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Variable Surface Protein Vmm of *Mycoplasma mycoides* subsp. *mycoides* Small Colony Type

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A variable surface protein, Vmm, of the bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* small colony type (*M. mycoides* SC) has been identified and characterized. Vmm was specific for the SC biotype and was expressed by 68 of 69 analyzed *M. mycoides* SC strains. The protein was found to undergo reversible phase variation at a frequency of 9×10^{-4} to 5×10^{-5} per cell per generation. The *vmm* gene was present in all of the 69 tested *M. mycoides* SC strains and encodes a lipoprotein precursor of 59 amino acids (aa), where the mature protein was predicted to be 36 aa and was anchored to the membrane by only the lipid moiety, as no transmembrane region could be identified. DNA sequencing of the *vmm* gene region from ON and OFF clones showed that the expression of Vmm was regulated at the transcriptional level by dinucleotide insertions or deletions in a repetitive region of the promoter spacer. *Vmm*-like genes were also found in four closely related mycoplasmas, *Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp . *capripneumoniae*, *Mycoplasma* sp. bovine serogroup 7, and *Mycoplasma putrefaciens*. However, Vmm could not be detected in whole-cell lysates of these species, suggesting that the proteins encoded by the *vmm*-like genes lack the binding epitope for the monoclonal antibody used in this study or, alternatively, that the Vmm-like proteins were not expressed.

Mycoplasma mycoides subsp. *mycoides* small colony type (*M. mycoides* SC) causes a severe respiratory disease in cattle, contagious bovine pleuropneumonia (CBPP). It is the only bacterial disease included in the A list of communicable animal diseases of the Office International des Epizooties (OIE) and is the most important animal disease in Africa, affecting at least 27 countries (3, 5, 82). CBPP disappeared from Europe at the end of the 19th century but reappeared sporadically and affected several countries in an epizootic in southern Europe between 1983 and 1999.

The CBPP agent was first isolated and described in 1898 (72), and the organism was classified into the genus *Mycoplasma* (32) nearly 70 years later. Mycoplasmas belong to the class *Mollicutes*, whose members lack a cell wall and are known as the smallest self-replicating organisms (62, 79). Phylogenetic classification has grouped *M. mycoides* SC with five closely related and highly pathogenic mycoplasmas into the *M. mycoides* cluster (26, 74, 94). The *M. mycoides* cluster comprises *Mycoplasma capricolum* subsp. *capricolum* (*M. capricolum*), *Mycoplasma capricolum* subsp. *capripneumoniae* (*M. capripneumoniae*), *M. mycoides* subsp. *capri* (*M. capri*), *M. mycoides* subsp. *mycoides* large colony type (*M. mycoides* LC), *M. mycoides* SC, and *Mycoplasma* sp. bovine serogroup 7. The species *Mycoplasma cottewii*, *Mycoplasma yeatsii*, and *Mycoplasma putrefaciens* are sometimes included in the phylogenetic *M. mycoides* cluster, which is based on the 16S ribosomal DNA sequences (43). In this article we will only refer to the classical *M. mycoides* cluster, which excludes the three latter species.

The members of the *M. mycoides* cluster share many antigenic properties, and serological cross-reactions are often observed. Yet these infectious agents are strictly host specific, and the typical lung lesions of CBPP are solely formed after infection by *M. mycoides* SC. Consequently, any antigenic epitope that is unique for *M. mycoides* SC may be of importance for the pathogenicity of this organism and is therefore an interesting subject for research. Hitherto, very little is known about the pathogenicity of *M. mycoides* SC. The polysaccharide capsule and oxidative damage from hydrogen peroxide production may play important roles in CBPP infection (18, 49, 58, 64, 69, 75). Recently, it was suggested that the lipoprotein encoded by the *lppB* gene affects the virulence of *M. mycoides* SC (92).

Although the mortality rate is high among animals affected by CBPP, many become chronic carriers of the disease, and it is clear that *M. mycoides* SC, like many other pathogenic mycoplasmas, must have sophisticated mechanisms for virulence and persistence in the host.

Variable surface antigens are widely described for mycoplasmas (23, 79) and for other bacteria (44). It is generally believed that variable surface proteins are a means to enhance colonization and to adapt to the host tissue environment at various stages of infection. They have been shown to play a role in adhesion, immunomodulation, and substrate binding (e.g., see references 57, 66, 89, 93, and 97). Variable surface proteins can undergo phase variation, i.e., reversible ON/OFF switch of the expression, or antigenic variation, meaning that alternative phenotypes of the protein are expressed in the offspring of a bacterial cell, thus creating a heterogeneous population. Anti-

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genic variation of proteins often involves DNA rearrangements of repetitive regions, thereby creating new combinations of structural domains and changes in size (12, 13, 24, 60, 61, 93, 95, 98, 99). Epitope masking (83, 88, 96) is another process that contributes to phase variation of proteins on the bacterial surface.

A number of different strategies to regulate phase variation have been reported for mycoplasmas. Some mechanisms are quite complex and involve site-specific DNA inversion, which causes an alternate expression of one gene in a gene family, as described for the *vsa* genes of *M. pulmonis* (11, 86). Also, the *vsp* genes of *Mycoplasma bovis* seem to be regulated by site-specific DNA inversions (59, 61), and the *avg/vpma* genes for *Mycoplasma agalactiae* involve gene rearrangements, although the exact mechanism is still unknown (37, 39). Expression of the *vlhA* genes of *Mycoplasma synoviae* is regulated by gene conversion (73). Frameshift mutations in poly(A) or repetitive motifs causing gene truncation were shown to control the ON/OFF switch in the *pvpA* gene of *Mycoplasma gallisepticum* (13), the *p78* gene of *Mycoplasma fermentans* (89), and the *vaa* gene of *Mycoplasma hominis* (97). The number of repetitive units in putative transcriptional activators caused the phase variation of the *pMGA* genes of *M. gallisepticum* (38) and the *vlhA* genes of *Mycoplasma imitans* (63). Regulation strategies where the length of the promoter spacer controls the expression have been described for the *maa2* gene of *Mycoplasma arthritidis* (93) and for the *vlp* genes of *Mycoplasma hyorhinis* (24, 95).

A phase-variable protein that contributes to a dynamic surface architecture of *M. mycoides* SC was characterized in this study. We suggest the name Vmm, for variable protein of *M. mycoides*, and describe the promoter mutation that causes the phase variation. Vmm was studied because it contains an epitope that is specific for *M. mycoides* SC and it gave the first evidence of intraclonal variability in *M. mycoides* SC.

TABLE 1. Type and reference strains of mycoplasmas isolated in ruminants that were used to assess the specificity of MAb 5G1

Organism	Strain[c]	Reference	NCTC no.[a]	ATCC no.[b]
Acholeplasma laidlawii	PG8^T	31	10116	23206
Acholeplasma axanthum	S743^T	91	10138	25176
Acholeplasma modicum	PG49^T	56	10134	29102
M. agalactiae	PG2^T	32	10123	
Mycoplasma alkalescens	PG51^T	56	10135	29103
Mycoplasma anatis	1340^T	81	10156	25524
Mycoplasma arginini	G230^T	8	10129	23838
M. bovigenitalium	PG11^T	32	10122	19852
M. bovirhinis	PG43^T	55	10118	27748
M. bovis	PG45 Donetta^T	41	10131	25523
Mycoplasma californicum	ST-6^T	52	10189	33461
Mycoplasma canadense	275C^T	54	10152	29418
M. capricolum subsp. *capricolum*	California Kid^T	90	10154	27343
M. capricolum subsp. *capripneumoniae*	F38^T	34	10192	
Mycoplasma conjunctivae	HRC 581^T	7	10147	25834
Mycoplasma gallinarum	PG16^T	32	10120	19708
M. gallisepticum	PG31^T	33	10115	19610
M. mycoides subsp. *capri*	PG3^T	30	10137	
M. mycoides subsp. *mycoides* LC	Y goat^R	27	11706	
M. mycoides subsp. *mycoides* SC	PG1^T	32	10114	
Mycoplasma ovipneumoniae	Y98^T	19	10151	29419
M. putrefaciens	KS1^T	90	10155	15718
Mycoplasma sp. bovine serogroup 11	2D^R	55		
Mycoplasma sp. bovine serogroup 7	PG50^R	55		
Mycoplasma verecundum	107^T	40	10145	27862
Ureaplasma diversum serogroup A	A417^T	48	10182	43321
U. diversum serogroup B	D48			
U. diversum serogroup C	T74	47		49783

[a] NCTC, National Collection of Type Cultures and Pathogenic Fungi (London, United Kingdom).
[b] ATCC, American Type Culture Collection.
[c] T, type strain; R, reference strain.

