Characterization of cleavage events in the multifunctional cilium adhesin Mhp684 (P146) reveals a mechanism by which Mycoplasma hyopneumoniae regulates surface topography

Daniel Bogema  
*University of Wollongong, drb10@uow.edu.au*

Ania T. Deutscher  
*University of Wollongong, atb05@uow.edu.au*

Lauren K. Woolley  
*University of Wollongong, lkw49@uowmail.edu.au*

Lisa M. Seymour  
*University of Wollongong, lh75@uow.edu.au*

Benjamin B. A Raymond  
*University of Technology, Sydney*

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Abstract
Mycoplasma hyopneumoniae causes enormous economic losses to swine production worldwide by colonizing the ciliated epithelium in the porcine respiratory tract, resulting in widespread damage to the mucociliary escalator, prolonged inflammation, reduced weight gain, and secondary infections. Protein Mhp684 (P146) comprises 1,317 amino acids, and while the N-terminal 400 residues display significant sequence identity to the archetype cilium adhesin P97, the remainder of the molecule is novel and displays unusual motifs. Proteome analysis shows that P146 preprotein is endogenously cleaved into three major fragments identified here as P50P146, P40P146, and P85P146 that reside on the cell surface. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) identified a semitryptic peptide that delineated a major cleavage site in Mhp684. Cleavage occurred at the phenylalanine residue within sequence 672ATEF↓QQ677, consistent with a cleavage motif resembling S/T-X-F↓X-D/E recently identified in Mhp683 and other P97/P102 family members. Biotinylated surface proteins recovered by avidin chromatography and separated by two-dimensional gel electrophoresis (2-D GE) showed that more-extensive endoproteolytic cleavage of P146 occurs. Recombinant fragments F1P146-F3P146 that mimic P50P146, P40P146, and P85P146 were constructed and shown to bind porcine epithelial cilia and biotinylated heparin with physiologically relevant affinity. Recombinant versions of F3P146 generated from M. hyopneumoniae strain J and 232 sequences strongly bind porcine plasminogen, and the removal of their respective C-terminal lysine and arginine residues significantly reduces this interaction. These data reveal that P146 is an extensively processed, multifunctional adhesin of M. hyopneumoniae. Extensive cleavage coupled with variable cleavage efficiency provides a mechanism by which M. hyopneumoniae regulates protein topography.

Keywords
mhp684, p146, characterization, reveals, cleavage, mechanism, which, mycoplasma, hyopneumoniae, regulates, surface, topography, events, multifunctional, cilium, adhesin, CMMB

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NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Camden, NSW, Australia; School of Biological Sciences, University of Wollongong, Wollongong, NSW, Australia; School of Chemistry and Molecular Biosciences and Australian Infectious Diseases Research Centre, University of Queensland, Brisbane, QLD, Australia; The ithree Institute, University of Technology, Sydney, NSW, Australia; School of Chemistry, University of Wollongong, Wollongong, NSW, Australia; and Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa, USA

ABSTRACT Mycoplasma hyopneumoniae causes enormous economic losses to swine production worldwide by colonizing the ciliated epithelium in the porcine respiratory tract, resulting in widespread damage to the mucociliary escalator, prolonged inflammation, reduced weight gain, and secondary infections. Protein Mhp684 (P146) comprises 1,317 amino acids, and while the N-terminal 400 residues display significant sequence identity to the archetype ciliation adhesin P97, the remainder of the molecule is novel and displays unusual motifs. Proteome analysis shows that P146 preprotein is endogenously cleaved into three major fragments identified here as P50P146, P40P146, and P85P146 that reside on the cell surface. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) identified a semitryptic peptide that delineated a major cleavage site in Mhp684. Cleavage occurred at the phenylalanine residue within sequence ^672ATEF2^QQ677, consistent with a cleavage motif resembling S/T-X-F→X-D/E recently identified in Mhp683 and other P97/P102 family members. Biotinylated surface proteins recovered by avidin chromatography and separated by two-dimensional gel electrophoresis (2-D GE) showed that more-extensive endoproteolytic cleavage of P146 occurs. Recombinant fragments F1P146→F3P146, that mimic P50P146, P40P146, and P85P146 were constructed and shown to bind porcine epithelial cilia and biotinylated heparin with physiologically relevant affinity. Recombinant versions of F3P146 generated from Mycoplasma hyopneumoniae strain J and 232 sequences strongly bind porcine plasminogen, and the removal of their respective C-terminal lysine and arginine residues significantly reduces this interaction. These data reveal that P146 is an extensively processed, multifunctional adhesin of Mycoplasma hyopneumoniae. Extensive cleavage coupled with variable cleavage efficiency provides a mechanism by which Mycoplasma hyopneumoniae regulates protein topography.

IMPORTANCE Vaccines used to control Mycoplasma hyopneumoniae infection provide only partial protection. Proteins of the P97/P102 families are highly expressed, functionally redundant molecules that are substrates of endoproteases that generate multifunctional adhesin fragments on the cell surface. We show that P146 displays a chimeric structure consisting of an N terminus, which shares sequence identity with P97, and novel central and C-terminal regions. P146 is endoproteolytically processed at multiple sites, generating at least nine fragments on the surface of Mycoplasma hyopneumoniae. Dominant cleavage events occurred at S/T-X-F→X-D/E-like sites generating P50P146, P40P146, and P85P146. Recombinant proteins designed to mimic the major cleavage fragments bind porcine cilia, heparin, and plasminogen. P146 undergoes endoproteolytic processing events at multiple sites and with differential processing efficiency, generating combinatorial diversity on the surface of Mycoplasma hyopneumoniae.
geographically widespread respiratory disease that inflicts severe economic losses to pig production (8, 9).

