ABSTRACT

MYCOPLASMA PNEUMONIAE PROTEIN P30: STABILITY, INTERACTIONS, AND FUNCTION

by Hailey Erin Riggs

Mycoplasma pneumoniae infections are a leading cause of community-acquired pneumonia. There has been a recent surge in the incidence of antibiotic-resistant M. pneumoniae infections. Consequently, developing new targets to fight infections has become a priority. Both cytadherence and gliding motility are required for M. pneumoniae to be pathogenic, but their underlying mechanisms are unclear. These processes are mediated through the attachment organelle, a membrane-bound projection of the cell. Transmembrane protein P30 localizes to the tip of the attachment organelle and is required for both processes. The C-terminal region of P30 contains a series of imperfect proline-rich repeats (PRRs). Mutant P30 lacking these PRRs is unstable. We hypothesize that the PRRs of the C-terminal region of P30 contribute to the stability and functions of P30 through facilitating interactions with itself and/or other proteins. Chemical cross-linking and immunoblot analysis were performed to determine the binding partners of P30. Site-directed mutagenesis was used to identify roles of specific proline residues in the stability, structure, and functions of P30. This work demonstrated that P30 is present in putative homomeric and heteromeric complexes, and there is a complicated relationship between the proline residues and their roles in the structure and function of P30.
MYCOPLASMA PNEUMONIAE PROTEIN P30: STABILITY, INTERACTIONS, AND FUNCTION

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by

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This Thesis titled

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INTRODUCTION

*Mycoplasma pneumoniae*, a member of the Mollicutes class of bacteria, has a significantly reduced genome and lacks a cell wall. With a genome size of 816 kilobases, *M. pneumoniae* lacks many metabolic capabilities (Himmelreich et al., 1997). This organism survives by obtaining nutrients from its host. *M. pneumoniae* is a human pathogen and a leading cause of community-acquired pneumonia. Though these infections typically occur in children, 100,000 adults in the United States are hospitalized annually due to *M. pneumoniae*. Infections with this pathogen can also cause bronchitis, lead to chronic lung disease, or develop into extrapulmonary disease and autoimmune conditions such as Stevens-Johnson syndrome (Atkinson et al., 2008).

Due to *M. pneumoniae* lacking a cell wall, classes of antibiotics that target peptidoglycan are ineffective at treating *M. pneumoniae* infections. Macrolides, tetracyclines, and fluoroquinolones are typically used to treat infections, but macrolides are most commonly used as the use of tetracyclines and fluoroquinolones in children is avoided due to side effects (Bébéar et al., 2011). Moreover, there has been a recent surge in incidence of macrolide resistance. In some regions of Japan and China, over 90% of *M. pneumoniae* isolates are macrolide resistant (Waites et al., 2017). Developing new therapeutics to treat these infections has become a priority. Various foci have been proposed as targets for developing narrow-spectrum antibiotics, including virulence-associated traits used by *M. pneumoniae* to cause disease (Balish & Distelhorst, 2016).

*M. pneumoniae* uses multiple virulence factors to contribute to disease. During infection, this organism damages host tissue through the actions of reactive oxygen species and an ADP-ribosylating toxin to disseminate and to obtain nutrients (Kannan & Baseman, 2006). Importantly, these virulence factors cannot damage host tissue unless *M. pneumoniae* cells have direct contact with the host epithelial layer (Collier et al., 1969; Hu et al., 1975; Hu et al., 1976; Kannan & Baseman, 2006). *M. pneumoniae* is able to cytadhere, or attach to host cells, by binding sialylated glycoproteins (Chandler et al., 1982). Once *M. pneumoniae* cells are attached, they are able to perform gliding motility, or smooth movement across a surface. This type of motility has been characterized in many different bacteria using various unrelated mechanisms. For example, in *Myxococcus xanthus*, the bacteria secrete polysaccharide slime to aid in movement (Yu & Kaiser, 2007).
contain proteins that cycle along a helical track on the cell body and create a propulsion force when they come in contact with the slime-coated gliding surface beneath them (Shapiro et al., 2002; Nan et al., 2011). *Mycoplasma* species that exhibit gliding motility lack the components present in *M. xanthus*, indicating that these species use vastly different mechanisms.

The gliding mechanisms of some mycoplasma species, like *Mycoplasma mobile*, have been studied. In these organisms, both cytadherence and gliding motility are mediated by a structure called the attachment organelle. The attachment organelle is a membrane-bound extension of the cell and the leading end of the cell during gliding motility (Balish & Krause, 2006). In *M. mobile*, three large extracellular proteins involved in gliding motility, Gli349, Gli521, and Gli123, localize to the base of the attachment organelle (Uenoyama et al., 2004). One of the proteins, Gli349, acts as a "leg" and cycles through directional binding and releasing of the gliding surface, powered by energy derived from ATP hydrolysis by other proteins. Propulsion force is potentially generated through a cycle of conformational changes to Gli349 (Tanaka et al., 2016). The other attachment organelle proteins, Gli123 and Gli521, are likely responsible for positioning gliding machinery at the base of the attachment organelle and transmitting energy from cytoplasmic ATPase to Gli349 to drive conformational changes in Gli349, respectively (Seto et al., 2005; Uenoyama et al., 2005).

Knowledge of *M. mobile* motility is unlikely to provide insight into the mechanisms behind *M. pneumoniae* gliding motility, as mycoplasma species that exhibit gliding motility, such as *M. mobile*, *Mycoplasma penetrans*, *Mycoplasma insons*, and *M. pneumoniae* use compositionally different tip structures (Relich et al., 2009; Miyata, 2010; Jurkovic et al., 2012; Nakane et al., 2015; Distelhorst et al., 2017). The distribution of these similar structures with varying compositions indicates that they may have arisen through various mechanisms and are a result of convergent evolution (Relich et al., 2009; Jurkovic et al., 2012; Distelhorst et al., 2017).

Although the mechanism behind motility and cytadherence in *M. pneumoniae* differs from that of *M. mobile*, the cells likewise contain attachment organelles that serve as the centers for motility and cytadherence machinery. The attachment organelle of *M. pneumoniae* consists of an electron-dense core surrounded by electron-lucent material (Seto & Miyata, 2003). Like cytoskeletal proteins of eukaryotic cells, the cytoskeletal
proteins that form the electron-dense core of the attachment organelle are insoluble in nonionic detergents such as Triton X-100 (Biberfeld & Biberfeld, 1970). The electron-dense core of the *M. pneumoniae* attachment organelle is composed of three distinct regions (Figure 1). The proximal end of the core is a bowl-shaped structure, which appears to interact with the DNA of the cell (Hatchel & Balish, 2008). It has been proposed that in addition to function in cytadherence and motility, the attachment organelle and its components may play a role in cell division (Balish, 2014). The bowl complex is attached to two parallel rod-like structures called the paired plates. At the distal end of the paired plates is the terminal button, which is likely in direct contact with the tip of the attachment organelle (Henderson & Jensen, 2006; Seybert *et al.*, 2006). Proteins responsible for generation of new attachment organelles, cytadherence, and motility localize to the attachment organelle and its electron-dense core.