MATERIALS AND METHODS

Mycoplasma strains and isolates. (i) Cultivation. All mycoplasma isolates and reference strains were propagated in liquid medium as described earlier (77), unless otherwise stated. *M. mycoides* SC strain M223/90 was cultured in F medium (14).

(ii) Strains used to assess specificity of MAb 5G1. The specificity of the monoclonal antibody (MAb) 5G1 was determined with 28 type and reference strains of mycoplasmas, ureaplasmas, and acholeplasmas that have been isolated in ruminants, as specified in Table 1. In all, 145 other isolates of the *M. mycoides* cluster were also used to test MAb 5G1, comprising 69 isolates of *M. mycoides* SC (of which 11 were from France, 5 from Portugal, 37 from Italy, 6 from Spain, and 10 from Africa), 32 isolates of *M. mycoides* LC, 4 isolates of *Mycoplasma* sp. bovine serogroup 7, 5 isolates of *M. capri*, and 35 isolates of *M. capricolum*. Also, two field isolates of *M. yeatsii* and three field isolates of *M. bovis* were used in the tests.

(iii) Strains used to prepare subclones of phenotypic variants. *M. mycoides* SC strain Afadé is a bovine isolate from 1968 that originated in Chad. The strain used in this study is from the CIRAD-EMVT culture collection (CIRAD-EMVT Animal Health Program, TA 30/G Campus International de Baillarguet, Montpellier, France). Strain Afadé was proved to be highly pathogenic by experimental infections (1, 9) and is used to prepare antigen for a complement fixation test, which is the reference test for CBPP that is presently recommended by OIE (4).

M. mycoides SC strain B17 was formerly used to prepare the antigen for the recommended complement fixation test. It was isolated from a zebu in Chad in 1967, and the pathogenicity level is still unknown. Also strain B17 was provided by CIRAD-EMVT.

M. mycoides SC strain T1/44 is widely used as live vaccine in Africa (4). This strain has low pathogenicity and is resistant to streptomycin. T1/44 is a derivative of T1, which was isolated in Tanzania in 1952. The strain used in this study was received from the CIRAD-EMVT collection, batch EMVT 002 PANVAC-MVT, June 1996.

(iv) Preparation of direct lineages of phenotypic variants. Direct lineages of subcloned variants were prepared as follows: fresh broth cultures of organisms from the strain stocks were passed through a 0.45-μm-pore-size filter and were thereafter serially diluted and plated on agar. After 4 days of incubation, single colonies that were positive for MAb 5G1 were located by aligning them with corresponding colored dots on a nitrocellulose membrane screened by colony immunostaining (see below). The colonies were picked with Pasteur pipettes and propagated at 37°C in 1 ml of broth medium for 4 days. After three successive steps of subcloning, resulting broth cultures were stored at −80°C for further characterization by Western blot analysis, except 100 μl that was replated as above. After 4 days of incubation, the few reverting negative colonies were located by aligning them with corresponding Ponceau-stained dots on colony blots and were subsequently purified by several rounds of subcloning as described above. Negative colonies of the third generation of 5G1 negative subclones, were propagated in 1 ml of broth and were stored at −80°C, except 100 μl that was replated. Reverting 5G1 positive subclones were prepared as above. Oscillating phenotypic switches were quantitatively monitored and were expressed as a fraction of switched phenotype per cell per generation.

(v) Strain used for construction of the phagemid library. A low-passage isolate of *M. mycoides* SC referred to as strain M223/90 was used to make a phagemid library. The aim was to obtain a library that could reveal pathogenic features of *M. mycoides* SC. Strain M223/90 is a bovine isolate from pleural fluid that originated in Tanzania in 1990 (15).

Antibodies and immunobinding assays. (i) MAbs. MAb 5G1 was prepared from *M. mycoides* SC type strain PG1 as described in reference 17, in collaboration between AFSSA-Site de Lyon and the Istituto Zooprofilattico della Lombardia e dell'Emilia. Mouse ascites fluid containing MAb 5G1 was sterile filtered

through a filter with a pore size of 0.1 μm, and it was subsequently used for the experiments in this study. The specificity of MAb 5G1 was first determined by dot immunobinding assay (77) with the type and reference strains in Table 1 (strain F38 excepted). It was further tested on the 145 isolates of the *M. mycoides* cluster mentioned above, on the two isolates of *M. yeatsii*, and on the three *M. bovis* isolates. The specificity of MAb 5G1 was also investigated by colony immunostaining and Western blotting on reference strains of the *M. mycoides* cluster. The affinity of MAb 5G1 for strain F38 was determined by Western blotting only. *M. mycoides* SC strains that were negative for MAb 5G1 by dot immunobinding were also reanalyzed by colony immunostaining.

(ii) Colony immunostaining. Freshly grown mycoplasma colonies were transferred to nitrocellulose membranes by placing the membranes on the surface of agar plates. The membranes were gently removed and blocked in Tris-buffered saline B (TBS-B) (TBS contains 50 mM and 0.2 M NaCl; TBS-B is TBS supplemented with 10% horse serum), before they were incubated with MAb 5G1 in TBS-B at a concentration of 9 μg/ml for 1.5 h at ambient temperature. Unbound antibody was removed by three washings in TBS-T (TBS and 0.05% Tween 20) and one washing in TBS. Thereafter, the membranes were incubated with peroxidase-conjugated rabbit anti-mouse antibodies (DAKO A/S, Glostrup, Denmark) in TBS-B (2.6 μg/ml) for 1.5 h, followed by three washes in TBS-T and one wash in TBS. Colonies expressing Vmm were specifically identified by an enzymatic color reaction with 4-chloro-1-naphthol that gives dark blue color (10, 83). The membranes were finally stained with Ponceau S solution (Sigma Diagnostics Inc., St. Louis, Mo.), which unspecifically stains proteins red, to reveal the colonies that were negative for MAb 5G1.

(iii) Western blotting, Triton X-114 fractionation, and supernatants. Western blotting was carried out as previously described (57) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5 to 15% gradient or in homogenous 18% polyacrylamide gels. Triton X-114 phase fractionation was performed as described earlier (78). Supernatants of mycoplasma cultures were obtained by two successive centrifugation steps at 31,000 × *g*, followed by one filtration through a 0.20-μm-pore-size filter and two filtrations through a 0.10-μm-pore-size filter and finally a fivefold concentration by dialysis against polyethylene glycol (PEG) 6000 in phosphate-buffered saline (PBS) solution.

