Molecular Characterization of the *Mycoplasma gallisepticum* pvpA Gene Which Encodes a Putative Variable Cytadhesin Protein

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A putative cytadhesin-related protein (PvpA) undergoing variation in its expression was identified in the avian pathogen *Mycoplasma gallisepticum*. The pvpA gene was cloned, expressed in *Escherichia coli*, and sequenced. It exhibits 54 and 52% homology with the P30 and P32 cytadhesin proteins of the human pathogens *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, respectively. In addition, 50% homology was found with the MGC2 cytadhesin of *M. gallisepticum* and 49% homology was found with a stretch of 205 amino acids of the cytadherence accessory protein HMW3 of *M. pneumoniae*. The PvpA molecule possesses a proline-rich carboxy-terminal region (28%) containing two identical directly repeated sequences of 52 amino acids and a tetrapeptide motif (Pro-Arg-Pro-X) which is repeated 14 times. Genetic analysis of several clonal isolates representing different expression states of the PvpA product ruled out chromosomal rearrangement as the mechanism for PvpA phase variation. The molecular basis of PvpA variation was revealed in a short tract of repeated GAA codons, encoding five successive glutamate residues, located in the N-terminal region and subject to frequent mutation generating an in-frame UAA stop codon. Size variation of the PvpA protein was observed among *M. gallisepticum* strains, ranging from 48 to 55 kDa and caused by several types of deletions occurring at the PvpA C-terminal end and within the two directly repeated sequences. By immunoelectron microscopy, the PvpA protein was localized on the mycoplasma cell surface, in particular on the terminal tip structure. Collectively, these findings suggest that PvpA is a newly identified variable surface cytadhesin protein of *M. gallisepticum*.

*Mycoplasma gallisepticum* is an important pathogen of chickens and turkeys of considerable economic importance to poultry producers throughout the world (20). *M. gallisepticum* infection has a wide variety of clinical manifestations, the most significant of which is chronic respiratory disease of chickens, causing pathology in the form of tracheitis and air sacculitis (20). Like that of the human mycoplasmas, the morphology of *M. gallisepticum* is characterized by a flask-shaped appearance and a specialized tip-like organelle which mediates cytadhesion to the tracheal epithelial cells (29). Recently, three putative cytadhesin molecules (MGC1, MGC2, and GapA) were identified in *M. gallisepticum* (8, 10, 12). MGC2 was shown to be clustered at the tip organelle and was functionally implicated in cytadhesin (10). Interestingly, comparison of the known cytadhesin accessory molecules from *M. pneumoniae* (P30) and *M. genitalium* (P32) with the analogous molecules in *M. gallisepticum* (MGC1, MGC2, and GapA) revealed the presence in all of a proline-rich C-terminal region containing repeated coding sequences, as well as amino acid sequence homology (4, 5, 8, 10, 12, 31). These findings suggest that these pathogenic mycoplasmas possess a family of conserved cytadhesin molecules used to colonize widely divergent hosts.

We recently identified in *M. gallisepticum* a surface protein designated PvpA (49), exhibiting the following features: PvpA (i) is an integral membrane surface protein with a free C terminus, (ii) possesses an epitope shared by three distinct variant surface lipoproteins of the bovine pathogen *Mycoplasma bovis* (1, 49), (iii) is subject to spontaneous high-frequency variation in expression, (iv) exhibits size variation among strains, and (v) is not a lipoprotein.

In this study, we have characterized the *M. gallisepticum* pvpA gene and investigated the molecular basis of PvpA phase variation. These findings suggest that PvpA is a newly identified variable surface cytadhesin protein of *M. gallisepticum*. The PvpA molecule possesses a proline-rich carboxy-terminal region (28%) containing two identical directly repeated sequences of 52 amino acids and a tetrapeptide motif (Pro-Arg-Pro-X) which is repeated 14 times. Genetic analysis of several clonal isolates representing different expression states of the PvpA product ruled out chromosomal rearrangement as the mechanism for PvpA phase variation. The molecular basis of PvpA variation was revealed in a short tract of repeated GAA codons, encoding five successive glutamate residues, located in the N-terminal region and subject to frequent mutation generating an in-frame UAA stop codon. Size variation of the PvpA protein was observed among *M. gallisepticum* strains, ranging from 48 to 55 kDa and caused by several types of deletions occurring at the PvpA C-terminal end and within the two directly repeated sequences. By immunoelectron microscopy, the PvpA protein was localized on the mycoplasma cell surface, in particular on the terminal tip structure. Collectively, these findings suggest that PvpA is a newly identified variable surface cytadhesin protein of *M. gallisepticum*.