Fluorescent protein tagging has revealed localization of attachment organelle proteins to specific parts of the electron-dense core (Figure 1). P24, TopJ, P200, P41, MPN387, and HMW2 localize to the proximal bowl complex (Nakane *et al.*, 2015). P24 plays a role in the gliding frequency of *M. pneumoniae* cells while P41 contributes to gliding speed and location for formation of new attachment organelles (Hasselbring & Krause, 2007a,b). Co-chaperone TopJ functions in the assembly and formation of the attachment organelle (Cloward & Krause, 2010). The paired plates are composed of HMW1, HMW2, and HMW3 (Nakane *et al.*, 2015). HMW1 and HMW2 are mutually stabilizing, as loss of one results in reduced steady-state levels of the other (Krause *et al.*, 1982; Willby *et al.*, 2004). The terminal button is comprised of P65 and HMW3 (Nakane *et al.*, 2015). Externally, the membrane of the attachment organelle is surrounded by a protein layer called the nap structure, which contains P90 (protein B), P40 (protein C), and adhesin P1. A transmembrane protein, P30, colocalizes with the nap layer but it is presently unclear if it contributes to the raised nap structure visualized through microscopy (Nakane *et al.*, 2015). The P1 adhesin has been suggested to function as a "leg" for *M. pneumoniae* gliding motility that catches and releases binding substrate. When cells are exposed to a particular monoclonal P1 antiserum, they exhibit reduced gliding speed followed by loss of the ability to bind surfaces (Seto *et al.*, 2005). The precise mechanisms powering cytadherence and motility are presently unclear, although it has been suggested that P1 and P30 potentially
Figure 1. Schematic of *M. pneumoniae* attachment organelle with localization of component proteins. The attachment organelle of *M. pneumoniae* contains an electron-dense core (dark gray) that is composed of three distinct regions: the bowl complex, the paired plates, and the terminal button. Attachment organelle proteins localize to specific regions of the structure. Externally, the membrane of the attachment organelle is surrounded by a protein layer called the nap structure. Figure adapted from Kawakita *et al.*, 2016.
Bowl complex
Lon, P24, TopJ, P200, P41, MPN387, (HMW2)

Paired plates
HMW1, HMW2, CpsG, (HMW3)

Terminal button
(HMW2), HMW3, P65

P1 adhesin, P90, P40
Nap structure

P30
interact, as *M. pneumoniae* adherence to host epithelial cells requires both proteins (Jordan et al., 2001).

The role of P30, a 30-kDa protein, in gliding motility and cytadherence is less well-understood, and analysis of its structure is expected to provide insight into the mechanisms that power cytadherence and gliding motility. The necessity of P30 for both cytadherence and motility is illustrated by the inability of the *M. pneumoniae* P30 null mutant II-3 to cytadhere (Baseman et al., 1987; Dallo et al., 1990). Furthermore, *M. pneumoniae* cells exposed to antibodies targeting P30 are also unable to adhere (Baseman et al., 1987). Its amino acid sequence can be divided into a signal peptide that is cleaved off, a short cytoplasmic N-terminal region, a transmembrane domain, and a large extracellular C-terminal region (Dallo et al., 1996; Chang et al., 2011) (Figure 2). The C-terminal region of P30 contains a series of imperfect proline-rich repeats (Hasselbring et al., 2005, Chang et al., 2011) (Figure 2). From the beginning of the repeats, 41% of the amino acid residues of the C-terminus are prolines (Dallo et al., 1990) (Figure 3). Proline-rich regions of other proteins function as binding modules for other proteins (Zarrinpar et al., 2003). When proteins bind to proline-containing motifs, these complexes can contribute to processes such as cytoskeletal rearrangement and signal transduction (Umekawa et al., 2013). Domain analysis of P30 revealed that increasing deletions from the C-terminus of P30 leads to decreasing steady-state levels of P30. These truncation mutants also exhibit reduced gliding speeds (Chang et al., 2011). Thus, we hypothesize that the imperfect proline-rich repeats of the C-terminal region of P30 contribute to the stability and functions of P30 by facilitating its potential interactions with itself and/or other proteins.

Evidence that the role of P30 in cytadherence is independent of its role in gliding motility can be observed in *M. pneumoniae* mutant II-3R, which produces a P30 that differs from wild-type P30 over a 17-amino acid stretch (residues 135-151) (Hasselbring et al., 2005). This mutant is capable of adherence, but not motility. The variant of P30 produced in this mutant, which we designate P30s, is present in higher-order complexes that can be detected through immunoblotting. These complexes are consistent in size with dimers and tetramers of P30 and are not resolvable by treatment with sodium dodecyl sulfate (SDS) or urea. This mutant exhibits little motility, suggesting that formation and disengagement
Figure 2. P30 Schematic. The C-terminal region contains a series of imperfect proline-rich repeats (PRRs).
Figure 3. Amino acid sequence of P30. P30 is a 274-amino acid protein containing a series of proline-rich repeats. The repeats begin at position 151. Proline residues 193, 217, and 272 were targeted for mutation.
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<td>B</td>
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<td>QAWFIPTVAG</td>
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<td>C</td>
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<td>RPGMPPQPGF</td>
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from these complexes is necessary for the motility process (Hasselbring et al., 2005). Purification of recombinant P30 also results in unresolvable homomeric complexes (Marotta, 2014), indicating that the formation and disassembly of these complexes is necessary for the functions of P30.

In this study, we determined whether native P30 is present in higher-order complexes using chemical cross-linking and immunoblot analysis. P30 was detected in putative homomeric and heteromeric complexes. To determine how the proline residues of P30 contribute to its protein interactions, stability, and functions, we used site-directed mutagenesis and random transposon mutagenesis to generate M. pneumoniae transformants that produce P30-6XHis with three prolines at positions 193, 217, and 272 mutated to alanines. Two of these transformants exhibited wild-type steady-state levels of P30 and all three exhibited wild-type hemadsorption properties, gliding speed, and P30-containing higher-order complexes compared to wild-type transformant M. pneumoniae 24A. Analysis of the structure of P30 provided insight into the functions of P30 in M. pneumoniae cytadherence and gliding motility.
MATERIALS AND METHODS

Bacterial growth conditions

*Mycoplasma pneumoniae* strains and transformants were grown in plastic tissue culture flasks containing SP-4 broth (Tully et al., 1979). *M. pneumoniae* strains and transformants were plated on SP-4 with 1% (w/v) Noble agar (Becton, Dickinson, and Co.). SP-4 media used to grow *M. pneumoniae* transformants was supplemented with 18 μg ml⁻¹ gentamicin (Sigma-Aldrich) Luria-Bertani (LB) broth and agar containing 30 μg ml⁻¹ kanamycin were used to culture *Escherichia coli* strains containing suicide vector pMT85 with transposon Tn4001mod (Hahn et al., 1999). *M. pneumoniae* and *E. coli* cultures were grown at 37°C.