Within the confines of commercial swine-rearing facilities, *M. hyopneumoniae* enters naïve animals via the inhalation of mucosal respiratory droplets expelled during bouts of coughing from *M. hyopneumoniae*-infected swine (10). Upon inhalation, *M. hyopneumoniae* must overcome the mucociliary escalator and traverse layers of heavily glycosylated mucins produced as decoys for bacterial adhesins that exploit surface glycoconjugates and extracellular matrix components during colonization. *M. hyopneumoniae* is highly adept at colonizing ciliated epithelial epithelia (11, 12) where it initially causes ciliostasis but eventually destroys cilia and induces epithelial cell death. Heparin effectively blocks (11, 12) where it initially causes ciliostasis but eventually destroys cellular invasion (17, 18). In immune responses and regulate both adhesion to host tissues and ciliary escalator (11, 13, 15, 16).

Consequently, the ability to adhere tightly to respiratory cilia in the porcine respiratory tract (14). Enzootic pneumonia is a *M. hyopneumoniae* infection, indicating that *M. hyopneumoniae* is highly adept at colonizing ciliated respiratory epithelia by bacterial adhesins that exploit surface glycoconjugates and extracellular layers of heavily glycosylated mucins produced as decoys for *M. hyopneumoniae*.

Within the confines of commercial swine-rearing facilities, *M. hyopneumoniae* enters naïve animals via the inhalation of mucosal respiratory droplets expelled during bouts of coughing from *M. hyopneumoniae*-infected swine (10). Upon inhalation, *M. hyopneumoniae* must overcome the mucociliary escalator and traverse layers of heavily glycosylated mucins produced as decoys for bacterial adhesins that exploit surface glycoconjugates and extracellular matrix components during colonization. *M. hyopneumoniae* is highly adept at colonizing ciliated epithelial epithelia (11, 12) where it initially causes ciliostasis but eventually destroys cilia and induces epithelial cell death. Heparin effectively blocks the binding of *M. hyopneumoniae* to porcine ciliary preparations (13), indicating that *M. hyopneumoniae* is reliant on the presentation of heparin-binding proteins on its cell surface. Proteoglycans with highly sulfated glycosaminoglycan (GAG) side chains are prominently displayed on the surface of ciliated epithelium lining the porcine respiratory tract (14). Enzootic pneumonia is a chronic disease, and adaptive immune responses must be evaded for *M. hyopneumoniae* to survive and proliferate within host tissues. Consequently, the ability to adhere tightly to respiratory cilia is a key strategy employed by pathogens to overcome the mucociliary escalator (11, 13, 15, 16).

Functional redundancy within key surface protein families is identified in multiple pathogens and may be a strategy to avoid immune responses and regulate both adhesion to host tissues and cellular invasion (17, 18). In *M. hyopneumoniae*, multifunctional adhesions belonging to the P97/P102 families include Mhp182 (P102), Mhp183 (P97), Mhp493 (P135), Mhp494 (P216), Mhp683 (P135), Mhp271, Mhp107, and Mhp108 (P116). These adhesins are prominently expressed during broth culture (19–29), and their expression in vivo is indicated by reverse transcription-PCR (RT-PCR) of *M. hyopneumoniae* mRNA recovered from the bronchoalveolar lavage (BAL) fluid of experimentally infected swine (30). P97/P102 paralog family members on the cell surface of *M. hyopneumoniae* are targets of endoproteolytic processing events that generate a substantial combinatorial repertoire of cleavage fragments. Fragments of P97, P216, P135, Mhp271, Mhp107, and Mhp108 have been shown to bind porcine cilia (21–26), underscoring their important role in colonizing the respiratory tracts of swine. Additionally, fragments derived from P97, P159, P216, P135, Mhp271, and Mhp107 are potent adhesins of heparin (19, 21, 22, 24, 26, 31).

Interactions between microbial virulence factors and host GAGs underpin a plethora of pathogenic mechanisms including adhesion, invasion, and tissue tropism (18). These findings suggest that endoproteolytic fragments derived from P97/P102 paralog family members display discrete functional domains with multifunctional attributes and are key molecules in the pathogenic arsenal of *M. hyopneumoniae*.

Endoproteolytic processing is a well-known mechanism used by bacterial pathogens to convert immature preproteins into mature functioning virulence molecules; however, in the *M. hyopneumoniae* P97/P102 adhesin families, posttranslational cleavage is extensive and unorthodox. Most of the P97/P102 family members previously examined undergo at least one cleavage event and in some cases two or more cleavage events (19–22, 28, 29). The archetype cilium adhesin P97 itself undergoes extensive processing, creating many novel endoproteolytic fragments, but many of these fragments have not been experimentally characterized (20). Cleavage fragments reside on the surfaces of *M. hyopneumoniae* cells despite the lack of putative transmembrane domains (19–27, 30). Recently, the identification of an endoproteolytic cleavage motif with sequence S/T-X-F ↓ X-D/E has facilitated the prediction of cleavage sites among members of the P97/P102 families (22); however, the biological rationale for cleavage and the protease(s) responsible are yet to be determined.

The P146 adhesin-like-protein (Mhp684) is a paralog of the P97 cilium adhesin, and its corresponding gene is situated within a two-gene operon with the cilium- and heparin-binding protein P135 (Mhp683) (6). The gene itself is transcribed in vivo (30) and has garnered interest as a target for genotyping *M. hyopneumoniae*, due to its extensive variability in strains (32). A polyserine repeat (P146R3) is observed to vary considerably among the sequenced strains of *M. hyopneumoniae* (5–7). In this study, we have characterized binding functions of P146 and determined how it is endoproteolytically processed.

## RESULTS

P146 is extensively processed on the surface of *M. hyopneumoniae*. In pathogenic strain 232 of *M. hyopneumoniae*, the p97 (mhp183) paralog p146 (mhp684) encodes a 148.2-kDa protein with a theoretical pI of 8.79. Homologs of p146 have been identified in the genome sequences of *M. hyopneumoniae* strains J (mhj_0663), 7448 (mhp7448_0663), and 168 (mhp168_0676) (5–7). Additionally, p146 homologs have been identified and sequenced in strains F7.2C and BQ14 and partially sequenced in strains PMS and 7422 (32, 33). The six full-length p146 gene sequences share >85% identity. The TMHMM algorithm identified a transmembrane domain (*P* = 0.999) spanning residues 7 to 29; however, SignalP 4.0 did not conclusively identify the presence of a putative signal peptide (D score of 0.44; signal peptide cutoff of D score of >0.45).

