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Attributing a function to Vsa phase variation in Mycoplasma pulmonis.

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ATTRIBUTING A FUNCTION TO VSA PHASE VARIATION IN *MYCOPLASMA PULMONIS*

by

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ABSTRACT OF DISSERTATION
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Title Attributing a Function to Vsa Phase Variation in *Mycoplasma pulmonis*

Mycoplasma pulmonis causes murine respiratory mycoplasmosis (MRM). Although MRM is the best-studied animal model of mycoplasmal disease, little is understood about its pathogenesis. Many mycoplasmas undergo high-frequency surface antigen variation, including both phase and size variation, that may have a role in pathogenesis. *M. pulmonis* contains a phase- and size-variable protein named Vsa. Phase variation may serve multiple purposes in vivo including aiding in adherence to specific tissues, avoiding the host immune system, and allowing for gene exchange between mycoplasma cells.

Initial work focused on gene transfer between cells of *M. pulmonis*. It was determined that mating occurred by cell fusion, which is a mechanism distinct to *Mycoplasma* species. However, studies examining a role for the Vsa proteins in this process concluded that phase variation is not a contributing factor.

The role of Vsa phase variation in avoidance of the mouse innate and adaptive immune systems, as well as its potential role to modulate tissue tropism, was also examined. To this end, wild-type, $RAG^{-/-}$, and $iNOS^{-/-}$ mice were infected with a strain of *M. pulmonis* that expressed >90% *vsaG*. At days 3, 14, and 21 postinoculation, mice were sacrificed and various tissues were collected and assayed for *M. pulmonis* CFU. Ten

colonies from each tissue of each mouse were analyzed by PCR for the *vsa* gene expressed by the majority of cells in the population.

As expected, isolates from wild-type mice demonstrated variation away from VsaG in all tissues. However, $RAG^{-/-}$ mice exhibited populations that varied little from the initial inoculum. Isolates collected from $iNOS^{-/-}$ mice resembled wild-type in that Vsa variation did occur, but analysis of all time points suggests that the time needed for variation is greater in these animals. We therefore conclude that phase variation of the Vsa proteins aids in avoiding primarily the adaptive immune system of the mouse. Subsequent analysis has also shown that Vsa phase variation does not modulate specific tissue tropism.

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LIST OF ABBREVIATIONS

bp	base pairs
ca.	circa
cat	chloramphenicol acetyltransferase gene (encodes chloramphenicol resistance)
CFU	colony-forming unit
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
Expt	experiment
g	gravity
h	hour(s)
HCl	hydrochloric acid
IACUC	Institutional Animal Care and Use Committee
iNOS	inducible nitric oxide synthase
kb	kilobases
Mg^{2+}	magnesium ions
$MgCl_2$	magnesium chloride
µg	microgram(s)
µl	microliter(s)
µm	micrometer(s)
ml	milliliter(s)

mM	millimolar
min	minute(s)
mol%	mole percent
MRM	murine respiratory mycoplasmosis
Nat.	natural
ND	not determined
No.	number
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	postinfection
R/M	restriction/modification
s	second(s)
SDS	sodium dodecyl sulfate
tetM	gene encoding tetracycline resistance
wk	week
wt	weight
vol	volume
vsa	gene encoding variable surface antigens
vrs	*vsa* recombination site
UAB	University of Alabama at Birmingham

INTRODUCTION

Basic Biology and Genetics of Mycoplasmas

Mycoplasmas are the smallest (0.2 μm) self-replicating prokaryotes known. Because they lack a cell wall, they are taxonomically divided apart from walled bacteria into the class *Mollicutes* (trivial name mycoplasmas). Within this class, there are eight recognized genera: *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Mycoplasma*, *Spiroplasma*, *Ureaplasma*, *Entomoplasma*, and *Mesoplasma* (22). These organisms are naturally pathogenic to a wide variety of species, including mammals, birds, reptiles, fish, plants, and insects (22). The primary site of infection in humans and animals is the mucous membranes of the respiratory and urogenital tracts, though infections of other sites, including joints, have been documented (71). Thus the lack of a cell wall has not been detrimental to the success of these organisms.

Mycoplasma genomes range in size from 580 kb in *Mycoplasma genitalium* to 1,380 kb in *Mycoplasma mycoides* subspecies *mycoides* strain LC (71). Having evolved from an extremely A + T-rich branch of the Gram-positive bacteria, some species have G + C content as low as 24 mol% (104). Perhaps to accommodate DNA with a lower G + C content, the codon TGA is no longer used as a stop codon but rather to code for tryptophan at a rate 10 times that of the codon typically used for tryptophan in other prokaryotes, TGG. Due to the small genome sizes of these bacteria, mycoplasmas lack many of the cellular processes found in other bacteria, including pathways for cell wall and amino acid synthesis, de novo synthesis of purines and pyrimidines, the tricarboxylic acid cycle,

and an electron transport chain system (18). They also require cholesterol for growth (71). As such, they live a parasitic lifestyle within their host and are difficult to culture in vitro without rich, undefined medium.

Many mollicute genomes have been sequenced to date. Among them are *Ureaplasma urealyticum* (29), *Mycoplasma pulmonis* (10), *M. genitalium* (27), *Mycoplasma pneumoniae* (12, 41), *Mycoplasma gallisepticum* (67), *Mycoplasma penetrans* (78), and *M. mycoides* subspecies *mycoides* strain SC (104). Many other mycoplasma genomes, too numerous to mention, are currently in various stages of sequencing.

From these genome sequences it has been noted that a large portion of the genome is devoted to lipoprotein genes. These proteins are anchored to the outer portion of the cell membrane and have been shown in vitro to serve a structural, transport, adhesive, or enzymatic function. Within the animal, mycoplasma lipoproteins are potent activators of the host immune system, particularly the humoral immune system (11). Of the lipoproteins found, it has been determined that many are both phase- and size-variable. Although the molecular mechanisms used to create variation can be quite diverse between species, the end result is a high degree of antigenic diversity. Functions for phase-variable lipoproteins in the genus *Mycoplasma* have only been described in vitro, thus it is necessary to decipher their role in vivo.

Infection With *M. pulmonis* as a Model of Respiratory Mycoplasmosis

In humans, *M. pneumoniae* accounts for up to 30% of all pneumonias in the general population (52). *M. pulmonis*, which causes murine respiratory mycoplasmosis (MRM), is the best-studied animal model of respiratory mycoplasmosis. Like *M. pneu-*

moniae, *M. pulmonis* results in a biphasic host response, characterized by an initial innate immune response consisting largely of alveolar macrophages and complement, followed by a later stage of acquired immunity (9, 106). Because MRM is a naturally occurring disease in mice and rats, it provides a convenient and economical tool for studying a variety of strains of *M. pulmonis* in different rodent models in an effort to ascertain the relationships that exist between mycoplasma and host (9, 13, 106).

In 2001, the complete genome sequence of *M. pulmonis* strain CT was published (10). This strain was found to contain a single, circular chromosome of 963,879 bp and had a G + C content of 26.6 mol%. Lipoproteins accounted for 7% of the total coding sequence of the genome, with a total of 56 lipoproteins. Evidence for two as yet uncharacterized phase-variable lipoproteins which vary by slipped-strand mispairing was presented. Additionally, the presence of the previously characterized phase- and size-variable lipoprotein V-1 and the phase-variable type I restriction/modification system encoded by the *hsd* loci, both of which phase vary by site-specific DNA inversion, was confirmed (5, 21, 23, 86). A type III restriction/modification system was also found with the completion of the genome (10), and it has also been predicted to phase vary (72).

Immune Responses to MRM

A primary goal of mycoplasma research has been to understand the relationships that exist between mycoplasmas and their host. To this end, the innate immune response to MRM infection has been investigated. The interaction between complement and mycoplasmas will be discussed in detail below. In regard to natural killer cells, little is known except that the activity of these cells increases 24 h after infection in response to

M. pulmonis, and their subsequent release of interferon-γ activates macrophages (53, 54). Alveolar macrophages in *M. pulmonis* infection have been studied in greater detail. Howard and Taylor provided some of the first evidence that macrophages are critical in response to MRM. Using five different strains of *M. pulmonis*, they compared the virulence of each strain following intranasal infection to the time necessary to eliminate mycoplasmas from the peritoneal cavity following intraperitoneal injection of organisms. Their results showed that the more virulent strains were more resistant to killing by peritoneal macrophages (44). Additional studies using nitrogen dioxide or toxic liposomes to deplete alveolar macrophages in vivo resulted in increased susceptibility to MRM and an increase in organism load in the lungs (14, 40, 68). Furthermore, it has been shown that surfactant protein A must be present for killing of the mycoplasma to occur within the alveolar macrophage, and killing is dependent on nitric oxide (39). More specifically, inhibitors of reactive oxygen species demonstrated that killing is accomplished by peroxynitrite, which is the reaction product of nitric oxide and superoxide (37, 38).

The adaptive immune system has been shown to play an important role in the chronicity of MRM. Thymectomized mice, which lack T cells, are more susceptible to *M. pulmonis* infection than wild-type mice. They demonstrate dissemination of organisms throughout the body (15), and these animals go on to develop severe arthritis (48). Severe combined immunodeficient mice, which lack mature B and T cells, develop wasting and arthritis. These animals develop few lung lesions, though the organism load in the lungs remains high (24, 77). X-linked immunodeficient mice, which possess B cells that respond abnormally to activating signals due to a mutation in Bruton's tyrosine kinase (70), do not develop lung lesions, though the organism load is also quite high in the lungs (77).

Furthermore, immunization of mice with viable organisms has been shown to protect the animals from a subsequent intranasal infection, whereas the transfer of immune serum to experimentally infected animals reduces the number of lesions which develop, though organism load is unchanged (91). From these results, it is apparent that adaptive immune responses are critical to keeping mycoplasmas from widespread dissemination, and these responses trigger inflammatory stimuli which ultimately result in tissue injury (8).

The V-1 Protein of *M. pulmonis* is Encoded by the *vsa* Locus

In 1987, the V-1 protein was first discovered after isolation of pure membrane fractions and subsequent two-dimensional polyacrylamide gel electrophoresis (43). The frequency of variation of V-1 was noted to be high with rates of 10^{-3} per cell per generation (102). Variation of the V-1 protein was demonstrated in vivo, and higher levels of variation correlated with increased severity of lung lesions in C3H/HeNCr mice (89). Later experiments demonstrated that the *M. pulmonis* chromosome underwent high-frequency rearrangements that correlated with changes in susceptibility to the mycoplasma phage P1 (4). These chromosomal rearrangements were shown to regulate the expression of a cluster of genes called the *vsa* (*v*ariable *s*urface *a*ntigen) locus, which encode the V-1 lipoprotein (hence V-1 and Vsa are used interchangeably to denote the protein). More specifically, site-specific DNA inversions occur at a 34-bp sequence termed the *vrs* (*v*sa *r*ecombination *s*ite) box. When this sequence is recognized by the enzyme HvsR (87), DNA inversion occurs which associates the single *vsa* expression site (which consists of the promoter, ribosome binding site, and the first 714 nucleotides of the *vsa* coding region) with a previously silent *vsa* gene, thus allowing expression of the gene. A

single cell will express only one *vsa* gene, whereas all other *vsa* genes remain silent. However, because of the high rate of DNA inversions, analysis of the *vsa* gene expressed by a mycoplasmal colony becomes a study of population genetics. In the KD735 strain of *M. pulmonis* (7), the *vsa* locus was found to contain the genes *vsaA*, *vsaB*, *vsaC1*, *vsaC2*, *vsaC3*, *vsaD1*, *vsaD2*, *vsaE1*, *vsaE2*, *vsaE3*, and *vsaF* (5, 82). The additional *vsaC*, *vsaD*, and *vsaE* genes are a result of gene duplication. The CT strain has also been analyzed and found to contain a slightly different array of *vsa* genes, in that it lacks the *vsaB* and *vsaD* genes and contains additional *vsaG*, *vsaH*, and *vsaI* genes. Gene duplication is not seen in strain CT (10, 82). A diagram depicting the *vsa* genes from strains KD735 and CT is shown in Fig. 1. The subsequent proteins, shown in Fig. 2, contain a conserved N-terminus of 242 amino acids followed by a spacer region of variable size and a C-terminus containing size-variable tandem repeats which are specific to each of the Vsa proteins. The VsaE and VsaH proteins of CT and the VsaE1 protein of KD735, however, do not contain tandem repeats (82).

