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Avoidance of the Host Immune System through Phase Variation in *Mycoplasma pulmonis*

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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. The Vsa surface proteins vary at a high frequency, the in vivo significance of which has yet to be determined. We investigated the role of Vsa phase variation in respect to tissue tropism and avoidance of the immune system in the mouse host. Mycoplasmas were cultured 3, 14, and 21 days postinoculation from the nose, lung, trachea, liver, and spleen of experimentally infected C57BL/6 (wild-type), C57BL/6–RAG-1$^{-/-}$ (RAG$^{-/-}$), and C57BL/6-inducible nitric oxide synthase (iNOS)$^{-/-}$ (iNOS$^{-/-}$) mice. In wild-type and iNOS$^{-/-}$ mice, a large number of Vsa variants were seen by 21 days postinoculation. In contrast, little Vsa variation occurred in all tissues of RAG$^{-/-}$ mice. Analysis of isolates from 14 days postinoculation revealed less variation of the Vsa proteins in iNOS$^{-/-}$ mice than in the wild type. Western blot analysis of isolates from each strain of mouse demonstrated that Vsa phase variation 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 avoidance of the immune system with no obvious contribution to tissue tropism.

Phase variation is a common mechanism thought to aid in microbial survival by allowing for the presence of diverse subpopulations that can quickly respond to changing environmental conditions (9, 13). Among the better-studied models of phase variation is the pilin protein of *Neisseria gonorrhoeae*, where phase variation affects competence for DNA transformation (24), adherence to epithelial cells (17, 23), and avoidance of the host immune system (2). Mycoplasmas possess an abundance of phase-variable proteins, especially surface-exposed lipoproteins (20, 30, 31). The in vivo significance of phase-variable lipoproteins from *Mycoplasma* species has not yet been established but may function in gene transfer, tissue tropism, or avoidance of the host immune system like that of pilin variation 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* (for variable surface antigen) genes. The amino terminus of the Vsa proteins is composed of a conserved domain of 242 amino acids, and the carboxy terminus is a variable domain usually consisting of numerous tandem repeats (25). Each cell will express the *vsa* gene that is associated with the single *vsa* expression site. This site contains 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 (29) at a 34-bp site termed the *vsa* recombination site (*vrs* box) (1). The CT strain of *M. pulmonis* used in these studies contains the *vsa* genes *vsaA*, *vsaC*, *vsaE*, *vsaF*, *vsaG*, *vsaH*, and *vsaI* (5, 25). Vsa size variation is thought to occur by slipped-strand mispairing within the tandem repeats of the *vsa* gene during DNA replication and has been shown to have a role in modulating the susceptibility of the cell to complement lysis. Isolates with a large number of tandem repeats are resistant to complement-mediated killing, whereas isolates with few tandem repeats are susceptible (26, 27).

Previous work in our laboratory noted that in rats infected intranasally 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 (12). We hypothesized that the shift seen in the lower respiratory tract at 7 and 14 days postinoculation (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 UAB CT that predominantly (>90% of cells) produced VsaG. To investigate whether phase variation was a means to avoid the adaptive immune system, C57BL/6–RAG-1$^{-/-}$ (RAG$^{-/-}$) mice, which lack B and T cells (21), were intranasally infected with *M. pulmonis*. At 3, 14, and 21 days PI, isolates were recovered from the nose, lung, trachea, liver, and spleen 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 the number recovered at the same time points and from the same tissues of wild-type C57BL/6 mice. Additionally, it has been shown that *M. pulmonis* is killed by macrophages in the presence of surfactant protein A and that mice deficient in inducible nitric oxide

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synthase (iNOS) are deficient in antimycoplasmal activity (14, 15). Therefore, iNOS-deficient mice (18) were used in the present study to determine the effect of this component of the innate immune system on Vsa variation. These experiments demonstrated that Vsa phase variants were frequently isolated from wild-type mice. However, in RAG$^{-/-}$ mice, the isolates recovered varied little from that of the initial inoculum, suggesting that the adaptive immune system is required to exert selection pressure to cause a shift in the Vsa protein produced. In C57BL/6 mice deficient in iNOS (iNOS)$^{-/-}$ mice), Vsa variation observed at 21 days PI was similar to that seen in wild-type mice. However, analysis of isolates recovered at 14 days PI revealed that less Vsa switching occurred in iNOS$^{-/-}$ mice than in wild-type mice, suggesting that selection pressure for Vsa variants was reduced in the iNOS$^{-/-}$ animals. No evidence for tissue tropism was found, indicating that the primary function of Vsa phase variation is avoidance of the immune system.