Electron microscopy. Colonies of *M. mycoides* SC strain Afadé were grown on agar plates, resuspended in PBS, and transferred to a microcentrifuge tube. The cells were concentrated by centrifugation and resuspended in a small volume of PBS. One drop of cell suspension was placed on a 400-mesh nickel grid for 1 min before being washed three times in PBS and blocked with 0.5% bovine serum albumin (BSA) in PBS (PBS-BSA) for 5 min. The grid was transferred to a 50-μl drop of MAb 5G1 in PBS (28 μg/ml) and was left for 1 h at room temperature. After three washes in PBS-BSA, the grid was incubated with the secondary antibody (15-nm colloidal gold-labeled goat-anti-mouse; optical density, 3.5; Amersham Pharmacia Biotech, Uppsala, Sweden) diluted 1:20 in PBS-BSA for 1 h at room temperature. Another three washes in PBS were performed, and the cells were fixed with 2% glutaraldehyde in PBS during 10 min, washed three times in PBS, and once quickly in sterile water, before negative staining with 1% ammonium molybdate. Finally the grids were dried and examined by electron microscopy.

[U-^{14}C]palmitic acid labeling of lipoproteins. Metabolic labeling of lipoproteins in *M. mycoides* SC strain PG1, which expresses Vmm, and in strain M223/90, which is Vmm deficient (see "Occurrence of Vmm in field isolates of *M. mycoides* SC and other mycoplasmas" in Results), was performed by growth in the prescence of [U-^{14}C]palmitic acid, as described by Cheng et al. (20). Whole-cell lysates containing the labeled proteins were analyzed by Western blotting, and the incorporation of [U-^{14}C]palmitic acid was detected with MAb 5G1 and exposure of Kodak Biomax MR-1 film (Amersham Pharmacia Biotech).

Phage display. (i) Phagemid vectors, suppressor strain, and helper phage. Two gene VIII-based phagemid vectors, pG8PL0 and pG8SPA0 (51), were used to produce a phagemid library. Vector pG8PL0 has the *lacZ* promoter (β-galactosidase of *Escherichia coli)* and the *pelB* signal sequence (pectate lyase of *Erwinia chrysanthemi*), while pG8SPA0 has a promoter and a signal sequence that originate from the *spa* gene region (protein A of *Staphylococcus aureus*). Both vectors contain a tag in fusion to gene VIII, which binds human serum albumin (HSA). The tag is out of frame with gene VIII, which means that some of the randomly fragmented mycoplasma inserts need to restore the open reading frame (ORF) to give an expression of phage protein VIII.

Phages and phagemids were propagated in an *E. coli* suppressor strain for the TGA codon, strain CDJ64/Δ14, with the genetic markers Δ(*lacpro*) *nalA rif valR thi trpT*(Su9)/F′*lacpro* (35, 84). The *E. coli* strain was grown in Luria broth (LB) or on Luria agar (LA) (Sigma), prepared according to the manufacturer's description. Selective media or agar plates contained ampicillin at a final concentration of 60 mg/liter unless otherwise stated. Soft agar consisted of LB medium and 0.5% agarose. Helper phage R408 (Promega Corp., Madison, Wis.) was used for the production of phage stocks.

(ii) Construction of the phagemid library. A whole-genome phage display library of *M. mycoides* SC strain M223/90 was constructed in a mixture of phagemid vectors pG8PL0 and pG8SPA0 (51). The phagemid vectors were digested with *Sna*BI and dephosphorylated with calf intestine alkaline phosphatase. Equal amounts of the two vectors were pooled. Genomic DNA from *M. mycoides* SC strain M223/90 was prepared and purified by proteinase K lysis and phenol and chloroform extractions. The DNA was randomly fragmented by sonication until the majority of the fragments had sizes ranging from 1,000 to 2,000 bp and were thereafter treated with T4 DNA polymerase to give blunt ends and were subsequently phosphorylated with T4 polynucleotide kinase. Approximately 25 μg of the blunt-ended and phosphorylated fragments was ligated with Ready-to-Go ligase to 2.5 μg of the vector mixture. The ligated DNA was purified by phenol and chloroform extractions and precipitated with sodium acetate in ethanol, and the phagemid vectors carrying the mycoplasma inserts were dissolved in 16 μl of sterile H_2O. *E. coli* strain CDJ64/Δ14 was transformed with the phagemid constructs by electroporation. The transformants were immediately transferred to 150 ml of LB medium supplemented with 2% glucose, and the culture was incubated at 37°C. After 1 h of incubation for phenotypic expression, 1 ml was removed to determine the titer by viable count. Ampicillin was added to the remainder to a final concentration of 50 mg/liter. The culture was grown overnight, and 2 ml of the culture was thereafter infected with helper phage R408 at a multiplicity of infection of 200. The infected *E. coli* was mixed with soft agar and poured on selective LA plates, which were incubated at 37°C overnight. Phagemid particles were eluted from the soft agar by standard procedures, and the phage stock was divided into aliquots and stored at −80°C until use.

(iii) Affinity pannings to identify the 5G1 epitope. Affinity selection of phage that displayed mycoplasma peptides recognized by MAb 5G1 was made by three subsequent pannings. Two Maxisorp microtiter wells (Nalge Nunc International, Roskilde, Denmark) were coated with 125 μg of protein G in 250 μl of coating buffer (0.05 M Na_2CO_3 [pH 9.7]) for 1 h at room temperature. The wells were rinsed three times with PBS containing 0.05% Tween 20 before addition of 250 μl of MAb 5G1 (4.5 mg/ml) diluted 1:75 in PBS. Serum from BALB/c mice was used instead of MAb 5G1 as a negative control. The MAbs and BALB/c serum were immobilized for at least 1 h, and the wells were thereafter rinsed six times with PBS-Tween and blocked with PBS-Tween for 10 min. Meanwhile, the HSA binding region of the phagemids was blocked by incubating the phage stock with HSA (100 μg/ml). After 1 h, 200 μl of the phage stock was transferred to each coated well, and the panning proceeded for 4 h at room temperature. To wash away unspecifically bound phage, wells were rinsed 30 times in PBS-Tween before elution of the captured phage with 200 μl of sodium citrate buffer (50 mM Na citrate, 140 mM NaCl, [pH 2]). The eluates were neutralized with 40 μl of 2 M Tris-HCl buffer (pH 8.7), serially diluted in LB medium, and immediately used to infect 150 μl of overnight culture of *E. coli* CDJ64/Δ14. The infected cultures were incubated for 30 min at room temperature and were thereafter spread on selective LA plates. After incubation of the plates overnight, the colonies were counted and 150 colonies were transferred to a new selective LA plate to perform colony blot screening. New phage stocks were produced by resuspending the rest of the colonies from the 5G1 panning in LB medium, infecting the bacteria with helper phage R408, and processing as described above. A conjugate of HSA and horseradish peroxidase (HRP) was used in the colony blot screening to detect the tag. Replica blots were screened with MAb 5G1 to identify the clones that expressed the 5G1 epitope.

Analysis of the *vmm* gene. (i) DNA sequencing. The sequences of the mycoplasma inserts from 27 phagemid clones that were positive for the tag and for MAb 5G1 by colony blot screening were determined. The phagemids were purified by the Wizard Plus SV Miniprep DNA purification system (Promega), and the inserts were first sequenced with the ALBP primer (all primers are listed in Table 2), which is complementary to the HSA binding region of the vector. Depending on in which vector the mycoplasma DNA was inserted, the phagemids were then sequenced with the Sasekv and Nypel primers that are complementary to the *spa* and *pelB* signal sequences, respectively. DNA sequencing in this study was performed with the ALFexpress DNA Sequencer and the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech), according to the manufacturer's description. DNA sequences were assembled with ASSEMGEL, software in the PC/Gene package (Intelligenetics Inc., Mountain View, Calif.).