Phase-Variable Lipoproteins in Other Mycoplasma Species

As mentioned previously, phase-variable lipoproteins are a common theme in the mycoplasma literature. After the discovery of Vsa, lipoproteins that vary by site-specific DNA inversions were also found in *Mycoplasma bovis* (Vsp protein) (55, 56) and *Mycoplasma agalactiae* (Vpma and Avg proteins) (26, 30). *Mycoplasma penetrans* also contains a family of phase variable lipoproteins named P35, which are encoded by the *mpl* genes. In total, 38 *mpl* genes have been discovered in the *M. penetrans* genome. Phase variation of P35 also occurs by site-specific DNA inversions in *M. penetrans*, but inver-

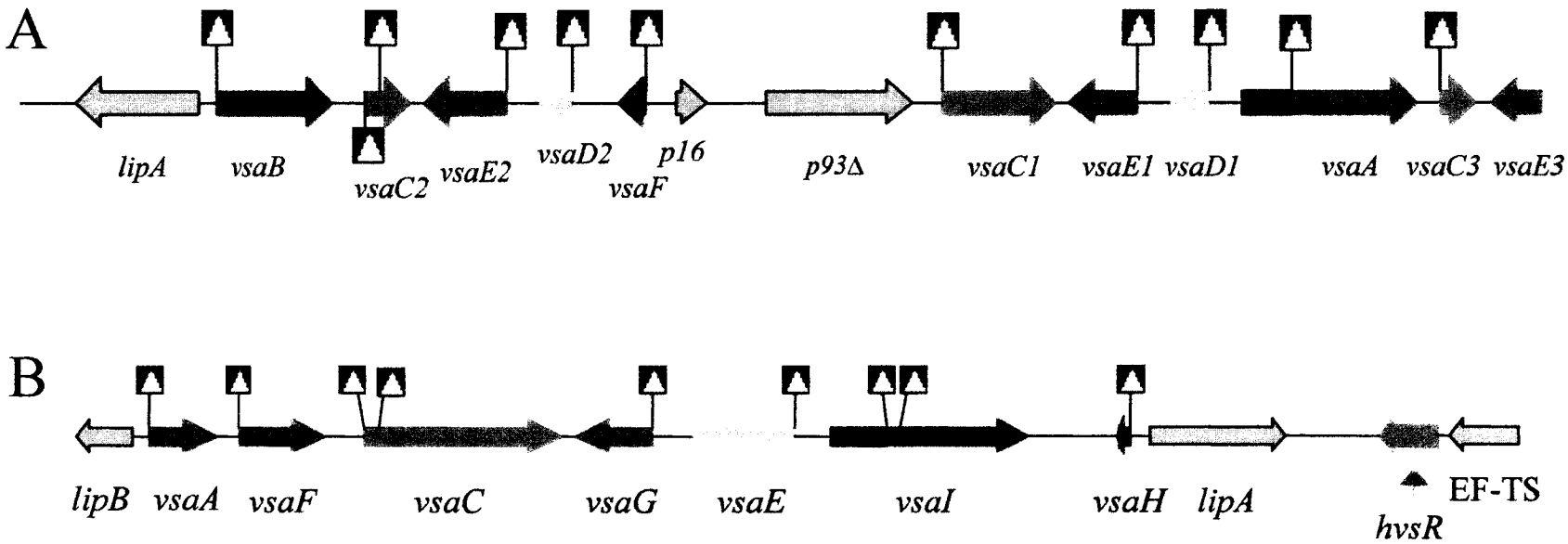

FIG 1. Schematic diagram of the *vsa* locus of strains KD735 (A) and CT (B). Genes are shown as thick arrows indicating directionality. Homologous *vsa* genes are shaded in the same color. The region shaded in black represents the *vsa* expression site which includes the promoter, ribosome binding site, and the first 714 nucleotides of the *vsa* coding region. Triangles denote locations of *vrs* boxes. Genes depicted in purple lack *vrs* boxes and therefore are not *vsa* genes. The recombinase, *hvsR*, is shown in gray.

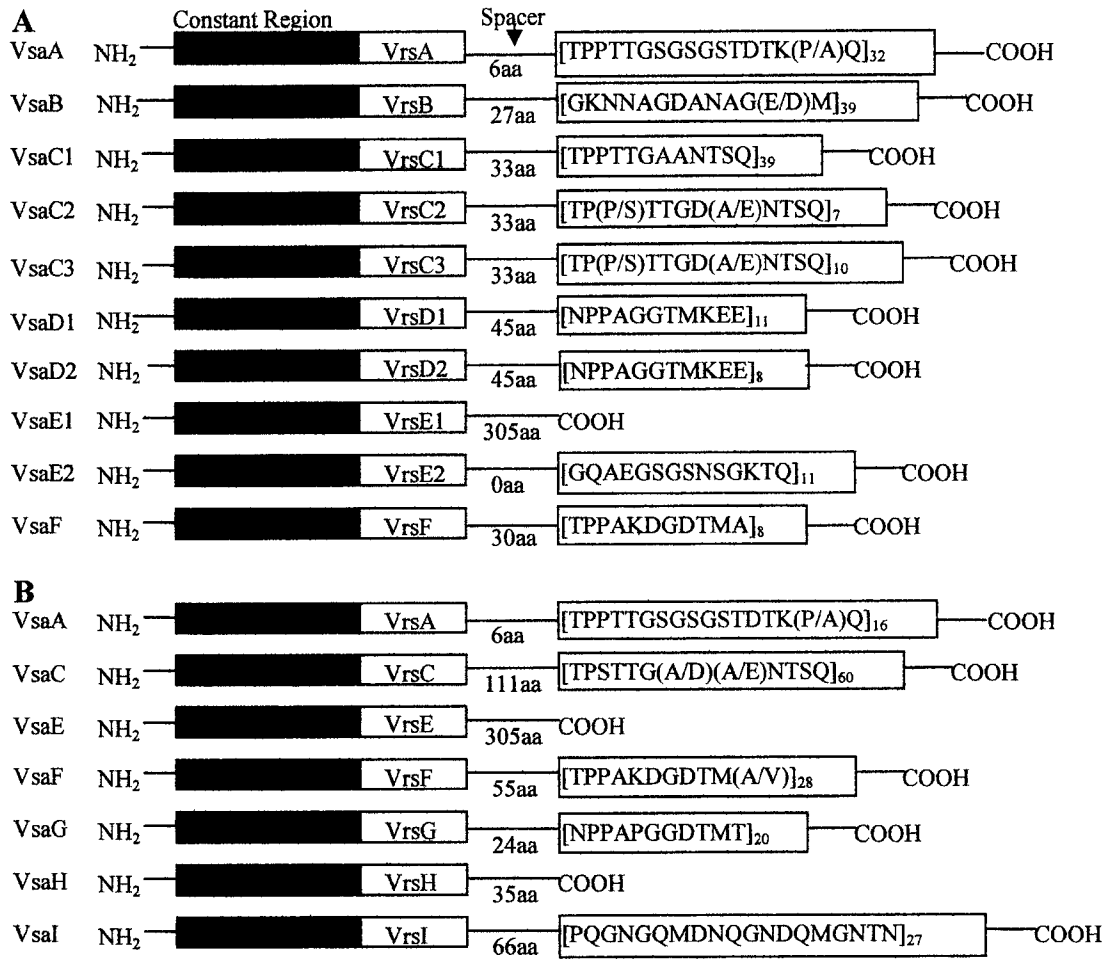

FIG 2. The Vsa proteins of strain KD735 (A) and CT (B) as modified from Reference 82. The N-terminus of all of the Vsa proteins is conserved, as shown by the solid black box. Each Vsa protein contains a unique spacer region and a variable C-terminus comprised of differing numbers of tandem repeat units.

sions in this organism have been reported specifically within the promoter region of one of the *mpl* genes, pepIMP13. Because most of the other *mpl* genes contain promoter-like sequences that are hypothesized to be invertible, it is thought that *M. penetrans* has the capacity to produce a large number of distinct lipoproteins which in turn generates an extremely high degree of antigenic diversity (42, 64, 74).

With the completion of the *M. pulmonis* genome sequence and from subsequent analysis of *M. bovis* and *M. agalactiae*, the recombinases responsible for site-specific DNA inversions have been identified for the *vsp*, *avg*, and *vsa* genes. These recombinases are all members of the lambda integrase family of tyrosine site-specific recombinases and share high homology to the XerD recombinase of *Escherichia coli* (73). The recombinase found in *M. pulmonis*, *hvsR*, also catalyzes site-specific DNA inversions at the *hsd* loci, making this the first bifunctional site-specific recombinase ever identified (87). Also of note, examination of the *U. urealyticum* genome has identified a gene encoding a Xer-like protein near the gene encoding the multiple banded antigen, a protein which has recently been shown to phase vary (62). Therefore, a wide variety of mollicutes use similar recombinases to achieve site-specific DNA inversion, and mycoplasmas use this to their advantage to create antigenic and restriction/modification system diversity.

Differing mechanisms of lipoprotein phase variation have been elucidated in other mycoplasma species as well. In *Mycoplasma hyorhinis*, the Vlp proteins are phase and size variable, and phase variation is controlled by insertions and deletions within a homopolymeric tract of adenine residues in the promoter region (107). Similarly, *Mycoplasma arthritidis* contains a phase- and size-variable lipoprotein named MAA2 (100), which was originally characterized based on its ability to adhere to several types of rat

tissues ex vivo (99). Again, phase variation is controlled by insertions and deletions in the promoter region, but, instead of adenine, the region contains a polythymine tract (101).

Lessons From Phase-Variable Surface Molecules of Other Prokaryotes

Phase variation is the random, reversible switching of gene expression between ON and OFF states. This process allows for the continued presence of subpopulations that are poised to respond to changing environmental conditions. The mechanisms by which phase variation occurs are numerous but include genome rearrangement by DNA inversion and homologous recombination, as well as slipped-strand mispairing during DNA replication and methylation of DNA sequences in the region of the gene's promoter (16, 36, 96). Examples of phase-variable surface molecules can be found in many micro-organisms, from mycoplasmas to Gram-positive and Gram-negative bacteria. For additional information considering phase-variable molecules not mentioned here, the reader is encouraged to review References 16, 36, and 96.

Phase-Variable Surface Molecules in Gram-Negative Bacteria

The presence of phase-variable surface molecules in Gram-negative bacteria has been well-documented. Though not mentioned in further detail, it should be noted that phase-variable lipopolysaccharide has been documented in numerous Gram-negative bacteria, including *Haemophilus influenzae*, *Helicobacter pylori*, and *Neisseria gonorrhoeae* (36), which affect antigenicity of the lipopolysaccharide. Additionally, phase-variable outer membrane proteins have been discovered in multitudes of Gram-negative bacteria, including *E. coli* (34, 35), *Neisseria* spp. (63, 95), and *H. pylori* (88).

Salmonella typhimurium has been shown to have a phase-variable flagellum. Phase variation occurs by inversion of a 996-bp segment of DNA (109, 110). Either end of the DNA segment contains sites recognized by the Hin recombinase, which catalyzes inversion (46). In the ON orientation, the gene encoding the H2 flagellin is transcribed along with another gene which represses H1 flagellin transcription. After inversion, the promoter needed for transcription of the gene encoding the H2 flagellin is moved so that transcription of this gene and of the repressor does not occur, and the gene which encodes the H1 flagellin is freely transcribed (84). Recent data using phase-locked mutants have shown that the virulence of this organism is affected by the phase produced, but invasion of epithelial cells was not. However, the importance of the phase of the flagellin to virulence has yet to be established (45).

The fimbriae of *E. coli* have been studied quite extensively because they are also phase-variable. The type I fimbriae vary by inversion of a 314-bp sequence, which contains the *fim* promoter that is located upstream of the *fimA* gene. In the ON orientation, the promoter is in position to allow transcription of the *fimA* gene, but, in the OFF orientation, transcription does not occur (1). Inversion is controlled by the FimB recombinase which allows for a reversible switch between ON and OFF orientations, as well as the FimE recombinase which only allows ON to OFF switching (49). Both FimB and FimE are responsive to temperature and growth medium, such that, at 37°C, FimB promotes inversions while FimE activity is decreased. As such, in humans during the course of an infection the ON orientation and the presence of fimbria are favored (28). Fimbriae from other organisms have also been shown to phase vary, such as that from *Moraxella bovis* (59), *Moraxella lacunata* (33), *S. typhimurium* (66), and *H. influenzae* (66).

The Phase-Variable Pilus of N. gonorrhoeae

The type IV pilus of the Gram-negative bacterium *N. gonorrhoeae* is one of the best-studied examples of RecA-dependent phase variation, whereby variants which produce a pilus can generate nonpiliated variants at high frequency. The expressed pilin gene is named *pilE*. Throughout the genome are several additional pilin genes (*pilS*), which are not expressed because they lack a promoter. Homologous recombination with RecA (51) can transfer one of the *pilS* genes so that it replaces sequences in the *pilE* gene. The only genes that are expressed are those which contain a complete promoter (5′ constant region) and 3′ variable region (32, 80).

Phase variation of the pilus has been shown to have many consequences, the first being that the presence of the pilus confers full competence for DNA transformation. *recA* mutants have been shown to have decreased transformation efficiencies (50, 61). By transposon mutagenesis, recent experiments have shown that the RecBCD pathway is responsible for gene transfer (60). This pathway requires RecA for full activity. In contrast, phase variation of the pilus requires the RecF pathway, which also requires RecA for full activity (60). Even though phase variation of the pilus and competence for DNA transformation occur by two different pathways, both are tied together by a common enzyme, and both are necessary to increase the organism's success.