MATERIALS AND METHODS

Mycoplasmas. The UAB CT strain of *M. pulmonis* was originally isolated from a mouse infected experimentally with strain UAB T2 (7). This strain was used to infect a male (6- to 8-week-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 previously described (8). Subcloning of strain CT-AD was performed as previously described (12).

Animal infections. Male (4- to 6-week-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-week-old mice, 12 mice of each strain per experiment) were infected intranasally 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 at 37°C in 10% CO_2. 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 (25, 29). 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 (12). To ensure that only the predominant *vsa* type was detected and that minor subpopulations within the culture were not detected, the number of cycles of PCR was limited to 25 (12). 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 × *g* for 3 min and suspended 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), and boiled for 3 min. Proteins (each, 20 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to nitrocellulose membranes (Osmonics) with Bio-Rad's Transblot SD Semi-Dry Transfer Cell System (27). The membranes were reacted with a 1:1,500 dilution of monoclonal antibody 7.1-2 (1) which recognizes the constant region conserved in all Vsa proteins. Sheep anti-mouse (immunoglobulin G heavy and light chains) antibody 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–nitroblue tetrazolium chloride and photographed. The characteristic Vsa ladder pattern was observed on the blots, and negative controls in which the primary antibody had been omitted were blank, as shown previously (1).

Statistical calculations. CFU data were first transformed to $\log_{10}$ values, and the mean, standard deviation, and an unpaired Student's *t* test were used to calculate any differences in mycoplasma load between C57BL/6 and either RAG$^{-/-}$ or iNOS$^{-/-}$ animals. To determine Vsa population differences, contingency table analyses including chi-square analyses were used. In all statistical tests, significance was defined as $P < 0.05$ (SigmaStat, version 2.; 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 various Vsa 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 (94%) subclones expressed *vsaG* and 5 (6%) expressed *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. The average $\log_{10}$ CFU per tissue is shown in Table 1. 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. Statistically significant differences in the number of mycoplasmas recovered was seen in some groups of animals, but none of these differences were reproduced between experiments. However, there was a trend in that immunodeficient mice had more CFU than did C57BL/6 mice at days 14 and 21 PI. 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 occupied the expression site. The results from experiments 1 and 2 are shown in Fig. 1 and 2, respectively. In each graph, the percentages of colonies that produced VsaA, VsaC, VsaG, VsaH, and VsaI are 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. Differing selection pressure for phase variants was indicated if the percentage of Vsa subpopulations recovered from the tissues of RAG$^{-/-}$ or iNOS$^{-/-}$ mice differed significantly from those isolated from wild-type mice.

In the first experiment, no significant differences were noted at day 3 PI between any of the strains of mice in any tissues, except in the trachea where a difference was seen between wild-type and iNOS$^{-/-}$ mice ($P = 0.001$). A sizeable VsaH population was seen in the trachea and, to a lesser extent, in the nose. In wild-type mice, variation was seen in all tissues at 14 and 21 days PI. A shift away from VsaG to a predominately VsaA-producing population was seen by day 14 and at day 21 in the wild-type mice, although VsaG populations reappeared in the trachea and, to a lesser extent, the nose by day 21. In RAG$^{-/-}$ mice, VsaG remained 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. However, isolates collected from day 14 lung ($P \leq 0.001$), day 14 and 21 trachea ($P \leq 0.001$), and day 21 spleen ($P = 0.021$)

TABLE 1. Summary of CFU recovered per tissue and number of mycoplasma colonies analyzed by PCR[a]

