JOURNAL OF BACTERIOLOGY, Sept. 1996, p. 5395–5401 Vol. 178, No. 18
0021-9193/96/$04.00+0

Phenotypic Switching of Variable Surface Lipoproteins in *Mycoplasma bovis* Involves High-Frequency Chromosomal Rearrangements

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Received 8 May 1996/Accepted 11 July 1996

***Mycoplasma bovis*, an important pathogen of cattle, was recently shown to possess a family of phase- and size-variable membrane surface lipoprotein antigens (Vsps). These proteins spontaneously undergo noncoordinate phase variation between ON and OFF expression states, generating surface antigenic variation. In the present study, we show that the spontaneously high rate of Vsp phenotypic switching involves DNA rearrangements that occur at high frequency in the *M. bovis* chromosome. A 1.5-kb *Hin*dIII genomic fragment carrying the *vspA* gene from *M. bovis* PG45 was cloned and sequenced. The deduced VspA amino acid sequence revealed that 80% of the VspA molecule is composed of reiterated intragenic coding sequences, creating a periodic polypeptide structure. Four distinct internal regions of repetitive sequences in the form of in-tandem blocks extending from the N-terminal to the C-terminal portion of the Vsp product were identified. Southern blot analysis of phenotypically switched isogenic lineages representing ON or OFF phase states of Vsp products suggested that changes in the Vsp expression profile were associated with detectable changes at the DNA level. By using a synthetic oligonucleotide representing a sequence complementary to the repetitive *vspA* gene region as a probe, we could identify the *vspA*-bearing restriction fragment undergoing high-frequency reversible rearrangements during oscillating phase transition of *vspA*. The 1.5-kb *Hin*dIII fragment carrying the *vspA* gene (on state) rearranged and produced a 2.3-kb *Hin*dIII fragment (OFF state) and vice versa. Two newly discovered *vsp* genes (*vspE* and *vspF*) were localized on two *Hin*dIII fragments flanking the *vsp* gene upstream and downstream. Southern blot hybridization with *vspE*- and *vspF*-specific oligonucleotides as probes against genomic DNA of VspA phase variants showed that the organization and size of the fragments adjacent to the *vspA* gene remained unchanged during VspA ON-OFF switching. The mechanisms regulating the *vsp* genes are yet unknown; our findings suggest that a recombinative mechanism possibly involving DNA inversions, DNA insertion, or mobile genetic elements may play a role in generating the observed high-frequency DNA rearrangements.**

The genus *Mycoplasma* consists of the smallest known self-replicating organisms phylogenetically related to gram-positive eubacteria (22, 23). The mycoplasmas lack a cell wall and contain a remarkably small genome that in some species consists of only 600 kb (4, 23). Nevertheless, these wall-less organisms occupy a diverse range of natural habitats which include a large variety of animal hosts (31, 38). Many mycoplasmas have been identified as infectious agents of humans and animals, and many are established pathogens. The successful adaptation of the mycoplasmas to the different and changing environment seems to depend on their remarkable ability to rapidly alter their antigenic surface components (40, 41). This strategy, by which different levels of population diversity are maintained, ensures the presence of a certain phenotype needed for survival in a sudden environmental change. It also enables the mycoplasma to evade the host immune response. Efforts during the last few years have been focused on membrane surface proteins of several mycoplasma species to identify and characterize variable proteins undergoing high-frequency phenotypic switching on the mycoplasma cell surface. Considerable evidence for surface antigenic variation has been reported for several pathogenic mycoplasma species (1, 20, 26, 27, 36, 39, 42). However, detailed descriptions of molecular mechanisms mediating high-frequency phenotypic switching have remained relatively unexplored.

Recently, a new system mediating surface antigenic variation was discovered in the bovine pathogen *Mycoplasma bovis* (1, 25). A set of variable membrane surface lipoproteins designated Vsps constitutes the Vsp protein family. Three members of the Vsp family (VspA, VspB, and VspC) have been described to date (1). Comparison of the Vsp system with the well-studied Vlp system of *Mycoplasma hyorhinis* (26, 27, 43, 44), a swine pathogen, and with the variable *vsa* gene of *Mycoplasma pulmonis* (32), a murine pathogen, reveals several closely similar characteristics. These include the following: (i) an N-terminal portion containing a prokaryotic lipoprotein signal sequence; (ii) a surface-exposed C-terminal region bearing repetitive structures; (iii) spontaneously high rates of noncoordinate phase variation; (iv) high-frequency size variation; and (v) Vsps and Vlps expressed as abundant amphiphilic proteins anchored on the mycoplasma membrane via a lipid moiety on an N-terminal Cys residue. Despite these similarities, one key feature clearly distinguishes the Vsp system from the others, namely, the presence of several distinct internal regions of in-tandem reiterated sequences extending from the N-terminal to the C-terminal portion of the Vsp molecule. This allows the generation of size variants as a result of additions or deletions of repetitive blocks throughout the *vsp* coding re-

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gions. Another system that has recently been described is the pMGA gene family consisting of a repertoire of pMGA-like genes. Like the Vlp and Vsp systems, the pMGA system shares the ability to switch ON and OFF particular coding sequences and was suggested to contribute to surface variation of the avian pathogen *Mycoplasma gallisepticum* (17).

