APR) domains, which are highly repetitive sequences of variable length and uncertain function (2).

**TABLE 1. *M. pneumoniae* strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Proteins absent</th>
<th>Proteins reduced or altered</th>
<th>Analysis</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>M129</td>
<td>None (wild type)</td>
<td>None</td>
<td>None</td>
<td>++ +</td>
<td>15, 17</td>
</tr>
<tr>
<td>I-2</td>
<td>MPN310</td>
<td>HMW2</td>
<td>HMW1, HMW3, P65, P41, P30, P28, P24</td>
<td>++ +</td>
<td>9, 22</td>
</tr>
<tr>
<td>II-3</td>
<td>MPN453</td>
<td>P30</td>
<td>–</td>
<td>–</td>
<td>4, 15, 22, 30</td>
</tr>
<tr>
<td>III-4</td>
<td>MPN142</td>
<td>B, C</td>
<td>–</td>
<td>–</td>
<td>17, 22, 36</td>
</tr>
<tr>
<td>IV-22</td>
<td>MPN141</td>
<td>P1, B, C</td>
<td>–</td>
<td>–</td>
<td>17, 22, 33</td>
</tr>
<tr>
<td>M6</td>
<td>MPN447 and MPN453</td>
<td>HMW1</td>
<td>HMW2 (P30 truncated)</td>
<td>–</td>
<td>17, 24</td>
</tr>
<tr>
<td>201G</td>
<td>MPN452::Tn4001</td>
<td>HMW3, P200</td>
<td>P65</td>
<td>–</td>
<td>37; this study</td>
</tr>
<tr>
<td>201GR1</td>
<td>MPN567::IS256</td>
<td>P200</td>
<td>–</td>
<td>++ +</td>
<td>37; this study</td>
</tr>
</tbody>
</table>

* ***+, Like wild type; +, substantially less than wild type; −, none; NT, not tested.*
TX-insoluble protein P200 likewise contains an APR domain, as well as six imperfect repeats of a 31- to 33-amino-acid motif of undetermined function, designated the EAGR (for enriched in aromatic and glycine residues) box (2, 28) (Fig. 1A). This motif is also found in HMW1 and an uncharacterized APR-domain-containing protein in \textit{M. pneumoniae} but is otherwise found in no other proteins to date except orthologs in other mycoplasma species (2). The deduced sequence and biochemical features of P200 suggest it might also be a terminal organelle protein with a role in cytadherence (3). Here we determined the subcellular location of P200 and characterized a mutant lacking P200.

We localized P200 by immunofluorescence microscopy as described elsewhere (19) except that cells incubated on coverslips for 3 h were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and blocked using PBS–5% (wt/vol) powdered milk–0.02% NaN₃–0.05% Tween 20 (pH 7.2), and primary antibody was used at 1:500. Cells of the wild-type \textit{M. pneumoniae} strain (26) generally exhibited a single polar fluorescent focus corresponding to the terminal organelle (Fig. 2). In contrast, a patchy fluorescence pattern was observed with the cytadherence mutants M6, I-2, II-3, III-4, and IV-22; this patchy distribution appeared to be more pronounced for mutant IV-22, suggesting a possible relationship between P1 and P200, but additional studies are required to examine this relationship.
further. However, to our surprise, no fluorescence was observed in the HMW3− mutant 201G or HMW3+ revertant 201GR1, described by us previously (37).

Western immunoblotting (11) revealed that P200 was present at wild-type levels in each mutant examined except 201G and 201GR1, the latter hereafter referred to as the P200− mutant (Fig. 1B). The MPN567 allele encoding P200 from this mutant was sequenced utilizing templates from multiple overlapping PCR encompassing the entire gene from mycoplasma genomic DNA. Sequence analysis revealed an IS256 insertion at a site corresponding to residue 657 (of 1,036; Fig. 1C) and an adjacent 8-bp duplication (not shown).