(ii) Analysis of the *vmm* flanking regions in subcloned phenotypic variants. A high-quality template for sequencing the *vmm* gene and its flanking regions of cloned phase variants was produced by nested PCR with the primers 5′f-5G1 and 3′r-5G1 in the first reaction and primers 5′-USP-5G1 and 3′-RSP-5G1 in the

TABLE 2. Oligonucleotides for DNA sequencing, PCR, Southern blotting, and 5′ RACE

Primer	Orientation	Sequence	5′ label
ALBP	Reverse	5′-GCCATACTGCTTTAGTTCATTGAT-3′	Cy5[a]
Sasekv	Forward	5′-TATCTGGTGGCGTAACACCTGCT-3′	Cy5
Nypel	Forward	5′-CCTATTGCCTACGGCAGCCGCTGG-3′	Cy5
5′f-5G1	Forward	5′-AGCAGCTAGAATTTATGCACT-3′	
3′r-5G1	Reverse	5′-ACAAAGATGATATTTTAGATCAG-3′	
5′-USP-5G1	Forward	5′-CGTTGTAAAACGACGGCCAGTTAGTCAGTTGATTAAGTGTAG-3′	
3′-RSP-5G1	Reverse	5′-CACAGGAAACAGCTATGACCCCATATCTAGTACTCTTATTC-3′	
USP	Forward	5′-CGTTGTAAAACGACGGCCAG-3′	Cy5
RSP	Reverse	5′-CACAGGAAACAGCTATGACC-3′	Cy5
5G1-insert probe	Forward	5′-GCGTGTGGTGATAGATCAA-3′	DIG
vmmRT1	Reverse	5′-GAATATGACCACTGTCATCATAATCAGC-3′	
vmmRT2	Reverse	5′-TGAATCAGCTGGTTTATCAGAAGTTCC-3′	
postlinkRT	Forward	5′-AAGGACTGCTATCAACGCAGAGTACGCGGG-3′	
postlinkPCR	Forward	5′-AAGGACTGCTATCAACGCAGAGT-3′	

[a] Cy5-labeled primers were used for DNA sequencing with the ALFexpress DNA sequencer.

second reaction. Amplifications were performed in a reaction mixture consisting of 10 mM Tris-HCl buffer (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.8 mM deoxynucleoside triphosphate, 10 pmol of each primer, 1 U of AmpliTaq DNA polymerase (Roche Molecular Systems, Inc., Branchburg, N.J.), and 1 μl of highly diluted DNA. The *vmm* gene region was amplified for 30 cycles, with denaturation for 20 s at 95°C, annealing for 20 s at 58°C for primers 5′f-5G1 and 3′r-5G1 or at 65°C for primers 5′-USP-5G1 and 3′-RSP-5G1, and elongation for 1 min at 72°C. The amplicons were sequenced with the USP and RSP sequencing primers, as described above.

(iii) mRNA analysis. Total RNA was isolated with Trizol Reagent (Life Technologies, Gaithersburg, Md.) from 200 ml of culture of *M. mycoides* SC strain PG1. First-strand synthesis and 5′ rapid amplification of cDNA ends (RACE) of the transcript were performed as follows: 5 μg of total RNA was incubated for 10 min at 70°C with 2 pmol of primer vmmRT1 and 4 pmol of primer postlinkRT in a total volume of 11 μl. The first strand was synthesized at 42°C for 90 min after addition of 2 μl of deoxynucleoside triphosphate (10 mM), 2 μl of dithithreitol (100 mM), 1 μl of PowerScript Reverse Transcriptase (Clontech, Palo Alto, Calif.), and 4 μl of the 5× first-strand buffer provided with the enzyme. Subsequently, the transcript was amplified in a seminested PCR, using 5 μl of the cDNA and primers vmmRT1 and postlinkPCR in the first reaction. The PCR mixture contained 10 mM Tris-HCl buffer (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.8 mM deoxynucleoside triphosphate, 10 pmol of each primer, and 1 U of AmpliTaq DNA polymerase. The reactions were amplified with 5 cycles of 94°C for 10 s and 72°C for 3 min, followed by 5 cycles of 94°C for 10 s, 70°C for 15 s, and 72°C for 3 min and finally 25 cycles of 94°C for 10 s, 68°C for 15 s, and 72°C for 3 min. Primers vmmRT2 and postlinkPCR were used in the second PCR, where 0.1 μl of the first amplicon served as template. The reaction mixture was prepared as in the first reaction; however, the primer postlinkPCR was not added until 15 cycles of 94°C for 15 s and 72°C for 1 min 20 s were completed. Another 25 cycles at 94°C for 15 s, 70°C for 15 s, and 72°C for 1 min 20 s were then performed. The final amplicon was DNA sequenced with the vmmRT2 primer and the Thermo Sequenase Cy5 Dye Terminator Kit (Amersham Pharmacia Biotech) as described by the manufacturer. The sequences were analyzed with the ALFexpress DNA sequencer.

(iv) Southern blot hybridizations. The occurrence of the *vmm* gene in 18 *M. mycoides* SC strains that represent the different IS*1296* hybridization patterns (21), as well as in three other *M. mycoides* SC strains, was assessed by Southern blotting. Chromosomal DNA was digested to completion with the restriction enzyme *Hin*dIII, and the fragments were separated by electrophoresis on 0.8% agarose gels. Southern blotting was performed as described elsewhere (60, 76). Hybridizations of the digoxigenin (DIG)-labeled probe 5G1-insert probe (Table 2) were carried out at 44°C. Stringent washes were performed at the hybridization temperature in 0.2× SSC (1× SSC buffer is 0.15 M NaCl plus 0.015 M sodium citrate) supplemented with 0.1% (wt/vol) SDS, and the hybridized probe was detected with the DIG nucleic acid detection kit, as described by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). The occurrence of the *vmm* gene in the type strains of the closely related species of the *M. mycoides* cluster and the species *M. putrefaciens*, *M. cottewii* (28), *M. bovis*, *M. agalactiae*, *Mycoplasma bovirhinis*, *Mycoplasma bovigenitalium*, and *Mycoplasma primatum* (29) (a primate mycoplasma closely related to *M. agalactiae* and *M. bovis*) was analyzed with the 5G1-insert probe as described above and with the 5G1-PCR probe at a hybridization temperature of 68°C. The DIG-labeled 5G1-PCR probe was produced by nested PCR with the primers 5′f-5G1 and 3′r-5G1 in the first reaction and DNA of strain PG1 as the template, followed by amplification with primers 5G1-insert probe and 3′-RSP-5G1 as described above. The reaction mixtures were prepared with the PCR DIG Probe Synthesis Kit (Roche Diagnostics).

Protein analyses of Vmm. (i) Preparation and Western blotting of phage proteins. To verify that the affinity pannings had selected phage that displayed the true target protein for MAb 5G1 and not a false epitope, the different hybrid proteins of the phage were analyzed by Western blotting. A crude protein preparation of phage was made by precipitating the phages with PEG, as follows: *E. coli* clones with the recombinant phagemids were inoculated in 15 ml of LB medium supplemented with ampicillin, and the cultures were grown overnight. The cells were pelleted and dissolved in 5 ml of fresh LB medium containing ampicillin, and the culture was then infected with 100 μl of helper phage R408 (10^{11} PFU/ml). Production of hybrid phage particles was allowed for 4 h at 37°C before the samples were centrifuged at 12,000 × *g*, and the supernatant was filtered through a 0.45-μm-pore-size sterile filter. Phages were precipitated by adding 500 μl of 20% PEG 8000 in 2.5 M NaCl to 3.5 ml of the phage stock, incubating the mixture at room temperature for 45 min, and centrifuging the samples at 12,000 × *g*. The pellet was dissolved in 25 μl of PBS before addition of 25 μl of 2× SDS gel loading buffer (85). Western blotting was performed after separation by SDS-PAGE in a 12% gel.

(ii) Protein prediction. Prediction of the structural organization of Vmm was performed with the SignalP V2.0 World Wide Web Server (70, 71), ProfileScan of PROSITE Patterns at the ISREC-Server of the Swiss Institute for Experimental Cancer Research and the Swiss Institute for Bioinformatics (45), and TMpred at the European Molecular Biology Network (46). Similarity searches were done with BLASTN and BLASTP at the server of the National Center for Biotechnology Information (2).

