The Vsa Proteins Modulate Susceptibility of *Mycoplasma pulmonis* to Complement Killing, Hemadsorption, and Adherence to Polystyrene

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Mycoplasmas cause a variety of acute and chronic diseases in animals and humans as a result of their ability to infect the respiratory tract, joints, the reproductive tract, and other tissues and to evade the host immune system (19, 26, 27, 35). Atypical pneumonia in humans, which is caused by *Mycoplasma pneumoniae*, is generally a self-limiting disease. During the course of severe infection, influx of neutrophils and edema into the airspaces (acute phase), large accumulations of lymphoid cells into the respiratory tract (chronic phase), small-airway hyperreactivity, and increased levels of inflammatory cytokines detected in the bronchoalveolar lavage fluids of patients are seen (26). Despite the presence of a strong immune response, *M. pneumoniae* can be detected or isolated from the respiratory tract for up to several months after resolution of the pneumonia (20). These features are typical of many mycoplasmal respiratory infections and highlight the importance of the host inflammatory and immune response in the disease. Other than the attachment tip structure possessed by some species such as *M. pneumoniae* (24, 25), mycoplasm factors that confer virulence and resistance to the host immune system are not well understood.

Murine respiratory mycoplasmosis (MRM) caused by *Mycoplasma pulmonis* represents a model of interactions between the murine host and its natural pathogen. MRM has features similar to the respiratory mycoplasmoses seen in humans and animals (7, 8). MRM has an acute, often fatal, phase which is associated with large influxes of neutrophils and edema into the terminal airspaces and a chronic phase that is associated with peribronchial accumulations of lymphocytes (27). *M. pulmonis* produces a set of phase-variable surface antigens (Vsa) which influence virulence (10, 40). The Vsa proteins modulate the ability of the mycoplasma to adhere to polystyrene, to adsorb red blood cells (48), and to adsorb mycoplasma virus P1 (13). The *vsa* locus of *M. pulmonis* strain UAB CT codes for a repertoire of seven different phase-variable Vsa proteins (VsaA, VsaC, VsaE, VsaF, VsaG, VsaH, and VsaI). Variation in *vsa* gene expression results from site-specific DNA inversions that combine one of the seven *vsa* genes with the *vsa* expression site (2, 34, 37). Most *vsa* genes have an extensive tandem repeat region at the 3′ end. In addition to phase variation, variation in the number of tandem repeats results in size variation of Vsa (2). The production of VsaH by *M. pulmonis* is associated with the ability to adhere to plastic (polystyrene adherence-positive [PA+] phenotype) and to hemadsorb (hemadsorption-positive [HA+] phenotype), while cells that produce VsaA are reported to be HA− and PA− (44, 45). Anecdotal reports link VsaH production to experimentally induced chronic airway infection in mice, while VsaA production is linked to the acute alveolar form of MRM (39, 48).

Little is known about how mycoplasmal surface proteins affect host interactions. Vsa variation, as well as the variation of surface molecules in other mycoplasmas, has been proposed to function in evasion of the host immune system and/or to function in tissue tropism (9, 34, 37, 49). Several cytadherence molecules of the *M. pneumoniae* tip structure mediate attachment to the host epithelium (23). Many species of mycoplasmas lack an attachment tip yet adhere well to epithelial surfaces, presumably as a result of the presence of surface molecules that mediate cytadherence. Many mycoplasmas are resistant to complement-mediated killing (3), but no studies have linked any mycoplasmal surface protein with serum resistance. In *Mycoplasma hyorhinis*, the length of the tandem...
Repeat region of the Vlp surface proteins is associated with resistance to metabolic inhibition by specific antibodies (9).

The Vsa proteins affect the surface properties of *M. pulmonis* and thereby have the potential to affect host-pathogen interactions. We report here on the analysis of variants of *M. pulmonis* strain UAB CT that produce a shortened form of the VsaA protein and are HA<sup>+</sup> and PA<sup>-</sup>. Thus, factors other than VsaA can mediate PA and HA. Additionally, we show that *M. pulmonis* variants that produce short forms of VsaA are highly susceptible to complement killing whereas variants that produce longer forms are serum resistant. Our studies are the first to associate a mycoplasmal protein (VsaA) with resistance to complement and with the modulation of other surface properties such as HA.