The pilus is also essential for adherence to human cells. Although the PilE protein is the major subunit of the pilus, other proteins also make up the overall structure. Two of those proteins, PilC and PilV, in addition to PilE, are required for adherence to human corneal epithelial cells (75, 76, 105). PilE has been shown to bind to human erythrocytes, whereas PilC does not (79), and PilC alone cannot act as an adhesin to corneal tissues

(47). Taken together, these data demonstrate a role of the pilus in adherence to human tissues, and, more specifically, each of the pilin proteins has differing tissue tropism which increases the number of tissues that can be infected.

There is also evidence that the pilus plays a role in avoidance of the host immune system. Variation of the pilus has been documented in vivo, whereby isolates taken from human volunteers vary away from that of the inoculum at each time point tested (81). The pili are a main target of the antibody-mediated immune response (92). In a vaccine study, though, the antibody response was directed toward the single type of pilus which was used for vaccination (93). Therefore, a single type of pilus failed to protect men from subsequent infections with *N. gonorrhoeae* because other antigenic variants were present within the *N. gonorrhoeae* population (6). Although these data do not prove a role for phase variation in immune avoidance, they are suggestive. Further studies of the role of pilin phase variation in immune avoidance have been severely hampered due to the lack of a good animal model for *N. gonorrhoeae* disease.

Phase-Variable Surface Molecules in Gram-Positive Bacteria

Phase-variable surface proteins in Gram-positive bacteria have been found to serve primarily an adherence function. In 2001, a novel protein, SclB, was identified in *Streptococcus pyogenes* which encodes a collagen-like surface molecule that is hypothesized to vary by slipped-strand mispairing. This protein was shown to adhere to human fibroblast cells ex vivo (69). In *Streptococcus pneumoniae* serotypes 3, 8, and 37, the *cap3A*, *cap8E*, and *tts* genes, respectively, have been shown to contain duplicate sequences within each of the genes which shifts the reading frame and results in a disrup-

tion of function. Phase variation in this system allows for the presence of acapsular variants that are thought to be more successful at tissues invasion, as well as capsular variants that effectively establish systemic infections (97, 98). Data from studies of phase variation in *Staphylococcus epidermidis* have established a mechanism for variation where the insertion sequence element IS*256* inserts and excises from the *icaC* gene. This gene encodes a key enzyme responsible for synthesis of the polysaccharide intercellular adhesin, which is necessary for intercellular adherence and the successful formation of biofilms. Thus, interruption of the *icaC* gene leads to the production of cells which cannot form biofilms and are free to disseminate (108). The alpha C protein of Group B Streptococcus, which is encoded by the *bca* gene, has also been shown to phase vary. Expansion and deletion within a pentanucleotide repeat region upstream of the start codon of the *bca* gene controls transcription. Without proper spacing, this virulence-associated protein will not be produced, though the exact function of this protein and its phase variants has not been established.

Defining the Function of the Vsa Proteins

Experiments to elucidate the exact function of Vsa phase and size variation have been ongoing. Initially, an isolate of *M. pulmonis* producing the VsaA protein with 40 tandem repeats was compared to an isolate which produced VsaH (no tandem repeats). The VsaH-producing isolate was found to adhere to polystyrene and red blood cells, whereas the VsaA-producing isolate did not adhere (103). These studies were unable to decipher whether size or phase variation was more important to this phenotype. Recent studies have determined that the size-variable nature of the VsaA protein accounts for its

ability to adhere to polystyrene and red blood cells and in turn regulates the susceptibility of the mycoplasma to complement killing. These studies demonstrated that a greater number of tandem repeats resulted in a decreased level of adherence but provided resistance to complement. However, fewer tandem repeats allowed for greater adherence, but these cells possessed an increased susceptibility to complement (85). These data have been extended to include isolates producing VsaG and VsaI, each containing many or a few tandem repeats, further demonstrating the function of Vsa size variation (W. S. Simmons, A. M. Denison, and K. Dybvig, manuscript submitted).

To date, the function of Vsa phase variation has not been determined. Preliminary studies to answer this question were performed in rats using an isolate of *M. pulmonis* strain KD735 in which >95% of the cells produced VsaA. After intranasal infection, the animals were sacrificed and mycoplasmas were isolated from the nose, trachea, and lung tissues. As verified by PCR and by immunoblotting, isolates recovered from the nose continued to produce VsaA at 7 and 14 days postinfection. However, 38% of the isolates recovered from the trachea and lung produced a protein other than VsaA by days 7 and 14 postinfection. Daily passage in broth medium of isolates that produced alternate Vsa proteins showed an eventual reversion to VsaA. Thus, these data suggested that selection pressures found in the lower respiratory tract, but not present in the nose or broth culture, were responsible for the shift away from VsaA. As such, it was hypothesized that phase variation functioned as a means for the mycoplasma to avoid the host immune system, whether it be innate or humoral immunity, or for adherence to specific tissues (tissue tropism) (31).

Gene Transfer in Mycoplasmas

Gene transfer in vivo is a significant problem, because it allows bacteria living in the same niche to transfer antibiotic resistance genes (83). Although gene transfer has been documented in *Spiroplasma* (2) and *Acholeplasma* (17, 57), it has not been shown in *Mycoplasma*. Circumstantial evidence does suggest that gene transfer has occurred between mycoplasmas within the animal host. *M. hyorhinis*, *Mycoplasma hyopneumoniae*, and *Mycoplasma flocculare*, all of which infect swine, contain the insertion sequence IS*1221* (25). Additionally, the avian pathogen *Mycoplasma gallisepticum* contains the *pMGA* genes. *Mycoplasma imitans* and *Mycoplasma synoviae*, which also afflict avian species, contain related genes, whereas mycoplasmas phylogenetically related to these species recovered from humans do not possess similar genes (58, 65).

Like phase variation in *N. gonorrhoeae* (60), another possible function of Vsa phase variation is to allow the transfer of chromosomal DNA between two mycoplasma cells that produce distinct Vsa proteins. Only recently have there existed a sufficient number of selectable markers that function in *M. pulmonis* that would permit such studies (20). This necessitated the development of protocols to assess if gene transfer could be achieved between cells of *Mycoplasma*, determination of the method by which gene transfer occurred, and finally determination of any role of the Vsa proteins in this interaction.

Gene transfer occurs in bacteria by three major mechanisms. The first, transformation, requires the release of DNA from one cell into the medium where it can then be taken up by another cell. Therefore, this mechanism is sensitive to DNase and requires the viability of both cells. Transduction is the transfer of genetic material from one cell to

another via a phage. Although phages of *M. pulmonis* have been identified (19), these phages are not lysogenic, and there is no evidence that the KD735 and CT strains of *M. pulmonis* contain phage DNA (94). The third mechanism, conjugation, results in a unidirectional transfer of genetic material from a host to a recipient cell. Although mycoplasmas can acquire conjugative transposons like Tn*916* from enterococci, the use of conjugation as a mechanism for transfer between two mycoplasmas has not been described (18, 22). The complete genome sequence of *M. pulmonis* also did not present evidence for the existence of conjugative machinery, thus this mechanism is not likely. A potential fourth mechanism, cell fusion, could exist in mycoplasmas due to the presence of only a single membrane surrounding the cell. Experimental evidence of cell fusion has been presented in *Mycoplasma capricolum* (90) and *Spiroplasma citri* (3) using polyethylene glycol (PEG), and, in *S. citri*, fusion by this method can result in gene transfer. These four mechanisms were tested using natural and PEG-mediated mating protocols in an attempt to establish the method by which gene transfer occurred in *M. pulmonis*.

Determining the Function of Vsa Phase Variation

These studies attempted to determine the function of Vsa phase variation. Three potential functions (gene transfer, immune avoidance, and tissue tropism) were examined. The studies described in the first manuscript demonstrate that gene transfer can occur between cells of *M. pulmonis* by cell fusion. Though not described in this manuscript, experiments to elucidate a role for Vsa phase variation were performed, and no correlation was found. However, a role for Vsa size variation in gene transfer may exist that to date remains unexplored. The second manuscript details efforts to examine the role of

Vsa phase variation within the mouse host as a means to avoid killing by alveolar macrophages and the adaptive immune system, as well as the potential role of variation in binding of mycoplasmas to specific tissues.

All animal experiments were performed in accordance with the policies set forth by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham (see Appendix for IACUC approval).

GENE TRANSFER IN *MYCOPLASMA PULMONIS*

by

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ABSTRACT

Experiments were undertaken to examine gene transfer in *Mycoplasma pulmonis*. Parent strains containing transposon-based tetracycline and chloramphenicol resistance markers were combined to allow transfer of markers. Two mating protocols were developed. The first consisted of coincubating the strains in broth culture for extended periods of time. The second protocol consisted of a brief incubation of the combined strains in a 50% solution of polyethylene glycol. Using either protocol, progeny that had acquired antibiotic resistance markers from both parents were obtained. Analysis of the progeny indicated that only the transposon and not flanking genomic DNA was transferred to the recipient cell. Gene transfer was DNase resistant and probably the result of conjugation or cell fusion.

INTRODUCTION

Transformation, transduction, and conjugation are the three primary mechanisms of horizontal gene exchange in bacteria. Because mollicutes lack a cell wall and are bound by a single membrane, a process involving membrane fusion is a fourth potential mechanism for gene exchange between these organisms. The first reports of chromosomal gene transfer in the class *Mollicutes* was in *Spiroplasma citri* (2, 3). Gene exchange has also been reported in *Mycoplasma pulmonis* (27), but it was later shown that the organism used for this study was actually *Acholeplasma* (11).

Exchange of chromosomal DNA between cells of the genus *Mycoplasma* has not been previously demonstrated. Mycoplasmas probably do not acquire DNA by natural transformation (12, 18). Although mycoplasma phages are known to exist, transduction

has not been described. Several mycoplasmas can acquire the conjugative transposon Tn*916* by mating with an enterococcal donor, but conjugal transfer of any genetic element, including Tn*916*, from a mycoplasmal donor has not been described (12, 18). However, circumstantial evidence suggests that horizontal gene exchange has occurred between species of *Mycoplasma*. The IS*1221* insertion sequence element is found in *M. hyorhinis*, *M. hyopneumoniae*, and *M. flocculare* (20). These three species are all parasites of swine, and IS*1221* could have spread by horizontal gene exchange in the animal host. The *pMGA* genes of *M. gallisepticum* have closely related genes in *M. imitans* and *M. synoviae* but not in phylogenetically related mycoplasmas from humans (28, 29). These three species are avian pathogens, leading again to the suggestion of horizontal gene exchange in animal hosts.

Genetic markers have recently become available to study gene exchange in the murine pathogen *M. pulmonis*. The *tetM* determinant of transposon Tn*916* has been a widely used antibiotic resistance marker in many mycoplasmas, including *M. pulmonis* (12, 18). We constructed a chloramphenicol acetyltransferase gene (*cat*) that functions in *M. pulmonis* and described the use of transposon Tn*4001* as a delivery vehicle to insert the *cat* and *tetM* genes into the mycoplasma chromosome (16). Two parent strains, one with *tetM* and the other with *cat*, were mixed in broth and incubated together in the absence of antibiotic selection, followed by incubation on agar with double antibiotic selection. Mating progeny that were resistant to both antibiotics were obtained at a very low frequency. Gene transfer was resistant to DNase, required both parents to be viable, and could be stimulated by polyethylene glycol (PEG). The strains of *M. pulmonis* used in

this study are thought to be free of phages. Thus, cell fusion or conjugation is the most likely mechanism of gene transfer.

MATERIALS AND METHODS

Mycoplasma Strains and Antibiotics

Mycoplasmas were grown in broth medium as described previously (16). *M. pulmonis* strain KD735-15 (KD) is a derivative of strain UAB 6510 (4), which has been shown to be virulent in rats (6). Strain CT causes severe respiratory disease in mice (5, 9). Genetic markers were introduced into both strains by PEG-mediated transformation with plasmids containing Tn*916* (pAM120), Tn*4001T* (contains *tetM*), or Tn*4001C* (contains the *cat* gene) as previously described (13, 16). Tetracycline (3 μg/ml) and chloramphenicol (15 μg/ml) were used for antibiotic selection for cells containing *tetM* and *cat*, respectively. A summary of the parent strains used for mating experiments is provided in Table 1.