Nucleotide sequence accession number. The nucleotide sequence for the *vmm* gene has been assigned the GenBank accession number AF428142.

RESULTS

Vmm is a phase-variable, surface-located lipoprotein. Colony immunostainings of *M. mycoides* SC with MAb 5G1 showed that this MAb targets a surface-exposed protein, Vmm, that undergoes high-frequency phenotypic variation (Fig. 1). Immunoblotting of whole-cell proteins from ON and OFF variants of direct clonal lineages (Fig. 2) resulted in a single band for stock culture and positive clones, whereas no protein band was detected in negative clones. This proved that the phenotypic variation observed in the colony immunostaining was a result of reversible phase variation of the Vmm expression and not a consequence of epitope masking or size variation. The switching rate of Vmm was similar in the three

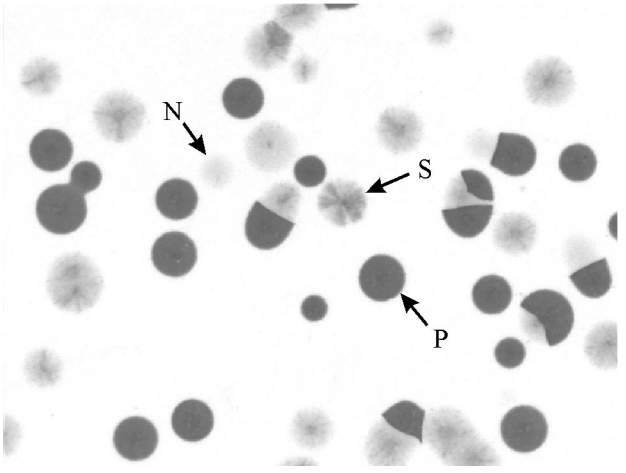

FIG. 1. Colony immunostaining with MAb 5G1 of *M. mycoides* SC strain B17, showing variable expression of the surface protein Vmm among and within colonies. The population consists of colonies that are positive for MAb 5G1 and express the Vmm protein (P), negative colonies (N), and sectored colonies (S) where mutations during growth have induced ON/OFF switching of the Vmm expression. The colonies are derived from a single colony that was cultured in broth, filtered, and plated on agar. The scanned colony blot was processed in CorelDRAW 9.0.

strains Afadé, B17, and T1/44, and it was calculated to be 7×10^{-4} to 9×10^{-4} per cell per generation for ON-to-OFF reversion and 5×10^{-5} to 9×10^{-5} per cell per generation for OFF-to-ON reversion.

Vmm migrated like a 16-kDa protein in SDS-PAGE, and the protein appeared to be identical for the analyzed strains Afadé, B17, and T1/44, as shown in Fig 2. Separation of Vmm on a high-resolution-gradient gel showed that the protein occurred in two sizes, approximately 15 and 17 kDa (Fig. 3). Vmm was present in the Triton X-114 phase and absent in the aqueous phase and the supernatant, suggesting that it is a membrane-associated protein. Metabolic incorporation of [U-^{14}C]palmitic acid in strains PG1 and M223/90 and analysis of whole-cell proteins by Western blotting proved that Vmm is a lipoprotein.

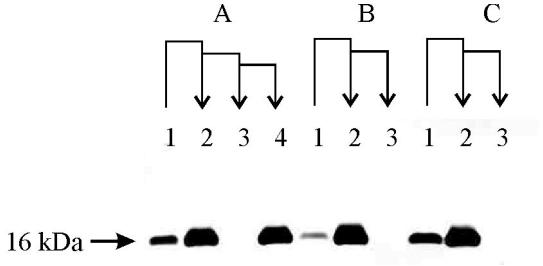

FIG. 2. Whole-cell proteins of serially subcloned phenotypic variants from three *M. mycoides* SC strains that were separated in an 18% polyacrylamide gel, immunoblotted, and detected with MAb 5G1. Sequential phenotypic transitions of the Vmm expression are indicated as arrows that represent direct lineages of the subcloned variants. Lanes 1 were stock populations of the strain; lanes 2 were ON-type subclones; lanes 3 were OFF-type revertant subclones; and lane 4 was an ON-type double revertant subclone. Panel A shows samples from strain Afadé, panel B shows strain B17, and panel C shows strain T1/44. The scanned blot was processed with Corel Photo-Paint 9.0 and CorelDRAW 9.0.

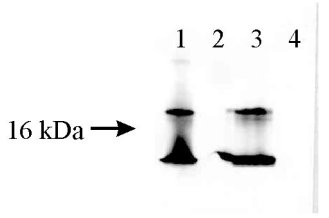

FIG. 3. Immunoblots detected with MAb 5G1, showing the occurrence of protein Vmm in whole-cell proteins (lane 1), culture supernatant (lane 2), Triton X-114 phase (lane 3), and aqueous phase (lane 4). The samples were prepared from *M. mycoides* SC strain T1/44. The scanned blot was processed with Corel Photo-Paint 9.0 and CorelDRAW 9.0.

Detection of the blot with MAb 5G1 gave a clear band of approximately 16 kDa for strain PG1 but no band for strain M223/90. Similarly, this band was observed among the lipoproteins of PG1 in the autoradiographic film but was absent from the lipoproteins of strain M223/90 (data not shown). The absence of Vmm in strain M223/90 is explained below.

Immunoelectron microscopy of strain Afadé showed that MAb 5G1 targets a protein at the surface of the mycoplasma membrane; hence, Vmm is a surface-located lipoprotein (Fig. 4). The phase variation of Vmm was clearly observed as some gold-labeled bacteria and some negative bacteria, all originating from one clone. It can also be concluded from the electron micrographs that the protein is expressed at low levels in strain Afadé, although it cannot be excluded that the capsular layer limits the accessibility of the membrane proteins, thereby hindering the antibodies from binding Vmm.

Occurrence of Vmm in field isolates of *M. mycoides* SC and other mycoplasmas. Analysis of 69 *M. mycoides* SC isolates by dot immunobinding assay showed that 31 of the isolates were clearly positive for MAb 5G1, including the type strain PG1. Thus, these strains were expressing Vmm. No significant reaction occurred with the other 38 *M. mycoides* SC strains; however, 37 of these revealed a positive subpopulation by colony immunostaining. A positive subpopulation was defined as expression of Vmm in at least 0.01% of the cells. Only strain M223/90 from Tanzania was negative by both methods, and it was therefore believed not to express Vmm at all. All of the 28

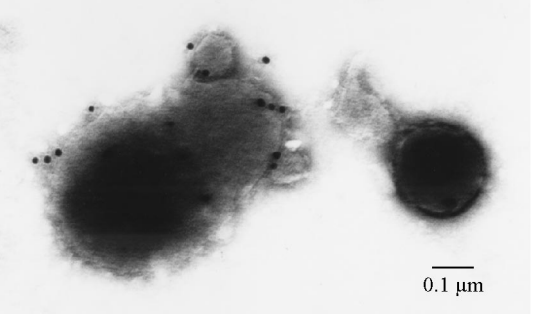

FIG. 4. Electron micrograph after immunostaining of *M. mycoides* SC strain Afadé with MAb 5G1. The antibody binds surface molecules of cells that express Vmm. The size of the gold particles is 15 nm. The scanned photo was processed with Corel Photo-Paint 9.0 and CorelDRAW 9.0.

reference strains of mycoplasma species that have been isolated in ruminants (Table 1) were negative for MAb 5G1 in dot immunobinding, except strain PG1. Also, the 76 field isolates belonging to the *M. mycoides* cluster (*M. mycoides* LC, *Mycoplasma* sp. bovine serogroup 7, *M. capri*, and *M. capricolum)*, as well as the two *M. yeatsii* and three *M. bovis* isolates, were negative for MAb 5G1 in dot immunobinding. No positive subpopulations could be detected among the reference strains of the *M. mycoides* cluster, as judged by colony immunostaining. The field isolates were not tested by colony immunostaining.