### MATERIALS AND METHODS

**Mycoplasma strains and characterization.** The murine pathogen *M. pulmonis* strain UAB CT (12) was passaged in mycoplasma broth 12 times and designated strain CTp12. *M. pulmonis* strains CT182 and CT228 are previously described (38, 42) mutants of strain CTp12 that contain transposon Tn<sub>4000</sub> (14) inserted in their genomes at nucleotide positions 652759 and 656621, respectively. In strain CT228, the transposon disrupts the gene encoding the HsvR recombinase that catalyzes vsa gene rearrangement (38). Thus, strain CT228 produces VsaA and cannot switch to production of an alternative Vsa protein. In strain CT182, Tn<sub>4000</sub> truncates the last codon of the vsaH gene. *M. pulmonis* strain CT39-6-2 was derived from UAB strain CT39-6, a VsaH-producing *M. pulmonis* variant that is associated with causing the airway form of MRM (39, 48). Some proper-

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Vsa gene</th>
<th>Mode HA score</th>
<th>PA</th>
<th>Complement susceptibility&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT182</td>
<td>VsaA R3</td>
<td>3</td>
<td>+</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Filter clones from CT182</td>
</tr>
<tr>
<td>CT182R3-1, -2, and -3</td>
<td>VsaA R3</td>
<td>2</td>
<td>−</td>
<td>S</td>
<td>Filter clones from CT182</td>
</tr>
<tr>
<td>CT182R40-1, -2, and -3</td>
<td>VsaA R40</td>
<td>1</td>
<td>−</td>
<td>R</td>
<td>R3 survivors from complement killing assay</td>
</tr>
<tr>
<td>CT182R3R40-1 and -2</td>
<td>VsaA R40</td>
<td>ND</td>
<td>ND</td>
<td>R</td>
<td>hvsR mutant of CTp12</td>
</tr>
<tr>
<td>CT39-6-2</td>
<td>VsaH</td>
<td>2</td>
<td>+</td>
<td>S</td>
<td>ND, not done.</td>
</tr>
<tr>
<td>CT228</td>
<td>VsaA R40</td>
<td>1</td>
<td>−</td>
<td>R</td>
<td>ND, not done.</td>
</tr>
<tr>
<td>Ctp12</td>
<td>VsaA R40</td>
<td>0</td>
<td>−</td>
<td>R</td>
<td>ND, not done.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Complement susceptibility presented as R for resistant and S for sensitive to complement.

<sup>b</sup> ND, not done.

PA. Frozen starter stocks (25 μl) of *M. pulmonis* strains UAB CTp12, CT182, and CT228 were used to inoculate 3 ml of mycoplasma broth in a 25-cm<sup>2</sup> tissue culture flask (Falcon catalog no. 350802). After overnight growth at 37°C, the broth was pipetted from the flask (nonadherent phase), the flask was gently washed three times with 5 ml of phosphate-buffered saline (PBS) at room temperature, and the attached mycoplasmas were scraped from the bottom of the flask (adherent phase) with a cell scraper (Falcon 350805) into 1 ml of mycoplasma broth. The nonadherent and adherent phases were assayed for CFU to determine the percentage of CFU that had adhered to the flask.

**HA.** The HA analysis procedure has been previously described (17). Briefly, the mycoplasmas were serially diluted in mycoplasma broth and grown on 60-mm-diameter mycoplasma agar plates for 5 to 7 days in a humidified incubator at 37°C. Agar plates with 30 to 100 colonies were overlaid with 3 ml of 0.5% sheep red blood cells (sRBC) in 1× PBS and incubated at 37°C for 30 min without rocking. The sRBC suspension was pipetted off, and the plates were gently washed three times by hand rocking with 3 ml of PBS. The colonies were observed with a Leica WILD M3Z dissecting microscope at ×6.5 to ×40 magnification as needed. A colony was assigned an HA score of 0 when few or no sRBC were attached to the colony, a score of 1 when up to 25% of the colony surface area was covered, a score of 2 when 25 to 50% of the colony was covered, a score of 3 when 50 to 75% of the colony was covered, and a score of 4 when 75 to 100% of the colony was covered. The mean HA score (standard error) and the median and mode HA scores for each strain were determined by pooling the data from several experiments. Images of sRBC adsorbing to representative colonies of the different strains were taken at various magnifications with a Leica HC microscope (Diagnostics Instruments, Inc.) and evaluated with SPOT imaging software.