TABLE 1. *M. pulmonis* strains used in this study

Strain	Characteristics
CT	Virulent pathogen of mice; no transposon
CT182	CT containing Tn*4001T* inserted into Mypu_5290 (encoding VsaH) at nucleotide position 652759
CT186	CT containing Tn*4001T* inserted at nucleotide position 678012, disrupting Mypu_5470
CT247	CT containing Tn*4001T* inserted at nucleotide position 640094, disrupting Mypu_5220 (encoding LipB)
CT G5	CT containing Tn*4001C* at undetermined location
KD735-15	Derivative of strain UAB 6510; no transposon
KD-T2	KD735-15 containing Tn*916* at undetermined location
KD305	KD735-15 containing Tn*4001T* at undetermined location
IVC-3	KD735-15 containing Tn*4001C* at undetermined location

Broth (Natural) Mating Protocol

M. pulmonis strains containing either Tn*916*, Tn*4001T*, or Tn*4001C* were grown in separate cultures containing the appropriate antibiotic to late-logarithmic or stationary-growth phase. Cells were harvested by centrifugation and suspended in the original volume of fresh medium without antibiotic selection. For mating of tetracycline-resistant KD with chloramphenicol-resistant KD, equal volumes (1 ml, usually ca. 10^8 CFU) of each parent culture were mixed. For mating between KD and CT, 10 ml of CT were mixed with 1 ml of KD to compensate for the generally low titers of CT (usually ca. 10^7 CFU/ml). The mixed culture was incubated overnight (ca. 16 h) unless indicated otherwise. Mating progeny were assayed on mycoplasma agar (16) selecting for resistance to both tetracycline and chloramphenicol. Colonies were picked, propagated in 1 ml of mycoplasma broth, and stored at -80°C for later analysis.

PEG-Mediated Mating Protocol

M. pulmonis cultures (1.5 ml for CT and 150 µl for KD) were grown to late-logarithmic or stationary-growth phase as described above, combined in a single tube, washed once in phosphate-buffered saline (PBS), and suspended in a final volume of 15 µl. The parental strains were combined in one microcentrifuge tube containing 135 µl of PEG (PEG 8000 or PEG 3350, Sigma; 50% [wt/vol] solution in PBS unless stated otherwise). Some experiments also included 10 mM $MgCl_2$ in the PEG-cell mixture. After vortexing to mix the cells and PEG, the mixture set at ambient temperature for 1 min. Fresh medium was added to dilute the mixture 10-fold, followed by a 2-h incubation at 37°C. Cells were harvested by centrifugation and suspended in 1 ml of medium. Mating

progeny were assayed on mycoplasma agar and preserved at -80°C as described above for further analysis.

Heat Killing of *M. pulmonis* Parents

M. pulmonis cultures were grown to late-logarithmic or stationary-growth phase, and a sample was removed to assay CFU prior to heat killing. A culture of one of the parental strains (CT or KD) was placed in a water bath for 15 min at 50°C. Samples were removed and assayed for CFU to confirm effective heat killing. The heat-killed culture was added to either natural or PEG-mediated mating mixtures as described above.

Nuclease Treatment of Mating Mixtures

DNase I (200 μg/ml; Sigma) was added at the time parent strains were mixed and coincubated in nonselective broth to determine whether gene transfer was nuclease resistant.

PCR Analysis of Mating Progeny

M. pulmonis genomic DNA was isolated by using the Easy-DNA Kit (Invitrogen, Carlsbad, Calif.). PCR amplification of several genes, including *tetM*, *cat*, *p93* (KD specific), and *lipB* (CT specific), was used to examine the genotype of the mating progeny (Table 2). For the parent strains and for some mating progeny, the precise location of Tn*4001T* was determined by the inverse PCR strategy diagrammed in Fig. 1. The inverse PCR methods were as described previously (1, 24, 31). Briefly, genomic DNA was digested with the restriction enzyme *Nla*III, the restriction fragments were circularized by

TABLE 2. Oligonucleotides and PCR conditions

Pair	Expected product (annealing temp [°C])	Primers
1	296-bp *lipB* fragment (60)	5′-CAAAAAGAATCAACTAACTTGTCTG 5′-TGCTTGTTTTCAGAAATTACAGC
2	390-bp *p93* fragment (60)	5′-AATTAGATTTGAAATCAATGATCC 5′-ATTCTAAAGCGTAATTTCCTTCAG
3	398-bp *tetM* fragment (55)	5′-TTATCAACGGTTTATCAGG 5′-CGTATATATGCAAGACG
4	700-bp *cat* gene fragment (55)	5′-GGAGGTACCATGGAGAAAAAAATCAC 5′-CACTTCTCGAGGCGTAGCACCAGG
5	267-bp product containing the junction of Tn*4001T* and the CT182 genome (50)	5′-ACCGATTCTAAATCTACACTGAG (o.8) 5′-GAGGAAGTGGCAAAACTGAAACTC
6	415-bp product containing the junction of Tn*4001T* and the CT186 genome (65)	5′-AGGACTGCATAACATCTTCCGCAG (o.6) 5′-TTCCAGGCTTTGATGATGAAGTTG
7	607-bp product containing the junction of Tn*4001T* and the CT247 genome (50)	o.8 5′-TGCTTGTTTTCAGAAATTACAGC
8	Inverse PCR product to determine nucleotide position of Tn*4001T* in CT transformants (65)	o.8 5′-CCCAATCCCATAGCCATACCTATC (o.5)

incubation with T4 DNA ligase, and the ligation products were PCR amplified with the primers o.5 and o.8. The nucleotide sequence of the junction between the transposon and the mycoplasma chromosome was determined by using o.6 as the primer. Sequence reactions used automated dye terminator methods at the Iowa State University DNA Synthesis and Sequencing Facility, Ames. Sequence analysis was performed by using MacVector and Sequencher software, and the basic local alignment search tool (BLAST [http://genolist.pasteur.fr/MypuList/]) for comparison to the complete CT genome.

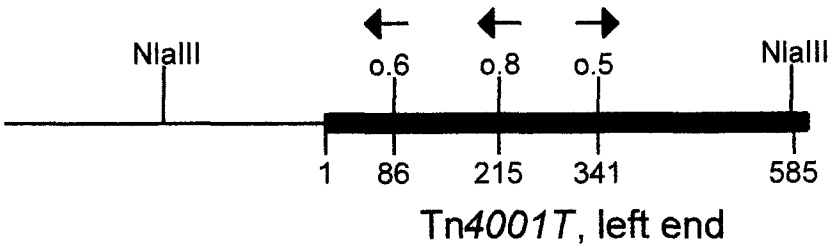

FIG. 1. Schematic diagram of the junction between the left end of Tn*4001T* (thick line) and *M. pulmonis* genomic DNA (thin line) showing the location of the o.5, o.6, and o.8 primer binding sites (the arrows indicate directionality) used to determine the nucleotide position of the transposon in the genome by inverse PCR. The numbers refer to nucleotide positions, with position 1 being the first nucleotide of Tn*4001T*. The *Nla*III site shown in *M. pulmonis* genomic sequences represents the *Nla*III site that is closest to the left end of Tn*4001T* for the particular transformant being analyzed.

For parent strains for which the precise nucleotide location of Tn*4001T* in the chromosome was determined, primers were designed to directly amplify the junction between the transposon and the mycoplasma chromosome. These primer pairs (summarized in Table 2) were used to determine by PCR whether flanking DNA in addition to the transposon was transferred to the mating progeny. Standard 25-μl PCRs were subjected to previously described cycling parameters (30) by using 30 amplification cycles and the annealing temperatures provided in Table 2.

RESULTS

Gene Transfer in the Absence of PEG

Several pilot experiments were performed to identify conditions under which gene transfer in *M. pulmonis* could be detected in the absence of PEG (natural mating). The parent strains for these experiments, KD-T2 and IVC-3, were both derived from KD735-15. Experiments designed to investigate gene transfer between cells grown on agar were not successful. Gene transfer in broth was rare and was detected in only a minority of the experiments if the mixture of parent strains was incubated for 6 h or less prior to applying antibiotic selection on agar. It was not until the parent strain mixtures were incubated overnight that gene transfer was consistently obtained. Thus, the mating protocol used for most experiments included overnight incubation. Also, gene transfer was more consistent when the cell density of the parent strains was high. Therefore, cultures of the parent strains were often grown to high titers ($>10^8$ CFU/ml) and likely in stationary-growth phase prior to being mixed. Conditions were not identified in which gene transfer was detectable if either parent was heat killed.

A variety of parent strains derived from KD and CT were examined for their ability to undergo gene transfer (Table 3). Both the *tetM* and *cat* markers for many experiments resided on Tn*4001* (Table 3, experiments 4 to 8). Thus, gene transfer was independent of the conjugative properties of Tn*916* (8). Gene transfer occurred between KD and CT regardless of whether *tetM* was in KD and *cat* in CT (Table 3, experiment 9) or vice versa (Table 3, experiments 6 to 8). Gene transfer was independent of the particular site where a transposon was located in the parental *M. pulmonis* chromosome. Gene transfer occurred in the presence of high concentrations of DNase I (Table 3, experiments

TABLE 3. Summary of gene transfer experiments

Expt	Protocol[a]	Parent 1	Parent 2	Parent 1 (CFU/ml)	Parent 2 (CFU/ml)	No. of progeny colonies	Presence or absence[b] of: Mg^{2+}	Presence or absence[b] of: DNase I
1	Nat.	IVC-3 Tn*4001C*	KD-T2 Tn*916*	2.2×10^8	1.1×10^8	4	-	-
2	Nat.	IVC-3 Tn*4001C*	KD-T2 Tn*916*	2.2×10^8	6×10^9	2	-	-
3	Nat.	IVC-3 Tn*4001C*	KD-T2 Tn*916*	4×10^8	8×10^8	9	-	-
3	Nat.	IVC-3 Tn*4001C*	KD-T2 Tn*916*	4×10^8	8×10^8	8	-	+
4	Nat.	IVC-3 Tn*4001C*	KD305 Tn*4001T*	6.0×10^8	1.3×10^9	10	-	-
5	Nat.	IVC-3 Tn*4001C*	KD305 Tn*4001T*	ND[c]	ND	2	-	-
6	Nat.	CT186 Tn*4001T*	IVC-3 Tn*4001C*	ND	ND	8	-	-
6	Nat.	CT186 Tn*4001T*	IVC-3 Tn*4001C*	ND	ND	9	-	+
7	Nat.	CT182 Tn*4001T*	IVC-3 Tn*4001C*	7.0×10^6	1.8×10^9	7	-	-
8	Nat.	CT247 Tn*4001T*	IVC-3 Tn*4001C*	3.0×10^7	2.7×10^9	5	-	-
9	Nat.	CT G5 Tn*4001C*	KD-T2 Tn*916*	6.0×10^6	1.0×10^8	11	-	-
10	50% PEG 3350	IVC-3 Tn*4001C*	KD-T2 Tn*916*	ND	ND	1,100	-	-
10	50% PEG 8000	IVC-3 Tn*4001C*	KD-T2 Tn*916*	ND	ND	700	-	-
11	50% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	6×10^7	6×10^7	>3,000	-	-
11	50% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	6×10^7	6×10^7	>3,000	+	-
11	5% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	6×10^7	6×10^7	0	-	-
11	5% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	6×10^7	6×10^7	0	+	-
12	50% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	ND	ND	96	-	-
12	50% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	ND	ND	80	+	-
13	50% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	2.5×10^7	4.0×10^7	>3,000	-	-
13	50% PEG 3350	CT186 Tn*4001T*	IVC-3 Tn*4001C*	2.5×10^7	4.0×10^7	>3,000	-	+
14	50% PEG 3350	CT182 Tn*4001T*	IVC-3 Tn*4001C*	4.5×10^6	8.5×10^7	2	-	-
15	50% PEG 3350	CT247 Tn*4001T*	IVC-3 Tn*4001C*	1.6×10^5	1.2×10^8	16	-	-

[a] Mating was done by the natural broth method (Nat.) or was PEG mediated at the indicated percentage and type of PEG.

[b] The presence (+) or absence (-) of Mg^{2+} and DNase I is shown.

[c] ND, not determined.

3 and 6). Also, growth of *M. pulmonis* strains in the presence of 10 μg of the Tn*916*-containing plasmid pAM120/ml did not result in any tetracycline-resistant progeny. Therefore, gene transfer did not result from natural transformation.

In three independent experiments not shown in Table 3, mating between strains CT G5 and CT186 was examined. No progeny that were resistant to both tetracycline and chloramphenicol were obtained. In each one of these experiments, there were 10^6 to 10^7 CFU of each parent in the mating mixture. Thus, the lack of mating progeny may have been a result of a low density of parent cells.

PEG-Mediated Gene Transfer

PEG is thought to enhance fusion of mycoplasma membranes, possibly with a requirement for magnesium ions (Mg^{2+}) (32). PEG was previously shown to enhance gene transfer in *S. citri* but to have no effect in *Acholeplasma* (2, 27). Using the PEG-mediated protocol described in Materials and Methods, gene transfer in *M. pulmonis* was readily detected at comparable frequencies with a 50% solution of either PEG 3350 or PEG 8000 (Table 3, experiment 10). Low concentrations of PEG (5%) did not promote gene transfer, and the presence of $MgCl_2$ had no effect regardless of the PEG concentration (Table 3, experiments 11 and 12). The inclusion of DNase I in the PEG-cell mixture also did not affect gene transfer (Table 3, experiment 13).