Tissue	Mouse strain	Expt	Day 3		Day 14		Day 21	
			Average $\log_{10}$ CFU/tissue ± SD	No. of colonies analyzed	Average $\log_{10}$ CFU/tissue ± SD	No. of colonies analyzed	Average $\log_{10}$ CFU/tissue ± SD	No. of colonies analyzed
Nose	C57BL/6	1	3.45 ± 0.49	30	3.70 ± 0.00	40	3.50 ± 0.88	39
	RAG-1$^{-/-}$	1	3.57 ± 0.19	37	3.68 ± 0.66	39	4.62 ± 0.43[b]	29
	iNOS$^{-/-}$	1	3.90 ± 0.55[b]	30	3.83 ± 0.81	40	3.84 ± 1.05	39
	C57BL/6	2	3.27 ± 0.47	40	4.87 ± 0.51	40	4.93 ± 1.00	40
	RAG-1$^{-/-}$	2	3.29 ± 0.76	40	5.51 ± 0.37	40	5.89 ± 0.49	40
	iNOS$^{-/-}$	2	3.73 ± 0.99	40	5.19 ± 0.60	40	5.50 ± 0.58	40
Lung	C57BL/6	1	2.45 ± 0.50	32	1.95 ± 1.43	28	2.55 ± 1.04	39
	RAG-1$^{-/-}$	1	0.50 ± 1.00[c]	5	2.84 ± 0.61	24	3.73 ± 1.37[b]	28
	iNOS$^{-/-}$	1	3.23 ± 0.48[b]	27	2.57 ± 1.84	18	3.61 ± 2.05	36
	C57BL/6	2	5.39 ± 0.72	40	5.32 ± 0.60	40	4.47 ± 1.15	37
	RAG-1$^{-/-}$	2	2.74 ± 3.17	40	4.10 ± 1.39	39	4.89 ± 1.20	40
	iNOS$^{-/-}$	2	3.56 ± 1.63	30	5.95 ± 0.87	40	5.42 ± 1.42	40
Trachea	C57BL/6	1	3.18 ± 1.04	39	2.77 ± 0.95	37	3.55 ± 0.31	40
	RAG-1$^{-/-}$	1	2.03 ± 1.36	25	3.09 ± 0.62	38	4.67 ± 0.61[b,c]	30
	iNOS$^{-/-}$	1	3.40 ± 0.51[b]	29	3.08 ± 1.19	34	4.05 ± 0.74	40
	C57BL/6	2	4.66 ± 1.24	40	5.29 ± 0.48	40	4.95 ± 1.12	38
	RAG-1$^{-/-}$	2	2.60 ± 2.14	30	4.77 ± 1.05	40	4.62 ± 1.18	39
	iNOS$^{-/-}$	2	3.98 ± 1.01	40	5.54 ± 0.77	39	4.75 ± 2.00	39
Liver	C57BL/6	1	0.54 ± 1.07	10	0	0	2.34 ± 0.71	32
	RAG-1$^{-/-}$	1	0	0	3.53 ± 0.36[c]	34	3.49 ± 0.44[b]	30
	iNOS$^{-/-}$	1	2.35 ± 0.21[b,c]	29	0.63 ± 1.25	9	2.45 ± 0.66	33
	C57BL/6	2	0.83 ± 1.66	10	1.96 ± 0.75	13	2.28 ± 0.62	32
	RAG-1$^{-/-}$	2	0	0	2.32 ± 1.97	19	3.57 ± 0.82[c]	36
	iNOS$^{-/-}$	2	0	0	3.08 ± 0.24[c]	40	3.02 ± 1.16	31
Spleen	C57BL/6	1	0.66 ± 0.78	9	0.83 ± 1.09	12	1.87 ± 1.02	22
	RAG-1$^{-/-}$	1	1.01 ± 0.83	2	2.50 ± 0.55[c]	37	2.70 ± 0.00[b]	30
	iNOS$^{-/-}$	1	1.19 ± 1.19[a]	12	0.35 ± 0.40	2	1.99 ± 0.75	28
	C57BL/6	2	0.66 ± 1.32	10	1.17 ± 1.02	16	1.48 ± 1.07	28
	RAG-1$^{-/-}$	2	0	1	1.72 ± 1.54	21	2.96 ± 0.60	40
	iNOS$^{-/-}$	2	0	0	2.11 ± 0.84	34	2.22 ± 1.49	24

[a] Four animals per group unless stated otherwise.
[b] Only three animals per group, due to an animal death.
[c] Significantly different ($P < 0.05$) from C57BL/6 control animals, as calculated by unpaired Student's *t* test.