Chemical cross-linking

For cross-linking with 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (Pierce), *M. pneumoniae* cultures were grown to late log phase. The cells were washed three times with 0.1 M sodium phosphate pH 7.0 and treated with 1 mM DFDNB in 0.1 M sodium phosphate for one hour at 37°C. The cross-linking reaction was stopped by bringing the DFDNB and sodium phosphate-containing solution to 2 mM glycine. The cells were washed twice with 0.1 M sodium phosphate solution, scraped into 1% SDS in 0.09 M sodium phosphate solution, and lysed at 68°C for 10 minutes.

For cross-linking cultures with bis[sulfosuccinimidyl] suberate (BS₃) (Pierce), *M. pneumoniae* cultures were grown to late-log phase. The cells were washed with phosphate-buffered saline (PBS) (150 mM NaCl, 3.2 mM NaH₂PO₄, 13.6 mM Na₂HPO₄, pH 7.2) three times and treated with BS₃ at 1 mM, 3 mM, or 5 mM for one hour at 4°C. The cross-linking reaction was stopped using 1 M Tris-HCl, pH 7.5 to bring the reaction to 20 mM Tris. The cells were washed with PBS two times, scraped into 1% SDS in 0.09 M sodium phosphate solution, and lysed at 68°C for 10 minutes.

Protein purification

Six tissue culture flasks containing 50 ml SP-4 broth were inoculated with *M. pneumoniae* transformant 24A (Relich & Balish, 2011) and grown to late-log phase. The spent medium was aspirated and cells were washed three times with 10 ml of PBS. Cells were scraped into PBS and pelleted at 17,400 x g for 20 minutes at 4°C. Each cell pellet was suspended in 1 ml lysing solution containing 1% (w/v) n-dodecyl β-D-maltoside (DDM), 20
mM sodium phosphate pH 8.0, 300 mM NaCl, 10% glycerol (v/v), and 20 mM imidazole and incubated at 4°C for 2 hours with agitation. Insoluble material and unlysed cells were pelleted through centrifugation at 100,000 x g for 50 minutes at 4°C. The supernatant was stored at -20°C for later applications.

A 1-ml HisTrap HP column (GE Healthcare) was prepared by washing with 10 ml ultra-pure water that had been passed through a 0.22-μm syringe filter and equilibrated with 10 ml equilibration buffer containing 1% DDM (w/v), 20 mM sodium phosphate, 600 mM NaCl, and 20 mM imidazole, pH 7.4. The *M. pneumoniae* 24A lysate was applied to the column and followed with a 5-ml wash with equilibration buffer. The column was then washed with a total of 20 ml washing buffer containing 1% DDM (w/v), 20 mM sodium phosphate, 600 mM NaCl, and 100 mM imidazole, pH 7.4, which was collected in two 5-ml aliquots and ten 1-ml aliquots. The bound protein was eluted from the column with 5 ml elution buffer containing 1% DDM (w/v), 20 mM sodium phosphate, 600 mM NaCl, and 500 mM imidazole, pH 7.4, and collected in 1-ml aliquots. Fractions collected from the column were stored at -20°C for further analysis.

**Transformation of *M. pneumoniae* II-3**

The plasmid p0061 (Marotta, 2014) was purified from *E. coli* DH5α using the Qiagen Plasmid Maxi Kit. The p0061 suicide vector contains a transposon Tn4001mod engineered to contain the genes coding for P21 and P30-His (Relich & Balish, 2011) with three point mutations that result in replacement of prolines at positions 193, 217, and 272 with alanines. Electrocompetent *M. pneumoniae* II-3 cells were transformed via electroporation with p0061 as previously described (Hedreyda et al., 1993). Electroporated cells were allowed to recover in SP-4 broth for one hour at 37°C. The cells were then diluted ten-fold, and 100-μl aliquots were plated on SP-4 plates. Plates were incubated for 6-7 days at 37°C. Several isolated colonies were picked and used to inoculate 1-ml aliquots of SP-4 broth with gentamicin (18 μg ml⁻¹). When the 1-ml cultures reached late log phase, the cultures were passed through 0.22-μm syringe filters, and then serially diluted samples were plated on SP-4 plates with gentamicin. The process of colony picking, culturing, and filtering was repeated a total of three times to ensure that clonal cultures were achieved. Stock cultures of these transformants were made after the third passage and stored at -80°C for further applications.
Immunoblot analysis

*M. pneumoniae* cultures were grown to late-log phase. Cells capable of attaching to the tissue-flasks were harvested by aspirating the media, washing three times with PBS, scraping, and pelleting at 17,400 x g for 20 minutes at 4°C. Unattached cells were harvested by pelleting at 17,400 x g for 20 minutes at 4°C and suspending the pellet in PBS three times. After washes, cell pellets were suspended in 1% SDS in 0.09 M sodium phosphate solution and cells were lysed at 68°C for ten minutes. The bicinchoninic acid protein assay kit (Pierce) was used to determine protein concentrations of cell lysates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on aliquots of cell lysates equivalent to required protein amounts for analysis with or without β-mercaptoethanol (βME) in the sample buffer (Laemmli, 1970). The gels were used to transfer the electrophoretically separated proteins to nitrocellulose membranes overnight at room temperature for immunoblotting (Towbin *et al.*, 1979). The nitrocellulose membranes were blocked for 2 hours at room temperature in 5% (w/v) skim milk in Tris-buffered saline (TBS) (50 mM Tris base, pH 7.5, 150 mM NaCl). Following blocking, the blots were probed with a 1:250 dilution of P30 rabbit antiserum (Romero-Arroyo *et al.*, 1999), a 1:500 dilution of rabbit α-6x-His (Immunology Laboratory Consultants), a 1:5,000 dilution of HMW3 rabbit antiserum (Stevens & Krause, 1992), a 1:1,000 dilution of P1 rabbit antiserum (Krause & Baseman, 1983), or a 1:100 dilution of P65 rabbit antiserum (Proft & Herrmann, 1994) for two hours at room temperature. Blots were then washed with TBS containing 0.05% (v/v) Tween 20 (TBS-T) for five minutes five times. Membranes were then probed with goat α-rabbit IgG (Fc) conjugated to alkaline phosphatase (Promega). Detection was performed using nitro-blue tetrazolium and 5-bromo-4-chloro-3’-indoylphosphate. Densitometry analysis was performed using AlphaView software.