Genetic Analysis of Mating Progeny

A series of PCR experiments was performed to examine the extent of gene transfer between CT and KD. Primer pairs that would amplify an internal fragment of the *cat*

and *tetM* genes were used to confirm that gene transfer had in fact occurred. By PCR analysis, all of the progeny described in this study had both the *tet* and *cat* markers and had not acquired antibiotic resistance by spontaneous mutation. The *lipB* and *p93* genes are specific for CT and KD, respectively (30). Thus, primer pairs that amplified an internal fragment of these genes were used to determine whether progeny colonies had a KD genetic background, a CT background, or a chimera of both parental genomes. The results of these PCR experiments are summarized in Table 4. No mating progeny had both *p93* and *lipB*, indicating that neither of these genes, which are unlinked to the transposon, was transferred to progeny cells. For the natural matings without PEG, all of the analyzed progeny had the *p93* gene but lacked *lipB*. Therefore, CT served as a donor for gene transfer to KD recipients. For the PEG matings, about half of the analyzed progeny also were KD recipients. The other progeny had *lipB* but not *p93* and are considered to be CT recipients. For each of the KD recipients, the junction between the CT genome and Tn*4001T* was not transferred to KD. Therefore, gene transfer apparently involved transfer of Tn*4001T* but not flanking CT sequences, a finding indicative of transposition of Tn*4001T* into the recipient genome.

The possibility of transposition of Tn*4001T* from CT donors to representative KD recipients was further examined by nucleotide sequence analysis of inverse PCR products containing the junction between Tn*4001T* and the KD genome. The nucleotide sequence was compared to that of the CT complete genome to determine the site of Tn*4001T* insertion. These data are summarized in Table 5. Each of four progeny obtained from experiment 7 of Table 3 had Tn*4001T* inserted into the genome at a different site, which was distinct from the site of the parent strain (nucleotide position 652759 in CT182). One of

TABLE 4. PCR analysis of KD X CT matings and the direction of gene transfer[a]

Mating	Total no. of progeny analyzed	No.	
		KD to CT[b]	CT to KD[c]
IVC-3 X CT182 (Nat.)	6	0	6
IVC-3 X CT182 (PEG)	3	1	2
IVC-3 X CT186 (Nat.)	8	0	8
IVC-3 X CT186 (PEG)	3	0	3
IVC-3 X CT247 (Nat.)	5	0	5
IVC-3 X CT247 (PEG)	15	10	5

[a] The primer pairs for these PCR experiments are presented in Table 2.

[b] CT recipients were each PCR positive for *cat* (primer pair 4), *tetM* (pair 3), and *lipB* (pair 1) and retained the original Tn*4001T*-mycoplasmal genome junctions of the CT parent (pair 5, 6, or 7, as appropriate). CT recipients were PCR negative for *p93* (pair 2).

[c] KD recipients were each PCR positive for *cat*, *tetM*, and *p93* and lacked the Tn*4001T*-mycoplasmal genome junction found in the CT parent. KD recipients were PCR negative for *lipB*.

TABLE 5. Genome location of Tn*4001T* transferred to IVC-3 recipients

CT parent	Expt[a]	Tn*4001T* nucleotide position(s)
CT182	7	77847 and 580731[b]
CT182	7	709875
CT182	7	529246
CT182	7	914001
CT182	14	83281
CT186	6	79030
CT186	6	205991
CT247	15	87533
CT247	15	32636
CT247	15	206410
CT247	8	284708
CT247	8	KD-specific sequence[c]

[a] As provided in Table 3.

[b] This progeny clone had two inverse PCR products, a finding consistent with two copies of Tn*4001T* in the IVC-3 genome as indicated.

[c] The sequence adjacent to Tn*4001T* in this progeny clone had no match in the CT genome sequence.

these progeny apparently had two copies of Tn*4001T*, located at two different genomic sites. Three progeny from experiment 15 of Table 3 and two from experiment 8 also had Tn*4001T* at sites distinct from the parental CT247 strain and distinct from one another. The sequence obtained for one of these progeny did not match the CT genome sequence, indicating that Tn*4001T* had transposed into a region of the KD genome that is missing in CT. The finding that no two progeny had Tn*4001T* at the same site in the KD genome suggests that the progeny arose from independent mating events and are not siblings.

DISCUSSION

It is highly unlikely that the mechanism of gene transfer in *M. pulmonis* by the natural route (i.e., no PEG) is transformation or transduction. The resistance of gene transfer to DNase I, the requirement that both parent strains be viable, and the failure of purified plasmid to transform cultures of *M. pulmonis* all argue against transformation. *M. pulmonis* is susceptible to infection by mycoplasma virus P1 (14). However, P1 is not thought to be lysogenic, and the KD and CT strains lack P1 DNA (33). Therefore, gene transfer was not a result of transduction by this phage. Even if the KD strain harbors an unknown lysogenic phage, it would be expected that KD cells might be resistant to super-infection by this phage. Therefore, gene transfer from KD to KD (Table 3, experiments 1 to 5) might be expected to not occur if transduction was the mechanism. Also, the CT genome sequence lacks any putative prophages (7). Gene transfer from CT to KD was independent of the site of Tn*4001T* in the CT genome and apparently did not involve the transfer of any DNA (hypothetical phage) other than the transposon.

One possible mechanism of gene transfer is conjugation, the transfer of DNA from donor to recipient cells in direct contact. In the absence of PEG, gene transfer was unidirectional from CT to KD. Although this result might suggest conjugation as a mechanism, the CT genome sequence lacks recognizable genes associated with conjugation in other bacteria. The frequency of gene transfer was low, suggesting that a highly evolved mechanism for gene transfer may not exist. The apparent unidirectionality of gene transfer may be a result of the dynamics of cell growth during overnight incubation of the combined cultures. KD grows more rapidly than CT. The doubling times of KD and CT are ca. 90 and 120 min, respectively (17). Thus, KD may have been more efficient at expression of a recently acquired antibiotic resistance gene than was CT.

Another possible mechanism of gene transfer is fusion, which we define as the partial or complete combining of the cytoplasm of two cells as a result of membrane fusion. Broth cultures of most species of mycoplasma, including *M. pulmonis*, often contain chains or clusters of cells that might be amenable to occasional fusion events. Because mycoplasmas lack a cell wall, fusion between mycoplasmas may perhaps be similar to protoplast fusion of gram-positive bacteria (23). Two cells may fuse to become a single cell with two chromosomes. Transposition of a transposon from one chromosome to another, followed by cell division to segregate the chromosomes into separate daughter cells, would account for the mating progeny obtained in this study. As above, the apparent unidirectionality of gene transfer from CT to KD may result from the superior growth of KD.

It is not known whether gene transfer in the absence of PEG occurs by the same mechanism as gene transfer by the PEG method. PEG can stimulate the transformation of

many mycoplasmas, including *M. pulmonis* (13, 15). However, the insensitivity of the PEG-mediated gene transfer to DNase I argues against transformation as the mechanism. PEG can stimulate protoplast fusion as well as fusion between mycoplasma membranes (23, 32), a finding consistent with a cell fusion model of gene transfer. However, it is also possible that PEG would stimulate conjugation, if it exists in this system.

M. pulmonis probably lacks a robust homologous recombination system. Attempts to specifically target *M. pulmonis* genes for mutagenesis by transformation with plasmids that contain an internal fragment of the gene to be targeted have not succeeded using strategies that were successful in other mollicutes (19, 21, 24). A lack of homologous recombination would restrict the incorporation of transferred genes into the recipient chromosome. Perhaps the only genes that could be recombined were those that did not require homologous recombination, e.g., genes located on a transposable element. Even if homologous recombination exists in *M. pulmonis*, it may be much less efficient than transposition of Tn*4001* and Tn*916* and have gone undetected in the current study. It would be interesting to perform mating experiments similar to those of the current study but in a species of *Mycoplasma* for which homologous recombination is known to be active, such as *M. genitalium* (10).

Tn*4001* can be used as a vector to insert genes into the mycoplasmal chromosome. For example, Tn*4001* has been used to express *lacZ* in *M. pulmonis*, *M. arthritidis*, and *M. gallisepticum* and cytadherence-associated genes in *M. pneumoniae* (16, 22, 25, 26). The protocols developed in the current study could be used to transfer genes within Tn*4001*, such as *lacZ*, from one strain of *M. pulmonis* to another. If interspecies gene

transfer proves feasible, this approach might significantly impact studies on mycoplasmal genetics.

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IMMUNE AVOIDANCE THROUGH PHASE VARIATION IN *MYCOPLASMA PULMONIS*

by

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ABSTRACT

Phase-variable lipoproteins are commonly found in *Mycoplasma* species. *Mycoplasma pulmonis* contains a family of extensively-studied phase- and size-variable lipoproteins encoded by the *vsa* locus. This surface protein varies at a high frequency, though the in vivo significance of this high rate of variation has yet to be determined. We investigated the role of Vsa phase variation in both tissue tropism and immune avoidance within the mouse host. Mycoplasmas were collected 3, 14, and 21 days postinfection from the nose, lung, trachea, liver, and spleen of experimentally infected C57BL/6 (wild-type), C57BL/6-RAG-1$^{-/-}$ (RAG$^{-/-}$), and C57BL/6-iNOS$^{-/-}$ mice (iNOS$^{-/-}$). In wild-type and iNOS$^{-/-}$ mice, a high rate of Vsa variation was seen by 21 days PI. In contrast, little Vsa variation occurred in all tissues of RAG$^{-/-}$ mice. Analysis of isolates from 14 days postinfection revealed less variation of the Vsa proteins in iNOS$^{-/-}$ mice than in wild-type. Western blot analysis of isolates from each strain of mice demonstrated that phase variation at the *vsa* locus occurred independently of size variation, indicating no obvious selection pressure for size variants. Additionally, these experiments provided no evidence that mycoplasmas producing particular Vsa proteins adhered only to specific tissues. The data strongly indicate that Vsa phase variation is a mechanism for immune avoidance and does not contribute to tissue tropism.

INTRODUCTION

Phase variation mediated by DNA recombination is a common mechanism thought to aid in microbial survival by allowing for the presence of populations that can quickly respond to changing environmental conditions (8, 12). Among the more well-

studied models of phase variation is the pilin protein of *Neisseria gonorrhoeae*, where phase variation affects competence for DNA transformation (23), adherence to epithelial cells (15, 22), and avoidance of the host immune system (3). Mycoplasmas possess an abundance of phase-variable proteins, especially surface-exposed lipoproteins (19, 30, 31). The in vivo significance of phase variation of lipoproteins from *Mycoplasma* species has not yet been established, though they may function in gene transfer, tissue tropism, or avoidance of the host immune system like that of the pilin protein of *N. gonorrhoeae*.

Mycoplasma pulmonis, the causative agent of murine respiratory mycoplasmosis, produces a family of phase- and size-variable surface-bound lipoproteins encoded by the *vsa* (*v*ariable *s*urface *a*ntigen) locus. Although the amino-terminus of the Vsa proteins is composed of a conserved domain of 242 amino acids, the carboxy-terminus consists of a variable domain often containing tandem repeats (24). Each cell will express the *vsa* gene that is associated with the single *vsa* expression site. This site encodes the *vsa* promoter and the first 714 nucleotides of the coding region. Different *vsa* genes can become expressed by site-specific DNA inversions, catalyzed by the HvsR recombinase (27), at a 34-bp site termed the v*sa* *r*ecombination *s*ite (*vrs* box) (2). The CT strain of *M. pulmonis*, which contains the *vsa* genes *vsaA*, *vsaC*, *vsaE*, *vsaF*, *vsaG*, *vsaH*, and *vsaI*, was used in these studies (4, 24). Vsa size variation occurs due to slipped-strand mispairing and has been shown to have a role in resistance to complement, where isolates with a greater number of tandem repeats are resistant to complement killing while isolates with few tandem repeats are susceptible regardless of the particular Vsa protein that is produced (25, 26).