tissues from iNOS$^{-/-}$ mice exhibited significantly less Vsa variation than that found in the wild-type populations. In tissues 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 of the first. The most notable difference was that the Vsa population shift 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 VsaA, VsaG, VsaH, and VsaI proteins after daily broth passage. To assess the stability of cells producing VsaA, VsaG, VsaH, and VsaI, isolates producing these proteins were passaged daily by a 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. The stability of VsaC in the X1048 strain of *M. pulmonis* was determined previously, in that cells switched to produce VsaA by passage 10 (12). As shown in Table 2, isolates producing VsaA, VsaG, and VsaI were relatively stable for 25 passages. However, VsaH-producing cells were quite unstable, producing VsaA by the fifth passage. It should be noted that even though VsaC- and VsaH-producing cells are unstable in broth culture, such isolates were often cultured from animal tissues. Nevertheless, the number of VsaC- and VsaH-producing isolates is potentially underrepresented.

Lack of evidence for tissue tropism of the Vsa proteins. The data shown in Fig. 1 and 2 from wild-type mice were also examined to determine if specific Vsa proteins are associated with specific tissues. In both experiments, day 3 isolates produced mostly VsaG, regardless of the tissue. In experiment 1, the population shifted in all tissues to production of 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

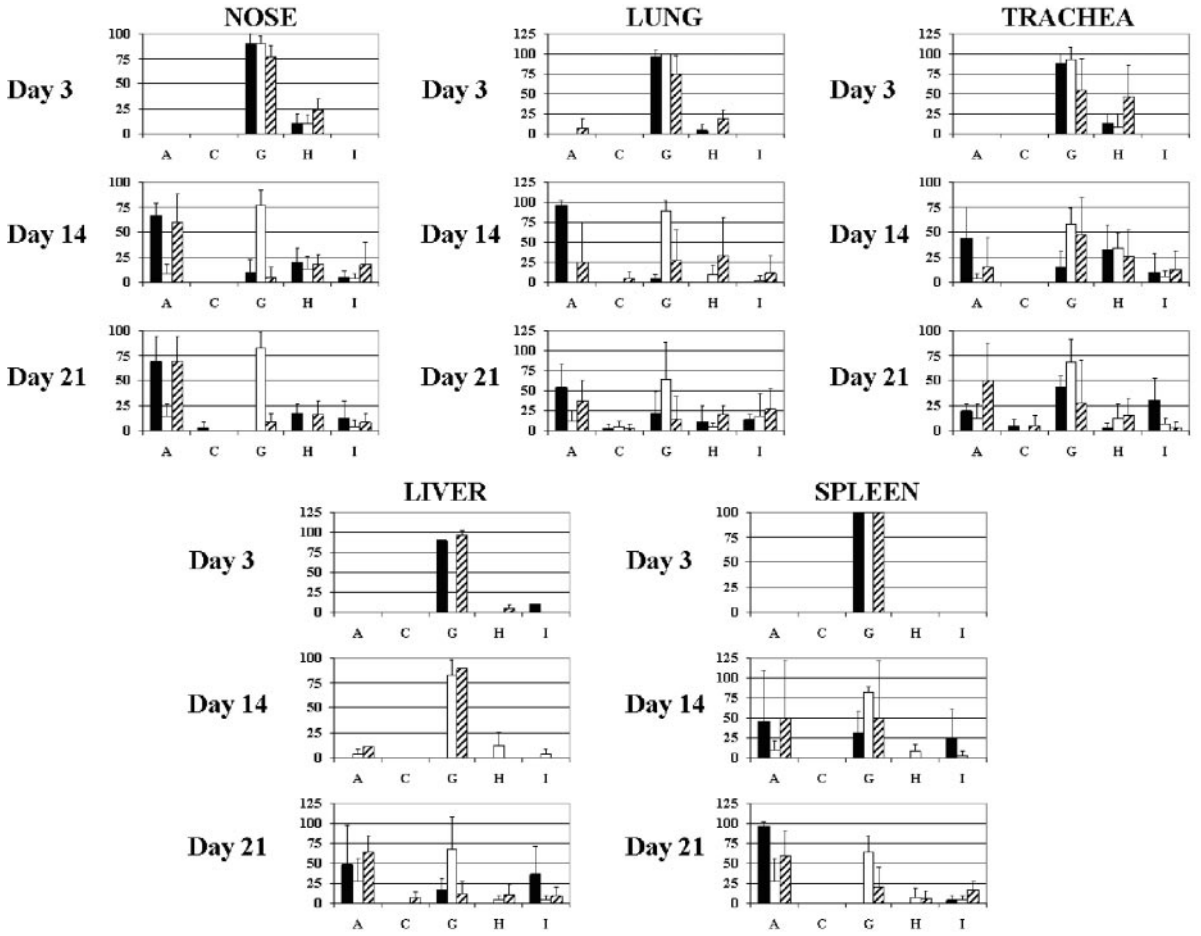

FIG. 1. The percentage of colonies that express each *vsa* gene in the cell majority as determined by PCR are shown. The colonies were isolated from various tissues over a 21-day period during the course of experiment 1. Isolates obtained from C57BL/6 (wild-type), $RAG^{-/-}$, and $iNOS^{-/-}$ animals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