Properties of the phagemid library. To reveal the mechanisms for phase variation of Vmm, we first used phage display to identify the *vmm* gene. A phagemid library that consisted of 10^7 independent clones and in which the phage stock had a titer of 10^{11} PFU/ml was produced. Approximately 95% of the phagemids contained mycoplasma inserts, and the sizes of the inserts varied from 100 to 1,500 bp. To verify that phages in the library indeed display hybrid proteins of mycoplasma peptides and the HSA binding tag, affinity pannings to HSA were performed. Phages cannot display the HSA binding tag on the surface unless a mycoplasma insert has restored the ORF between gene VIII and the tag (51). More than 10^7 phages were immobilized in the first panning, which is considerably more than the control pannings against newborn calf serum that captured approximately 10^3 phages. Mycoplasma inserts of clones that were positive for HSA by colony blot screening were subsequently sequenced, and it was found that more than one-third of the inserts contained one or more TGA codons. It was therefore concluded that the suppressing ability of *E. coli* CDJ64/Δ14 was efficient enough to allow expression of peptides containing several TGA codons. The experiments showed importantly that hybrid peptides were present on the phage surface and that the detection of tag expression could be used as a ligand independent screening system. There were as many phagemids of type pG8PL0 as of pG8SPA0 in the library; however, after one panning to HSA, most of the HSA-positive clones contained the *spa* promoter and signal sequence, indicating that the pG8SPA0 vector is more efficient than the vector containing the *lacZ* promoter and *pelB* leader.

Identification of the *vmm* gene. Affinity pannings of the phage display library to MAb 5G1 resulted in an accumulation of phage expressing the target epitope for MAb 5G1. The total amounts of recovered phage from the first, second, and third pannings were 5.4×10^4, 3.0×10^4, and 4.0×10^6 CFU, respectively, as determined by viable count of *E. coli* infected with the eluted phage. The corresponding figures for the control pannings to BALB/c serum were 1.3×10^4, 6.4×10^3, and 1.2×10^3 CFU. Colony blot screenings for expression of the tag in 150 colonies resulted in 2, 99, and 140 positive colonies in each affinity panning, whereas the original phage stock had two colonies that were positive for HSA out of 150. The majority of the colonies that were positive for HSA were also positive for MAb 5G1. Twenty-seven of these clones were selected at random, the phagemids were isolated, and the mycoplasma inserts were sequenced. When the sequences were aligned, it was obvious that 20 of the inserts originated from the same gene region, the *vmm* gene region. Although some of the clones were multiples of the same recombinant, there were five different size variants of the gene segment. The remaining seven sequences were from other parts of the *M. mycoides* SC genome. It was possible to determine the full sequence of the *vmm* gene and to design primers in the flanking regions after searching the genome database of *M. mycoides* SC strain PG1 with the consensus of the alignment (J. Westberg, A. Persson, A. Holmberg, E. Björkvall, K.-E. Johansson, M. Uhlén, and B. Pettersson, Program Abstr. 12th Int. Organ. Mycoplasmology Conf., p. 151-152, 1998). By coincidence, one of the recombinant clones contained the full *vmm* gene, except for the stop codon TAA.

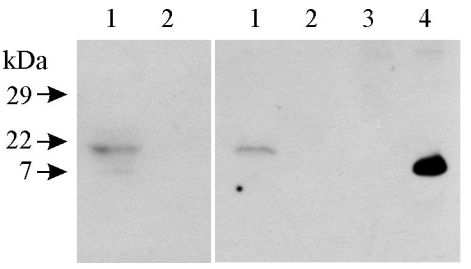

FIG. 5. Western blot of total phagemid proteins. The left panel was detected with an HSA-HRP conjugate, i.e., the tag, and the right panel was detected with MAb 5G1. Lanes 1, phagemid containing the entire *vmm* gene fused to gene VIII; 2, helper phage R408; 3, whole-cell lysate of *M. mycoides* SC strain M223/90; and 4, whole-cell lysate of *M. mycoides* SC strain PG1. The scanned blots were processed with Corel Photo-Paint 9.0 and CorelDRAW 9.0.

When the panning experiments were repeated, the first and second pannings resulted in 3.0×10^2 and 3.0×10^3 CFU with MAb 5G1 and 1.4×10^3 and 4.0×10^2 CFU with BALB/c serum. A third panning was not performed. The number of HSA-positive colonies increased from 31 in the first panning to 82 in the second panning. Inserts from five positive clones were DNA sequenced; four of these contained *vmm* gene fragments in three different size variants.

Hybrid proteins from five different phagemid recombinants containing *vmm* fragments were expressed in *E. coli.* The clones were infected with helper phage, and phage stocks were produced. Phages were precipitated, and the proteins were analyzed by SDS-PAGE and Western blotting with MAb 5G1 and HSA-HRP (Fig. 5). Four of the five clones clearly expressed the 5G1 epitope and the tag in a band whose size corresponded to the size of the hybrid protein as calculated from the sequence data (data not shown). Although all samples were prepared from similar amounts of phage, the fifth clone produced considerably smaller amounts of the hybrid protein, and the expression was too low to be detected on the Western blot, as neither detection for the tag with HSA-HRP nor the specific detection with MAb 5G1 resulted in a clearly visible band. Because all clones had inserts from the same gene, the result was still a confirmation that the phage display technique really selected phages that displayed the 5G1 epitope of Vmm and not an unspecific selection or artifact in the colony blot screening.

Structure of the *vmm* gene. The structural features of the *vmm* gene region, as deduced from the DNA sequences of the type strain PG1 and the phagemid clones of strain M223/90, are outlined in Fig. 6. The *vmm* gene has an ORF of 177 bp, encoding 59 amino acids (aa). A typical promoter with the −35 motif TTGACA and the −10 region TATAAT is located 71 bp

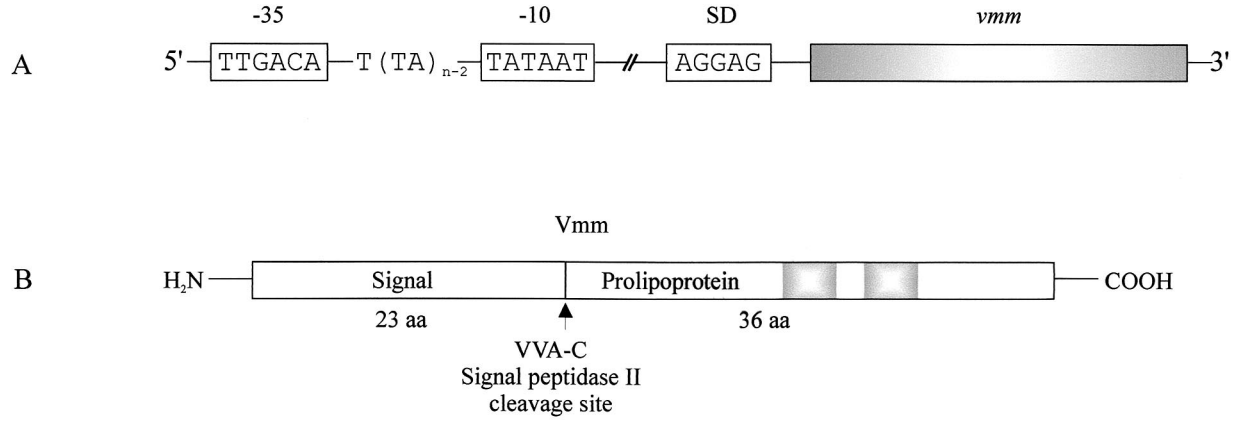

FIG. 6. Schematic illustrations of the *vmm* gene with the promoter region and the Vmm precursor. (A) The *vmm* gene is preceded by a Shine-Dalgarno (SD) sequence and a promoter that consists of −10 and −35 regions that are identical to the consensus δ^{70} promoter of *E. coli*. A dinucleotide $(TA)_n$ repeat of variable length makes up the promoter spacer and part of the Pribnow box, and it was shown that active promoters have a total number (*n*) of 10 repetitions. Note that the illustration is not drawn to scale. (B) The *vmm* gene encodes a protein precursor of 59 aa, which has a prolipoprotein signal peptidase site, VVA-C, at positions 21 to 24, indicating that lipid modification will take place at the cysteine moiety and that the signal peptide of 23 aa will be spliced off. The shaded boxes represent two repetitive motifs of KPAD.

