

How Some Mycoplasmas Evade Host Immune Responses

Variable surface antigen proteins are key for how these microorganisms evade host immune responses

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Even though free-living, mycoplasmas depend on their hosts for key nutrients, including purines, pyrimidines, several amino acids, and sterols, which are incorporated into the membranes of these wall-less bacteria. These requirements reflect their relative genomic simplicity. The 580-kb genome of *Mycoplasma genitalium*, for example, is comparable in size to that of a large virus.

Nonetheless, this highly successful group containing both commensals and pathogens withstands robust host immune responses. Mycoplasmal diseases are generally inflammatory, including mycoplasma-induced asthma episodes, pelvic inflammatory disease, and bronchiectasis. The Centers for Disease Control and Prevention estimate that walking pneumonia,

caused by *Mycoplasma pneumoniae*, accounts each year for 2 million cases and 100,000 hospitalizations in the United States. Mycoplasmas often can be detected in individuals long after their symptoms disappear.

Although many factors contribute to disease chronicity, the mycoplasmas appear to evade immune surveillance by varying their antigenic patterns and by shielding themselves from components of the immune system. In focusing on the murine pathogen *M. pulmonis* as a model, we learned that one key feature for how this group of microorganisms avoids host immune responses is the abundant variable surface antigen (Vsa) proteins that undergo high-frequency phase and size variation. The length of these proteins affects the adherence properties of the mycoplasma as well as its degree of shielding from the immune system.

Summary

- The C-terminal variable region of the mycoplasma variable surface antigen (Vsa) proteins contains up to 60 tandem repeat units, and this variable length proves a major factor affecting adherence properties and shielding these bacteria from host immune responses.
- The cell-surface Vsa proteins constitute as much as 10% of total mycoplasma protein, helping to shield cells against environmental factors.
- When grown on solid surfaces and producing short Vsa proteins, *M. pulmonis* forms biofilms that confer partial resistance to immune system components.
- Mycoplasmas form biofilm-like aggregates on host tissues, where they might also form tower structures to protect against innate immune system responses.

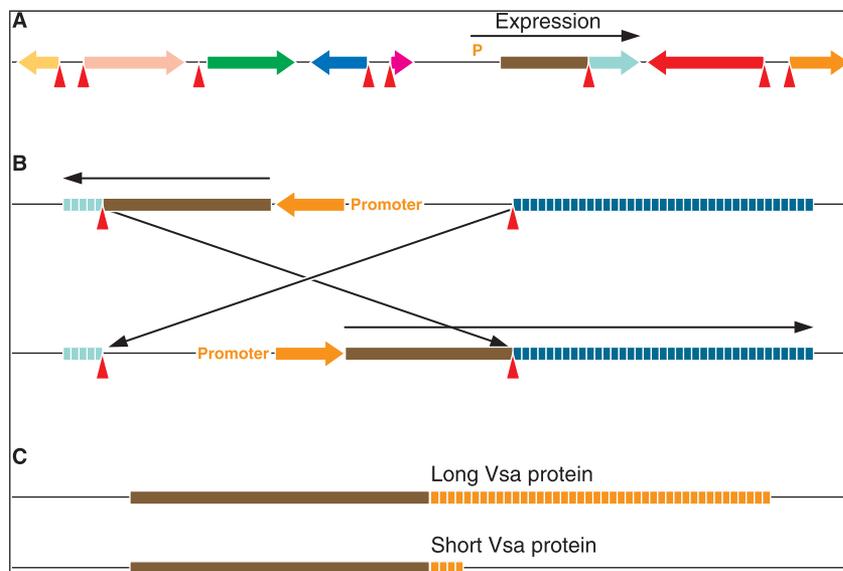
Mycoplasmas Avoid Adaptive Immune Responses through Vsa Phase Variation

Vsa lipoproteins have a 242-amino-acid N-terminal region and a C-terminal variable region containing as many as 60 tandem repeat units ranging in size from 10–19 amino acids (Fig. 1A and B). Each cell transcribes only one *usa* gene. Silent *usa* genes are missing sequences that code for the conserved N-terminal region. Each of these silent genes contains little more than a *usa* recombination site (*vrs*) followed by a tandem repeat sequence. Through gene rearrangement between *vrs* sequences, each of the silent *usa* genes is capable of recombining into the *usa* expression site. All of the