population produced VsaG, although substantial subpopulations producing VsaA and VsaI were also present. At day 21, the results of the second experiment demonstrated 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 any of the Vsa proteins can colonize each of the tissues.

Significant Vsa size variation is absent in experimental isolates. Because of the high-frequency gain or loss of *vsa* tandem repeats by slipped-strand mispairing, cultures of *M. pulmonis* contain subpopulations of cells producing Vsa size variants. When analyzed by Western blotting, the population of Vsa protein size variants forms a ladder pattern in which each rung of the ladder represents the gain or loss of a single repeat unit (1, 27, 28). To determine if phase variation was a means by which the mycoplasma could elongate the tandem repeat region (size varies) in response to host selection, isolates obtained from animals at days 3 and 21 PI were examined via immunoblotting. It was noted in both experiments that no differences existed in the size of the predominant uppermost band of the Vsa ladder of 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 bands from these isolates compared to the initial inoculum (representative results are shown in Fig. 3). Immunoblots of some isolates did contain bands of lower-molecular-weight Vsa proteins that were relatively intense (Fig. 3). These intense bands of lower-molecular-weight represent subpopulations of mycoplasmas that produce a Vsa protein containing fewer tandem repeats than 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 to support this hypothesis. 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* (19, 20), the Vpma and Avg proteins of *Myco-*