Time-lapse microcinematography analysis

Motility stocks of *M. pneumoniae* transformants were prepared and analysis of motility was performed as previously described (Hatchel *et al.*, 2006). Briefly, thawed motility stocks and SP-4 motility media were combined in various volumes to fill chamber slides with 800 μl of liquid. The chamber slides were then incubated for 3 h at 37°C to allow cells to attach to the bottom of the chamber slides. Attached cells were visualized using a
Leica DM IRB inverted microscope under 100X objective in a chamber heated to 37°C. Phase-contrast images of the cells were captured every second for 27 consecutive frames using SPOT software. SPOT software was used to merge the frames and Adobe Photoshop CS manipulation was used to color-code movement of cells. SPOT software was then used to trace cell paths and quantify movement.

**Hemadsorption assay**

A ten-fold serial dilution was performed on stocks of *M. pneumoniae* strains and transformants and diluted cells were plated on SP-4 media with or without gentamicin (18 \( \mu \text{g ml}^{-1} \)). Plates were incubated for 6 days at 37°C until colonies of adequate size for visualization were formed. Hemadsorption assays were performed on plate cultures as previously described (Sobeslavsky *et al.*, 1968) with sheep red blood cells in Alsever's solution diluted 1:200 in saline and three washes with PBS. Immediately following the washes, colonies were imaged with a 40X objective lens under an inverted microscope.
## RESULTS

**Table 1.** Bacterial strains and plasmids used in this study.

<table>
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<th>Strain or Plasmid</th>
<th>Description</th>
<th>Source/Reference</th>
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<td><strong>Bacterial Strains</strong></td>
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<td>Used for p0061 plasmid propagation</td>
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<td>Laboratory Stock</td>
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<td><em>Mycoplasma pneumoniae</em> 24A</td>
<td><em>M. pneumoniae</em> II-3+<em>M. pneumoniae</em> M129 P30-6XHis operon, HA*</td>
<td>Relich &amp; Balish, 2011</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em> 48C</td>
<td><em>M. pneumoniae</em> II-3+<em>M. pneumoniae</em> M129 P30-6XHis{P272A} operon, HA*</td>
<td>Marotta, 2014</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em> 58C</td>
<td><em>M. pneumoniae</em> II-3+<em>M. pneumoniae</em> M129 P30-6XHis{P169,272A} operon, HA*</td>
<td>Marotta, 2014</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em> 61B,G,I</td>
<td><em>M. pneumoniae</em> II-3+<em>M. pneumoniae</em> M129 P30-6XHis{P193,217,272A} operon, HA*</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p0061</td>
<td>p0024 with P30-6XHis{P193,217,272A}</td>
<td>Marotta, 2014</td>
</tr>
</tbody>
</table>
P30 in *Mycoplasma pneumoniae* strains and transformants is detectable in higher-order complexes after cross-linking

To determine if P30 is present in higher-order complexes in native conditions, DFDNB chemical cross-linking of *M. pneumoniae* M129 cells was performed, then followed with SDS-PAGE and immunoblot analysis using P30 antiserum (Romero-Arroyo *et al.*, 1999). Monomeric P30 was detected around 30 kDa. Higher-order complexes were detected in the DFDNB-treated cells in addition to monomeric P30, but due to the non-specific binding exhibited by the P30 antiserum, it was difficult to determine if these higher-order complexes contained P30 (Figure 4). The cross-linking procedure was repeated with *M. pneumoniae* transformant 24A (Relich & Balish, 2011), which produces a C-terminally 6X-histidine tagged P30 (P30-6XHis), and the immunoblot analysis was performed using α-6x-His antibody (Immunology Laboratory Consultants) to visualize P30-containing complexes specifically. When *M. pneumoniae* 24A was treated with DFDNB and SDS-PAGE was performed without βME, P30 was detected in a very large complex. When βME was added to the sample, this large complex was present, in addition to smaller subcomplexes as well as a 30-kDa species consistent with a monomer. These smaller subcomplexes were consistent in migration with homodimers and homotetramers of P30 (Figure 5). P30-containing complexes formed through DFDNB cross-linking remained insoluble after a variety of treatments, including detergent and urea (data not shown).

*M. pneumoniae* II-3R produces a variant of P30 that differs from wild-type P30 over a 16-amino acid stretch from position 135-151(P30R). P30R is present in monomeric form and unresolvable higher-order complexes (Hasselbring *et al.*, 2005). To determine if cross-linking would result in an increase in higher-order complexes consistent in migration with those naturally occurring in II-3R, DFDNB cross-linking was performed on *M. pneumoniae* II-3R cells and followed with immunoblot analysis using P30 antiserum. Although the results were difficult to interpret due to the non-specific reactivity of the P30 antiserum, monomeric P30R was detected in II-3R non-cross-linked and cross-linked samples around 30 kDa. The cross-linked sample contained much less monomeric P30R and an increase in potentially P30R-containing higher-order complexes, of which some are present in faint amounts in the non-cross-linked II-3R sample (Figure 6).
Figure 4. Immunoblot of P30-containing complexes in *M. pneumoniae* M129 after cross-linking with DFDNB. Whole cell lysates were separated through electrophoresis through a 9% SDS-polyacrylamide gel and probed with P30 antiserum at a 1:250 dilution. Twenty micrograms of protein were loaded in each lane. Molecular weight standards are indicated at left.
Figure 5. Immunoblot of P30-6XHis-containing complexes in *M. pneumoniae* 24A after cross-linking with DFDNB. Whole cell lysates were separated electrophoretically through a 10% SDS-polyacrylamide gel with or without βME and probed with α-6X-His antibody at a 1:500 dilution. Twenty micrograms of protein was loaded in each well. Molecular markers are indicated at right.
Figure 6. Immunoblot of P30-containing complexes in *M. pneumoniae* M129 and II-3R after cross-linking with DFDNB. Whole cell lysates were separated through electrophoresis through a 9% SDS-polyacrylamide gel and probed with P30 antiserum at a dilution of 1:250. Each lane was loaded with 75 μg of protein. Molecular weight standards are indicated on the left.
Mycoplasma genitalium produces a protein orthologous to P30, P32 (Reddy et al., 1995). The sequences share 43% identity and a region of proline-rich imperfect repeats. The repeats in P32 are different and less regular than the repeats in P30. M. pneumoniae transformant 17B, which produces a C-terminally 6X-histidine tagged P32 in a P30 null background (Relich & Balish, 2011), exhibits wild-type motility and cytadherence. To determine if P32 produced in M. pneumoniae 17B is present in similar higher-order complexes to P30 in M. pneumoniae 24A, DFDNB cross-linking was performed on both transformants followed by immunoblot analysis with α-6X-His antibody. Immunoblot analysis revealed that P30 and P32 produced in a P30 null background were present in similar higher-order complexes (Figure 7).