The terminal organelle in wild-type M. pneumoniae has a characteristic electron-dense core (5), which is atypical in mutant 201G, often separating at the proximal end to yield a chevron shape (37). Cells were examined by transmission electron microscopy as described previously (37), except that fixed mycoplasmas were enrobed in 3% noble agar at 58 to 60°C before postfixation, and samples were embedded in Epon-Araldite plastic by using flatbed molds and allowed to polymerize at 75°C overnight. The electron-dense cores in the P200− mutant were indistinguishable from the wild type and distinct from the atypical cores found in mutant 201G (Fig. 3), indicating that the loss of P200 had no obvious impact on the appearance of the core and confirming that the core defect in the HMW3− mutant (37) was indeed a result of the loss of HMW3.

M. pneumoniae cells glide intermittently in the direction of the terminal organelle (29). We examined cell gliding as described previously (15), with the P200− mutant exhibiting both a slower velocity and a higher percentage of time resting than the wild type (Table 2). Impaired gliding was not a function of glass binding, which for the mutant was at least as high as the wild type (data not shown).

In order to assess the requirement for P200 in cytadherence, we measured the binding of radiolabeled mycoplasmas to erythrocytes in suspension (15) and to the A549 human lung adenocarcinoma cell line in submerged culture. Briefly, A549 cells were plated at 5 × 10⁵ cells/cm² on Transwell-clear culture inserts (12 mm, 0.4-μm pore size; Costar, Cambridge, MA) thin coated with rat-tail collagen type I (Collaborative Research, Bedford, MA) and fed apically and basally in F12K medium (Gibco-BRL, Rockville, MD) with 10% fetal bovine serum but without antibiotics for 4 to 5 days until the monolayer was confluent. The apical medium was removed, the apical surface was washed with 500 μl of prewarmed Hanks balanced salt solution (HBSS; Sigma Chemical, St. Louis, MO), and radiolabeled mycoplasmas in 150 μl of Hayflick medium were added, followed by incubation for 4 h. The apical liquid was then gently aspirated, and the membranes were washed four times with PBS, kept overnight at ambient temperature to dry, separated from the plastic Transwell supports, added to scintillation vials containing 300 μl of 1% sodium dodecyl sulfate, incubated overnight at 37°C, and processed for liquid scintillation counting. The P200− mutant was indistinguishable from wild-type M. pneumoniae with respect to binding to erythrocytes or A549 cells (Fig. 4A and B).

We also examined the ability of the P200− mutant to colonize NHBE cells in air-liquid interface culture (23). When cultured as described, NHBE cells have functional cilia, secrete mucins, and establish tight junctions (10), unlike A549 cells (38). NHBE cells were cultured submerged on Transwell inserts as described above for 5 to 7 days, the apical medium was removed, and incubation was continued with the cells exposed to medium only on the basal surface. Prior to infection cells were washed on the apical surface with 500 μl of prewarmed HBSS to remove excess mucus and then infected immediately or after incubation for the indicated times. Mucin produced by NHBE cells accumulates in the Transwell inserts over time. To control for mucus depth, we tested M. pneumoniae colonization at different time points after washing the NHBE cell apical surface. The P200− mutant colonized NHBE cells at substantially lower levels than did wild-type M. pneu-

![FIG. 3. Transmission electron micrographs of thin sections of wild-type M. pneumoniae (A), 201G (B), and 201GR1 (C). Note the chevron shape to the core in panel B. Scale bar, 100 nm.](https://example.com/fig3.jpg)

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**TABLE 2. Characterization of cell gliding by wild-type M. pneumoniae, P200− mutant 201GR1, and 201GR1 complemented with the recombinant wild-type P200 allele.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean ± SD*</th>
<th>Corrected gliding velocity (μm/s)</th>
<th>% Time resting</th>
<th>Sample size (no. of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.29 ± 0.01</td>
<td>29.0 ± 2</td>
<td></td>
<td>62</td>
</tr>
<tr>
<td>201GR1</td>
<td>0.11 ± 0.01*</td>
<td>46.0 ± 4*</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>201GR1 + P200</td>
<td>0.25 ± 0.04</td>
<td>35.0 ± 14</td>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>

* *, P < 0.05 relative to the wild type.
moniae (Fig. 4C). Mycoplasma attachment dropped with length of incubation after washing, corresponding to increased mucus depth, but at similar rates for wild-type and mutant M. pneumoniae.