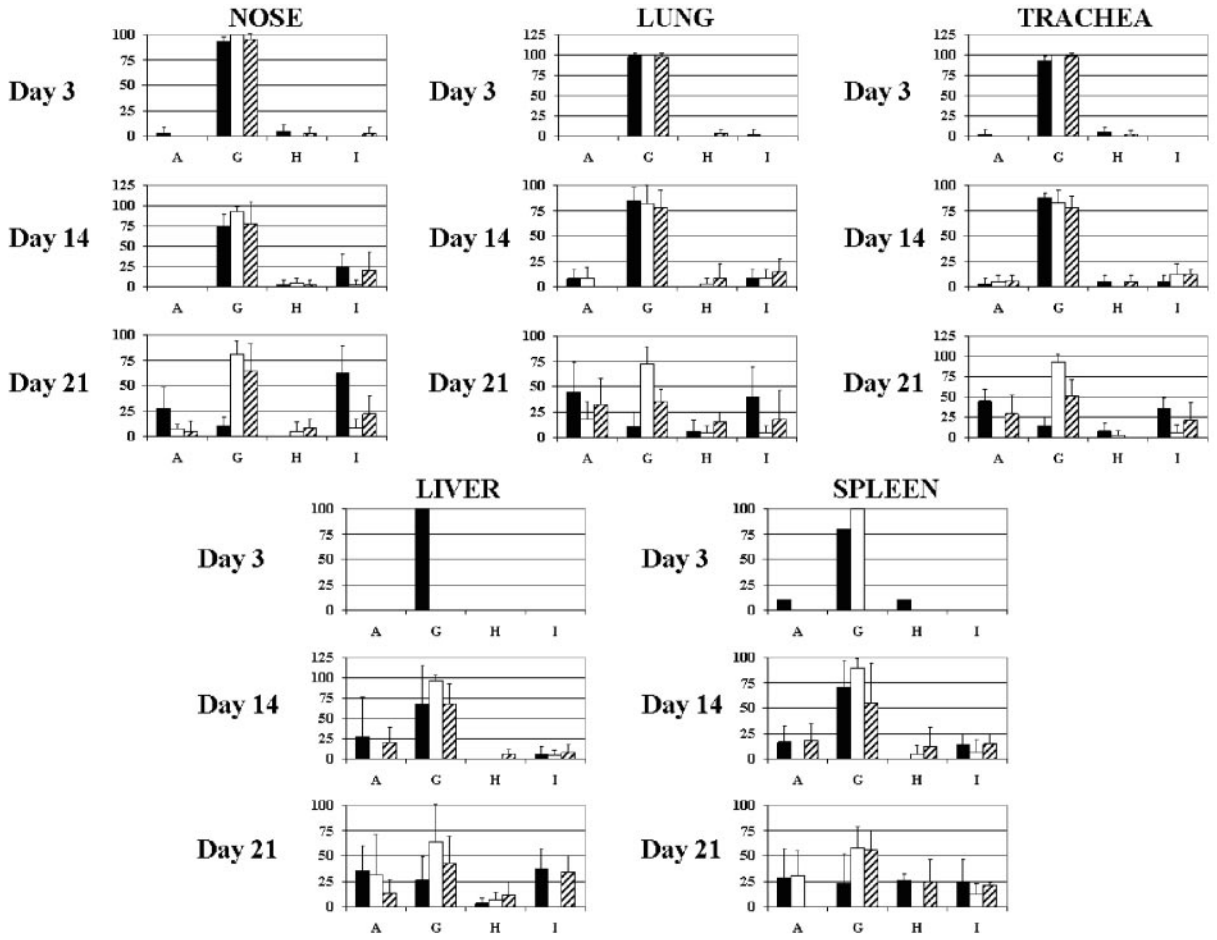

FIG. 2. The percentage of colonies that express each *vsa* gene in the cell majority as determined by PCR are shown. The colonies were isolated from various tissues over a 21-day period during the course of experiment 2. Isolates obtained from C57BL/6 (wild-type), $RAG^{-/-}$, and $iNOS^{-/-}$ animals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

plasma agalactiae (10, 11), the P35 family of proteins from *Mycoplasma penetrans* (16), 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 it has been shown to phase vary (22), and a gene encoding a predicted site-specific DNA recombinase is present near the locus that encodes the MB antigen (http://cbi.labri.fr/outils/molligen/). No isolates in the present study produced VsaE or VsaF. In contrast, a previous study involving the experimental infection of rats with *M. pulmonis* strain X1048 resulted in numerous isolates that produced VsaE (12). The configuration of the *vsa* genes in strains X1048 and CT-AD has not been determined. DNA inversions only occur between *vrs* box recombination sites that are in an inverted orientation relative to one another (1, 25). Perhaps isolates producing VsaE or VsaF were not obtained in the present study because the *vsaE* and *vsaF* genes in CT-AD are in the same (direct) orientation in the genome as is the *vsaG* gene that occupies the *vsa* expression site.

Variation in bacterial surface proteins is common and is usually assumed to contribute to avoidance of the immune system and/or tissue tropism; but it could function in other roles as well. Our studies provide direct in vivo evidence of the function of phase variation of a mycoplasmal lipoprotein. Phase variation provides a mechanism for the mycoplasma to cope with the host's adaptive immune response, and avoidance of the immune system likely increases the success of the infection, disease chronicity, and dissemination of organisms to other hosts. The lack of phase variants in $RAG^{-/-}$ mice indicates that phase variants are selected in wild-type mice either by the production of specific antibody or by T-cell immunity.

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

Isolate[a]	No. of passages					
	0	5	10	15	20	25
R1L-D21-3	A	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

[a] Each isolate was recovered from experimentally infected mice during the course of these studies. R1L-D21-3 was isolated from the lung of a $RAG^{-/-}$ mouse at day 21 PI. M3T-D3-16 and M2N-D3-11 were isolated from wild-type mice at day 3 from the trachea and nose, respectively. 17B2 was isolated from the nose of an $iNOS^{-/-}$ mouse at day 14 PI.