**Complement killing assays.** Complement killing of *M. pulmonis* strains was performed on the basis of the results of assays by Taylor-Robinson et al. (41). Briefly, hypolipidized guinea pig complement (Colorado Serum Company, Denver, Colo.) was rehydrated, aliquoted, and stored at −80°C in 1.5-ml aliquots. The vsa gene that occupied the vsa expression site of the *M. pulmonis* strain in a cell culture was determined by semiquantitative PCR using methods and primers previously described (18). PCR analysis with a forward primer (o.6666) that binds to the vsa expression site (18) and a vra-specific reverse primer (5′-TTTGTGCTATATTTGAAGTCT-3′) was used to determine the nucleotide length of the vra tandem repeat region. The nucleotide sequence of the resulting PCR product was determined using oligonucleotide primers o.6666 and L4.667 (5′-AAAAACATACAAATATGAACAGG-3′). Also, PCR products from the semiquantitative reactions using the vraA primer set were sequenced with primer o.6666. The sequence data were analyzed with an ABI PRISM model 377 software package (DNA Synthesis and Sequencing Facility, Iowa State University, Ames, Iowa).

**Immunoblot analysis.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Bio-Rad) by the method of Towbin et al. (43). All immunological reactions were performed at room temperature as previously described (47). The Vsa-specific monoclonal antibody (MAB) 7.1-2 [immunoglobulin G (IgG)] (44, 47) was diluted 1:500 and the alkaline phosphatase-conjugated secondary sheep anti-murine immunoglobulin G (Serotec, Kidlington, Oxford, United Kingdom) was diluted 1:2,000. The Vsa protein bands were visualized by development of the immunoblots with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as the substrate.

**Statistical analysis.** Statistical analysis was performed with a Sigma Stat version 2.03 software package (SPSS Inc., Chicago, Ill.). Data were analyzed by analysis of variance by comparing the complement killing or polystyrene attachment of all the strains producing VsaA R40 to those producing the short VsaA R3 and to those producing VsaH. Analysis of variance was also used to compare the level of killing of each strain to those of all other strains. All pairwise multiple
RESULTS

*M. pulmonis* strain CT182 produces a short (R3) form of VsaA. Immunoblot analysis of the *M. pulmonis* strains using the Vsa-specific MAb showed the typical Vsa ladder pattern (46, 47), with the apparent molecular mass of the uppermost, major band being in excess of 200 kDa (strain CTp12), about 180 kDa (strain CT228), and about 30 kDa (strain CT39-6-2) (Fig. 1). Strain CT182 had a predominant Vsa band that migrated at an apparent molecular mass of 60 kDa in addition to other major bands indicative of a mixed population of cells producing Vsa protein variants. Subcloning of this parental CT182 strain yielded progeny producing a variety of Vsa molecules ranging from 60 to more than 200 kDa (Fig. 1B). Strains CT182R3-1, CT182R3-2, and CT182R3-3 are subclones of strain CT182 that produced a Vsa protein (results for representative strain CT182R3-1 are shown in Fig. 1B, lane -R3-1) that comigrated with the 60-kDa major band of the parental CT182 strain (Fig. 1A). Strain CT182R40-1 produced a Vsa protein of 150 kDa, while strains CT182R40-2 and CT182R40-3 produced a VsaA protein of about 200 kDa.

Semiquantitative PCR analysis using a battery of *vsa* oligonucleotide primers indicated that more than 95% of the *M. pulmonis* cells in all stock cultures of CT182 and its progeny tested had the *vsaA* gene occupying the *vsa* expression site. Thus, they produced VsaA (18). The nucleotide sequence of the PCR product obtained by PCR amplification using primers that flanked the 3’ *vsaA* repetitive region revealed that strain CT182 and its R3 progeny had only three tandem copies of the 51-bp *vsaA* repeat. Thus, these strains are referred to as CT182R3 variants. Most strains of *M. pulmonis* have 30 to 40 of these tandem repeat units (2, 34, 37). For this reason, we refer to the CT182 strains producing the long form of VsaA as strain CT182R40 variants. Strain CT182 and its R3 variants represent the first isolation of cells producing a short R3 form of VsaA.

*M. pulmonis* strains that produce the R3 form of VsaA hemadsorb and adhere to polystyrene. *M. pulmonis* colonies grown on agar were scored on their ability to adsorb sRBC (Materials and Methods). Their mean, median, and mode HA scores are shown in Table 2. Of the *M. pulmonis* strain CTp12 colonies, almost none adsorbed sRBC (mode HA score = 0) and virtually all remained HA even when the PBS washes of the culture plates were omitted. The *hvsR* mutant strain CT228, which exclusively produces VsaA, had a mode HA