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FIGURE 1



Phase and size variation of the *vsa* gene. (A) The *vsa* locus contains a single *vsa* expression site (brown) and the promoter (P), several different silent *vsa* genes (colored arrows) with tandemly repeated sequences, and *vsa* recombination sites (*vrs* sequences, red arrows). Through random inversions between *vrs* sequences, the *vsa* expression site recombines with one of several different silent *vsa* genes (colored arrows). Here the *vsa* expression region is depicted as recombining to one silent gene, resulting in expression of this *vsa* gene. (B) The mechanism of phase variation. The *vsa* expression site contains the conserved 726-bp 5' region of the *vsa* open reading frame and the *vsa* promoter (orange) and ends with a *vsa* recombination site (*vrs*; red arrows). DNA inversion, shown by the crossed arrows, associates the expression site with a different 3' tandem repeat region, a formerly silent *vsa* gene. (C) *Vsa* size variation results from slipped-strand DNA replication in the *vsa* tandem repeat region. This increases or decreases the number of tandem repeats (orange) in a *Vsa* protein.

identified gene rearrangements are DNA inversions that serve to replace the tandem repeat region of the formerly expressed gene with the tandem repeat region of a newly expressed gene.

The repertoire of *Vsa* proteins (*Vsa* types) available to *M. pulmonis* strain CT is *Vsa*A, C, E, F, G, H, and I. Although each cell has a single *vsa* expression site and produces only one *Vsa* type at a time, subpopulations of cells in culture or during animal infections produce each of the alternative *Vsa* types. Phase switching occurs at a frequency of about 10^{-3} per CFU per generation and likely contributes to disease chronicity.

For example, when mice with normal immunity are infected with *M. pulmonis* that predominantly consists of a single *Vsa* type (few phase variants present in the inoculum), the *Vsa* population remains unchanged early during infection (day 3) but many phase variants arise within 2 or 3 weeks. If the mice lack B and T

cells because of a *rag* mutation, the *Vsa* population remains unchanged throughout the experiment. These data suggest that the immune system exerts selection pressure for *Vsa* phase variants in ordinary mice. There is no evidence of tissue tropism as the *Vsa* types are the same in various tissues and blood. *Vsa* phase variants arise only after the onset of a specific antibody response, apparently helping these bacteria to avoid that adaptive immune response.

The Mycoplasmal Shield

The cell surface *Vsa* proteins constitute as much as 10% of the total cellular protein, helping to shield cells against environmental factors. Variations in size modulate mycoplasmal cell surface properties.