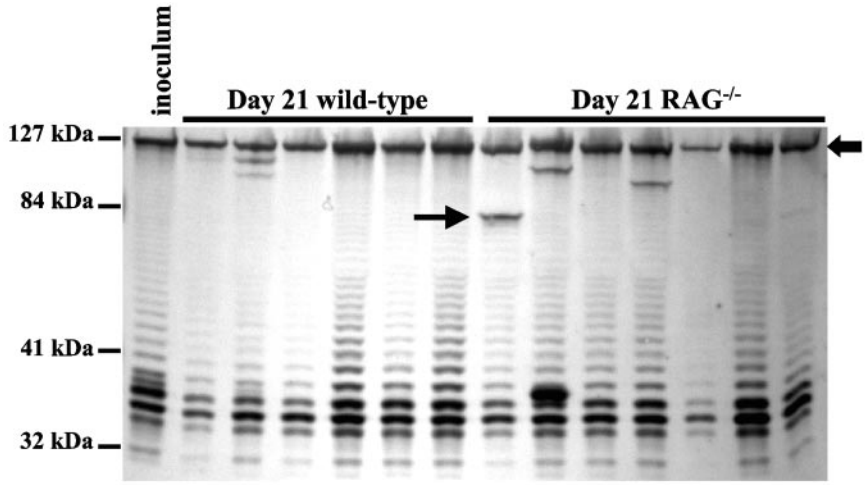

FIG. 3. Western blot of the initial inoculum used in animal infections and several VsaG-producing isolates obtained from both wild-type and $RAG^{-/-}$ animals on day 21 of experiment 1. The arrow at the top right refers to the uppermost band of the Vsa ladder. The arrow in the central region points 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. Numbers on the left refer to the positions of standard mass markers. However, as is the case for many other repetitive proteins of mycoplasmas, the Vsa proteins migrate slowly and are smaller than predicted from the mass markers (1).

Specific antibody is produced in wild-type mice around 7 to 14 days PI (4). The timing of the appearance of phase variants at day 14 or 21 but not at day 3 is consistent with specific antibody being the selecting force.

Phase variation may also provide the mycoplasma a mechanism by which it avoids macrophage killing. Without Vsa phase variation, specific antibody would presumably opsonize the mycoplasma, leading to phagocytosis and killing. Therefore, the delay in the appearance of Vsa variants in isolates from $iNOS^{-/-}$ mice may be due to weakened selection pressures to vary the Vsa proteins because of a deficiency in mycoplasma killing within the alveolar macrophage. Specific antibody may also act in inhibiting growth of the mycoplasma, as has been seen with the Vlp proteins of *Mycoplasma hyorhinis* (6). Specific antibody likely provides selection pressure for phase variants but is insufficient to clear the infection, as VsaG-producing isolates reappeared by 21 days PI in the first experiment.

While the recoverable CFU were variable throughout these experiments, we observed a trend in which immunodeficient mice had more recoverable CFU than did controls. Previous work by Hickman-Davis et al. (14) demonstrated that $iNOS^{-/-}$ mice have 100 times more CFU in the lungs after intranasal infection with the UAB CT strain of *M. pulmonis* than wild-type controls. While our results demonstrate slightly higher numbers of mycoplasmas recovered from the lungs at 14 and 21 days PI, we were unable to reproduce the 100-fold differential previously reported. The trend towards increased CFU in immunodeficient mice would also seem to disagree with the results of Cartner et al. (3), where no differences in recoverable mycoplasmas were seen between infected severe combined immunodeficient (SCID) and wild-type animals. The dissimilarity between our results and those of Cartner et al. may be due to several differences in experimental protocol. Among those differences was the use of color-changing units as a means to determine cell numbers. Our use of the more accurate CFU assay likely detected minor differences in mycoplasma load between wild-type and immunodeficient mice that would be missed with a color-changing unit assay.

Although Vsa size variation is undoubtedly required for the organism's success, size variation was apparently not necessary for avoidance of the immune system under the experimental conditions of this study. It was possible that analysis by immunoblotting would have revealed that, over time, isolates gain an even larger 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 (26), and no selection 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 is hypothesized that size variation would have occurred over time to generate mycoplasmas with a large number of tandem repeats to acquire resistance to complement killing or the growth-inhibitory effects of specific antibody (6).

Isolates possessing subpopulations producing few tandem repeats were not found in immunodeficient mice at a higher frequency than in wild-type mice. This would suggest that subpopulations having few tandem repeats serve an in vivo function aside from avoidance of the immune system. 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 (27). Thus, the in vivo function of a subpopulation of cells producing a short Vsa protein may be adherence to the epithelium.

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