upstream of the start codon ATG, but there is no obvious hairpin loop structure for transcription termination 3′ of *vmm*. Analysis of the *vmm* transcript in strain PG1 by 5′ RACE and DNA sequencing showed that the transcription is initiated at the 8th nucleotide downstream of the −10 region, starting with the sequence ATCT. A Shine-Dalgarno sequence, AGGAG (68), is located 13 bp 5′ of the start codon. The *vmm* gene did not contain any TGA-Trp codons.

Southern blot hybridizations of *Hin*dIII-digested genomic DNA with the 5G1-insert probe showed that the *vmm* gene, or at least the probing region, was present in 18 tested *M. mycoides* SC strains that represent different geographical origins and IS*1296* patterns. Ten of these are shown in Fig. 7. The *M. mycoides* SC strains had identical hybridization patterns with the 5G1-insert probe, characterized by a single 6,550-bp band, indicating that there is only one copy of the *vmm* gene and that the chromosomal location is similar. The existence of only one *vmm* gene was also confirmed by Southern blotting of chromosomal DNA digested with *Alu*I from eight strains (not shown), and we know from the genome sequence database that strain PG1 has one gene. Also, the strain that did not express the Vmm possessed the gene that encodes this phase-variable surface protein. This is noteworthy because the phagemid library was coincidentally made of this strain and because the phage display assay could not have been used to select for the 5G1 epitope unless strain M223/90 possessed the *vmm* gene.

Characterization of the *vmm* gene product. Protein prediction analyses of the amino acid sequence recognized Vmm as being a lipoprotein where the only putative transmembrane helix is located in the signal sequence. The likeliest cleavage site for the prolipoprotein-specific signal peptidase is between aa 23 and 24, at the VVA-C motif (Fig. 6) (16, 25), leaving a mature lipoprotein of 36 aa. The Western blot of Vmm after separation on a high-resolution SDS-PAGE showed two sizes for the protein (Fig. 3), the bigger being approximately 17 kDa and a smaller peptide of almost 15 kDa. Theoretically, the signal peptide is 2.5 kDa, suggesting that the signal peptide is being spliced off. The mature protein is hydrophilic, as judged from the amino acid sequence. Characteristic repetitive regions like those for, e.g., the variable surface proteins (Vsps) of *M. bovis* (61) and variant lipoproteins (Vlps) of *M. hyorhinis* (24, 95) were not found; only a short motif of 4 aa, KPAD, was repeated twice. Similarity searches by BLASTp showed 54% identity and 79% positive matches between the Vmm signal peptide and that of spiralin of *Spiroplasma citri* (22). The rest of the Vmm was not similar to any protein in the databases.

The molecular basis for phase variation of Vmm. To find the mutation that causes phase variation of Vmm, the DNA sequence of the full *vmm* gene with flanking regions was deter-

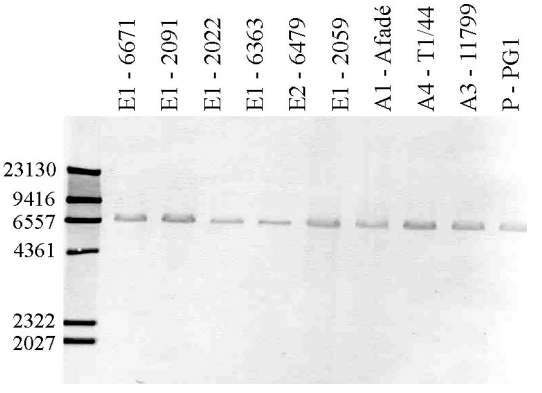

FIG. 7. Southern blot showing the presence of the *vmm* gene in some *M. mycoides* SC isolates from different geographical origins. The strains also represent different IS*1296* hybridization patterns as determined according to reference 21. Insertion sequence patterns are indicated before the strain designation. The Southern blots were prepared from *Hin*dIII-digested chromosomal DNA, and hybridization was performed with the 5G1-insert probe. Strain 6671 originated in Italy in 1993, strain 2091 in France in 1984, strain 2022 in France in 1984, strain 6363 in Spain in 1991, strain 6479 in Italy in 1992, strain 2059 in Spain in 1984, strain Afadé in Chad in 1968, strain T1/44 in Tanzania in 1952, and strain 11799 in Senegal in 1988. Type strain PG1 has an unknown origin. The scanned blot was processed with Powerpoint 7.0 and CorelDRAW 9.0.

TABLE 3. Number of dinucleotide repeats in the *vmm* promoter for individual clones of *M. mycoides* SC strains B17, Afadé, T1/44, and M223/90

Name[c]	Expression of Vmm	No. of $(TA)_n$ repetitions in the *vmm* promoter			
		Clone 1	Clone 2	Clone 3	Clone 4
B17	Positive	10	10	10	10
B17 revertant	Negative	12	10	12	12
Afadé	Positive	10	10	10	10
Afadé revertant	Negative	12	12	13	12
Afadé double revertant	Positive	10	10	10	10
T1/44	Negative	12	12	12	12
T1/44 revertant	Positive	10	10	10	10
M223/90	Negative[a]	6	7	ND[b]	ND

[a] Strain M223/90 does not express Vmm.
[b] ND = not determined.
[c] Revertants and double revertants were direct lineages of the original colonies.

mined for 28 subcloned phase variants as summarized in Table 3. The table also contains data from strain M223/90 that were found when phagemid clones were sequenced. Strain M223/90, as mentioned earlier, does not express Vmm at all. All sequences were identical in the coding region, including the sequence of M223/90. The sequences were also identical in the flanking regions except for the size of the promoter spacer that separates the −35 region from the −10 region. The spacer consists of repetitive units known as $(TA)_n$, where *n* is the total number of repetitions and the last two repeats were part of the −10 region (Fig. 6). Clones that express Vmm and thus have an active promoter were found to have a spacer of 17 bp in the promoter, i.e., there were 10 dinucleotide repeats. Any other number of repeats was found to disrupt the functionality of the promoter. There was, however, an exception in one revertant clone of strain B17 that was found negative by colony blotting but contained 10 TA repeats in the promoter.