Previous work in our laboratory noted that, in rats intranasally infected with a strain of *M. pulmonis* that produced VsaA, selection pressures caused a shift of the mycoplasma population toward cells that produced a Vsa protein other than VsaA. Switching of the Vsa protein that was produced occurred in the lower respiratory tract but not in the nose (11). We hypothesized that the shift seen in the lower respiratory tract, which was seen at 7 and 14 days postinfection (PI), was due to selection pressures from the host's immune response. The alternative explanation was that particular Vsa proteins contribute to adherence to specific host tissues (tissue tropism). To investigate the role of the immune system in selecting for Vsa variants, experiments were performed using several strains of mice and an isolate of *M. pulmonis* strain CT that predominately (>90%) produced VsaG. To investigate whether phase variation was a means to avoid the adaptive immune system, $RAG^{-/-}$ mice, which lack B and T cells (20), were intranasally infected with *M. pulmonis*. At 3, 14, and 21 days PI, isolates were recovered from the nose, lung, trachea, liver, and spleen of $RAG^{-/-}$ mice and assayed to identify the Vsa protein produced by the majority of cells in each isolate. The number of Vsa variants isolated from $RAG^{-/-}$ mice was compared to that found in isolates recovered at the same time points and from the same tissues of wild-type mice. Additionally, it has been shown that *M. pulmonis* is killed, in the presence of surfactant protein A, by peroxynitrite within the alveolar macrophage (13, 14). Because nitric oxide and superoxide combine in vivo to form peroxynitrite, mice deficient in inducible nitric oxide synthase (iNOS) (16) were used in these experiments to determine the effect of this component of the innate immune system on Vsa variation. These experiments demonstrated that, as expected, Vsa phase variants were frequently isolated from wild-type mice. However, in $RAG^{-/-}$ mice, the iso-

lates recovered varied little from that of the initial inoculum, suggesting that the adaptive immune system is required to exert selection pressure to cause a switch in the Vsa protein produced. In iNOS$^{-/-}$ mice, Vsa variants were isolated at 21 days PI similar to that seen in wild-type mice. However, analysis of isolates recovered at 14 days PI suggests that more time is required for Vsa switching to occur in iNOS$^{-/-}$ mice than in wild-type mice. No evidence for tissue tropism was found, indicating that the primary function of Vsa phase variation is immune avoidance.

MATERIALS AND METHODS

Mycoplasmas

The UAB CT strain of *M. pulmonis* was isolated from a mouse experimentally infected with strain UAB T2 (5). This strain was used to infect a male (6- to 8-wk old) RAG$^{-/-}$ BALB/c mouse (C.129S6(B6)-*Rag2*^{tm1}N12, Taconic, Germantown, N.Y.). A colony (strain CT-AD) was isolated from the lung of the mouse. Strain CT-AD was propagated in mycoplasma broth and analyzed for CFU on mycoplasma agar as described (7).

Animal Infections

Male (4- to 6-wk old), specific pathogen-free C57BL/6, C57BL/6-iNOS$^{-/-}$ (B6.129P2-*Nos2*^{tm1Lau}), and C57BL/6-RAG$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom}) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). In two independent experiments, mice (6- to 8-wk old, 12 mice of each strain per experiment) were intranasally infected with 3.5×10^8 CFU of *M. pulmonis* strain CT-AD in a 50 µl total volume. On days 3, 14, and 21 PI, four animals of each strain were sacrificed. Nose, lung, trachea, liver, and

spleen tissues were collected and placed in 1 ml of mycoplasma broth (2 ml for lungs and liver) in a glass vial. The tissues were minced and sonicated for 30 s at 90% output (cuphorn; model 450 Sonifier; Branson Ultrasonics Corporation). Serial dilutions were assayed for CFU after incubation at 37°C in a 10% CO_2 incubator. Mycoplasma colonies were picked, grown in 1 ml of mycoplasma broth, and stored at -80°C for further analysis.

PCR Analysis of *vsa* Genes

PCR was used to identify the *vsa* gene associated with the *vsa* expression site in the majority of cells in each isolate obtained from the animal infections. The nucleotide sequence and binding site of the expression site primer and primers C, E, F, G, H, and I have been described previously (24, 27). An additional A.2 primer (5'-GATCCACTTCCTGTAGTTGG-3') was also designed. The expression site primer binds to the *vsa* expression site, and primers A.2, C, E, F, G, H, and I bind to the coding regions of *vsaA*, *vsaC*, *vsaE*, *vsaF*, *vsaG*, *vsaH*, and *vsaI*, respectively. Cycling conditions were described previously (11). To ensure that only the predominant *vsa* type was detected and minor subpopulations within the culture were not detected, the number of cycles of PCR was limited to 25 (11). PCR products were visualized on a 1.4% agarose gel stained with ethidium bromide and photographed.

Immunoblotting of Experimental Isolates

Frozen 1 ml stocks of *M. pulmonis* isolates were thawed, and 300 μl was removed. Cells were harvested by centrifugation at 14,000 x *g* for 3 min and were sus-

pended in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 200 mM dithiothreitol, and 0.2% bromophenol blue), boiled for 3 min, and separated on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes (Osmonics). The membranes were reacted with a 1:1,500 dilution of the monoclonal antibody 7.1-2 (2) which recognizes the constant region conserved in all Vsa proteins. Sheep-anti-mouse (Immunoglobulin G heavy and light chains) conjugated to alkaline phosphatase (Serotec) was used as the secondary antibody at a 1:2,000 dilution. The proteins were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride and photographed.

Statistical Calculations

Statistics were calculated using contingency table analyses including chi-square analysis with significance defined as $P \leq 0.05$ (SigmaStat version 2.0, SPSS Inc.).

RESULTS

Variation in *M. pulmonis* Strain CT-AD

Strain CT-AD was chosen for use as the inoculum in these experiments. Since each isolate of *M. pulmonis* consists of cells with varied phenotypes, it was necessary to characterize the population of the stock that would be used for infections. Eighty-four colonies (subclones) were assayed by PCR to determine the *vsa* gene that was associated with the *vsa* expression site in the cell majority. Seventy-nine subclones expressed *vsaG* and 5 expressed *vsaH*. Thus, about 94% of the CT-AD cells produced VsaG and 6% produced VsaH.

Isolation of Mycoplasmas From Infected Mice

In two independent experiments, isolates were collected from nose, lung, trachea, liver, and spleen tissues on days 3, 14, and 21 PI. There were no differences in the total number of CFU recovered between wild-type, $RAG^{-/-}$, or $iNOS^{-/-}$ mice. Mycoplasmas were recovered from nose, lung, and tracheal tissues at all time points but variably recovered from spleen and liver tissues, especially at day 3. Over 2,600 isolates were analyzed during the course of both experiments. The total numbers of isolates analyzed from each tissue of the three strains of mice at each time point are summarized in Table 1.

Vsa Variation of Isolates Obtained From *M. pulmonis*-Infected Mice

Isolates were analyzed by PCR to determine the *vsa* gene that occupies the expression site. The results from experiment 1 and experiment 2 are shown in Figs. 1 and 2, respectively. In each graph, the percentage of colonies isolated that produced VsaA, VsaC, VsaG, VsaH, and VsaI is shown for each of the three strains of mice at each time point in nose, lung, trachea, liver, and spleen tissues. Isolates producing VsaE and VsaF were not found in either experiment, and VsaC-producing isolates were notably missing from the second experiment.

In the first experiment, no significant differences were noted between any of the strains of mice in any tissues at day 3 PI, except in the trachea where a difference was seen between wild-type and $iNOS^{-/-}$ mice ($P = 0.001$). The sizeable VsaH population seen in the trachea was also noted to a smaller extent in the nose, though the reason for the large number of these cells is unknown. In wild-type mice, variation was seen in all tissues at days 14 and 21 PI. A shift away from VsaG to a predominately VsaA-producing

TABLE 1. Total number of mycoplasma colonies analyzed from mouse tissues

Tissue	Mouse strain	Expt	Day 3	Day 14	Day 21	Expt 1 total	Expt 2 total
Nose	C57BL/6	1	30	40	39	109	
	C57BL/6	2	40	40	40		120
	$RAG^{-/-}$	1	37	39	29	105	
	$RAG^{-/-}$	2	40	40	40		120
	$iNOS^{-/-}$	1	30	40	39	109	
	$iNOS^{-/-}$	2	40	40	40		120
Lung	C57BL/6	1	32	28	39	99	
	C57BL/6	2	40	40	37		117
	$RAG^{-/-}$	1	5	24	28	57	
	$RAG^{-/-}$	2	40	39	40		119
	$iNOS^{-/-}$	1	27	18	36	81	
	$iNOS^{-/-}$	2	30	40	40		110
Trachea	C57BL/6	1	39	37	40	116	
	C57BL/6	2	40	40	38		118
	$RAG^{-/-}$	1	25	38	30	93	
	$RAG^{-/-}$	2	30	40	39		109
	$iNOS^{-/-}$	1	29	34	40	103	
	$iNOS^{-/-}$	2	40	39	39		118
Liver	C57BL/6	1	10	0	32	42	
	C57BL/6	2	10	13	32		55
	$RAG^{-/-}$	1	0	34	30	64	
	$RAG^{-/-}$	2	0	19	36		55
	$iNOS^{-/-}$	1	29	9	33	71	
	$iNOS^{-/-}$	2	0	40	31		71
Spleen	C57BL/6	1	9	12	22	43	
	C57BL/6	2	10	16	28		54
	$RAG^{-/-}$	1	2	37	30	69	
	$RAG^{-/-}$	2	1	21	40		62
	$iNOS^{-/-}$	1	12	2	28	42	
	$iNOS^{-/-}$	2	0	34	24		58
Daily Totals			677	893	1,039	1,203	1,406

FIG 1. The percent of colonies that express each *vsa* gene in the cell majority as determined by PCR are shown. The colonies were isolated from various tissues over a 21-day period during the course of experiment 1. Isolates obtained from C57BL/6 (wild-type) animals are represented by the black bars, $RAG^{-/-}$ animals are represented by the open bars, and $iNOS^{-/-}$ animals are represented by the hatched bars.