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variation as well as its size variation. The structural features of the PvpA protein, its surface localization, and its high homology to other mycoplasmal cytadhesin accessory molecules suggest that PvpA is a newly identified variable cytadhesin protein of M. gallisepticum.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. M. gallisepticum strains R, F, HHT5, K703, and A5969 were obtained from the Jerusalem laboratory collection; their origin, properties, and growth conditions are described elsewhere (48). E. coli K2101 were obtained from the Georgia laboratory collection. The Escherichia coli strains used were DH5αMC (Gibco BRL, Life Technologies, Inc., Gaithersburg, Md.) and Y1090 (Promega, Madison, Wis.). Recombinant clones were constructed in the plasmid vector pBluescript II KS+ (Stratagene, La Jolla, Calif.).

Chemicals, media, and growth conditions. E. coli cultures for plasmid and bacteriophage isolation were grown with shaking at 37°C in Luria-Bertani broth (49). E. coli cultures for expression of proteins under T7 promoter control (40) were grown at 30°C with shaking in M9 medium (34) supplemented with an amino acid mixture. Restriction enzymes, T4 ligase, and T4 polynucleotide kinase were purchased from Promega and used according to the manufacturer’s recommendations. β-Galactosidase (1-β-d-galactopyranosyl-1-thio-β-D-galactopyranoside (β-gal), isopropyl-1-thiogalactopyranoside (IPTG), ampicillin, kanamycin, and rifampin were purchased from Sigma Chemicals, St. Louis, Mo. [α-32P]dCTP and [35S]methionine were purchased from Amersham, Little Chalfont, United Kingdom.

Genomic library construction. A recombinant phage library was constructed in the phage vector λgt11 (Promega) using partially digested EcoRI chromosomal fragments from M. gallisepticum strain R expressing the 55-kDa protein of PvpA (49). Viable phage particles were produced by in vitro packaging of recombinant phage DNA using a commercial in vitro lambda DNA packaging system (Promega). Phage plaques were generated in E. coli strain Y1090 on NZCYM plates containing 0.6% (wt/vol) agarose (Gibco BRL).

Immunoscreening of the M. gallisepticum genomic library. Agar plates (80-mm diameter) containing approximately 3 × 10^7 PFU were grown at 42°C for 3.5 h. Plates were then overlaid with nitrocellulose filters saturated with 10 mM IPTG and incubated at 37°C for an additional 3.5 h. Filters were then washed in TBST buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 0.05% Tween 20) and incubated with TBST containing 20% fetal calf serum for 30 min at room temperature to saturate nonspecific protein binding sites. The filters were incubated overnight at 4°C with monoclonal antibody (MAb) 1E5 at a dilution of 1:100 as the primary antibody. The filters were then washed for 15 min at room temperature using three changes of TBST buffer and then incubated for 3 h at room temperature in peroxidase-conjugated goat antiserum to mouse immunoglobulin M at a dilution of 1:1000 (Jackson ImmunoResearch Laboratories, West Grove, Pa., and Nordic, Tilburg, The Netherlands). Filters were developed with the enzyme substrate o-dianisidine (Sigma). Positive phases expressing the PvpA protein were picked, replated at low density, and again immunoscreened. After two rounds of plaque purification, a positive clone was isolated for further analysis.

DNA preparation, labeling, and manipulation. Genomic DNA from M. gallisepticum strains as well as from M. gallisepticum clonal isolates was extracted and purified as previously described (49). The DNA was digested to completion by restriction enzymes, electrophoresed, and subcloned to Southern blot hybridization as previously described (22, 49). Labelling of DNA probes was performed using the HexaLabel DNA labeling kit (MBI Fermentas, Vilnius, Lithuanian). Isolation of bacteriophage DNA was performed using the method for rapid, small-scale isolation of bacteriophage λ DNA, as described elsewhere (34).

RNA isolation and Northern blot analysis. RNA was extracted from mid-logarithmic-phase cultures of M. gallisepticum variants using the RNasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. Briefly, M. gallisepticum cells from overnight culture were incubated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Three hundred fifty microliters of lysis buffer (provided by the manufacturer) and 250 μl of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added, and the sample was loaded onto an RNeasy mini-spin column. RNA bound to the membrane was eluted in water. Fifteen micrograms of total RNA was denatured for 10 min at 65°C in the sample buffer, containing 250 μl of formamide, 83 μl of 37% (vol/vol) formaldehyde, 50 μl of 10% morpholinopropanesulfonic acid buffer (MOPS), and bromophenol blue. Total RNA was separated by electrophoresis in a 1% agarose gel containing 6% (vol/vol) formaldehyde in 1 X MOPS. After electrophoresis, the region containing the RNA marker R (Boehringer, Mannheim, Germany) was stained with ethidium bromide and photographed. The electrophoresed RNA was transferred onto a nylon membrane (Schleicher & Schuell Nytran; Midwest Scientific, St. Louis, Mo.) and baked for 2 h at 80°C.