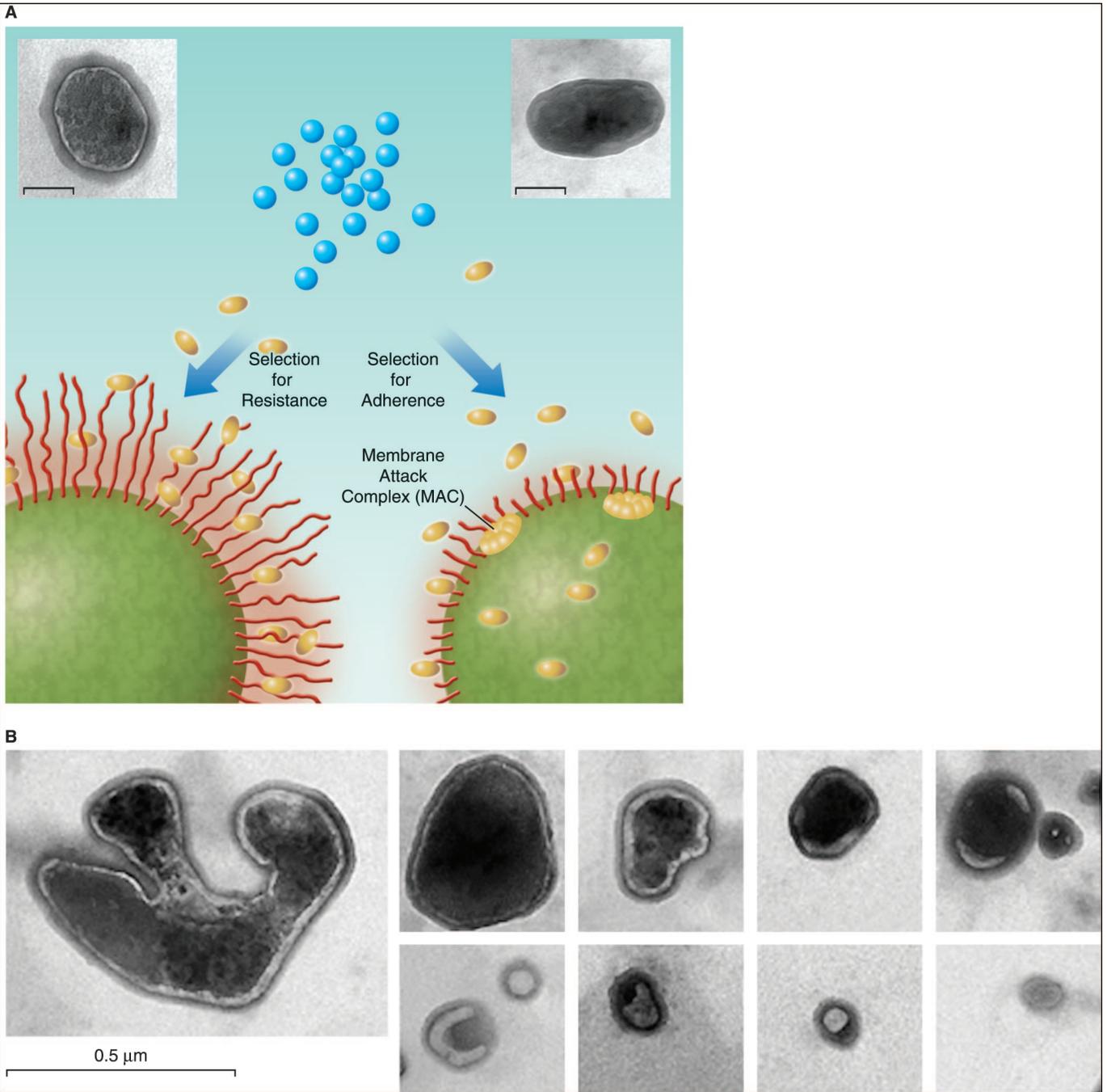
Vsa size varies when slipped-strand mispairing occurs during DNA replication (Fig. 1C), either increasing or decreasing the number of tandem repeats. A short *Vsa*A protein containing three tandem repeat units is referred to as *Vsa*A-R3 (an R3 protein). The long form of a *Vsa* protein is often referred to as an R40—typically with 40 tandem repeats but sometimes with as

many as 60. Like phase variation, size variation occurs stochastically at a frequency of about 10^{-3} per CFU per generation.

When observed by transmission electron microscopy, mycoplasmal cells appear to be surrounded by a nap (Fig. 2, Panel A). The length of the *Vsa* proteins correlates with the thickness of the surface of the mycoplasma. For example, for mycoplasmas that produce a R40 *Vsa* protein, that nap is about 26 nm thick, whereas it is about 16 nm thick on mycoplasmas producing a R3 protein.