To determine if interactions of the extracellular or intracellular region of P30 are responsible for cross-linking, M. pneumoniae 24A was treated with membrane-impermeant cross-linker BS³ at increasing concentrations. SDS-PAGE followed by Coomassie Blue staining revealed a very minimal effect of BS³ treatment on the protein profile of M. pneumoniae 24A as opposed to treatment with membrane-permeant DFDNB (Figure 8), consistent with BS³ having fewer targets because it does not cross the cell membrane. As revealed by blotting with α-6X-His antibody, both treatments resulted in a large P30-containing complex and a smaller species consistent in migration with a homodimer of P30. Treatment with BS³ resulted in two species of P30-containing complexes. Treatment with higher concentrations of BS³ resulted in increased amounts of the largest P30-containing complex (Figure 9).

To determine if other proteins became cross-linked with P30-6XHis, immunoblot analysis on lysate from BS³ treated cells was performed with HMW3 antiserum (Figure 10A), P1 antiserum (Figure 10A), or P65 antiserum (Figure 10B). HMW3, a strictly cytoplasmic protein (Nakane et al., 2015), was not present in higher-order complexes in lysate from BS³-treated cells as opposed to in DFDNB-treated cells, consistent with BS³ not entering cells (Figure 10A). Like P30-6XHis, P1, which is a transmembrane protein (Baseman et al., 1982), and P65, some of which is surface-exposed (Proft et al., 1994), were detected in discrete higher-order complexes in BS³-treated cells (Figure 10A, B), distinct from the smear of complexes observed in DFDNB-treated cells. To determine if P1 and P30
Figure 7. Immunoblot of P30-6XHis or P32-6XHis-containing complexes in M. pneumoniae 24A and 17B after cross-linking with DFDNB. Whole cell lysates were separated electrophoretically through a 10% SDS-polyacrylamide gel and probed with α-6X-His antibody at a 1:500 dilution. Fifty micrograms of protein was loaded into each lane. Molecular weight standards are indicated at left.
Figure 8. Coomassie Blue staining of whole-cell lysate from *M. pneumoniae* 24A after cross-linking with DFDNB or BS₃. Twenty-five micrograms of protein from whole-cell lysate was separated electrophoretically through a 10% SDS polyacrylamide gel and stained with Coomassie blue. Molecular weight standards indicated at left.
Figure 9. Immunoblot of P30-6His-containing complexes in *M. pneumoniae* 24A after cross-linking with BS³. Whole cell lysates were separated electrophoretically through a 10% SDS-polyacrylamide gel and probed with α-6X-His antibody at a 1:500 dilution. Twenty-five micrograms of protein was loaded in each lane. Molecular weight standards are indicated at left.
Figure 10. Immunoblots of HMW3, P1, P65, and P30-6XHis after cross-linking with BS3. Whole-cell lysates were separated by SDS-PAGE through 5% gels, transferred to a nitrocellulose membrane, separated into sections, and probed with A) 1:5,000 dilution of HMW3 antiserum, 1:1,000 dilution of P1 antiserum, or 1:500 dilution of α-6x-His; B) 1:100 dilution of P65 antiserum or 1:500 dilution of α-6x-His; C) 1:500 dilution of α-6x-His or 1:1,000 dilution of P1 antiserum. Twenty-five micrograms of protein was loaded in each lane. Molecular weight standards indicated at left or right. Boundary of SDS-polyacrylamide gel during transfer of protein to nitrocellulose membrane was marked for C.
were in the same very large complex, immunoblots were performed on a gel with adjacent BS\(^3\)-treatment lanes. The large complexes in the immunoblot performed with P1-antiserum and immunoblot performed with α-6x-His antibody were at the gel boundary (Figure 10C).

**Purification of P30-6XHis from *M. pneumoniae* 24A**

Purification of P30-6XHis from *M. pneumoniae* 24A was attempted for future experiments. DDM detergent extraction was performed on *M. pneumoniae* 24A cell pellets followed by ultracentrifugation. Solubilized material was hand-fed into an equilibrated 1-ml HisTrap HP column (GE Healthcare) using a syringe. The column was washed with equilibration buffer and washing buffer. Bound material was then eluted with elution buffer. An aliquot from each fraction collected from the column was analyzed through SDS-PAGE and SYPRO Ruby staining. A protein band of approximately 30 kDa was present in low concentrations in the wash fractions and was most abundant in the first two elution fractions. This band was confirmed to be P30-6XHis after immunoblot analysis (data not shown). An unidentified protein of approximately 55 kDa was also present in high concentrations in the first two elution fractions (Figure 11).

**Triple point mutations to prolines in P30-6XHis do not reduce P30-6XHis steady-state levels relative to *M. pneumoniae* transformant 24A**

Plasmid p0061 was generated in *E. coli* MFB61 (Marotta, 2014) and contains the promoter for P21, the gene for P21, and the gene for P30 with a C-terminal 6X-His tag and the three prolines at positions 193, 217, and 272 switched to alanine. The gene for P21, which is located immediately upstream of the gene for P30 in the *M. pneumoniae* genome, and its products have been found to have no effect on the phenotype of *M. pneumoniae* II-3 (Relich & Balish, 2011). The Qiagen Plasmid Maxi kit was used to purify p0061 from *E. coli* MFB61. *M. pneumoniae* II-3, which does not produce P30 due to a frameshift mutation (Romero-Arroyo et al., 1999), was transformed with p0061, which resulted in random transposon mutagenesis, as previously described (Hedreyda et al., 1993). The transformed cells were plated on SP-4 agar with gentamicin selection. Three rounds of filter cloning were completed to ensure clonality of cultures and three transformants were selected for further analysis (Table 1). Random transposon mutagenesis using p0061, a derivative of suicide vector pMT85 (Zimmermann & Herrmann, 2005), containing Tn\(^{4001}\)mod as a tool
Figure 11. SYPRO Ruby staining of fractions from purification of P30-6XHis from *M. pneumoniae* 24A. Forty-microliter aliquots of each fraction from syringe-fed His-Trap HP column purification of *M. pneumoniae* 24A were separated electrophoretically through a 10% SDS-polyacrylamide gel and stained with SYPRO Ruby. Molecular weight standards are indicated at left. The band observed around 30 kDa was confirmed to be P30-6XHis via immunoblotting with α-6X-His antibody (data not shown). FT, flow-through, E1-E5, elution fractions 1-5.
to incorporate gene(s) of interest into the *M. pneumoniae* genome is necessary because this species cannot propagate this plasmid.