Prehybridization was performed at 42°C with agitation for 2 h in solution containing 50% formamide, 5 X Denhardt solution (34), 0.1% sodium dodecyl sulfate (SDS), 200 μg of salmon sperm DNA per ml, and 5 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaPO4, pH 7.2, 1 mM EDTA). Hybridization was performed overnight at 42°C in solution containing 50% formamide, 2.5 X Denhardt solution, 0.1% SDS, 100 μg of salmon sperm DNA per ml, 5 × SSPE, and the labeled DNA probe. Membranes were washed twice for 15 min at room temperature in 6 × SSPE-0.1% SDS and once for 15 min at 37°C in 1 × SSPE-0.1% SDS. The membranes were air dried and autoradiographed with Super RX Fuji X-ray film (Fuji, Tokyo, Japan).

DNA sequence analysis. DNA sequence analysis of both strands was performed by the deoxyxero chain termination method (35). Overlapping sets of deletion mutants were generated from the recombinant plasmid carrying the pvpA gene by graduated directions of the Erase-A-Base debase deletion kit (Promega). As sequencing primers, the T7 promoter sequence and the T3 sequences located on the plasmid vector as well as pvpA-related sequences were used. Sequencing was performed with the automatic sequencer dye-terminator cycle sequencing model ABI PRISM 377 (Perkin-Elmer, Foster City, Calif.). Sequence data were analyzed using the computer software Assembligen and MacVector 6.0.

PCR and oligonucleotides. The pvpA gene from M. gallisepticum strains was amplified by PCR. Reactions were carried out in 100 μl containing 10 ng of template DNA, 5 U of Vent DNA polymerase in 1 X Thermopol buffer (New England Biolabs), 2 mM deoxyoligonucleotide triphosphate mixture, and 500 ng of genomic DNA as a template. PCR amplified DNA was subjected to Southern blot hybridization as previously described (22, 49). Labeling of DNA probes was performed in TBST buffer (34) containing 0.1% SDS, 100 μg of salmon sperm DNA per ml, 5 × SSPE, and 50 μg of calf thymus DNA. The resultant products were purified by High Pure filter columns (Boehringer Mannheim GmbH, Indianapolis, Ind.) and directly sequenced. PCR sequence-specific oligonucleotides, used as PCR primers, were synthesized at the interdepartmental facility of the Hebrew University-Hadassah Medical School on a model 380B DNA synthesizer (ABI, Foster City, Calif.). The 26-nucleotide (nt) sequence designated pvpA-3′ oligonucleotide was 5′-GGATTCCTCAAGTCGTGTTAATTC-3′. The 28-nucleotide sequence designated pvpA-5′ oligonucleotide was 5′-CGGAGTATCACCGGAGGACAA-3′. The 17-nucleotide sequence designated pvpA-3′ oligonucleotide was 5′-GGATTCCTCAAGTCGTGTTAATTC-3′.

RESULTS

Cloning and expression of M. gallisepticum pvpA gene. A genomic library of M. gallisepticum strain R expressing a 55-kDa product of PvpA (49) was constructed in the bacteriophage λgt11. The library was immunoscreened with MAb 1E5, which has been previously shown to recognize a common epitope present on three distinct variable surface proteins of M. bovis (1) but also on a phase-variable surface protein (PvpA) of M. gallisepticum (49). One phage, designated MG1t, showing strong immunostaining was purified. Extract-
tion of DNA from MGgtl and digestion with EcoRI restriction enzyme revealed a 0.7-kb DNA insert. In order to detect a larger genomic fragment which might carry the entire pvpA gene and its flanking sequences, the 0.7-kb DNA insert was used as a probe in Southern blot hybridization against restricted genomic DNA of M. gallisepticum strain R. A 1.9-kb EcoRV restriction fragment was identified (data not shown). Gel-excision EcoRV fragments from that region were subcloned into the EcoRV site of the pKS plasmid vector, and E. coli cells harboring the recombinant plasmids were screened using the 0.7-kb DNA insert as a probe. A recombinant plasmid carrying a 1.9-kb EcoRV fragment that hybridized to the probe was selected in this process. To obtain expression of the pvpA gene in E. coli, the T7 RNA polymerase promoter system (40) was utilized. The 1.9-kb EcoRV insert was subcloned in both orientations into the EcoRV site of the pKS plasmid vector, yielding the recombinant plasmids pKP.V1 and pKP.V2, in which the mycoplasma DNA insert was placed under the control of the T7 promoter of this vector. T7 RNA polymerase encoded on a second plasmid (pGP1-2) was induced to selectively initiate transcription of the gene cloned downstream of the T7 promoter. Rifampin was used to inhibit the host RNA polymerase in order to observe protein synthesis from the DNA insert without concurrent synthesis of host proteins. Expressed mycoplasma proteins metabolically labeled with [35S]methionine were separated by SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotted with MAb 1E5, and autoradiographed. A radiolabeled polypeptide band of 55 kDa synthesized under T7 promoter induction from the pKP.V2 plasmid in that orientation only (Fig. 1A, left panel) was strongly recognized by MAb 1E5 (Fig. 1A, right panel). Notably, the size of the recombinant protein in E. coli was similar to that of the authentic PvpA protein expressed in the mycoplasma. These results confirmed that we have cloned and expressed the pvpA gene of M. gallisepticum strain R.