***vmm*-like genes in other species of the *M. mycoides* cluster.** Similarity searches for the *vmm* with BLASTn detected a very similar gene in contig MC438 of *M. capricolum.* Unfortunately, this sequence was not complete, so it was not possible to fully compare the two genes. It was confirmed by Southern blot analysis with the DNA probes 5G1-insert probe and 5G1-PCR probe that, among the reference strains of the *M. mycoides* cluster, *M. mycoides* SC, *M. capricolum*, and *M. capripneumoniae* have one *vmm* or *vmm*-like gene, while *Mycoplasma* sp. bovine serogroup 7 may have two copies of *vmm*-like genes (Fig. 8). *M. putrefaciens* had only one barely visible band after hybridization with the oligonucleotide 5G1-insert probe, suggesting that there is one *vmm*-like gene that poorly hybridizes with this probe, although, when hybridized with 5G1-PCR probe, there were two clear bands (not shown). Reference strains from *M. mycoides* LC, *M. capri*, *M. yeatsii*, and *M. cottewii* seem to lack *vmm*-like genes, as none of the probes hybridized to DNA from these species. *M. yeatsii* was, however, analyzed only with the 5G1-insert probe. Although our data show that there are *vmm*-like genes in other mycoplasma species, they need to be further investigated and the sequences should be determined and compared to that of *M. mycoides* SC. Interestingly, the observations agree well with the phylogenetic relationship between these species, where *M. mycoides* SC forms an intermediate branch between the *M. capricolum*

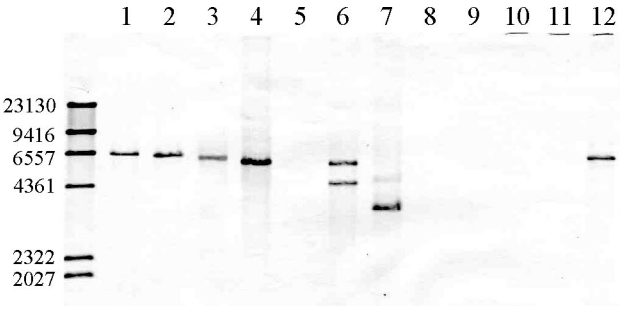

FIG. 8. Southern blot showing the occurrence of *vmm* or *vmm*-like genes in nine representative strains of the phylogenetic *M. mycoides* cluster. Genomic DNA was digested with *Hin*dIII before separation in agarose gel, and the Southern blot was hybridized with 5G1-insert probe. The samples were loaded in the following order: *M. mycoides* SC strain Afadé (lane 1), *M. mycoides* SC strain PG1 (lane 2), *M. capricolum* strain California Kid (lane 3), *M. capricolum* strain PP goat 189 (lane 4), *M. putrefaciens* strain KS1 (lane 5), *Mycoplasma* sp. bovine serogroup 7 strain PG50 (lane 6), *M. capripneumoniae* strain F38 (lane 7), *M. mycoides* LC strain Y goat (lane 8), *M. capri* strain PG3 (lane 9), *M. yeatsii* strain GHI (lane 10), *M. cottewii* strain VIS (lane 11), and *M. mycoides* SC strain 4813 (lane 12). The scanned blot was processed with Corel Photo-Paint 9.0 and CorelDRAW 9.0.

species group and the *M. capri* species group (74). The *M. capricolum* species group comprises *M. capricolum*, *M. capripneumoniae*, and *Mycoplasma* sp. bovine serogroup 7, while *M. mycoides* LC and *M. capri* are found in the *M. capri* species group. *M. putrefaciens*, *M. yeatsii*, and *M. cottewii* are more distantly related (43). Western blots to whole-cell lysates of the reference strains of all the *M. mycoides* cluster members, including *M. putrefaciens*, *M. cottewii*, and *M. yeatsii*, were negative for MAb 5G1, *M. mycoides* SC excepted. Thus, the *vmm*-like genes may be silent or their gene products do not contain the binding epitope for MAb 5G1.

DISCUSSION

This study has shown that *M. mycoides* SC possesses a variable surface protein, Vmm, that undergoes high-frequency phase variation. The phase variation was caused by a dinucleotide insertion or deletion in a repetitive region of the promoter. The active *vmm* promoter with the TTGACA −35 region and the TATAAT −10 region separated by a 17-bp spacer is identical to the consensus promoter that is recognized by σ^{70}-type prokaryotic sigma factors (80). Insertions or deletions of the spacer may cause changes of the promoter that affect the recognition and binding of the sigma factor. Alternatively, changes in DNA bending and the extent of supercoiling as a result of mutations in the promoter will affect the thermodynamics of DNA opening and may prevent the formation of a stable, open RNA polymerase-promoter complex and thereby disable transcription initiation. In *E. coli*, the promoter strength may be greatly influenced by the length of the spacer, mainly due to effects on the kinetics on the open complex formation as reviewed in reference 80. It should be noted that only a single sigma factor has been identified in mycoplasmas and that these organisms will therefore not regulate expression of any genes by alternative sigma factors (79).

Regulation strategies that are similar to the phase variation in the *vmm* gene, where the length of the promoter spacer

controls the expression, have been described for the *maa2* gene of *M. arthritidis* (93) and for the *vlp* genes of *M. hyorhinis* (24, 95). In both species, it is an insertion/deletion mutation in a homopolymeric nucleotide stretch that determines the length of the spacer.

When 28 selected clones of phenotypic lineages were analyzed by sequencing the *vmm* gene, 27 clones were consistently showing that the length of the spacer affects the Vmm expression as described above, a number that is too high to be a mere coincidence. However, there was one clone of the Vmm OFF phenotype that had the characteristic 10 (TA) repetitions that were found in all clones that express Vmm. The frequency of phase variation is high, 5×10^{-5} to 9×10^{-5} per cell per generation, and when this particular clone was checked for reversion, it was obvious that a considerable number of cells in the clone had switched phenotype. The PCR amplification may furthermore have exponentially increased the disproportion between ON- and OFF-type genes in this revertant clone of strain B17, which generated a misleading sequencing result.

Generally, it was concluded during this work that the phage display library in combination with the genome database was a very efficient means for identifying a particular protein epitope and its corresponding gene. The successful use of phage expression libraries to identify genes for mycoplasma surface proteins has been demonstrated earlier (89). There is an obvious risk of creating false epitopes in fusion proteins that can be the targets for ligands or antibodies in affinity pannings (36, 67). In this study, the affinity pannings selected five different insert variants of the same gene. The epitopes created by the fusion to gene VIII and the HSA binding region were different for the five inserts, and it is therefore unlikely that the result is an artifact from mimic epitopes. Furthermore, the fusion proteins of the phagemids were recognized by MAb 5G1 on Western blots, which confirmed identification of the true *vmm* gene.

The observation that there were two sizes of Vmm when separated by gradient SDS-PAGE was explained as the presence of two different states of processing of the protein, one prolipoprotein with the signal peptide still attached and one mature lipoprotein where the signal peptide was spliced off. Whether there is an aggregation of lipid-modified prolipoprotein in the cell, e.g., due to an inefficient prolipoprotein signal peptidase, needs to be further investigated (16, 42, 50). Comparison of the theoretical Vmm size as converted from the ORF (6.5 kDa including the signal peptide) to the apparent size observed by immunoblotting (17 kDa) may not seem to provide very good agreement. However, Vmm cannot be expected to migrate in the SDS-PAGE according to the standard relationship between the molecular weight and electrophoretic mobility, due to the smallness of the protein, the hydrophilic structure, and the lipid modification (6, 53, 65, 87). Moreover, the apparent size of the phage-expressed Vmm in SDS-PAGE is in accordance with the theoretical size (19 kDa for the fusion protein in Fig. 5, including the Vmm signal sequence, the tag, and protein VIII).

To study the virulence and pathogenicity mechanisms of important pathogens such as *M. mycoides* SC is, needless to say, crucial for the development of efficient vaccines and treatments of the disease. The surface components involved in attachment to the host tissues, in transport of metabolites or toxic substances across the membrane, and in immune evasion are key factors for pathogenicity. Although the function of Vmm in *M. mycoides* SC is still unknown, characterization of its structural features and the mechanism for phase variation is one step toward understanding the flexible surface of *M. mycoides* SC. Vmm is a small lipoprotein, and it will be important to find out if it interacts with other proteins in a complex system, thus regulating a set of processes by its phase variation. Similarly, it is of great interest to investigate if the switch of phenotype correlates to different stages of CBPP infection. It is also relevant to examine whether Vmm is a member of a large protein family, as commonly found for other mycoplasma surface proteins.

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