To determine how the point mutations in P30 affect the steady-levels of P30, SDS-PAGE was performed on whole-cell lysate of transformants and followed by immunoblotting with α-6X-His antibody (Immunology Laboratory Consultants). Fifty micrograms of protein was loaded for each transformant lysate and equal loading was confirmed with densitometry analysis (Figure 12). *M. pneumoniae* II-3 served as a negative control for P30. Densitometry analysis was used to determine steady-state levels of P30 in transformants and calculated as an average amount of P30-6XHis in each transformant relative to *M. pneumoniae* 24A and normalized for total amount of protein loaded. As previously observed, transformant 48C, which produces P30-6XHisP272A, had a lower average steady-state level than transformant *M. pneumoniae* 24A, which produces P30-6XHis without point mutations. Transformant 58C, which produces P30-6XHisP169,272A, had an even more reduced average steady-state level of P30 when compared to *M. pneumoniae* 24A (Marotta, 2014) (Figure 13, Table 2). Two of the 61-series transformants, *M. pneumoniae* 61B and 61G, had slightly reduced average steady-state levels, whereas the other 61-series transformant, *M. pneumoniae* 61I, exhibited wild-type average steady-state levels of P30.

**Triple point mutations to prolines in P30-6XHis do not reduce gliding speeds of *M. pneumoniae* transformants**

To determine if the gliding speeds of the 61-series transformants were affected by the point mutations in P30, time-lapse microcinematography was performed. The gliding speeds we compared to the *M. pneumoniae* transformant 24A which produces P30-6XHis without mutations. The ranges of gliding speeds and percentage of cells that were motile were also compared. All three of the 61-series transformants maintained wild-type gliding speed (Table 3, Table 4). Previously observed results in the 48- and 58-series transformants, where changing one or two prolines to alanines decreased the gliding speed of the transformants (Marotta, 2014).
Figure 12. Coomassie Blue staining of whole-cell lysates of *M. pneumoniae* transformants with densitometry analysis showing equal loading. Whole cell lysate of the strains and transformants were separated by electrophoresis through a 10% SDS-polyacrylamide gel. Fifty micrograms of protein was loaded for each sample. Densitometry values were determined as a percentage of wild-type *M. pneumoniae* transformant 24A. Molecular weight markers are indicated at left.
Figure 13. Immunoblot and densitometry analysis of P30-6XHis in *M. pneumoniae* transformants and null mutant II-3. Whole cell lysates of each strain and transformant were separated electrophoretically through a 10% SDS-polyacrylamide gel and probed with α-6X-His antibody at a 1:500 concentration. Fifty micrograms of protein was loaded for each sample. Densitometry values were determined as a percentage of wild-type *M. pneumoniae* transformant 24A. Molecular weight markers are indicated at left.
Table 2. One-way ANOVA analysis of P30-6XHis steady-state levels in transformants.

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Comparison</th>
<th>Mean difference</th>
<th>95% Confidence Interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. pneumoniae</em> 24A</td>
<td>vs. 48C</td>
<td>-40.1466</td>
<td>-72.5648</td>
<td>0.0130</td>
</tr>
<tr>
<td></td>
<td>vs. 58C</td>
<td>-54.5767</td>
<td>-86.9949</td>
<td>0.0012</td>
</tr>
<tr>
<td></td>
<td>vs. 61B</td>
<td>-30.3906</td>
<td>-62.8088</td>
<td>0.0709</td>
</tr>
<tr>
<td></td>
<td>vs. 61G</td>
<td>-20.6589</td>
<td>-53.0771</td>
<td>0.3301</td>
</tr>
<tr>
<td></td>
<td>vs. 61I</td>
<td>-20.6589</td>
<td>-53.0771</td>
<td>0.5772</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> 48C</td>
<td>vs. 58C</td>
<td>14.4301</td>
<td>22.6621</td>
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</tr>
<tr>
<td></td>
<td>vs. 61B</td>
<td>9.7561</td>
<td>22.6621</td>
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</tr>
<tr>
<td></td>
<td>vs. 61G</td>
<td>19.4877</td>
<td>-12.9305</td>
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<tr>
<td></td>
<td>vs. 61I</td>
<td>56.1970</td>
<td>23.7788</td>
<td>0.0009</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> 58C</td>
<td>vs. 61B</td>
<td>24.1862</td>
<td>-8.2320</td>
<td>0.1967</td>
</tr>
<tr>
<td></td>
<td>vs. 61G</td>
<td>33.9178</td>
<td>1.4996</td>
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<tr>
<td></td>
<td>vs. 61I</td>
<td>70.6271</td>
<td>38.2089</td>
<td>0.0001</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> 61B</td>
<td>vs. 61G</td>
<td>9.7316</td>
<td>-22.6865</td>
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</tr>
<tr>
<td></td>
<td>vs. 61I</td>
<td>46.4409</td>
<td>14.0228</td>
<td>0.0044</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> 61G</td>
<td>vs. 61I</td>
<td>36.7093</td>
<td>4.2911</td>
<td>0.0236</td>
</tr>
</tbody>
</table>
Table 3. Gliding motility parameters of wild-type and transformant strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean gliding speed (nm s(^{-1})) ± SD</th>
<th>Range of speeds (nm s(^{-1}))</th>
<th>% cells that are motile</th>
<th># of prolines mutated (Mutation)</th>
<th>Data Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. pneumoniae M129</td>
<td>337±59</td>
<td>220-479</td>
<td>N/A</td>
<td>N/A</td>
<td>Marotta, 2014</td>
</tr>
<tr>
<td>M. pneumoniae 24A</td>
<td>304±61</td>
<td>190-443</td>
<td>61</td>
<td>N/A</td>
<td>This study</td>
</tr>
<tr>
<td>M. pneumoniae 48C</td>
<td>245±60</td>
<td>121-422</td>
<td>N/A</td>
<td>1 (P30-6XHis(^\text{P272A}))</td>
<td>Marotta, 2014</td>
</tr>
<tr>
<td>M. pneumoniae 58C</td>
<td>253±51</td>
<td>149-405</td>
<td>N/A</td>
<td>2 (P30-6XHis(^\text{P169,272A}))</td>
<td>Marotta, 2014</td>
</tr>
<tr>
<td>M. pneumoniae 61B</td>
<td>307±58</td>
<td>146-432</td>
<td>65</td>
<td>3 (P30-6XHis(^\text{P193,217,272A}))</td>
<td>This study</td>
</tr>
<tr>
<td>M. pneumoniae 61G</td>
<td>289±46</td>
<td>165-383</td>
<td>63</td>
<td>3 (P30-6XHis(^\text{P193,217,272A}))</td>
<td>This study</td>
</tr>
<tr>
<td>M. pneumoniae 61I</td>
<td>309±49</td>
<td>178-421</td>
<td>64</td>
<td>3 (P30-6XHis(^\text{P193,217,272A}))</td>
<td>This study</td>
</tr>
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</table>
Table 4. One-way ANOVA analysis of gliding motility speed of wild-type and transformant strains.