We believe that the shield is contained within the nap. When mycoplasmas produce a long *Vsa* protein, the thick nap partly blocks access to the outer membrane of mycoplasmal cells. When mycoplasmas produce a short *Vsa* protein, however, there is ready access to the cell surface. *Vsa* length and shielding affect phenotype. For instance, *M. pulmonis* strains that produce a long

FIGURE 2



(A) The length of the Vsa repeat region modulates the mycoplasma shield. A population of mycoplasma cells (center) contains mixtures of cells that produce Vsa proteins of different types and lengths. Two such cells are shown on the right and the left of the illustration. The Vsa tandem repeat units are shown in red. Individual cells that produce a long Vsa protein containing about 40 to 60 repeat units (the cell shown on the left; TEM image shown in insert; scale bars are 100 μm) are shielded from the molecules that form the membrane attack complex of complement (shown in tan). The long repeat region results in an increased thickness of the nap that surrounds the cell and sterically blocks the complement components from reaching the mycoplasma cell membrane. The long Vsa proteins cover adhesins or hydrophobic moieties on the surface resulting in a cell with diminished capacity to adhere. When the cells produce a short Vsa protein containing few tandem repeat units (the cell shown on the right; TEM image in the insert), the complement molecules have access to the membrane, and the membrane attack complex forms and lyses the cells. Adhesins or hydrophobic moieties on the surface of the mycoplasma are exposed when Vsa is short, and the cells are highly adherent. (B) Electron and phase microscopic studies have shown that mycoplasma cells can form filaments and protrusions from the cell membrane. These constrictions have the potential to shed blebs or vesicles from the mycoplasma. This series of TEM images shows a continuum of sizes of mycoplasma cells and vesicles formed in culture.



Vsa have a longer doubling time than do strains that produce a short Vsa. Cells producing a long Vsa protein may lack access to key nutrients because the shield interferes with cell surface degradative enzymes, preventing them from breaking down host macromolecules.

Vsa length correlates with the ability of mycoplasmal colonies to adsorb sheep erythrocytes (hemadsorb) and to grow on polystyrene or glass. Mycoplasmas that produce a short VsaA, hemadsorb well and grow readily on polystyrene or glass. Mycoplasmas that produce a long Vsa protein do not hemadsorb or attach to plastic or glass. These findings suggest that molecules involved in adherence extend beyond the shield and promote adherence only if Vsa is short.

The shield modulates the susceptibility of individual cells to killing by complement (Fig. 2, Panel A). When Vsa is short, the mycoplasmas are highly sensitive to the complement membrane attack complex (MAC), while cells producing long Vsa are protected, even though complement component C3 deposits on the mycoplasma surface and MAC forms in both cases. Thus, *M. pulmonis* activates the pathway yet resists lysis when the shield is thick. Similarly, the length of the tandem repeat region of the Vlp lipoprotein is associated with shielding *M. hyorhinae* from growth-inhibiting antibodies, according to Kim Wise and his group at the University of Missouri.

However, the shield does not protect individual mycoplasmas against the pore-forming antimicrobial peptide gramicidin. Independent of the length or type of Vsa protein, individual mycoplasmal cells are killed efficiently by gramicidin at 100-fold-lower levels than are needed to kill *Mycoplasma mycoides*. The shield apparently allows small molecules to pass through it.

Hemadsorption, adherence to polystyrene, and susceptibility to lysis by MAC may seem unrelated, but these phenotypes all reflect interactions that are taking place at the surface of the mycoplasma. The finding that Vsa shielding affects these properties independent of the Vsa-type indicates that the modulation of the surface interactions is a generalized, nonspecific process.

In our view, slipped-strand mispairing within the *usa* repeat region generates short-Vsa subpopulations with minimal shielding and long-Vsa subpopulations with maximal shielding.

Minimally shielded cells are highly adherent and grow well. Maximally shielded cells resist complement and perhaps other components of innate immune defenses. However, even a thick shield does not fully protect mycoplasmas against adaptive immunity, necessitating phase variation of the shield.

Mycoplasmas Form Encapsulating Biofilms

When grown on solid surfaces, mycoplasmas form biofilms that confer partial resistance to innate host defenses. In the case of *M. pulmonis*, biofilms form only if the Vsa protein is short and the shield thin. Typical of many biofilms, the *M. pulmonis* biofilm contains an extracellular matrix containing protein, lipid, and polysaccharide. *M. pulmonis* biofilms have honeycombed regions containing numerous cavities that allow cells free access to the surrounding medium. In this region, the mean distance between a cell and its three closest neighbors is about 9 μm .