Several members of the P97 and P102 families undergo extensive posttranslational cleavage (19–22, 24, 25, 28, 29). Expression of P146 by *M. hyopneumoniae* strain J in broth culture was established by using liquid chromatography with tandem mass spectrometry (LC-MS/MS) to identify tryptic peptides derived from proteins separated by SDS-PAGE (Fig. 1). Consistent with previ-
ously characterized P97/P102 family members and proteomic studies of *M. hyopneumoniae* (28, 29), we were unable to identify any peptides unique to P146 near its predicted mass of 148.2 kDa. Tryptic peptides spanning P146 mapped to three endoproteolytic fragments, indicating that P146 is cleaved twice. A protein that migrated with mass between 40 and 53 kDa (slice 8) mapped to the N-terminal region of P146 (Fig. 1A) (P50P146). Peptides matching the central region of the P146 sequence were identified in slices 8 (40 to 53 kDa) and 9 (39 to 42 kDa) (Fig. 1B) (P40P146), and peptides matching the C-terminal region were identified in slice 6 (70 to 90 kDa) (Fig. 1C) (P85P146). Recently, we proposed a cleavage motif with sequence S/T-X-F2X-D/E that can be used to predict cleavage sites in the P97 and P102 paralog families (22). Two sequences that closely resemble this motif were identified within the cleavage regions defined by mass spectrometry. The first of these sequences, TYFAE, resides between amino acids 315 and 366 that separate matched peptides defining P50P146 and P40P146. However, we have been unable to experimentally confirm cleavage at the phenylalanine residue in TYFAE. The second motif, with sequence TEFQQ, resides between residues 545 and 595. Figure 1D shows the mass spectrometry analysis of P146. *M. hyopneumoniae* strain J proteins were examined using the following two methods. In method 1, lysates were subjected to 1-D SDS-PAGE, the gels were sectioned into pieces representing 14 molecular mass ranges, and the proteins in each gel slice were examined using LC-MS/MS. In method 2, surface proteins were labeled by surface biotinylation and enriched by affinity chromatography of the cell lysate. Purified biotinylated proteins were separated by either 1-D SDS-PAGE or 2-D GE and identified by LC-MS/MS. Peptides identified by LC-MS/MS are shown in bold type and underlined. The numbers above or below the sequence refer to amino acid positions. (A to C) Peptides unique to three regions of the P146 sequence were identified in both experiments. Terminal sequences matching an *M. hyopneumoniae* cleavage motif defined previously are underlined but not bold. (A) Fragment P50P146 and peptides unique to the N-terminal region of P146. (B) Fragment P40P146 and peptides unique to the central region of P146. (C) Fragment P85P146 and peptides unique to the C-terminal region of P146. The N terminus of P85P146 was identified from a semitryptic peptide. (D to F) Additional proteins were identified by method 2 at masses significantly lower than those identified by method 1. Terminal sequences matching or almost matching an *M. hyopneumoniae* cleavage motif defined previously (22) are underlined but not bold. (D) Peptides unique to P50P146 were identified in a 1-D SDS-polyacrylamide gel slice containing proteins migrating at ~25 kDa. (E) Peptides unique to one-half of P50P146 identified in a 2-D GE protein spot migrating at 25 kDa and pI of ~5.5. (F) Peptides unique to P85P146 identified in 2-D GE protein spots migrating at 50 kDa and pI of ~5.0.
that separate matched peptides that define P40\textsubscript{P146} and P85\textsubscript{P146}. Analysis of spectra derived from P85\textsubscript{P146} revealed a semitryptic peptide 676QQQDANSTNSSPTSPSAPSSPPSSP\textsubscript{P146} that defines the N terminus of P85\textsubscript{P146} (see Fig. S1 in the supplemental material) and confirms the TEP \downarrow QQ cleavage site.

To determine whether fragments of P146 reside on the surface of the bacterium, we biotinylated intact\textit{M. hyopneumoniae} cells and enriched biotin-conjugated proteins from whole-cell extracts using a monomeric avidin column. Biotinylated surface proteins were then separated using both 1- and 2-D GE. Peptides unique to P50\textsubscript{P146}, P40\textsubscript{P146}, and P85\textsubscript{P146} were identified from 2-D GE at molecular masses similar to those from previous 1-D SDS-PAGE with LC-MS/MS experiments (Fig. 1A to C). However, peptides unique to P146 fragments were also identified at masses significantly lower than those identified previously. Tryptic peptides mapping to P50\textsubscript{P146} were identified from proteins with masses of \( \sim 25 \text{ kDa} \) (Fig. 1D), indicating that a second, less efficient cleavage event may occur to separate the P50\textsubscript{P146} fragment into two halves. Confirming this, peptides unique to the second half of P50\textsubscript{P146} were identified from 2-D GE of biotinylated surface proteins at \( \sim 25 \text{ kDa} \) (Fig. 1E). A peptide sequence, TKSFQT, similar to but not matching the S/T-X-F \( \downarrow \) X-D/E cleavage motif, is found within the P50\textsubscript{P146} fragment, indicating a possible cleavage site. Peptides unique to the P85\textsubscript{P146} fragment were also identified from 2-D GE of biotinylated surface proteins in spots at \( \sim 50 \text{ kDa} \) (Fig. 1F), indicating further cleavage of these fragments.