Structural features of the pvpA gene and its deduced protein. The nucleotide sequence of the 1.9-kb EcoRV genomic fragment was determined. Within the sequenced fragment, two open reading frames (ORFs) were identified (Fig. 2A). One complete ORF (PvpA) contained 1,149 nt with a predicted molecular mass of 40.8 kDa. The second and partial ORF, localized upstream from the pvpA gene, extended 231 nt from the EcoRV site to a TAG stop codon and exhibited high homology of 74 and 71% identity to the elongation factor EF- G (51) of M. pneumoniae and M. genitalium, respectively (Fig. 2A).

Examination of the pvpA nucleotide sequence and of the deduced amino acid sequence revealed several notable features (Fig. 1B). The PvpA N-terminal region contains a typical prokaryotic signal sequence which begins with a sequence containing four positively charged Lys residues followed by an adjoining core of 25 hydrophobic amino acids (aa) which terminates with an Ala residue that could serve as a putative secretory signal peptide type I cleavage site (44). Another hydrophobic region exists between aa 74 and 105 of the PvpA molecule (Fig. 1B and 3). This structural feature suggested that the PvpA sequence contained sufficient intrinsic information for its translocation across the plasma membrane and confirmed our earlier observations indicating that PvpA is an integral surface membrane protein (49).

A second noteworthy feature is that the PvpA protein possesses a proline-rich carboxy-terminal region. Sixty proline residues are present within 202 aa (aa 176 through 378) which comprise 53% of the entire PvpA molecule, resulting in a significantly (28%) proline-rich region (Fig. 1B). The high concentration of proline residues may lead to abnormal migration (23) of the PvpA protein, 55 kDa as measured by SDS-PAGE (49), in comparison to a 40.8 kDa molecular mass calculated from the deduced amino acid sequences. In addition, this region was glycine rich (13%) and glutamate rich (14%). Within the proline-rich region, two identical and directly repeated amino acid sequences consisting of four amino acids, PRPX (X is Met, Gln, or Asn), which appears 14 times within the PvpA C-terminal region is highlighted by boldface. The fourth codon, within a tract of five glutamine residues and in which a nonsense mutation has occurred, is marked by an arrowhead. Sixty proline residues within the C-terminal region are marked by asterisks.

![FIG. 1. (A) Expression of the PvpA protein in E. coli under T7 promoter control. E. coli DH5α-MCR cells harboring the recombinant plasmid pKP.V.2 were subjected to selective induction of the T7 promoter. An SDS-PAGE autoradiograph of [35S]methionine-labeled proteins expressed in E. coli (left panel) or a Western blot analysis of the same proteins as depicted in the left panel with MAb 1E5 (right panel) is shown. Cells in both panels were used without induction (lanes 1), with induction of the T7 promoter (lanes 2), or with induction in the presence of rifampin (lanes 3). A labeled arrow indicates the recombinant PvpA 55-kDa product expressed in E. coli. (B) Amino acid sequence and structural features of the PvpA protein from M. gallisepticum strain R. Amino acid residues are numbered on the right. A broken line marks the putative PvpA signal peptide. Labeled arrows show the position and direction of two directly repeated amino acid sequences (DR-1 and DR-2). A repeated motif consisting of four amino acids, PRPX (X is Met, Gln, or Asn), which appears 14 times within the PvpA C-terminal region is highlighted by boldface. The fourth codon, within a tract of five glutamine residues and in which a nonsense mutation has occurred, is marked by an arrowhead. Sixty proline residues within the C-terminal region are marked by asterisks.](http://iai.asm.org/)
cytadhesin accessory proteins of two human pathogenic mycoplasmas. Homologies of 54.2 and 49% were found with the P30 protein and with a stretch of 205 aa (aa 284 through 489) of the HMW3 cytadherence accessory molecule of \textit{M. pneumoniae}, respectively (4, 24), and 52.5% homology was found with the P32 protein of \textit{M. genitalium} (31). In addition, 50% homology was found with the recently described MGC2 cytadhesin molecule of \textit{M. gallisepticum} (10). The relatedness of PvpA to the mycoplasmal adhesin-related proteins is also reflected in their remarkably similar hydrophilicity plots (Fig. 3). All four adhesin-related proteins possess, in addition to the hydrophobic signal peptide region, a second hydrophobic domain at about the same position between aa 70 and 100. The rest of the molecule in all four proteins is hydrophilic and proline rich and contains reiterated coding sequences (Fig. 3).