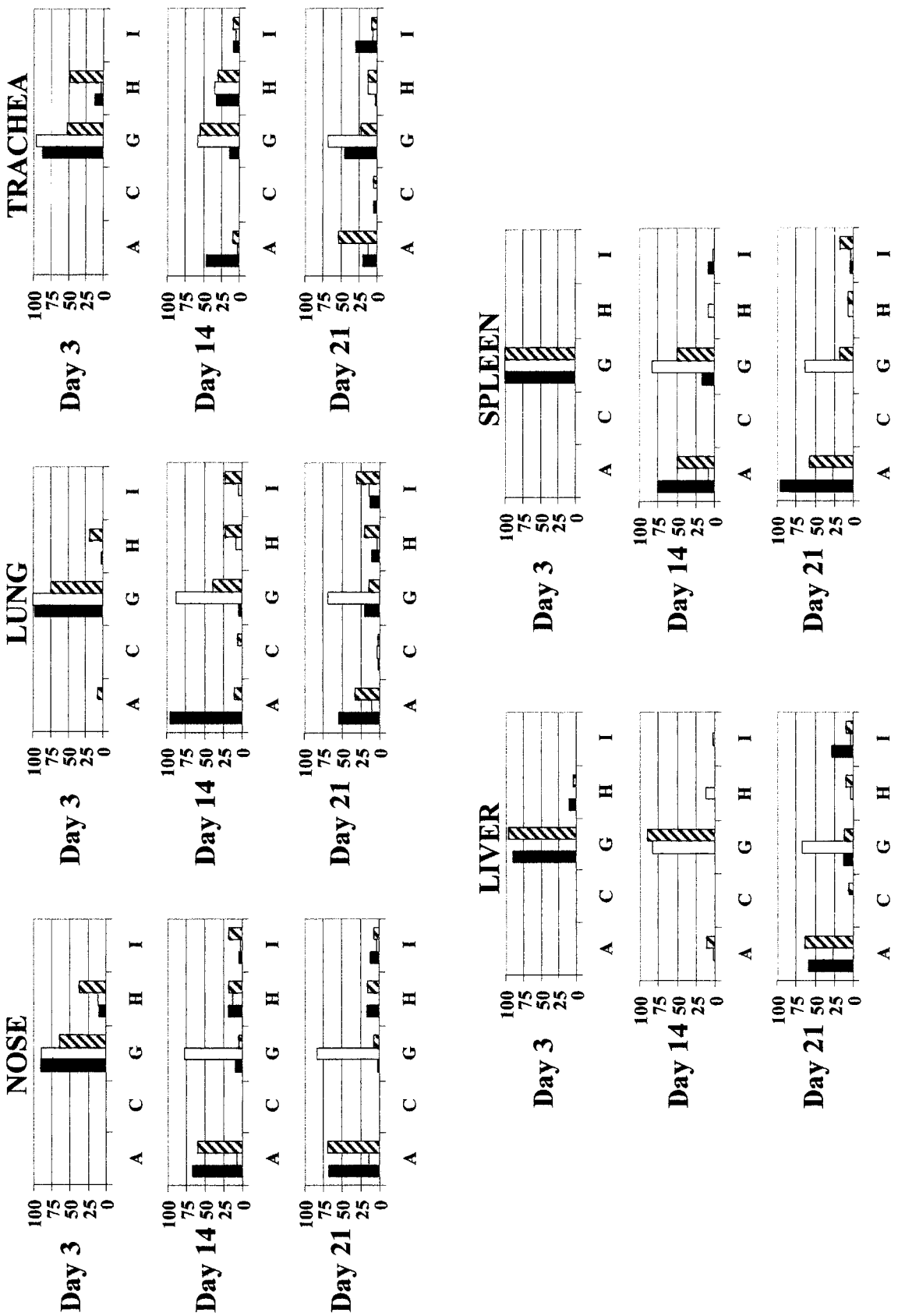
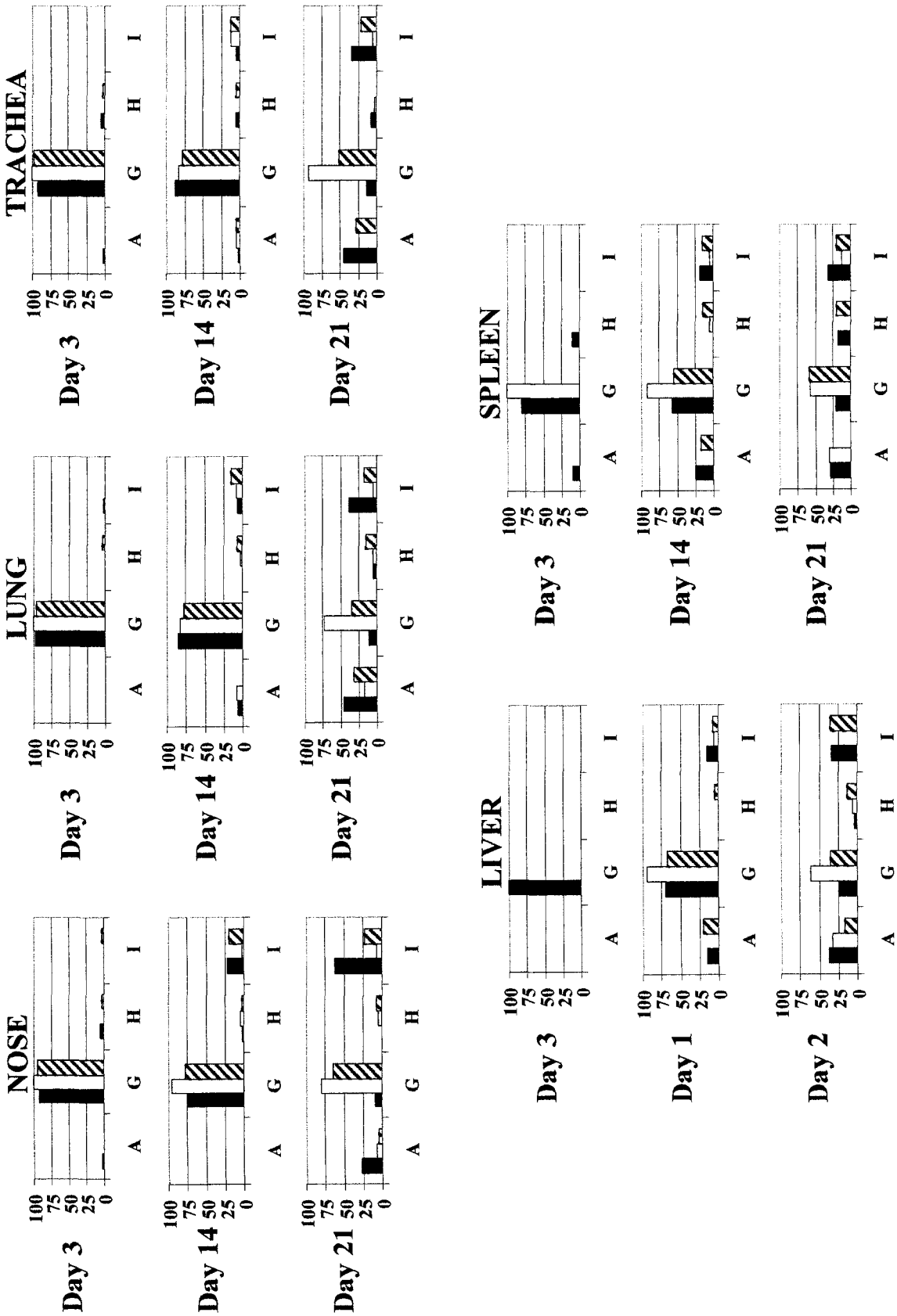


FIG 2. The percent of colonies that express each *vsa* gene in the cell majority as determined by PCR are shown. The colonies were isolated from various tissues over a 21-day period during the course of experiment 2. Isolates obtained from C57BL/6 (wild-type) animals are represented by the black bars, $RAG^{-/-}$ animals are represented by the open bars, and $iNOS^{-/-}$ animals are represented by the hatched bars.



population was seen by day 14 and at day 21 in the wild-type mice, though VsaG populations reappear in the trachea and, to a lesser extent, the nose by day 21. In $RAG^{-/-}$ mice, VsaG remains the predominant protein produced at all time points and in all tissues ($P \leq 0.023$). Mycoplasmas isolated from $iNOS^{-/-}$ mice also shifted by day 21 as seen in the wild-type mice. However, isolates collected from day 14 lung ($P \leq 0.001$), day 14 and 21 trachea ($P \leq 0.001$), and day 21 spleen ($P = 0.021$) exhibited significantly less Vsa variation than that found in the wild-type populations. In these tissues isolated from $iNOS^{-/-}$ mice, the mycoplasma populations required more time (21 days) to shift to producing an alternative Vsa protein.

Results from the second experiment were similar to those seen in the first. The most notable difference was that the shift seen at day 14 in wild-type mice in the first experiment was not seen until day 21 in the second experiment. At day 21, populations isolated from $RAG^{-/-}$ mice were again significantly different from those isolated from wild-type mice in all tissues ($P \leq 0.002$). At day 14, only nose ($P = 0.022$) and spleen ($P = 0.024$) tissues exhibited differences in Vsa populations between $RAG^{-/-}$ and wild-type mice. To further support the idea that a lack of iNOS results in less variation of the Vsa proteins, significantly less Vsa variation was observed in populations isolated from $iNOS^{-/-}$ mice than those from wild-type mice at day 21 in lung, trachea, and spleen tissues ($P \leq 0.016$).

Stability of the VsaG, VsaH, and VsaI Proteins After Daily Broth Passage

The stability of cells producing VsaA and VsaC in broth culture was established previously (11). To assess the stability of cells producing VsaG, VsaH, and VsaI, isolates

producing these proteins were passaged daily by 1:50 dilution of grown culture into 1 ml of fresh mycoplasma medium followed by incubation at 37°C. The remainder of the culture was stored at -80°C for later analysis of *vsa* expression by PCR. As shown in Table 2, isolates producing VsaG and VsaI were relatively stable after 25 passages. However, VsaH-producing cells were quite unstable, producing VsaA by the fifth passage. It should be noted that, even though VsaH-producing cells are unstable in broth culture, such isolates were often cultured from animal tissues. Nevertheless, the number of VsaH-producing isolates are potentially underrepresented.

TABLE 2. Vsa phenotype of isolates after daily passage in broth medium

	Number of passages					
Isolate	0	5	10	15	20	25
R1L-D20-3	H	A	A	A	A	A
M3T-D3-16	G	G	G	G	G	G
M2N-D3-11	H	A	A	A	A	A
17B2	I	I	G	I	I	I

Lack of Evidence for Tissue Tropism of the Vsa Proteins

The data from wild-type mice shown in Figs. 1 and 2 were also examined to determine if specific Vsa proteins only bind to certain tissues. In both experiments, day 3 isolates produced mostly VsaG regardless of the tissue. In experiment 1, the population shifted in all tissues to producing VsaA by day 14. In experiment 2, the population continued to produce mostly VsaG at day 14 in all tissues. For day 21, the first experiment showed that VsaA still predominated in the nose, lung, and liver. However, in the trachea the most predominant population was producing VsaG, though substantial amounts of VsaA and VsaI were also produced. At day 21, the results of the second experiment dem-

onstrated about equal proportions of VsaA and VsaI in all tissues examined. Therefore, these data do not suggest a predominance of any Vsa type in any single tissue. Rather, it seems that mycoplasmas producing each of the Vsa proteins can colonize any of the tissues.

Significant Vsa Size Variation is Absent in Experimental Isolates

To determine if phase variation was a means by which the mycoplasma could elongate its tandem repeat region (size vary) to avoid the host immune system, isolates from day 21 wild-type and $RAG^{-/-}$ mice that expressed *vsaG* by PCR were examined via immunoblotting (Fig. 3). No differences were found in the size of the uppermost band of the Vsa ladder in any of the isolates as compared to the initial inoculum. No variation in the size of the uppermost band was seen between VsaG-producing isolates collected from wild-type, $RAG^{-/-}$, or $iNOS^{-/-}$ mice at day 3 or day 21, and there was no difference in size of the uppermost band from these isolates as compared to the initial inoculum. Some isolates did contain bands of lower molecular weight Vsa proteins that were relatively intense, as is indicated by the arrow in Fig. 3. These intense bands of lower molecular weight represent subpopulations of mycoplasmas that produce a Vsa protein containing fewer tandem repeats as compared to the large number of repeats produced by the cell majority (the uppermost band). We had hypothesized that subpopulations containing fewer tandem repeats may be present more often in isolates obtained from immunodeficient mice than immunocompetent mice. This could occur due to a lack of selection pressure to maintain a large number of tandem repeats in immunodeficient animals. However, after analysis of approximately 100 isolates by immunoblotting, no evidence was found

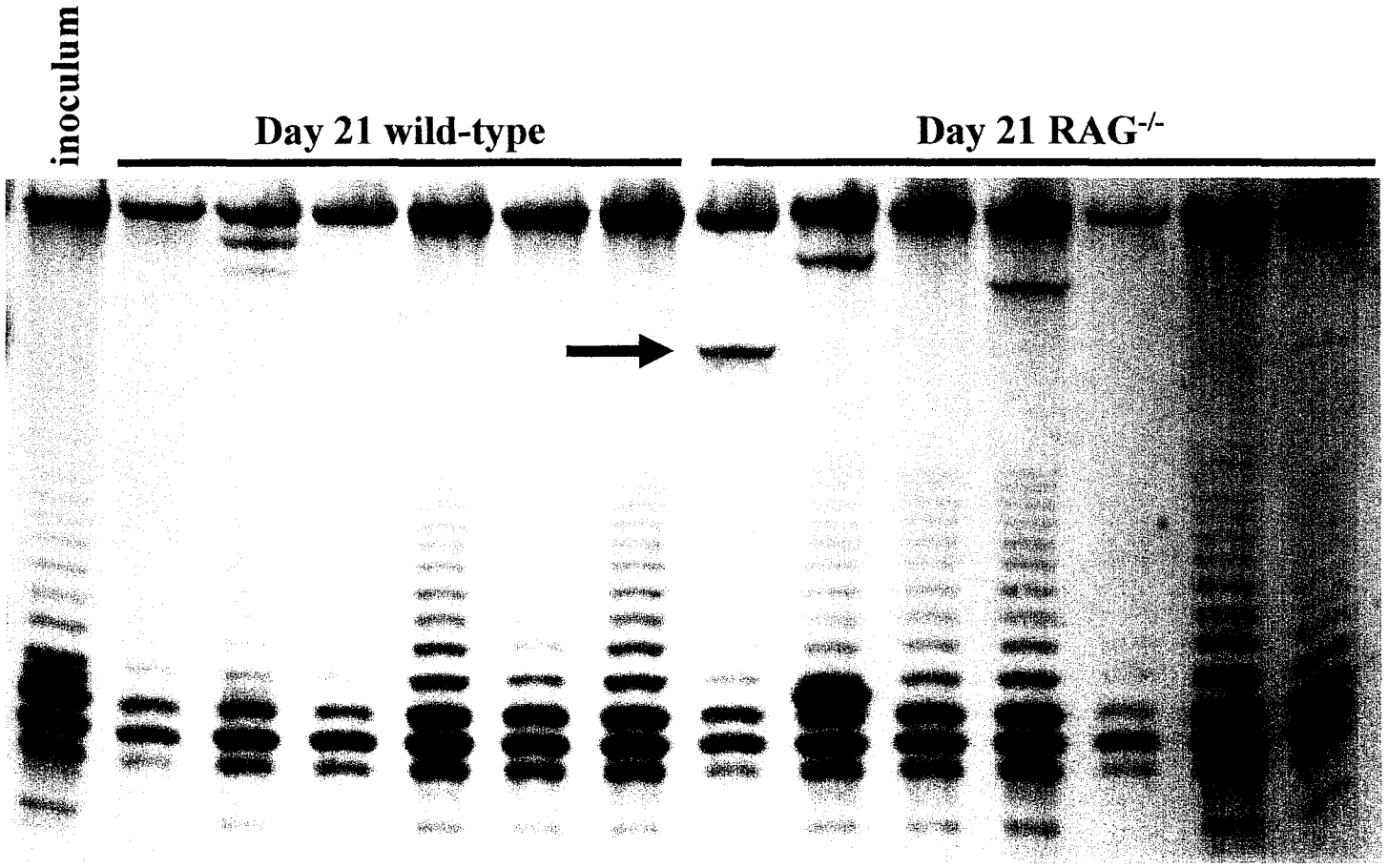


FIG. 3. Western blot of the initial inoculum used in animal infections and several VsaG-producing isolates obtained from both wild-type and RAG$^{-/-}$ animals on day 21 of experiment 1. The arrow points to a representative intense band on the blot which corresponds to a subpopulation of cells producing fewer tandem repeats than the cell majority represented by the uppermost band.

to support this hypothesis, because subpopulations containing fewer tandem repeats were found equally in isolates from wild-type and immunodeficient animals.