While surface exposure of P146 endoproteolytic fragments was observed in biotinylation experiments, we also performed whole-cell digestion experiments with trypsin. Antiserum generated against recombinant polyhistidine-tagged proteins F1\textsubscript{P146}, F2\textsubscript{P146}, and F3\textsubscript{P146}, which recognize P50\textsubscript{P146}, P40\textsubscript{P146}, and P85\textsubscript{P146}, respectively (Fig. 2), were used to probe blots containing lysates of\textit{M. hyopneumoniae} strain J exposed to different concentrations of trypsin for 15 min. The blots show that all P146 fragments are degraded by trypsin, confirming their surface location (see Fig. S2A in the supplemental material). In all immunoblots, trypsin digestion products are observed in concentrations as low as 0.5 \( \mu \text{g ml}^{-1} \), and major cleavage products are almost completely digested at a concentration of 50 \( \mu \text{g ml}^{-1} \). Immunoblots containing identical lysates probed with antiserum raised against the ribosomal protein L7/L12 showed that this protein was still detected at a trypsin concentration of 300 \( \mu \text{g ml}^{-1} \). Consistent with previous experiments (19, 21, 22, 25–27), these data indicate that the cell membrane remained intact. We also performed mass spectrometry on peptides released from the surface of\textit{M. hyopneumoniae} after trypsin digestion. Multiple peptides unique to all major cleavage fragments of P146 were identified by LC-MS/MS (see Fig. S2B in the supplemental material). Live/dead staining and flow cytometry of\textit{M. hyopneumoniae} cells confirmed the absence of excessive cell lysis during trypsin and surface biotinylation experiments (data not shown).

**Antibodies from vaccinated, challenged, and convalescent pigs recognize P146.** To determine whether P146 sequences were recognized by the porcine humoral immune response, sera from pigs recovering from porcine enzootic pneumonia (convalescent-phase sera) were used to probe immunoblots of P146 recombiant proteins F1\textsubscript{P146}, F2\textsubscript{P146}, and F3\textsubscript{P146}. The results were compared with results for serum from a pig from a healthy herd (high-health-status sera) (Fig. 2D). Immunoblots probed with high-health-status sera (negative control) showed a strong reaction to F2\textsubscript{P146} and weak reactions to F1\textsubscript{P146} and F3\textsubscript{P146}. Immunoblots probed with sera from two separate convalescent pigs both showed increased reactions to all three recombinant proteins compared to the high-health-status sera. In addition to convalescent porcine sera, we also examined serum from a single pig vaccinated with the commercial bacterin vaccine Suvaxyn and serum from the same pig after subsequent challenge with a pathogenic strain of\textit{M. hyopneumoniae} (Fig. 2E). Immunoblots probed with sera collected prior to vaccination again showed a strong reaction to F2\textsubscript{P146}, but no significant reactions to F1\textsubscript{P146} and F3\textsubscript{P146}. All three recombinant proteins showed strong reactions when probed with sera collected after treatment with Suvaxyn and 6 weeks after challenge with virulent\textit{M. hyopneumoniae} strain Hillcrest.

**P146 is processed variably in\textit{M. hyopneumoniae} field isolates.** To determine whether the cleavage observed in P146 was consistent across multiple\textit{M. hyopneumoniae} strains, cell lysates of laboratory strains and field isolates from varied geographical locations were analyzed by immunoblotting with anti-F1\textsubscript{P146}, anti-F2\textsubscript{P146} and anti-F3\textsubscript{P146} sera. Processing of the P85\textsubscript{P146} strain P50\textsubscript{P146} and P40\textsubscript{P146} fragments varied among strains (Fig. 3A). In strain J and field isolates 2-22421 and 95MP1509, strong bands at 40 kDa and 50 kDa were dominant when the blots were probed with anti-F1\textsubscript{P146} and anti-F2\textsubscript{P146} sera, respectively. However, in strain 232 and field isolates OMZ407, C1735/2, and 00MP1301, dominant bands were also observed at \( \sim 80 \) and \( \sim 125 \text{ kDa} \) with these antisera, indicating that cleavage of P146 may be less efficient in these strains. An \( \sim 80\)-kDa band that appears in immunoblots with anti-F1\textsubscript{P146} and anti-F2\textsubscript{P146} sera suggests inefficient cleavage between P50\textsubscript{P146} and P40\textsubscript{P146} whereas a \( \sim 125\)-kDa band that appears in immunoblots with anti-F2\textsubscript{P146} and anti-F3\textsubscript{P146} sera suggests inefficient cleavage between P40\textsubscript{P146} and P85\textsubscript{P146}. To confirm this, we performed SDS-PAGE on a cell lysate of\textit{M. hyopneumoniae} strain 232, sectioned the gel with molecular mass range from 75 to 125 kDa into 6 slices, digested them with trypsin, and examined the tryptic peptides eluted from each slice using LC-MS/MS (Fig. 3B). In gel slice 1 (\( \sim 110 \) to 125 kDa), peptides unique to both P40\textsubscript{P146} and P85\textsubscript{P146} were identified, indicating that an uncleaved fragment made of P40\textsubscript{P146} and P85\textsubscript{P146} was present (Fig. 3C). In gel slices 5 and 6 (\( \sim 75 \) to 85 kDa), peptides unique to P50\textsubscript{P146}, P40\textsubscript{P146}, and P85\textsubscript{P146} were identified, indicating that an uncleaved fragment made of P50\textsubscript{P146} and P40\textsubscript{P146} was present migrating at a molecular mass similar to that of fragment P85\textsubscript{P146} (Fig. 3D). In gel slice 4 (\( \sim 85 \) to 90 kDa), only peptides unique to fragment P85\textsubscript{P146} were detected. Analysis of\textit{M. hyopneumoniae} genome sequences reveals that both the TEFQQ and putative TY-FAE motifs are present and unchanged in both strains J and 232 (data not shown).

**Molecular analysis of P146.** P146 contains many striking sequence features, including three variable repeat regions, a KEKE repeat motif, and in strain J, a C terminus with the sequence SSS-SAPAAAAAKAAK (Fig. 4A). Three repeat regions identified previously include a proline-glutamine repeat (P146R1) spanning residues 415 to 444 of the strain 232 sequence, a proline-serine repeat (P146R2) spanning residues 696 to 722, and a serine repeat (P146R3) spanning residues 1065 to 1085 (33). All three regions vary in length in strains (33). The serine repeat varies from 44 consecutive residues in strain 7448, 19 in strain J, and 21 residues in strain 232 (6, 7).