We have previously shown by the colony immunoblot technique, using MAb 1E5, that PvpA is surface exposed (49). By using MAb 1E5 in immunoelectron microscopy, we could detect the PvpA protein on the mycoplasma cell surface, in particular on the bleb structure of the mycoplasma cell, which has been shown to mediate cytadherence (Fig. 4). Collectively, the structural features of the PvpA molecule, its surface localization and its homology to other mycoplasmal adhesin molecules, suggest a possible role in \textit{M. gallisepticum} cytadherence.

**Localized nonsense mutation in a poly-GAA tract of the \textit{pvpA} coding region determines PvpA antigenic variation.** PvpA...

**FIG. 2. Frameshift mutation implicated in PvpA phase variation. (A)** Schematic representation, structural features, and partial restriction map of the \textit{pvpA} gene and its flanking region. The solid thick line represents a 1.9-kb EcoRV genomic fragment cloned from \textit{M. gallisepticum} strain R. The position of the \textit{pvpA} gene within that fragment is shown by a labeled rectangle. The positions of EcoRV (EV), AccI (A), HincII (H), and CiaI (C) restriction sites are marked. A shaded block labeled S represents a putative signal peptide-encoding region of the \textit{pvpA} gene. The location and direction of two identical repeated sequences (DR-1 and DR-2) within the \textit{pvpA} structural gene are shown by hatched blocks with arrows. The location and direction of a partial ORF, upstream of the \textit{pvpA} gene, which exhibits high identity (74%) to the EF-G protein of \textit{M. pneumoniae} are shown. (B) Nucleotide sequences and their deduced proteins, from PvpA phase variants, spanning the poly-GAA tract located 364 nt downstream of the PvpA initiation codon. A single mutation, occurring in the fourth GAA codon and generating a UAA stop codon, is marked in boldface and indicated by an arrow. (C) Autoradiogram of the poly-GAA region from two PvpA phase variants (on to off). The nucleotide sequences are shown on the left. The position of a point mutation (thymine instead of guanine) in the off variant is marked by an arrowhead. (D) Western blot analysis with MAb 1E5 of total cell proteins from two sequential isolates (depicted in panel C) representing a direct switch of the PvpA from on (lane 1) to off (lane 2). The 55-kDa variable PvpA and the invariant P41 protein (an aqueous protein [see reference 49]) are indicated by arrows. (E) Northern blot analysis of total cell RNA from the two sequential isolates depicted in panel D. The blot was hybridized with a labeled PCR product spanning the \textit{pvpA} coding region as a probe. Two transcripts, of 1.4 and 2.6 kb, are marked by arrows.
was shown to undergo variation in expression at a frequency of about $10^{-3}$ to $10^{-4}$ per cell per generation (49). To analyze the genetic basis of PvpA phenotypic switching, we first examined whether genomic rearrangements (such as inversion or deletion) that might affect PvpA expression could be detected. Genomic DNAs from clonal isolates representing different expression states of the PvpA product were digested with several restriction enzymes and subjected to Southern blot hybridization with the $pvpA$ gene probe. No changes in the $pvpA$ gene or in its flanking regions were observed during phase variation (data not shown). These initial results suggested that the complete $pvpA$ gene is likely to be present at a constant chromosomal site in PvpA phase variants regardless of the expression state and that detectable DNA rearrangements are not associated with PvpA expression.

To determine whether sequence differences occurred between the $pvpA$ gene in the on phase and the gene in the off phase, the nucleotide sequence of the $pvpA$ gene, isolated from clonal isolates exhibiting different expression states of the PvpA product during several generations, was determined and compared. A single nucleotide substitution was detected in a short tract of five repeated GAA codons, encoding five successive glutamate residues, located 303 nt downstream of the ATG start codon (Fig. 1B and 2B and C). In PvpA-negative phenotypes, the guanine residue of the fourth glutamine codon was replaced with the nucleotide thymine, resulting in an in-frame UAA stop codon (Fig. 2B and C). This would result in premature termination of translation and abolition of PvpA expression. It is noteworthy that, in several PvpA$^-$ clonal isolates examined for the nature and location of the nonsense mutation, the mutation was consistently found within the fourth GAA codon and was the only sequence change detected. This suggests that the fourth GAA codon may be a strongly preferred site for such mutation within the $pvpA$ structural gene.

Supporting evidence suggesting that variation in PvpA expression is regulated at the translational level was obtained when the presence of $pvpA$ mRNA was monitored in PvpA-positive and -negative phenotypes. A 910-bp region representing most of the $pvpA$ coding region was amplified by PCR, and

![Hydrophilicity plots of the deduced amino acid sequences of PvpA (M. gallisepticum strain R), P30 (M. pneumoniae), MGC2 (M. gallisepticum), and P32 (M. genitalium). Positive numbers on the left indicate increased hydrophilicity, while negative numbers represent increased hydrophobicity. Amino acid residues are numbered along the bottom of each panel.](http://iai.asm.org/)

**FIG. 3.** Hydrophilicity plots of the deduced amino acid sequences of PvpA (M. gallisepticum strain R), P30 (M. pneumoniae), MGC2 (M. gallisepticum), and P32 (M. genitalium). Positive numbers on the left indicate increased hydrophilicity, while negative numbers represent increased hydrophobicity. Amino acid residues are numbered along the bottom of each panel.