<table>
<thead>
<tr>
<th>Transformant/Strain</th>
<th>Comparison</th>
<th>Mean difference</th>
<th>95% Confidence Interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
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<td>M. pneumoniae M129</td>
<td>vs. 24A</td>
<td>33.0000</td>
<td>-62.7996 - 3.2004</td>
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<tr>
<td></td>
<td>vs. 48C</td>
<td>92.0000</td>
<td>-123.2224 - 60.7776</td>
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<tr>
<td></td>
<td>vs. 58C</td>
<td>-84.0000</td>
<td>-112.0119 - 55.9881</td>
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<tr>
<td></td>
<td>vs. 61B</td>
<td>-30.0000</td>
<td>-59.3838 - 0.6162</td>
<td>0.0418</td>
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<td></td>
<td>vs. 61G</td>
<td>-48.0000</td>
<td>-79.7040 - 16.2960</td>
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</tr>
<tr>
<td></td>
<td>vs. 61I</td>
<td>-28.0000</td>
<td>59.7040 - 3.7040</td>
<td>0.1237</td>
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<tr>
<td>M. pneumoniae 24A</td>
<td>vs. 48C</td>
<td>-59.0000</td>
<td>-89.3885 - 28.6115</td>
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<td></td>
<td>vs. 58C</td>
<td>-51.0000</td>
<td>-78.0794 - 23.9206</td>
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<td>vs. 61B</td>
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<td>-25.4962 31.4962</td>
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<td>vs. 61G</td>
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</tr>
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<td>vs. 61G</td>
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<tr>
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<td>vs. 61I</td>
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<td>26.8380 85.1620</td>
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<td>M. pneumoniae 61B</td>
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<td>-48.4822 12.4822</td>
<td>0.5834</td>
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<tr>
<td></td>
<td>vs. 61I</td>
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<td>-28.4822 32.4822</td>
<td>1.0000</td>
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<tr>
<td>M. pneumoniae 61G</td>
<td>vs. 61I</td>
<td>20.0000</td>
<td>-12.7246 52.7246</td>
<td>0.5419</td>
</tr>
</tbody>
</table>
Triple point mutations to prolines in P30-6XHis do not detectably affect cytadherence capabilities

To determine if the triple point mutations to the prolines at positions 193, 217, and 272 of P30-6XHis affect cytadherence capabilities of the 61-series transformants, a hemadsorption (HA) assay was used with *M. pneumoniae* 24 as a positive control and *M. pneumoniae* II-3 as a negative control. Colonies of all three transformants of the 61-series were able to bind red blood cells and were thus HA-positive (HA+) (Figure 14).

**Triple point mutations to prolines in P30-6XHis do not detectably affect cross-linking of P30**

To determine if the point mutations in the 48-, 58-, and 61-series transformants affected the presence of P30 in higher-order complexes, chemical cross-linking was performed on representative transformants from each series and followed with immunoblot analysis. All transformants exhibited similar P30-containing higher-order complexes as observed in wild-type, *M. pneumoniae* 24A. The transformants that contained reduced steady-state levels of P30 also had reduced amounts of these higher-order complexes (Figure 15, Figure 16). The effect of these point mutations on the exact abundance of these higher-order complexes is difficult to determine with available densitometry methods.
Figure 14. Hemadsorption analysis of *M. pneumoniae* transformants. Strains and transformants are indicated below each panel. Scale bar, 10 μm.
Figure 15. Coomassie Blue staining of whole-cell lysates of *M. pneumoniae* transformants after cross-linking with DFDNB. SDS-PAGE was performed on whole-cell lysates from each transformant using a 10% gel and stained with Coomassie Blue. Fifty micrograms of protein was loaded in each lane. Molecular weight marker is indicated at left.
Figure 16. Immunoblot analysis of P30-6XHis in *M. pneumoniae* transformants after cross-linking with DFDNB. Fifty micrograms of protein from whole cell lysates of each transformant were separated through electrophoresis through a 10% SDS-polyacrylamide gel and probed with α-6X-His antibody at a 1:500 concentration. Molecular weight standards are indicated at left.
DISCUSSION

This study aimed to elucidate how the structure of P30 is related to its functions in the *M. pneumoniae* virulence-associated traits of attachment and gliding motility. The extracellular C-terminal region of P30 is comprised of a series of imperfect proline-rich repeats (Dalco *et al.*, 1996; Chang *et al.*, 2011). Because the proline-rich region of P30 contributes to the stability of P30 (Chang *et al.*, 2011), and proline-rich regions of proteins are involved in protein-protein interactions (Zarrinpar *et al.*, 2003), we hypothesized that the proline-rich region of P30 is involved in protein-protein interactions that are important for the stability and function of P30.

In this study, we used chemical cross-linking and immunoblot analysis to determine if native P30 forms homomeric complexes in addition to interactions with other proteins. DFDNB cross-linking revealed that P30 is present in higher-order complexes consistent in migration with homomeric complexes, as well as heteromeric complexes (Figure 5). These complexes remained insoluble after various treatments, including detergents and urea. BS³ cross-linking revealed two species of P30-containing higher-order complexes, one consistent in migration with a homodimer, and another large complex that possibly contains transmembrane adhesin P1 and/or attachment organelle protein P65, which mainly localizes to the internal tip of the attachment organelle, though a fraction is surface-exposed (Figure 9, Figure 10).

Based on these data and previous studies, we have proposed a model for the mechanism of gliding motility in *M. pneumoniae* (Figure 17). P30 is localized to the tip of the attachment organelle through various associations with core proteins mediated by its intracellular region, accounting for the heteromeric complexes revealed through DFDNB cross-linking. We predict that P30 in its low-energy conformation exists in multimers of various sizes. These multimers exit in equilibrium with the homodimeric form of P30. Homodimeric P30, after receiving an input of energy from an unknown source, changes to higher-energy conformation and dissociates into monomers. In our model, a molecule of P30 in high-energy conformation interacts with a very large higher-order complex containing P1. P1 and accessory proteins B and C exist as an adhesin complex (Krause & Balish 2004, Nakane *et al.*, 2011). We envision that when the P30 monomer interacts with the P1-containing complex, energy is transferred from the P30 monomer to the
Figure 17. Model of mechanism for *M. pneumoniae* gliding motility. P30 exists as a multimer of various sizes in its low-energy conformation (P30ₙ; dark blue). The P30 multimer exists in equilibrium with smaller homocomplexes of P30, including homodimers (1). When the P30 molecules of a homodimer (P30₂) receive an input of energy, they change to a high-energy conformation (light blue), causing them to dissociate into P30 monomers (2). One high-energy P30 molecule binds the P1-containing complex in a low-energy conformation (P1, B, C; dark green; 3). Energy is transferred from P30 to the P1-containing complex, which changes conformation to a higher energy state (light green) (4). This conformational change allows the P1-containing complex to bind substrate on the gliding surface (5) and also causes the P30, now in low-energy conformation, to dissociate from the complex (6) and return to a P30 multimer (7). Without P30, the P1-containing complex returns to its low-energy conformation while bound to substrate, in a motion that leads to forward displacement of the complex (red arrow, 8). The P1 complex is now in position to engage the next substrate molecule on the surface (9).
P1-containing complex, resulting in conformational change in the P1-containing complex. The conformational change allows the P1-containing complex to bind substrate on the gliding surface and simultaneously leads to the dissociation of P30, now in low-energy conformation. When the P1-containing complex is unbound from P30, it returns to its original low-energy conformation while bound to substrate, leading to a forward displacement of the P1-containing complex. The coordination of many of these P1-containing complexes returning to low-energy conformation at once creates the force necessary on the cell for the gliding movement of *M. pneumoniae*. It is unlikely that the power required for the motility process in *M. pneumoniae* is derived from ATP hydrolysis as observed in *M. mobile*, as motility is not inhibited in *M. pneumoniae* cells treated with ATP-depleting sodium arsenate (Jurkovic et al., 2013).