P146 shares significant sequence identity with P97 family...
members of the \textit{M. hyopneumoniae} genome, but this is essentially restricted to a region spanning \textapprox{} 400 amino acids from the N terminus. The remainder of the P146 protein is largely unique (6).

Outside of the \textit{M. hyopneumoniae} genome, BLASTP analysis determines that P146 shares the most similarity with hypothetical proteins of \textit{Mycoplasma conjunctivae}: MCJ\_004230, MCJ\_004150, MCJ\_005100, MCJ\_003150, and the LppS adhesin; this is unsurprising considering previously reported similarity between adhesins of these \textit{Mycoplasma} species (34). Analysis of P146 with coiled-coil prediction algorithms reveals two putative coiled-coil regions spanning residues 939 to 975 (coil 1) and 1210 to 1245 (coil 2) of the strain 232 sequence. The Paircoil2 (35) (coil 1 \textit{P} score = 0.0078, coil 2 \textit{P} score = 0.0153; coiled-coil predicted at a \textit{P} score of \textless{} 0.025) and COILS algorithms (36) (coil 1 \textit{P} score = 0.802, coil 2 \textit{P} score = 1.000) both indicated that coil 1 and coil 2 regions form coiled-coil structures. The coil 2 region corresponds to a KEKE motif, and interestingly, a putative coiled-coil linked to a KEKE motif has been observed previously in the P146 operon partner Mhp683 (22).

Previously we have shown that dominant cleavage sites in P97 and P102 paralogs were located within regions of predicted protein disorder (22). Five regions of significant structural disorder were predicted in P146 using the VSL1 algorithm (37) spanning residues 324 to 508, 589 to 742, 917 to 1016, 1045 to 1098, and 1224 to 1317 (Fig. 4B). Cleavage motifs TYFAE (P50\_P146/P40\_P146) and TEFQQ (P40\_P146/P85\_P146) reside within two predicted disor-
ordered regions spanning amino acids 324 to 508 and 589 to 742. Interestingly, most of P40 P146 is predicted to be disordered, and this fragment is clearly the most unstable compared to P50P146 and P85P146. This prediction suggests that P40P146 displays greater accessibility to endoproteolytic cleavage. P146 fragments bind heparin. Members of the P97/P102 families have a propensity to bind sulfated GAGs (19, 21, 22, 24, 26, 31). Recombinant fragments F1P146/232 and F3P146/232 bound heparin in a dose-dependent and saturable manner with physiologically relevant affinity constants, as assessed in microtiter plate-based enzyme-linked immunosorbent assays (ELISAs) (see Fig. S3A in the supplemental material). F2P146/232 did not significantly bind heparin in microtiter plate assays (data not shown). F1P146/232 and F3P146/232 binding to heparin was largely specific, as the interactions were almost completely (F1 P146/232) or mostly (F3P146/232) inhibited by the addition of a 50-fold excess unlabeled heparin. Affinity constants ($K_d$) of $5 \times 10^{-5}$ and $520 \times 10^{-5}$ nM were calculated for F1 P146/232 and F3 P146/232, respectively. Pre-in incubation of biotinylated heparin with unlabeled heparin or fucoidan inhibited binding of F1P146/232 and F3 P146/232, while a band found at a similar mass in strain 232 immunoblots. (D) Slices 5 and 6 (~75 to 85 kDa) were found to contain peptides unique to all major fragments of P146, suggesting that a cleavage event separated the protein into two separate ~80-kDa fragments.

FIG 3 P146 is processed variably in M. hyopneumoniae laboratory strains and field isolates. (A) Immunoblots of whole-cell lysates of different M. hyopneumoniae strains and field isolates. The blots were separately probed with anti-F1P146 (αF1P146), anti-F2bP146 (αF2bP146), and anti-F3P146 (αF3P146) sera in order to assess the consistency of protein expression and processing. Cleavage fragments equivalent to P50P146, P40P146, and P85P146 are conserved across all isolates in this study, but some also show distinct bands at higher masses that correspond to fragments that would result from inefficient cleavage. (B) 1-D SDS-polyacrylamide gel (molecular mass region ~75 to 125 kDa) of an M. hyopneumoniae strain 232 cell lysate was sectioned into 6 slices, and each slice was examined using LC-MS/MS. (C) Slice 1 (~110 to 125 kDa) was found to contain peptides unique to regions of P146 corresponding to P40P146 and P85P146 fragments and matched a band found at a similar mass in strain 232 immunoblots. (D) Slices 5 and 6 (~75 to 85 kDa) were found to contain peptides unique to all major fragments of P146, suggesting that a cleavage event separated the protein into two separate ~80-kDa fragments.
preincubation with porcine mucin or chondroitin sulfate B had limited or no significant effect (see Fig. S3B in the supplemental material).

**F3**<sub>P146</sub> binds plasminogen. In *M. hyopneumoniae* and other bacterial pathogens, surface proteins possessing a C-terminal lysine residue have been correlated with the ability to bind the serine protease precursor plasminogen (25–27, 38). P146 homologs in strains J, F7,2C, and BQ14 possess a C-terminal lysine residue (−SSSSAPAAAAAAKAAK); however, in strains 232, 7448, and 168, the terminating residue is an arginine (−SSSSAPAAAAAAKAR). To determine whether this sequence variability influences interactions with plasminogen, we cloned F3<sub>P146</sub> from both strains 232 and J and examined their binding to plasminogen using dot blotting and surface plasmon resonance (SPR). In dot blotting experiments, the J and 232 versions (F3<sub>P146/J</sub> and F3<sub>P146/232</sub>) bound biotinylated plasminogen similarly (see Fig. S1A and S1B in the supplemental material). SPR experiments indicated that both also bound plasminogen in a dose-dependent manner with *K<sub>d</sub>* = 39 ± 3 nM and association rate (*k<sub>a</sub>*) = (4.6 ± 0.5) × 10<sup>4</sup> M<sup>−1</sup>s<sup>−1</sup> for F3<sub>P146/J</sub> and *K<sub>d</sub>* = 46 ± 6 nM and *k<sub>a</sub>* = (1.7 ± 0.5) × 10<sup>4</sup> M<sup>−1</sup>s<sup>−1</sup> for F3<sub>P146/232</sub> (see Fig. S1C and S1D in the supplemental material). This suggests that whether the C-terminal basic residue is arginine or lysine has little effect on binding affinity; it does modestly affect the kinetics of interaction, with recognition of arginine being ~4-fold more efficient than lysine.