![Immunogold labeling of M. gallisepticum PvpA cytadhesin-related protein. M. gallisepticum cells (strain R) were examined by immunoelectron microscopy for PvpA expression with MAb 1E5 after negative staining. Secondary antibodies containing gold particles distributed mainly on the bleb organelle are shown by an arrow.](http://iai.asm.org/)

**FIG. 4.** Immunogold labeling of M. gallisepticum PvpA cytadhesin-related protein. M. gallisepticum cells (strain R) were examined by immunoelectron microscopy for PvpA expression with MAb 1E5 after negative staining. Secondary antibodies containing gold particles distributed mainly on the bleb organelle are shown by an arrow.
the resultant product was used as a probe in Northern blot analysis of total RNA from clonal isolates displaying different expression states of the PvpA product (Fig. 2D and E, lanes 1 and 2, respectively). Interestingly, two transcripts, one of about 1.4 kb, which fits the size of the pvpA gene and its flanking regions, and a second of about 2.6 kb, were observed in both phenotypes, regardless of the PvpA expression state (Fig. 2E).

Deletions within the 3′ end of the pvpA gene cause size variation of PvpA. By using the 1E5 MAb in Western blot analysis, we have shown that the PvpA antigen exhibits size polymorphism among M. gallisepticum strains (49). MAb 1E5 was also shown to recognize a 41-kDa invariant protein (49). As shown in Fig. 5A, size variation of the PvpA protein ranges from 48 to 55 kDa. Notably, in strain A5969 expression of the PvpA product was not detected. To investigate this variation, genomic DNAs of six strains, depicted in Fig. 5A, were restricted with the EcoRV restriction enzyme and subjected to Southern blot hybridization with the 1.9-kb EcoRV fragment, carrying the pvpA gene, as a probe. A single EcoRV genomic fragment differing in size was observed among the strains tested (Fig. 5B). With the exception of variant strain K703 (Fig. 5B, lane 3), size differences of the EcoRV fragment correlated with the size of the expressed protein, suggesting that variation within the pvpA structural gene affects the size of the expressed PvpA protein (Fig. 5).

To further analyze the molecular basis of PvpA size variation, the pvpA gene of seven M. gallisepticum strains was amplified by PCR and sequenced. Comparison of the resultant nucleotide sequences to the pathogenic R strain (depicted in Fig. 1B) revealed that several types of major deletions have occurred within the pvpA structural gene. Interestingly, the deletions were localized at the proline-rich C-terminal region and within the direct repeated regions DR-1 and DR-2 of the pvpA gene (Fig. 6). The extent of the deleted regions with respect to the pvpA gene of strain R was 231, 93, 93, and 48 nt in strains F, HHT5, K703, and ts-11, respectively. Notably, strains HHT5 and K703 possess an additional and identical stretch of 18 nt at nucleotide positions 714 to 732 which is not present in the other strains. Strain K703 had, in addition, another stretch of 15 nt at nucleotide positions 696 to 711, and strain K2021 had a deletion of 18 nt within the N-terminal region (nucleotide positions 372 to 390) (Fig. 6).

The A5969 strain, in which expression of the PvpA product was not detected (Fig. 5A, lane 6), possesses a full-length pvpA structural gene similar in size to the pvpA gene from strain R (Fig. 5B, lane 6, and Fig. 6). Interestingly, however, a stop codon was identified in this strain at nucleotide positions 793 to 795 within the DR-1 repeat (Fig. 6, indicated by an arrow). Taken together, the occurrence of deletions within the C terminus-encoding region of the pvpA gene in M. gallisepticum strains results in size variation of the expressed PvpA antigen.

FIG. 5. Polymorphism of the PvpA protein and of the pvpA gene among M. gallisepticum strains. (A) Western blot analysis of M. gallisepticum strains. Whole organisms were subjected to SDS-PAGE and immunoblotted with MAb 1E5. Three differently sized PvpA protein bands, of 55 (lane 1), 50 (lanes 2 and 3), and 48 (lanes 4 and 5) kDa, are indicated by labeled arrows. The invariant P41 protein (lanes 1 to 6) is also shown. M. gallisepticum strains included R, 227, K703, F, K2320, and A5969 (lanes 1 to 6, respectively). (B) Southern blot hybridization of M. gallisepticum strains depicted in panel A. Chromosomal DNAs (about 4 µg) were digested to completion with the restriction enzyme EcoRV and probed with the 1.9-kb EcoRV fragment (Fig. 2A) carrying the pvpA gene. The molecular size marker is shown on the left.