We cloned a 3.8-kbp FspI fragment containing the MPN567 gene encoding P200 from wild-type M. pneumoniae chromosomal DNA into the Smal site in a Tn4001 derivative within pKV104 (12). The resulting construct was confirmed by PCR sequencing (Integrated Biotech Laboratories, University of Georgia) and electroporated into M. pneumoniae for transposon delivery; transformants were cultured in Hayflick medium plus 10 or 24 μg of chloramphenicol/ml (12). The recombinant MPN567 allele produced P200 at wild-type levels (data not shown) and restored gliding velocity and resting frequency in the P200 mutant (Table 2), and these transformants colonized NHBE cells at wild-type levels (Fig. 4).

Given its TX insolubility and similarity in structure to other M. pneumoniae cytadherence-associated proteins, it came as no surprise that P200 localized to the terminal organelle in wild-type M. pneumoniae and had an altered subcellular localization in several cytadherence mutants. For these reasons, it was therefore surprising that P200 was dispensable for HA and binding to A549 cells. Nevertheless, this finding is consistent with a recent report that the P200 ortholog in the closely related Mycoplasma genitalium is likewise not required for HA (27).

At least two models might account for the impaired colonization of NHBE cells by this mutant. In the first model, P200 might function as an adhesin that binds receptors absent from erythrocytes and A549 cells but present on NHBE cells. In support of this model, preliminary analysis of protease sensitivity suggests that P200 is surface accessible (data not shown), although P200, like P65 and HMW1, has no obvious signal or transmembrane sequences. Clearly, the presence of supplementary receptor-binding mechanisms and the complexity of mycoplasma-host cell interactions has been documented (1, 8, 20).

Alternatively, the mucociliary activity of differentiated NHBE cells cultured as described presents a formidable barrier to mycoplasma attachment not found with erythrocytes and A549 cells. Estimates of the thickness of the mucus layer over airway epithelium vary from 5 to 7 μm to perhaps as much as 10-fold higher, while the upward flow rate toward the trachea is approximately 4.2 to 7 mm/min (6, 31, 34). The NHBE cell cultures used here produced approximately 1.25 μl of mucus/h/cm², and ciliary action swept fluorescent beads to the periphery in Transwell inserts (data not shown). Therefore, we favor a model that affords an explanation whereby impaired adhesion is concomitant with mucus depth and mucociliary activity.

FIG. 4. Quantitation of adherence to erythrocytes (A), A549 cells (B), and differentiated NHBE cells in air-liquid interface culture (C) by wild-type M. pneumoniae, the P200− mutant (201GR1), and the P200− mutant producing recombinant P200 (P200− + MPN567). In each case, % WT refers to the (percent attachment of mutant/percent attachment of wild type) × 100; the percent attachment is defined as follows: (cpm bound/total cpm added) × 100. Samples were tested in triplicate for binding to erythrocytes and in quadruplicate for binding to A549 and NHBE cells. For panel C, the adherence was assessed at the indicated time points after the accumulated mucus was removed and is expressed relative to the wild type at the 0-h time point. Error bars indicate the standard deviation.
gliding by the P200 mutant limited its ability to access NHBE cell receptors. While attachment by both wild-type and mutant mycoplasmas decreased as mucus accumulated on the apical surface, the P200 mutant was not impacted more severely than the wild type; thus, the mucus barrier alone may not account for impaired colonization by the mutant. Analysis of other gliding-deficient mutants and the spatial and temporal aspects of NHBE cell colonization are required to elucidate further the relationship between gliding and colonization of the respiratory mucosa.

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REFERENCES