To examine this further, we cloned strain J and 232 versions of F3<sub>P146</sub> without their respective C-terminal residues (F3<sub>P146/3AK</sub> and F3<sub>P146/232AK</sub>); these two proteins have near identical C-terminal sequences. Both displayed 5-fold-reduced affinity for plasminogen and as expected showed identical binding affinity and kinetics; *K<sub>d</sub>* = 210 ± 33 nM and *k<sub>a</sub>* = (1.3 ± 0.2) × 10<sup>4</sup> M<sup>−1</sup>s<sup>−1</sup> for F3<sub>P146/3AK</sub> and *K<sub>d</sub>* = 220 ± 33 nM and *k<sub>a</sub>* = (1.3 ± 0.2) × 10<sup>4</sup> M<sup>−1</sup>s<sup>−1</sup> for F3<sub>P146/232AK</sub> (see Fig. S2E and S2F in the supplemental material). These data clearly quantify the significance of having a C-terminal basic residue (a 5-fold contribution to affinity) and whether it is a lysine or an arginine (an effect on binding kinetics).

### P146 fragments bind porcine cilia

A microtiter plate-based assay used previously to identify cilium-binding proteins showed that P146 recombinant proteins P1<sub>P146/232</sub> and F3<sub>P146/232</sub> reproducibly bound cilia (see Fig. S5 in the supplemental material). The recombinant protein F4<sub>Mhp385</sub>, derived from the C terminus of Mhp385 and previously observed to display low cilium-binding properties (39) did not bind porcine cilia and was included as a negative binding control in these studies. F2<sub>P97</sub>, a recombinant protein that carries the R1 cilium-binding domain of cilium adhesin P97, was used as a positive control and bound to porcine cilia as expected.

### DISCUSSION

Members of the P97/P102 paralog families are secreted to the surface of *M. hyopneumoniae* where they play key roles in interactions with host cells, extracellular matrix components, and circulating host proteins (19, 21–27, 30). There are three overarching features that define members of these two protein families. The first feature is the presence of a single putative transmembrane domain at the N terminus that is not removed by signal peptidase 1 (19, 20, 22). Second, these molecules undergo endoproteolytic processing whereby N-terminal cleavage fragments presumably remain tethered to the cell membrane via the N-terminal transmembrane domain while central and C-terminal endoproteolytic fragments are released. Despite the absence of transmembrane domains distant from the N terminus, central and C-terminal cleavage fragments remain attached to the cell surface of *M. hyopneumoniae*, presumably by protein–protein interactions that are poorly understood. Third, P97 and P102 paralogs are multifunctional proteins that bind host cell-associated and circulatory molecules and present a variety of structural motifs with largely unknown functions (19–22, 24–27).

Here we show that P146, a paralog of P97, contains several structurally distinctive motifs including a polyserine repeat of unknown function and a serine/alanine-rich C-terminal motif [SSSSAin motif (bin motif)] whose terminal K or R (strain-dependent) residue plays an important role in binding plasminogen (see Fig. S4 in the supplemental material). We also identified two putative coiled-coil regions, one of which carries a KEKE motif, and...
arginine in strains 232, 7448, and 168 and lysine in strains J, F7.2C, lysine residues (25–27). P146 is particularly interesting due to the plasminogen-binding proteins, three of which contain C-terminal invasion and systemic infection. To date, including P146, we have plasminogen on its cell surface and facilitate its conversion to pneumoniae activity is significantly increased in the BAL fluid of pigs following lian plasminogen activators (e.g., tPA). Importantly, plasmin ac-gen in a process that facilitates conversion to plasmin by mamma-lian plasminogen via a C-terminal arginine present at the throm-bin cleavage site (42).

While the binding of plasminogen by proteins with C-terminal S/T-X-F | X-D/E cleavage motif described recently (22) were identified in regions spanning tryptic peptides that define these cleavage fragments. We confirmed the identity of one cleavage site by LC-MS/MS analysis of the semitryptic peptide 676QQQDANSTNSSPTSPSPTSPSAPSSPSPTSPK712 which defines the N terminus of the C-terminal cleavage fragment P85P146. The cleavage motif 673TEFQOQ777 displays a S/T residue in the +3 position, and cleavage occurs at the Phe residue residing at position −1. Although no D/E is present in the +2 position, these features are in accord with cleavage sites in other members of the P97 and P102 paralogs (22). We were unable to experimentally confirm the putative cleavage site 351TYFAE355 predicted to generate P50P146 and P40P146.

Immunoblots of cell lysates of M. hyopneumoniae strains from different geographic locations probed separately with anti-F1P146 and anti-F3P146 sera showed that cleavage in strain 232 and field isolates OMZ407, C17735/2, and 00M1301 is less efficient than in strain J and field isolates 2-22421 and 95MP1509. We have previously indicated that P97 (Mhp183) (20) and P159 (Mhp494) (19) undergo strain-specific processing events. However, the molecular identities of the strain-specific products were not identified. For the first time, we show here that strains of M. hyopneumoniae vary in their ability to efficiently cleave P146. We identified large endopeptidolytic products comprising a C-terminal 120-kDa fragment (P85P146 plus P40P146), derived from an inefficient cleavage event at the 673TEFQOQ777 site and an N-terminal P90P146 fragment (P50P146 plus P40P146), derived from inefficient processing at the putative 351TYFAE355 cleavage site (Fig. 5). P120P146 and P90P146 sequences were identified from high-molecular-mass regions of cell lysates of strain 232 by LC-MS/MS (Fig. 1B to D) and by probing cell lysates with anti-F1P146 and anti-F3P146 sera (Fig. 3A).