DISCUSSION

Phase-variable lipoproteins are commonly found in many different species of mycoplasma. The Vsp proteins of *Mycoplasma bovis* (18, 19), the Vpma and Avg proteins of *Mycoplasma agalactiae* (9, 10), the P35 family of proteins from *Mycoplasma penetrans*, and the Vsa proteins of *M. pulmonis* all use DNA inversion as a mechanism for phase variation. The MB antigen of *Ureaplasma urealyticum* likely uses a similar mechanism, because recently it has been shown to phase vary (21), and a gene encoding a predicted site-specific DNA recombinase is present near the locus which encodes the MB antigen (http://cbi.labri.fr/outils/molligen/). The phase-variable lipoproteins MAA2 from *Mycoplasma arthritidis* (28, 29) and P29 from *Mycoplasma fermentans* (17) have been shown in tissue culture systems to play a role in adherence to host cells, but these data have not been extended to animal models of disease.

Variation in bacterial surface proteins is common and has been hypothesized to contribute to immune avoidance and/or tissue tropism but could function in other roles as well. These experiments provide direct in vivo evidence of the function of phase variation in a mycoplasmal lipoprotein. We have determined that the phase-variable nature of the Vsa proteins provides the mycoplasma a mechanism to avoid the host's adaptive immune response. Immune avoidance likely increases disease chronicity, the success of the infection, and dissemination of organisms to other hosts.

Phase variation of the Vsa proteins may also provide the mycoplasma a mechanism by which it avoids macrophage killing, in that antigenic changes on the surface of the mycoplasma minimize opsonization of the mycoplasma by specific antibody. Without Vsa phase variation, specific antibody would opsonize the mycoplasma, leading to phagocytosis and killing. Therefore, the slowed Vsa variation seen in isolates from $iNOS^{-/-}$ mice may be due to the existence of lessened selection pressures to vary the Vsa proteins due to a deficiency in mycoplasma killing within the alveolar macrophage.

Comparatively little Vsa variation was seen in previous experiments in rats infected with *M. pulmonis* strain KD735. KD735, which is a derivative of UAB 6510 originally isolated from a rat (1, 6), contains a different repertoire of Vsa proteins in that it lacks VsaG, VsaH, and VsaI but contains the additional VsaB and VsaD proteins. Also, the *vsaC*, *vsaD*, and *vsaE* genes are found in multiple copies due to gene duplication in KD735 (24). The differences seen between the previously published rat experiments and the current experiments may reflect the presence of strain specificity in that the KD735 strain may be best suited to cause disease in rats and UAB CT may be best suited for mice.

Although Vsa size variation is undoubtedly required for the organism's success, these data suggest that size variation is not a means to avoid the mouse's adaptive immune response. It was possible that analysis by immunoblotting would have revealed that, over time, isolates gain an even greater number of tandem repeats to avoid the complement system. However, because the initial inoculum produced a Vsa protein with a large number of tandem repeats, the mycoplasma was already resistant to complement lysis (W. S. Simmons, A. M. Denison, and K. Dybvig, manuscript submitted), and no se-

lection pressure was evidently present to promote further expansion of the tandem repeats. If the initial inoculum had been a mycoplasma population producing a Vsa protein with few tandem repeats, it would be hypothesized that size variation would have occurred over time to generate mycoplasmas with a large number of tandem repeats to acquire complement resistance. We are currently testing this idea in our laboratory.

Isolates possessing a large number of subpopulations producing few tandem repeats were not found in immunodeficient mice as compared to those isolated from wild-type mice. This would suggest that subpopulations having few tandem repeats may serve an in vivo function aside from immune avoidance. Mycoplasmas producing a short Vsa protein with few tandem repeats efficiently hemadsorb, and mycoplasmas producing a Vsa protein with many tandem repeats do not hemadsorb (26). Thus, the in vivo function of a subpopulation of cells producing a short Vsa protein may be adherence to the epithelium. Therefore, the high rate of phase variation seen in wild-type but not in $RAG^{-/-}$ mice is a result of selection pressure for phase variation, not size variation, of the Vsa proteins, and phase variation of the Vsa proteins functions to allow the mycoplasma a mechanism by which it can avoid the adaptive immune system of the host.

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SUMMARY AND FUTURE DIRECTIONS

Mycoplasmas are unique pathogens of many species, and determining the interactions that occur between mycoplasma and host are critical to combating the diseases they cause. Phase variation is a common mechanism used by mycoplasmas to accomplish a successful infection. This work focused on determining the purpose of phase variation of the Vsa proteins of *Mycoplasma pulmonis*. It was hypothesized that phase variation could aid in successful infections by allowing mycoplasmas to exchange genetic information within the host, avoid the host's innate or adaptive immune system, or bind to specific host tissues.

Previously, gene transfer had not been demonstrated in *Mycoplasma* species. This work demonstrated that mating could occur between cells of the same and from different strains of *M. pulmonis* using natural and PEG-mediated mating protocols. Both parents had to be viable for mating to occur, and DNase had no effect on the ability of *M. pulmonis* cells to exchange genetic material, thus eliminating transformation as a means for gene transfer in these organisms. Tn*4001* is not a conjugative transposon and therefore would only be able to move by classical conjugation between cells of *M. pulmonis* if it were linked to sequences of genomic DNA. Because these experiments determined that only the transposon was transferred to the progeny and no conjugative machinery has been elucidated from the complete genome sequence, conjugation can be ruled out as the mechanism of gene transfer. These strains were also free of phages, leading to the conclusion that the method by which gene transfer occurs is not transduction. Consistent with

gene transfer being stimulated by PEG, we conclude that cell fusion is the most likely mechanism.

Experiments to identify if cells producing a specific Vsa protein mated with cells producing the same or a different Vsa protein were conducted. However, no correlation could be made between the Vsa protein produced and the likelihood of a subsequent mating event. Therefore, phase variation of the Vsa protein does not occur for gene transfer purposes. Recent data indicate that the number of tandem repeats present at the C-terminus of the Vsa protein affect the ability of the complement cascade to effectively kill mycoplasma cells in vitro, in that cells producing fewer tandem repeats are more easily killed by insertion of complement's membrane attack complex into the mycoplasma membrane. Cells with longer repeat regions are not killed, even though complement components are deposited on the cell (W. S. Simmons, A. M. Denison, and K. Dybvig, manuscript submitted). It is possible that, with a greater number of tandem repeats, large molecules like the membrane attack complex have a decreased ability to enter the cell due to a shield effect created by long Vsa proteins. Thus, fewer tandem repeats may be needed for large molecules, perhaps even DNA, to gain entry into the cell. Therefore, experiments to elucidate if the length of the tandem repeats (size variation) plays a role in the cell's ability to transfer genetic material should be explored further.

Attempts to accomplish gene transfer between different species of *Mycoplasma* were also made. Though not reported, these never resulted in successful progeny. Growth requirements are quite varied between mycoplasmas, and, although medium to compensate for this was prepared to minimize any potential effect, this may have played a part in the inability to achieve gene transfer. Differences in growth rates between species also

hindered the prospects of finding successful mating progeny. Learning more about the basic biochemistry of mycoplasmas as well as further defining the nutrients required or most beneficial for growth will ultimately assist in determining if interspecies mating can occur.

The second manuscript details efforts to determine if phase variation of the Vsa protein is a means by which the mycoplasma avoids the immune system or binds to specific tissues. In two independent experiments using a >90% VsaG-producing isolate to intranasally infect C57BL/6 mice, it was found that variation away from VsaG occurs 14 to 21 days postinfection in nose, lung, trachea, liver, and spleen tissues. In C57BL/6-RAG$^{-/-}$ mice, which lack B and T cells, even after 21 days no variation was seen in any tissue collected. This suggests that B or T cells are causing the shift in the Vsa population, and thus phase variation is a means to circumvent the adaptive immune system. Passive and active immunization experiments are currently underway to determine if Vsa phase variation is a means by which the mycoplasma avoids the B- or T-cell response of the adaptive immune system.

Experiments were also performed in mice (C57BL/6-iNOS$^{-/-}$) that are deficient in killing mycoplasmas within alveolar macrophages due to a lack of peroxynitrite. These data indicate that, while variation still occurs in these mice, variation is slower to come about in lung, trachea, and spleen tissues. This suggests that phase variation may be playing a role in circumventing the mycoplasmacidal activity of alveolar macrophages. More specifically, phase variation of the Vsa proteins may provide the mycoplasma a mechanism by which it avoids macrophage killing, in that antigenic changes on the surface of the mycoplasma minimize opsonization of the mycoplasma by specific antibody. Without

Vsa phase variation, specific antibody would opsonize the mycoplasma, leading to phagocytosis and killing. Therefore, in iNOS$^{-/-}$ mice there exists less selection pressure to vary the Vsa proteins because there is a deficiency in mycoplasma killing within the alveolar macrophage, and, subsequently, Vsa variation is slower to come about.

The data from these experiments also provide evidence that phase variation is not a means by which the mycoplasma binds to specific tissues. Similar populations were recovered from the nose, lung, trachea, liver, and spleen of wild-type mice. Regardless of the time point, no significant differences were seen between the populations isolated from any of the tissues. This, however, does not rule out the possibility that the Vsa proteins bind nonspecifically to tissues. As mentioned previously, size variation of the Vsa proteins may play a role in adherence, in that those with fewer repeats adhere to polystyrene and red blood cells more efficiently (W. S. Simmons, A. M. Denison, and K. Dybvig, manuscript submitted). Because these indicators are artificial measures of adherence to host tissues, it is still necessary to correlate these findings in an in vivo model.

Size variation of the Vsa proteins was also investigated by immunoblotting. It was determined that no differences in the sizes of the uppermost Vsa bands were evident in VsaG-producing isolates obtained from wild-type, RAG$^{-/-}$, or iNOS$^{-/-}$ animals. Therefore, phase variation was not a mechanism by which the mycoplasma could more quickly obtain a greater number of tandem repeats in its repeat region. Subpopulations of cells producing few tandem repeats were found in isolates obtained from each of the animal strains, suggesting that regardless of the immune status of the animal there is a necessity for subpopulations producing few tandem repeats. These subpopulations may serve essential functions apart from avoiding the host's immune system and may perhaps allow

the mycoplasma to bind to host tissues in a manner independent of the Vsa protein that is produced. Subsequently, the adherence properties of cells that produce few tandem repeats should be investigated further, particularly in animal models, but also in endothelial and immune cell lines.

Previously published experiments in rats (31) demonstrated that variation of the Vsa proteins occurred in the lower respiratory tract, but a similar percentage of isolates also had active restriction/modification (R/M) systems. The experiments described in the second manuscript did not examine the status of the R/M systems in the collected isolates. Future examination of these isolates for R/M system status may provide interesting insights into the in vivo purpose behind restriction systems. More specifically, in *M. pulmonis* Vsa and R/M system phase variation are controlled by the same enzyme (HvsR) and may work in concert. A determination of how these systems work together in the animal host to cause disease may prove valuable for discerning effective control measures.

Along similar lines, the rat data also demonstrated that variation did not occur in the nose, though it was prominent in the lower respiratory tract. The rat experiments used the KD735 strain of *M. pulmonis*, while the mouse experiments used the CT strain. These strains were originally isolated from the animal it was reintroduced to, and experiments to infect mice with the KD735 strain resulted in little variation of the Vsa proteins. Because each of these strains possesses a slightly different repertoire of Vsa proteins, this may suggest that there is a certain level of host specificity of each of the strains. Experiments to examine the differences that exist in the Vsa repertoire of other *M. pulmonis* strains and how that affects host specificity may provide interesting results.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL

DATE: September 5, 2003

TO: Kevin F. Dybvig, Ph.D.
KAUL-720A 0024
FAX: 975-4418

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Antigenic Variation in Mycoplasmas
Sponsor: NIH
Animal Project Number: 030906921

On September 5, 2003, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	150

Animal use is scheduled for review one year from September 2003. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 030906921 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Amy M. Denison

Graduate Program Pathology

Title of Dissertation Attributing a Function to Vsa Phase Variation in *Mycoplasma pulmonis*

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Kevin F. Dybvig	**, Chair**	
William H. Benjamin		
David E. Briles		
Susan K. Hollingshead		
Trenton R. Schoeb		

Director of Graduate Program Victor Darley-Usmar

Dean, UAB Graduate School

Date