The biofilms also contain tower structures, and within the towers are channels. The cells in the towers are so densely packed that the distances between the mycoplasmas cannot be measured. Lipid and polysaccharide appear to be most abundant in the tower regions of the biofilm, filling the channels within the towers.

The polysaccharide composition of the matrix is complex, reacting with lectins WGA and GS-II that bind to N-acetylglucosamine, and GS-I, which binds to α -linked galactose. Lipophilic fluorescent probes identify membrane within the matrix between cells. VsaA epitopes within the matrix of the honeycombed region suggests that Vsa lipoprotein is anchored to the membrane material in the matrix. Antibodies to the VsaA protein detect epitopes on the external surfaces of the tower structures but not within the internal regions, suggesting that the density of material is too high for the antibody to penetrate.

Long Vsa-producing mycoplasmas form free-floating biofilms that are held together by a flexible extracellular matrix containing lipid and polysaccharide in addition to Vsa. The volume of this extracellular matrix is much greater than that of attached biofilms, and the mean distance between cells in the microcolonies is 21

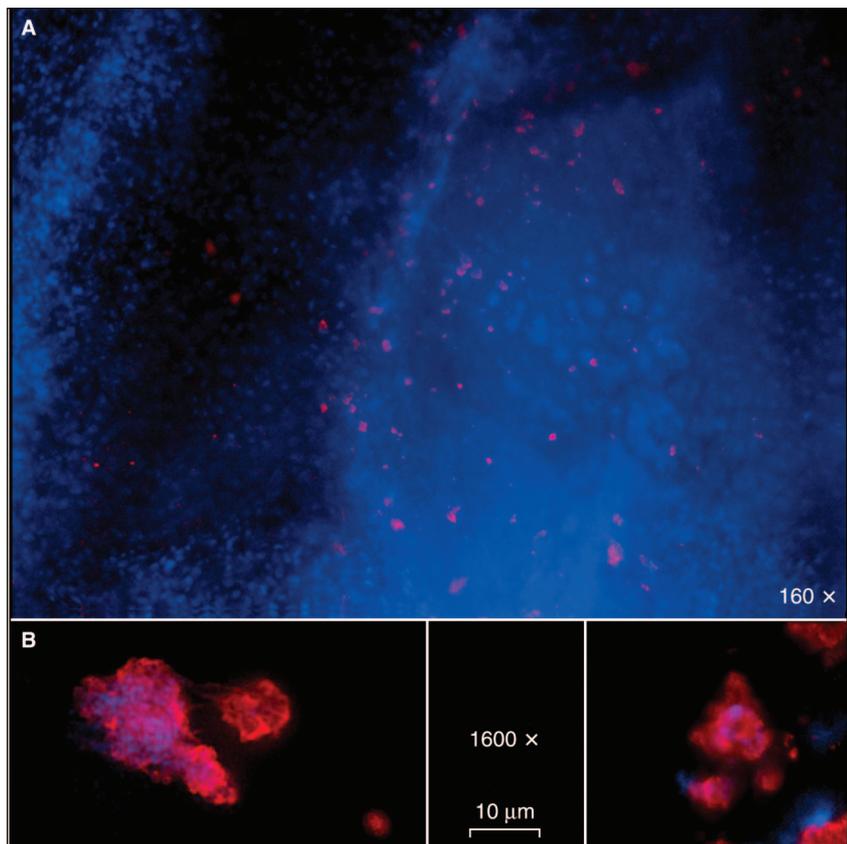
μm, more than twice the distance between cells of ordinary biofilms.

A plausible explanation for the differences in mean distances between cells within a microcolony and a biofilm is that the cells of the biofilm are restricted to close positions by virtue of their attachment to glass or plastic surfaces. However, when biofilm-forming cells are grown in polypropylene tubes, a surface to which they do not attach, the cells are too dense to measure distances between them. These results suggest that long Vsa proteins interact with other components of the extracellular matrix in such a way that greater volumes of matrix material accumulate between cells.