We also identified minor endopeptidolytic cleavage fragments with masses smaller than the dominant cleavage species P50P146, P40P146, and P85P146 by enriching biotinylated proteins with avi-din chromatography (Fig. 5). LC-MS/MS of biotinylated proteins resolved by 2-D gel electrophoresis confirmed that P50P146 (pre-

determine whether these residues have a role in F3P146 adherence to plasminogen.

LC-MS/MS of proteins resolved by 1- and 2-D gel electrophoresis identified endopeptidolytic cleavage fragments representing P50P146, P40P146, and P85P146. Two sequences that conform to the S/T-X-F | X-D/E cleavage motif described recently (22) were identified in regions spanning tryptic peptides that define these cleavage fragments. We confirmed the identity of one cleavage site by LC-MS/MS analysis of the semitryptic peptide 676QQQDANSTNSSPTSPSPTSPSAPSSPSPTSPK712 which defines the N terminus of the C-terminal cleavage fragment P85P146. The cleavage motif 673TEFQOQ777 displays a S/T residue in the +3 position, and cleavage occurs at the Phe residue residing at position −1. Although no D/E is present in the +2 position, these features are in accord with cleavage sites in other members of the P97 and P102 paralogs (22). We were unable to experimentally confirm the putative cleavage site 351TYFAE355 predicted to generate P50P146 and P40P146.

Immunoblots of cell lysates of M. hyopneumoniae strains from different geographic locations probed separately with anti-F1P146 and anti-F3P146 sera showed that cleavage in strain 232 and field isolates OMZ407, C17735/2, and 00M1301 is less efficient than in strain J and field isolates 2-22421 and 95MP1509. We have previously indicated that P97 (Mhp183) (20) and P159 (Mhp494) (19) undergo strain-specific processing events. However, the molecular identities of the strain-specific products were not identified. For the first time, we show here that strains of M. hyopneumoniae vary in their ability to efficiently cleave P146. We identified large endopeptidolytic products comprising a C-terminal 120-kDa fragment (P85P146 plus P40P146), derived from an inefficient cleavage event at the 673TEFQOQ777 site and an N-terminal P90P146 fragment (P50P146 plus P40P146), derived from inefficient processing at the putative 351TYFAE355 cleavage site (Fig. 5). P120P146 and P90P146 sequences were identified from high-molecular-mass regions of cell lysates of strain 232 by LC-MS/MS (Fig. 1B to D) and by probing cell lysates with anti-F1P146 and anti-F3P146 sera (Fig. 3A). We also identified minor endopeptidolytic cleavage fragments with masses smaller than the dominant cleavage species P50P146, P40P146, and P85P146 by enriching biotinylated proteins with avi-din chromatography (Fig. 5). LC-MS/MS of biotinylated proteins resolved by 2-D gel electrophoresis confirmed that P50P146 (pre-
dicated pI of 9.13) is cleaved to generate two fragments ~25 kDa in size. The C-terminal half of P50p146 is enriched in acidic amino acids (D or E residues). LC-MS/MS of a protein spot that migrated with a mass of ~25 kDa and pI of 5.5 confirmed that the protein represented the C terminus of P50p146. We have not determined the precise site of cleavage in P50p146. Minor fragments of P50p146 and P40p146 with different molecular masses were detected with anti-F1p146 and anti-F2b p146 sera, respectively (Fig. 3). This may indicate variation at the P50p146 C terminus and P40p146 N terminus caused by minor cleavage events (Fig. 5). LC-MS/MS identified a 50-kDa N-terminal fragment of P85p146 with a pI of ~5.0. It is interesting that the minor cleavage fragments identified by LC-MS/MS display predicted pI values that are considerably more acidic than those of P50p146 (pI = 9.13), P40p146 (9.33), and P85p146 (7.07), and of intact P146 (8.90). These data suggest that products of these minor cleavage events are not random breakdown products but represent domains enriched in acidic amino acids embedded within larger preproteins with strongly basic pIs. Furthermore, as noted for P159 (Mhp494) and P216 (Mhp493), regions enriched in acidic amino acid residues are a feature of members of the P97 and P102 paralog families (19, 21) and underscore their modular structure.

In total, we identified nine proteolytic cleavage fragments representing different regions of P146. These fragments are sensitive to digestion by trypsin, and many were recovered by avidin chromatography after the surfaces of M. hyopneumoniae cells were labeled with biotin. These data show that complex endoproteolytic processing events occur either on the surface of M. hyopneumoniae or prior to transport across the cell membrane. Processing is modulated by the efficiency of cleavage at dominant cleavage sites because different strains present different cleavage fragments on their cell surface. The mechanism by which M. hyopneumoniae regulates endoproteolytic cleavage remains unknown. Nonetheless, we have provided further evidence that cleavage often occurs at sites that conform to the S/T-X-F-X-D/E cleavage motif described recently (22). P97 is also subject to extensive endoproteolysis, but so far the events that precipitate cleavage and the precise composition of many of the endoproteolytic fragments have not been experimentally determined (20). Endoproteolytic processing occurs with all members of the P97 and P102 paralog families, and in at least two cases (P97 and P146), processing is more extensive than originally envisaged. Endoproteolytic processing significantly increases surface protein diversity by altering the presentation of functional domains on the cell surface and may be a mechanism used to regulate adhesion to host tissues, potentiate presentation of functional domains on the cell surface and may be a significantly increases surface protein diversity by altering the pre-