In this model the P1-containing complex and P30 are the terminal components of the gliding motility machinery. The number of functional motors in a cell and rate at which the components are able to assemble and disassemble in a coordinated manner would determine gliding speed. A correlation between reduced steady-state levels of P30 and reduced gliding speed has been observed in P30 truncation mutants (Chang et al., 2011), transformants with underexpression of the P30-6XHis gene under the *ldh* promoter (Relich, 2011), transformants engineered to produce truncated P65 (Hasselbring et al., 2012), and P30 point mutation transformants (Marotta, 2014). Evidence of conformational changes in P30 and P1 would provide further support for this model as well as evidence that P30 and P1 interact in a conformation-dependent manner directly or indirectly, as it has been proposed that protein P65 may serve as a linker between P30 and P1 (Hasselbring et al., 2012). This would account for the presence of P65 in a very large higher-order complex after cross-linking (Figure 10B).

Results from DFDNB cross-linking of *M. pneumoniae* II-3R were difficult to interpret but suggested that cross-linking results in an increase in putative P30$_R$ homodimers (Figure 6). The gliding defects observed in *M. pneumoniae* II-3R could be explained by the failure of P30$_R$ to disengage from protein-protein interactions. If monomeric P30$_R$ is disfavored due to aspects of its biochemistry, P30$_R$ would spend more time engaged in interactions as a multimer or with the P1-containing complex. The P1-containing complex would remain capable of binding substrate, but the infrequent dissociation of P30$_R$ from the
complex would not allow for the coordinated effort of the P1-containing complexes that
effectuates motility of the cell. Generation of *M. pneumoniae* transformations producing
P30R-6XHis and chemical cross-linking could provide clearer analysis of the mutant protein
dynamics.

DFDNB chemical cross-linking was performed on *M. pneumoniae* transformant 17B,
which produces the 6XHis-tagged P30 ortholog from *M. genitalium*, P32-6XHis, in a P30
null background. Immunoblot analysis of the cross-linked cells revealed P30-6XHis and
P32-6XHis produced in a P30 null background are present in similar higher-order
complexes (Figure 7). These data suggest that the ability of P32 to restore motility and
cytadherence to the P30 null mutant are due to its similarities to P30 in both structure and
ability to form higher-order complexes.

Future research should be conducted on solubilization of the P30-containing higher
order complexes and use of other methods to purify P30-6XHis. Coupled with mass
spectrometry these efforts will further elucidate the structure of P30, identify its binding
partners, and ultimately provide insight into the mechanisms behind *M. pneumoniae* gliding
motility and cytadherence. Although the purification protocol in this study did not result in
purified P30, it serves as a starting point for further optimization.

To determine how the proline residues of the C-terminal region of P30 contribute to
the structure, stability, and functions of P30, these residues were targeted for mutation
using site-directed mutagenesis. P30 null mutant *M. pneumoniae* II-3 and p0061 were used
to generate the 61-series transformants, which produced P30 with three proline residues
changed to alanines. The steady-state levels of P30-6XHis in the 61-series were compared
to representative transformants from the 48- and 58- series and 24A. Transformants 48C,
58C, and 61B exhibited reduced steady-state levels of P30-6XHis, possibly indicating
reduced stability of P30 when prolines in the C-terminal region are changed to alanines.
Definitive conclusions are difficult to make as transformants of the 61-series had
statistically significant steady-state levels of P30-6XHisP193,217,272A (Figure 13, Table 2).
Analysis of motility parameters revealed that the 61-series transformants did not have
observable motility defects. This result contrasts with previous observations, in which one
or two proline residues of P30 were changed to alanines in the 48- and 58-series
transformants resulted in reduced gliding speed (Table 3, Table 4; Marotta, 2014). The HA
assay showed that the cytadherence capabilities of the 61-series transformants were not affected (Figure 14). Like the 48- and 58-series transformants from a previous study, the colony morphologies of the 61-series transformants were indistinguishable from the wild-type transformant, 24A. The HA assay is qualitative and therefore cannot detect small differences between transformants. Quantitative analysis of HA can be assessed using radiolabeled mycoplasmas (Hasselbring et al., 2005) but was determined unnecessary for the purpose of this study. Representative transformants from the 48-, 58-, and 61-series also displayed wild-type patterns of chemical cross-linking, although higher-order complexes were observed in reduced amounts in the transformants that displayed reduced steady-state levels of P30 (Figure 16).

These results indicate that there is a complicated relationship between the proline residues and their relation to the structure and functions of P30. Although we have established that P30 is involved in protein-protein interactions, the role of the proline residues in these interactions, and the contribution of these interactions to stability of P30 remain unclear. More detailed structural analysis would provide further insight into the relationship of the proline residues and their contribution to P30 structure and function. Purified P30 is required for most feasible structural analyses, further emphasizing the importance of developing a protocol to purify P30. Comparing the structures of mutated P30 and the motility and cytadherence defects in transformants producing the mutated P30 will further elucidate how the proline-rich region of P30 is important for P30 protein-protein interactions, functions, stability, and structure. The process of generating these mutants would be more efficient using oligonucleotide synthesis and subcloning. Analysis of more transformants from each series generated would also provide more conclusive results.

This study has helped to elucidate the structure of P30, its protein-interactions, and its functions. Further analysis of P30 and its binding partners could continue to uncover the mechanisms of the \textit{M. pneumoniae} virulence-associated traits of gliding motility and cytadherence. In the face of increasing antibiotic resistance of \textit{M. pneumoniae} infections, elucidating the components and mechanisms behind these virulence-associate traits may provide vital insight for development of new drugs and/or therapeutics.
REFERENCES