<table>
<thead>
<tr>
<th>CT strain(s)</th>
<th>HA result</th>
<th>PA result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean score</td>
<td>SE</td>
<td>Median</td>
</tr>
<tr>
<td>Score (att.)</td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>CT182</td>
<td>1.6</td>
<td>0.14</td>
</tr>
<tr>
<td>CT39-6-2</td>
<td>2.5</td>
<td>0.08</td>
</tr>
<tr>
<td>CT228</td>
<td>1.4</td>
<td>0.05</td>
</tr>
<tr>
<td>CTP12</td>
<td>0.0</td>
<td>0.01</td>
</tr>
<tr>
<td>CT182R3-1,-2, and -3</td>
<td>2.5</td>
<td>0.13</td>
</tr>
<tr>
<td>CT182R40-1,-2, and -3</td>
<td>1.3</td>
<td>0.10</td>
</tr>
</tbody>
</table>
scores of 1. Although some strain CT228 colonies did not adsorb SRBC and other colonies scored more than 1, the typical pattern of SRBC adsorption was to the margin of the colony (referred to here as ring adsorption). The remainder of the M. pulmonis strains adsorbed SRBC to greater degrees. The mode HA scores were 3 for strain CT182, 2 for the CT182R3 strains, 1 for the CT182R40 strains, and 2 for the CT39-6-2 strain. The results were reproducible over several different experiments. The assays for the CT182R40 colonies (n = 806) versus the CT182R40 colonies (n = 726) were performed separately from the assays for the colonies of strains CT182 (n = 562 colonies), CT228 (n = 680), CTp12 (n = 743), and CT39-6-2 (n = 450).

Strains that readily hemadsorbed also adhered strongly to the polystyrene surface of tissue culture flasks. Less than 6% of the cells in cultures of M. pulmonis strains CT182R40, CT228, and CTp12, all producing the R40 form of VsaA, adhered to the polystyrene during growth (Table 2). More than 50% of the populations of strains that produced the R3 form of VsaA (CT182R3 and CT182) and VsaH (CT39-6-2) adhered to the polystyrene.

**M. pulmonis** strain CT variants that produce Vsa R3 are susceptible to complement killing. As the above-described experiments indicated that VsaA size variation changed the cell surface of the mycoplasma, we reasoned that mycoplasma-host interactions might be affected. Analyses of the efficiency of killing of *M. pulmonis* by complement in vitro have given mixed results (21, 41). However, a recent comparison of the Vsa proteins produced during the infection of immunocompetent versus immunocompromised mice indicated that Vsa protects the mycoplasma from the host immune system (A. M. Denison and K. Dybvig, unpublished data). We hypothesized that Vsa might affect the susceptibility of *M. pulmonis* to complement. Table 3 shows the percentages of CFU recovered from reactions in which *M. pulmonis* strains that produced different forms of Vsa were incubated in 10% dilutions of guinea pig serum (without the addition of *M. pulmonis*-specific antibody). Only 12% of the CFU of the CT182R3 strains were recovered, while about 94% of the CT182R40 strains were recovered (a statistically significant [P < 0.001] result). This indicates that CT182R3 strains were killed more efficiently than CT182R40 strains. Killing was abolished by heat inactivation of the serum.

<table>
<thead>
<tr>
<th>CT strain(s)</th>
<th>% Recovered after complement treatment (SE)</th>
<th>% Recovered after HIA serum treatment (SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT182R3-1 and -2</td>
<td>12 (2.0)</td>
<td>103 (6.9)</td>
<td>13</td>
</tr>
<tr>
<td>CT182R40-1 and -2</td>
<td>74 (8.9)</td>
<td>109 (8.6)</td>
<td>13</td>
</tr>
<tr>
<td>CT39-6-2</td>
<td>26 (6.9)</td>
<td>97 (5.7)</td>
<td>6</td>
</tr>
<tr>
<td>CT228</td>
<td>107 (13)</td>
<td>105 (15)</td>
<td>7</td>
</tr>
<tr>
<td>CTp12</td>
<td>102 (6.2)</td>
<td>95 (5.5)</td>
<td>6</td>
</tr>
<tr>
<td>CT182R3R40-1 and -2</td>
<td>101 (5.5)</td>
<td>116 (8.1)</td>
<td>14</td>
</tr>
</tbody>
</table>

* A total of 76% of strain CT182R40-1 CFU were recovered (n = 6), while a total of 109% of strain CT182R40-2 CFU were recovered (n = 7). The difference between the results for these two strains was not statistically significant. These data are combined, and the strains are referred to as CT182R40 strains in the text.