FIG. 6. Deletions within the C terminus-encoding region of the pvpA gene. The pvpA gene from different M. gallisepticum strains is shown by gray rectangles. The name of each strain is given on the left of each rectangle. The length of each ORF (in nucleotides) is given on the right. The location of two directly repeated sequences (DR-1 and DR-2) in the C terminus-encoding region of the pvpA gene from strain R is shown by labeled brackets. Gaps within the pvpA genes represent various types of deletions in comparison to strain R. Small dark rectangles indicate nucleotide sequences within the pvpA gene of strain HHT5 and K703 which are not present in the R strain. The numbers at the beginning of each deletion indicate the nucleotide position. Open rectangles in the vaccine strain ts-11 represent regions which were not sequenced.
DISCUSSION

Genetic analyses in this study provide evidence that PvpA is a putative variable adhesin molecule of *M. gallisepticum*. Several noteworthy features, which delineate the unique characteristics of PvpA, deserve further attention. The *pvpA* gene, which exists as a single chromosomal copy, is present in all strains tested but exhibits size variation affecting the size of its product. Although PvpA was shown to undergo variation in expression, its size variation does not represent high-frequency gain or loss of reiterated coding sequences, as was shown previously for several variable surface proteins (11, 22, 33, 37, 50, 52, 54). Sequence analysis of the *pvpA* gene from several *M. gallisepticum* strains has shown that PvpA size variation is a result of deletions occurring at the proline and glutamine (PQ)-rich C-terminal region and within the two direct repeat sequences (DR-1 and DR-2) (Fig. 1B). Proteins containing proline-rich repeat units are increasingly identified in a variety of prokaryotic and eukaryotic pathogens as major immunogenic surface antigens. Most of these amino acid repeats are involved in pathogen-host cell interaction and in the binding of a protein to specific ligands (6, 13, 14, 25–27, 47). For example, proline-rich repeated proteins were observed in the malarial parasite *Plasmodium yoelii* (17); in the causative agent of whooping cough, * Bordetella parapertussis* (21); and in PRA, a major immunogenic antigen of *Mycobacterium leprae* (42), the etiological agent of leprosy. Each of these proteins is thought to interact with the eukaryotic cell surface and to be important in the pathogenicity of these organisms.

The high concentration of proline residues within a surface-exposed domain contributes to the protein folding and to its overall conformation (2, 23), which may facilitate pathogen-host interaction. The presence of 60 proline residues within a surface-exposed domain at the carboxyl-terminal end of PvpA (Fig. 1B) and the conservation of this motif among the adhesins of other pathogenic mycoplasmas (3, 5, 8, 10, 12, 24, 39) suggest an important role of this external module in determining the functionality of PvpA as an adhesin molecule. The findings that *M. gallisepticum* isolates differ in their adherence and pathogenicity properties (19, 46) and show variation of the PvpA carboxy-terminal region (Fig. 6) suggest that this domain may be under selective pressure in the natural host. Extensive analysis of the cytadherence process in *M. pneumoniae* has demonstrated that this process is multifactorial, involving the coordinate action of primary adhesin molecules (P1 and P30) in concert with an array of high-molecular-weight accessory membrane proteins (15, 16). It is postulated that variation within PvpA, as one of the putative accessory membrane proteins of *M. gallisepticum*, could affect the specificity or affinity of adherence and may provide the mycoplasma more flexibility within different niches in the host where distinctive receptors may be required for optimal colonization.

PvpA was shown previously to undergo variation in expression (49). A single nonsense mutation was found in a short tract of five repeated GAA codons encoding the amino acid glutamate. The location of the nonsense mutation at the N-terminal end led to premature termination of *pvpA* translation and to abolition of expression. The lack of sequence changes in the *pvpA* upstream region of clonal isolates exhibiting different expression states of the PvpA product and the fact that *pvpA* mRNA was detected irrespective of the PvpA expression state indicate that PvpA variation in expression is controlled at the level of transcription. Notably, despite the fact that the *pvpA* gene was shown to exist as a single chromosomal copy, Northern blot analysis has shown the presence of two transcripts hybridizing to the *pvpA* gene (Fig. 2E). One transcript was about 1.4 kb in size, which fits with the size of the *pvpA* gene (1,149 bp) and its flanking regions. However, the presence of an additional transcript of about 2.6 kb in size argues for a second and functional site for *pvpA* transcription initiation localized approximately 1.2 kb upstream of the first site and upstream of or within the gene encoding the elongation factor EF-G (Fig. 2A). The function of the overlapping transcripts generated is not known. A recent study with *M. pneumoniae* has shown that the genes comprising the *hmw* gene cluster, including the ribosomal *rpsD* gene, constitute an operon expressed from overlapping transcripts (45). The possibility that the *pvpA* gene is part of an operon expressed from overlapping transcripts cannot be ruled out.