The accumulation of the Vsa protein and lipid in the extracellular matrix may result from blebbing of mycoplasmal cell membrane into this space. When observed by phase and transmission electron microscopy (TEM), mycoplasmal cells form filaments and other cellular protrusions (Fig. 2B). Membrane constrictions could lead to shedding of membranous blebs from the mycoplasma. At some point along this continuum, the size of the bleb becomes too small to contain a chromosome. Using the size of the *Escherichia coli* chromosome (4.6 Mbp) and the volume of the *E. coli* cell as a guide, we estimate that the minimal mycoplasmal cell has a diameter of about 0.2 μm, which is close to the smallest pore size through which mycoplasmal CFU can be filtered. Below this value, a self-replicating mycoplasmal cell is not likely to exist.

An abundance of small vesicles, some with diameters of 50 nm or less, are observed in cultures of *M. pulmonis*. Such vesicles, which may account for some of the Vsa and lipid in the extracellular environment, might function as decoys that tie up components of the immune system. Vesicles may fuse with mycoplasmal cells, transferring nutrients and other material between cells. If such vesicles sometimes contain DNA as found for outer membrane vesicles of some species of gram-negative bacteria, they may contribute to gene transfer between mycoplasmal cells.

FIGURE 3



Mycoplasma biofilm structures in tracheal organ culture. When whole mouse tracheas were incubated with biofilm-forming mycoplasmas, structures that resembled the towers of the biofilms were observed on the luminal surface of the trachea. These trachea were fixed in formalin and incubated with the DNA-specific stain Hoescht 33342 (shown in blue) and with antibodies that recognize the VsaA protein (shown in red). The top panel shows many mycoplasma towers (red) on the surface of the trachea (blue) at a power of 160 X. The structure of the towers in tracheal organ culture at a magnification of ×1,600 (B).

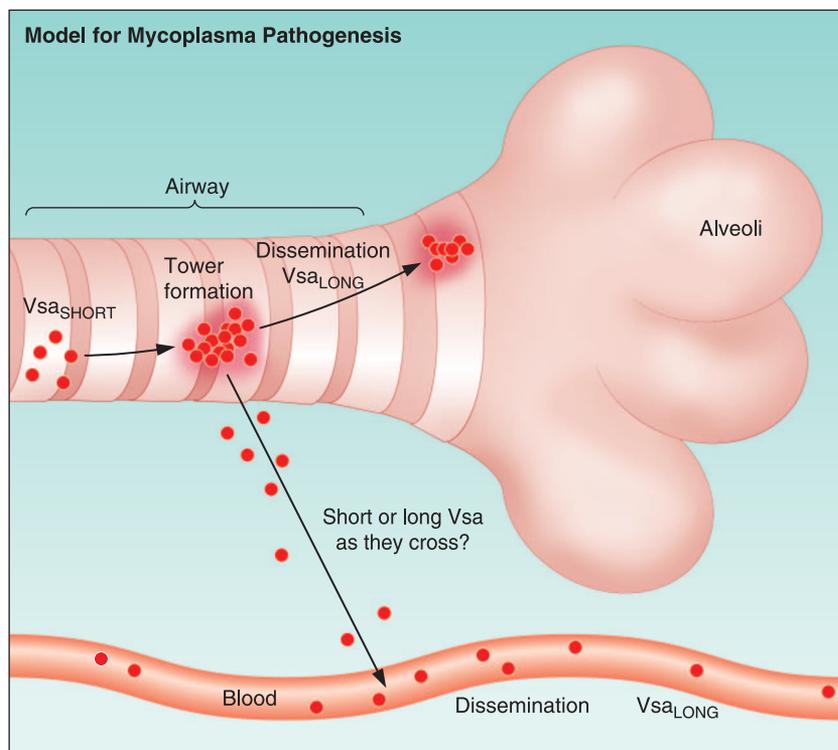
Biofilms Protect Mycoplasmas against Various Agents, Treatments

Bacteria within biofilms resist antimicrobial agents for several reasons. For one, lower growth rates in biofilms reduce the effectiveness of antibiotics that require high growth rates to kill. For another, biofilms reduce the diffusion of some antibiotics, such as aminoglycosides. Further, biofilms protect against antimicrobial peptides.