While the structural features contributing to Vsp phenotypic switching have been defined (25), the molecular mechanisms underlying Vsp antigenic, structural, or phase variation have not. In this report, we demonstrate that the chromosome of *M. bovis* undergoes rearrangements at a high frequency and that these rearrangements are associated with Vsp phenotypic switching. The *vspA* gene, a representative variable surface lipoprotein of the Vsp family, was cloned, sequenced, and analyzed by Southern blot hybridization of clonal isolates exhibiting different expression states of the VspA product. We provide evidence that the *vspA* gene is subjected to high-frequency reversible DNA rearrangements occurring during oscillating phase transition of VspA.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *M. bovis* strain used in this study was the PG45 type strain. Its origin and growth conditions have been described recently (25). The *Escherichia coli* strains used were DH5αMCR (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.) and KW251 (Promega, Madison, Wis.). Recombinant plasmids were constructed with the vector pBluescript II KS$^+$ (Stratagene, La Jolla, Calif.).

Chemicals, media, and growth conditions. *E. coli* cultures for plasmid and bacteriophage isolation were grown with shaking at 37°C in Luria-Bertani (LB) broth (16). *E. coli* cultures for expression of proteins under T7 promoter control were grown at 30°C with shaking in M9 medium (16) supplemented with an amino acid mixture. Restriction enzymes, T4 ligase, and T4 polynucleotide kinase were purchased from Promega and used as recommended by the manufacturer. 5-Bromo-4-chloro-3 indolyl-β-D-galactopyranoside (X-Gal), ampicillin, kanamycin, and rifampin were purchased from Sigma Chemical Co., St. Louis, Mo. [γ-^{32}P]ATP and [α-^{32}P]CTP were purchased from Amersham, Little Chalfont, United Kingdom.

Genomic library construction. A recombinant phage library was constructed with the phage vector λGEM-12 (*Xho*I half arms; Promega) with partially digested *Mbo*I chromosomal fragments from a clonal isolate of *M. bovis* PG45 with the phenotype VspA$^+$ VspB$^+$ VspC$^-$ (represented in Fig. 1C, lane 1). Viable phage particles were produced by in vitro packaging of recombinant phage DNA with a commercial in vitro lambda DNA packaging system (Promega). Phage plaques were generated with *E. coli* KW251 on NZCYM plates (16) containing 0.6% (wt/vol) agarose (Gibco BRL).

Immunoscreening of phage library and phage purification. Agar plates (80-mm diameter) containing approximately 2×10^3 PFU were overlaid for 10 min with nitrocellulose filters (0.45-μm pore size; Schleicher & Schuell, Dassel, Germany). The filters were removed and blocked with TS buffer (150 mM NaCl, 10 mM Tris [pH 7.4]) containing 3% (wt/vol) bovine serum albumin (BSA; fraction V; Sigma) and incubated for 18 h at 4°C with monoclonal antibody (MAb) 1E5 diluted in phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.0 mM NH_2PO_4 [pH 7.2]) as the primary antibody. The filters were washed three times in TS buffer and then incubated with horseradish peroxidase-conjugated goat antiserum to mouse immunoglobulin M (Jackson Immuno Research Laboratories, Inc., West Grove, Pa.) diluted 1:1,000 in TS buffer for 2 h at room temperature. Filters were developed with the substrate *o*-dianisidine as described previously (1, 25). Positive phages expressing Vsp antigens were picked, replated at low density, and again immunoscreened. After two rounds of plaque purification, three distinct phages were isolated for further analysis.

DNA preparation and cleavage. Genomic DNA from *M. bovis* strains and clinical isolates was extracted and purified by the method of Marmur (18). The isolation of bacteriophage DNA was done with a method for the rapid, small-scale isolation of bacteriophage λ DNA, as described elsewhere (16). The DNA was digested by restriction enzymes and electrophoresed as described previously (42).