**DISCUSSION**

The innate immune system appears to provide the antmycoplasma host defense in the lungs (4, 5), while adaptive immunity contributes to lung lesion severity in mice infected with *M. pulmonis* (6, 36). The nature of the lung lesions suggests that the host inflammatory response contributes to MRM, while the chronicity of the disease suggests that *M. pulmonis* evades the immune system despite an intense immune response. The data we present in this study indicate that variation in the length of the Vsa repetitive region affects *M. pulmonis* interactions with surfaces (PA and HA) and the innate immune system component complement. Our data also extend previous observations in which VsaA was associated with the HA− and PA− phenotypes and VsaH was associated with the HA+ and PA+ phenotypes (37, 45, 48). These studies indicate that the length of VsaA affects the ability of *M. pulmonis* to adhere to surfaces. The correlation between HA and PA and their association with both VsaH and VsaA R3 production suggest that the Vsa proteins can exert a general (nonspecific) effect on mycoplasma surface properties. Possibly, Vsa proteins with long repetitive regions hinder access to binding moieties on the mycoplasma surface. These moieties could mediate specific or nonspecific interactions such as hydrophobic attractions (45). Such a nonspecific mechanism would not preclude specific effects mediated by Vsa.

Our data also indicate that VsaA functions in resistance to complement. *M. pulmonis* strain CT isolates that produced short R3 forms of the VsaA or VsaH were sensitive to killing by guinea pig serum. This killing was eliminated when the serum was heat inactivated. In contrast, strains that produced the R40 form of VsaA were significantly resistant to complement. Resistance to complement was slight, but not statistically significantly, reduced in strain CT182R40-1 compared to that in strain CT182R40-2. This result could be due to the somewhat smaller VsaA protein (150-kDa major band) in strain CT182R40-1. Most complement-mediated killing of gram-positive bacteria is the result of enhanced opsonophagocytosis resulting from C3 deposition on the bacterial surface (32). Our assays were cell-free. Therefore, the mechanism of killing was mediated by cell lysis, presumably by the insertion of the complement’s terminal membrane attack complex into the mycoplasma membrane. The lack of a cell wall should make mycoplasmas inherently susceptible to complement lysis.
It follows that pathogenic species of mycoplasma must produce factors, such as the R40 form of VsaA, to evade complement.

The mechanism by which the R40 form of VsaA mediates resistance to complement killing is presently under investigation. It may be that M. pulmonis variants producing any of the other Vsa types (e.g., VsaI) with long C-terminal repetitive regions (34) are also resistant to complement. There are many strategies employed by bacteria to confer complement resistance. Capsular material or surface proteins can hinder the access of many complement components to the surface of the cell or inhibit activation of the complement pathway (32). Feeney et al. (15) determined that intermolecular aggregation was more favorable with peptide sequences with long, perfect, tandem-repetitive sequences than with those with short, imperfect sequences. The VsaA C-terminal repetitive region is comprised of 17 amino acid units that are nearly perfect in sequence. Possibly, the resistance of R40-producing cells to complement killing is due to steric hindrance imparted by the formation of a barrier by VsaA. Alternately, VsaA could bind C3 but inhibit further activation of the complement cascade.

Microorganisms that are resistant to opsonophagocytosis demonstrate an increased virulence in animal models (1, 29). Additionally, mice and humans that lack an intact complement system are susceptible to pulmonary infections and systemic dissemination (16, 33). These relationships underscore the importance of complement as a pulmonary host defense and the importance of resistance to complement as a microbial virulence factor. This is especially so when the mechanism of resistance involves phase-variable molecules. As the Vsa system is a phase-variable system, M. pulmonis may use a strategy in which increased abilities to adhere to surfaces, even in the case of increased susceptibility to complement, are beneficial.

Whether the resistance of M. pulmonis to complement killing can alter the outcome of MRM remains to be determined, but the implications are clear. Mycoplasma cells that are resistant to complement killing could still be opsonized but not cleared by alveolar phagocytes. As alveolar macrophages have been shown to be an important host defense for clearing M. pulmonis from the respiratory tract (30, 31), mycoplasma-complement interactions might have a profound effect on MRM pathogenesis. Thus, defining the interactions between phagocytes, M. pulmonis, and complement will better define the role of the Vsa proteins in mycoplasmoses. The implications go further in that mycoplasmal resistance to complement killing might enhance the ability of mycoplasmas to disseminate to other tissues (4, 22).

Mycoplasmal resistance to complement killing has significance for human respiratory and systemic mycoplasmoses. Respiratory infections with mycoplasmas lead to exacerbations of airway hypersensitivity in asthmatics (28, 50), increased influx of polymorphonuclear cells in the airspaces, and chronic infections that are difficult to clear (27). Activated (surface-bound) complement results in the release of anaphylatoxins and chemotactic agents (e.g., C3a and C5a) (16, 33). The ability of a mycoplasma to resist immune killing despite complement deposition and the resultant release of immunomodulatory substances could define a potential host-pathogen mechanism that is responsible for the lung injury seen in human mycoplasmoses.

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REFERENCES


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