The *pvpA* switching mechanism appears to be distinct from other currently known examples of genetic mechanisms mediating antigenic variation of mycoplasma surface molecules. Translational control of phase-variable proteins was documented for P78 of *Mycoplasma fermentans* (41) and for the Vaa adhesin of *Mycoplasma hominis* (53). In these examples, a homopolymeric tract of identical nucleotides [poly(A)] serves as a hot spot in the DNA sequence, allowing the occurrence of a reversible frameshift mutation at a high frequency. A poly(A) motif was also found in *vlp* genes of *Mycoplasma hyorhinis* (50). However, its location within the promoter region suggests that the *vlp* genes are regulated at the level of transcription. Usually, rec-A-independent slipped-strand mispairing during DNA replication has been proposed as the mechanism of high-frequency mutations within a hot spot site (18). In contrast, the mutation within the *pvpA* structural gene was a nonsense mutation in which the nucleotide guanine was replaced with the nucleotide thymidine (Fig. 2B and C). The nucleotide substitution took place consistently within the fourth GAA codon of the GAA tract in several clonal isolates tested. This suggests that this site is preferred for the occurrence of such a mutation. Although the mechanism is unknown, such a mutation cannot be explained simply by slipped-strand mispairing during DNA replication, and it may be linked to our inability to identify a reversible switching event. In several generations tested, colony immunoblotting of phenotypically PvpA-positive cells of *M. gallisepticum* strain R, using MAb 1E5, allowed identification of variant colonies, displaying the PvpA-negative phenotype, at a frequency of about 10^−3 to 10^−4 per cell per generation. However, platting of organisms with the negative phenotype did not allow the identification and isolation of progenies exhibiting the positive phenotype. These results suggest that occurrence of the observed nonsense mutation either is irreversible or occurs at a low frequency. Interestingly, the trinucleotide GAA motif has been implicated in the regulation of the pMGA genes (7). The GAA repeat is localized 5′ to the pMGA promoter, and variation in the number of the repeated units regulates the pMGA expression, apparently at the level of transcription.

Another interesting finding is related to *M. gallisepticum* strain A5969. This strain possesses a complete *pvpA* gene similar in size to its counterpart in *M. gallisepticum* strain R (Fig. 5B, lanes 1 and 6), and the gene is transcribed (data not shown). However, while strain R expresses a 55-kDa product, as was detected with MAb 1E5, no expression of the PvpA product was obtained from the whole population of strain A5969 (Fig. 5A, lanes 1 and 6, respectively). The presence of a nonsense mutation at nucleotide position 793 within the repeat DR-1 of the *pvpA* gene of strain A5969 may cause premature termination of PvpA translation, leading to expression of a truncated PvpA lacking most of the proline-rich C-terminal end (Fig. 6). The inability of MAb 1E5 to recognize the truncated PvpA protein can be attributed to the lack of the corre-
sponding epitope localized apparently within the C-terminal and repetitive region of the PvpA molecule. Strain R is known to be a virulent strain causing respiratory disease, while strain A5969 is a nonpathogenic strain unable to adhere to and colonize the tracheal lumen of the chicken host (19). Since PvpA is apparently an adhesin-related surface molecule of M. gallisepticum, it is intriguing to speculate whether lack of PvpA expression or the expression of the truncated form of the PvpA molecule contributes in part to the deficiency in the adherence capabilities of strain A5969.

PvpA was shown by colony immunoblotting, Triton X-114 phase fractionation, and digestion experiments with trypsin and carboxypeptidase to be an integral membrane protein with a surface-exposed C terminus (49) and apparently is localized at the tip organelle (Fig. 4). The mechanism by which PvpA is anchored to the mycoplasma membrane may be explained by the presence of a hydrophobic domain (aa 75 to 105) (Fig. 1B and 3) that could serve as a transmembrane domain. Notably, a similar hydrophobic domain at about the same position was also found in the MGC2 adhesin molecule of M. gallisepticum and in the P30 and P32 adhesins of M. pneumoniae and M. genitalium, respectively (Fig. 3). An alternative possibility is that the PvpA signal peptide sequence is able to mediate translocation but is incapable of being cleaved by mycoplasma signal peptidase enzyme. Notably, a recognizable signal peptidase I homolog has not been detected in the complete genome sequence of M. pneumoniae and M. genitalium (30). The PvpA protein could then be anchored in the membrane by the hydrophobic signal sequence and yet be exposed on the exterior membrane surface.

PvpA, a putative adhesin-related molecule identified in this study, joins an extended list of adhesin molecules (MGC1, MGC2, and GapA) of the avian pathogen M. gallisepticum, indicating that the cytadherence process in this species is multifactorial, as has been elegantly shown for M. pneumoniae (15, 16). The high homology and the conservation of structural features observed among the adhesin molecules of M. gallisepticum, as well as of the human pathogens M. pneumoniae and M. genitalium, strongly imply that a conserved but also divergent family of adhesins was acquired during evolution by the mycoplasmas and is being utilized successfully to adhere to host target cells.

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REFERENCES


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