**MATERIALS AND METHODS**

Porcine sera and Mycoplasma hyopneumoniae strains and culture. Convalescent porcine sera were obtained from finisher pigs from a herd infected with M. hyopneumoniae in an area where M. hyopneumoniae is endemic (Lockad, Australia). High-health-status serum was obtained from a high-health-status herd and tested negative for M. hyopneumoniae infection using a commercial ELISA kit. Pretreatment (negative-control) serum was obtained from a 9-week-old pig that tested negative to M. hyopneumoniae by quantitative PCR (qPCR) (44). The pig was subsequently vaccinated at 9, 12, and 15 weeks with Suvaxyn. Postvaccination serum was collected 15 weeks after the third vaccination. The pig was challenged with M. hyopneumoniae strain Hillcrest at week 17 and post-challenge serum was collected at week 23. The sources and conditions used to culture and harvest M. hyopneumoniae strains J, 232, and Hillcrest and field isolates OMZ407, 00MP1301, 2-2241, C1735-2, and 95MP1509 are as previously described (22, 24, 45).

**Proteomics and surface localization.** The materials and methods used for 1- and 2-D GE, immunoblotting, trypsin digestion, Zip-Tip cleanup, reduction, and alkylation have been previously described (20, 24, 46–48) with the following adjustments. For 2-D GE, first-dimension immobilized pH gradient (IPG) strips (ReadyStrip IPG strips, 110 mm long, pH 3 to 10; Bio-Rad) were used. The strips were rehydrated overnight with 300 μg of biotinylated surface protein in 210 μl of solubilization buffer [7 M urea, 2 M thiourea, 1% (wt/vol) 3-(4-heptyl)phenyl-3-hydroxypropyl]dimethylammonio]propanesulfonate] and overlaid with paraffin oil. M. hyopneumoniae proteins were reduced prior to 1-D SDS-PAGE for immunoblotting and reduced and alkylated with iodoacetamide prior to 1-D SDS-PAGE for LC-MS/MS. Gel slices prepared from 1-D SDS-PAGE for LC-MS/MS analysis were processed as previously described (24). LC-MS/MS analysis of 1- and 2-D GE excisions were done as previously described (22, 49), as were graded trypsin digestions of intact M. hyopneumoniae cells (22) and trypsin digestion experiments for mass spectrometry and biotinylation of the M. hyopneumoniae surface (22). Cell integrity for trypsin and biotinylation experiments was evaluated by flow cytometry with a BDTM LSRII (BD Biosciences, NJ) and YO-PRO-1 iodide dead cell dye (Invitrogen, CA) with data analyzed using BD FACSDiva software (BD Biosciences). Bioinformatic analysis of P146 used online resources: BLASTP (50), ProtParam (51), COILS (36), Paircoil2 (35), TMHMM (52), SignalP (53), and PONDR VSL1 (37).

**Expression of recombinant proteins and creation of polyclonal antisera.** Cloning of p146 was performed by using three fragments of different lengths (Fig. 2A) and based on the M. hyopneumoniae strain 232 genome sequence (6); fragments were constructed to mimic the P146 cleavage pattern as determined by preliminary mass spectrometry experiments. The fragments were denoted F1p146/232, F2p146/232, and F3p146/232 and ranged from amino acids 53 to 372, 376 to 690, and 691 to 1317, respectively. F3p146/232 and F3p146/232 were generated from M. hyopneumoniae strain J genome sequence (7). In-frame TGA codons were substituted by TGG and C-terminal residues (F3p146/232 and F3p146/232) were removed by use of overlap extension PCR or site-directed mutagenesis using mutagenic primers (see Table S1 in the supplemental material). The fragments were amplified by PCR from M. hyopneumoniae strain 232 chromosomal DNA using Pfu polymerase (Agilent Technologies, CA) and cloned into the pET100/GW/D-TOPO vector (Invitrogen) as described previously (24). Protein expression was performed using Escherichia coli BL21 Star (DE3) as previously described (27) with the following alterations: protein expression was performed using either LB broth, Terrific broth, or 2X Terrific broth depending on the amount of proteolytic cleavage observed in preliminary experiments. Expression of recombinant proteins was initiated by incubating cells at 18°C for 30 min prior to the addition of isopropyl-β-d-thiogalactopyranoside (IPTG), followed by incubation at 18°C overnight. Recombinant proteins were purified natively by nickel affinity chromatography and dialyzed in phosphate-buffered saline (PBS) (Fig. 2B); their concentrations were estimated as described previously (31). Polyclonal antisera (anti-F1p146/232 and anti-F3p146/232 sera) to recombinant proteins F1p146/232 and F3p146/232 were prepared by immunization of New Zealand White rabbits as previously described (54). Polyclonal antisera anti-F2p146/232 was prepared similarly with a recombinant protein representing residues 586 to 690 of P146. For some immunoblots, purified anti-F1p146/232 antibodies were used to remove cross-reactions and were prepared as described previously (55). All antisera were tested for activity by immunoblotting with recombinant protein (Fig. 2C).

**Binding assays.** Heparin-binding, inhibition, and competitive immunoassays were performed in 96-well, flat-bottomed microtitre plates (Linbro/Titertek; ICN Biomedicals Inc., Aurora, OH) as described elsewhere (31). The binding of F1p146/232, F2p146/232 to porcine cilia was examined using a microtitre plate adherence assay developed for the identification of the calcium-binding protein P97 (21). SPR analyses were performed using...
a BIAcore T100 instrument (Biacore AB, Sweden), operated at 20°C. The preparation of analyte proteins, ligand immobilization, kinetics assays, porcine plasminogen purification, and biotinylation of plasminogen have been described previously (25). Plasminogen was immobilized as a ligand for SPR assays; binding of recombinant P146 proteins was measured with a concentration range of 0 to 200 nM. A blank immobilized flow cell was used as a reference cell. F3-gal association kinetics with plasminogen were analyzed as the sum of two exponentials for a heterogeneous surface as described previously (24). Plasminogen dot blots were performed (24) with 5 µg ml⁻¹ biotinylated porcine plasminogen as described previously.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org

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