Less is known about how biofilms protect mycoplasmal cells against antimicrobial agents and physical treatments. For example, although *M. bovis* biofilms survive desiccation or 40-minute exposure to 50°C, both *M. bovis* and *M.*



FIGURE 4



Model for mycoplasma pathogenesis in the lungs. The illustration depicts mycoplasmas in the lungs and subsequent dissemination to the bloodstream. The mycoplasmas enter the airways of the lungs. Mycoplasmas that produce a short Vsa protein adhere to the airway epithelium. Here they are susceptible to the immune system but can form aggregates and establish an infection in the form of towers. As the towers develop, there is random switching of the Vsa-types resulting in subpopulations of the mycoplasmas producing a Vsa protein of varied length. Aggregates containing mixtures of Vsa types break off from the tower and disseminate to new sites on the epithelium. The phase switching to longer Vsa proteins may also facilitate the spread of the mycoplasmas into the bloodstream. As complement proteins are abundant in the blood, it would be an advantage to the mycoplasmas to produce a long form of the Vsa protein.

putrefaciens biofilms remain susceptible to fluoroquinolones and oxytetracycline, according to Laura McAuliffe and colleagues at the Veterinary Laboratories Agency in Surrey, United Kingdom.

However, we find that biofilms partly protect *M. pulmonis* from molecules that form pores in membranes. Whereas complement and gramicidin kill individual, dispersed cells of *M. pulmonis*, those cells are protected when they are encased in biofilms. For instance, more CFU are recovered from biofilms that are left intact and incubated with complement or gramicidin than are recovered from biofilms that are dispersed prior to incubation. Perhaps cells in towers within biofilms acquire resistance to comple-

ment by producing long Vsa proteins. In any case, when complement-exposed mycoplasmas from a biofilm are dispersed and again exposed to complement, 96% are killed. Thus biofilm-conferred resistance to complement is not an acquired trait. Moreover, only those mycoplasmas in the towers of the biofilm are protected, based on analyzing complement-treated biofilms with Hoechst dye, which stains all cells, and propidium iodide, which stains only dead cells.

These studies prove that the structure of the biofilm is protective. We believe that the high density of cells and matrix material in biofilm towers partly blocks access of complement and gramicidin to cell membranes. An alternative possibility is that those agents reach the membrane but cannot assemble pore-forming complexes.

How Biofilms Might Help Mycoplasmas Evade Host Immune Responses

How well does the shield hypothesis apply to survival in hosts? We know that mycoplasmas form biofilm-like aggregates and heavy layers on mucosal tissue of the trachea and the genitourinary tract. Further, we identified structures that resemble mycoplasma tower structures on the luminal side of tracheal explants (Fig. 3).

Experiments involving confocal scanning laser microscopy will soon help to determine whether these structures constitute genuine biofilms.

Aggregates of biofilm-forming mycoplasmas attach securely to host epithelium and grow to establish tower structures, thus protecting those cells from the innate immune system, according to our model for how the shield and the biofilm affect mycoplasma pathogenesis (Fig. 4). Random Vsa phase and size variation produce subpopulations of nonadherent mycoplasma cells. Although many nonadherent cells remain embedded in towers, some detach to establish other foci of infection. Even though vulnerable when disseminating, the long Vsa proteins could

partly protect individual cells and clusters within microcolonies.

A striking feature of Vsa proteins is their extensive proline-rich, tandem repeat region, much like surface antigens in other pathogens, including other mycoplasma species and in gram-positive bacteria phylogenetically related to mycoplasmas. Bacterial proteins with extensive tandem repeats that could extend beyond the wall and interact with host molecules and possibly contribute to shielding in-

clude the alpha C proteins of group B streptococci, several proteins of *Mycobacterium tuberculosis*, and various proteins of African trypanosomes and the malaria pathogen *Plasmodium falciparum*. The tandem repeats of these proteins generally are also proline-rich. Many of these antigens are thought to be protective against host defenses. For group B streptococci, the tandem domains of the alpha C proteins likely contribute to evasion of host immunity.

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