Protein expression under T7 promoter control. Expression of *M. bovis* genes in *E. coli* utilized the T7 polymerase-promoter system of Tabor and Richardson (35). *E. coli* DH5αMCR(pGP1-2) was transformed with the recombinant plasmid $pKA_{63.1}$ or $pKA_{63.2}$ carrying the *vspA* gene in two orientations. Transformants were grown in selective LB medium containing 50 μg of ampicillin and 40 μg of kanamycin per ml at 30°C. At an A_{590} of 0.5, cells from 300-μl cultures were washed with 5 ml of M9 medium and resuspended in 1.0 ml of M9 medium supplemented with 20 μg of thiamine per ml and 0.01% (wt/vol) of amino acid mixture and incubated at 30°C for 1 h. The temperature was then shifted to 42°C for 15 min for selective induction of T7 DNA polymerase expression, rifampin was added to a final concentration of 200 μg/ml, and incubation was continued for an additional 10 min. Cells were harvested, resuspended in cracking buffer (62.5 mM Tris [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate [SDS], 5% [vol/vol] 2-mercaptoethanol, 3% [wt/vol] urea, 10% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue). Protein samples were boiled for 3 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (14), electrophoretic transfer to nitrocellulose (37), and immunoblot analysis as described elsewhere (43).

DNA sequence analysis. DNA sequence analysis of both strands was performed by the dideoxy chain termination method of Sanger et al. (28). Overlapping sets of deletion mutants were generated from the recombinant plasmid pKA_{63} by graded directional exonuclease III digestion with the Erase-A-Base deletion kit (Promega). As a sequencing primer, the T7 promoter sequence located on the pKS plasmid vector was used. Sequence data were analyzed with the Genetics Computer Group Wisconsin sequence analysis package (6).

^{32}P-labeled oligonucleotide probes and DNA hybridization. *vsp* sequence-specific oligonucleotides were synthesized at the interdepartmental facility of the Hebrew University-Hadassah Medical School on a model 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The three 20-bp oligonucleotides were as follows: R_A4.1, 5′-CAAGGTGCAGGAACTAAACC-3′; R_E1, 5′-GCA CCACAACAAGGTACAGG-3′; R_F2, 5′-CCTTTAGGTGTTTCAGGTCC-3′. About 100 ng of oligonucleotide was ^{32}P labeled with 15 U of T4 polynucleotide kinase at 37°C for 1 h in 25 μl of a reaction mixture containing 40 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 2.5 μl of [γ-^{32}P]ATP (3,000 Ci/nmol). Unincorporated nucleotides were removed from the labeling reaction with NucTrap push columns (Stratagene) as described in the recommendations of the manufacturer. Electrophoresis of genomic DNA and Southern blot procedures were described elsewhere (42). Prehybridization was carried out at 50°C for 12 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) containing 0.2% polyvinylpyrrolidone (PVP-360; Sigma), 2% BSA, 2% Ficoll (type 400; Sigma), and 10 μg of denatured salmon sperm DNA per ml (type III, Sigma). Hybridization was carried out for 24 h at 46°C in the same buffer containing the labeled oligonucleotide. Blots were then washed at 46°C by soaking and shaking for 30 min with two changes in 6× SSC plus 0.1% SDS followed by two changes in 5× SSC plus 0.1% SDS at 50°C for 15 min. After air drying, the nitrocellulose sheets were exposed at −80°C to Fuji medical X-ray film with an intensifying screen.

Nucleotide sequence accession number. The nucleotide sequence data in this report have been assigned GenBank accession number L81118.

RESULTS

Cloning and expression of the *M. bovis vspA* gene in *E. coli*. To detect expression of *M. bovis* PG45 Vsp antigens in *E. coli*, a genomic library was constructed with the bacteriophage λGEM-12 from a clonal isolate with the phenotype VspA$^+$ VspB$^+$ VspC$^-$ (63 kDa of VspA and 46 kDa of VspB) (Fig. 1B, lane 1) and immunoscreened with MAb 1E5. This MAb has been shown previously to recognize a common epitope present on three distinct Vsps: VspA, VspB, and VspC (1). Three phages containing distinct DNA inserts were isolated by this screening process. One phage, designated λMbA1, which showed very strong immunostaining, was analyzed further. Extraction of DNA from λMbA1 and digestion with *Hin*dIII restriction enzyme revealed an 11.2-kb mycoplasma DNA insert containing five *Hin*dIII fragments of 3.8, 2.9, 1.8, 1.5, and 1.2 kb. Each of the five *Hin*dIII fragments was subcloned separately in both orientations into the pKS plasmid vector, placing the mycoplasma DNA insert under the control of the T7 promoter of this vector. T7 RNA polymerase encoded on a second plasmid (pGP1-2) was induced to selectively initiate transcription of genes cloned downstream of the T7 promoter. Expressed mycoplasma proteins were separated by SDS-PAGE and immunoblotted with MAb 1E5. A polypeptide band of 63 kDa was synthesized from a plasmid (pKA_{63}) containing the 1.5-kb *Hin*dIII fragment (Fig. 1B, lane 2). The size of the expressed protein in *E. coli*, by use of the T7 promoter system, was similar to that of the authentic VspA protein band expressed in the mycoplasma. This indicated that the intact *vspA* gene is present on the 1.5-kb *Hin*dIII fragment (Fig. 1B, lanes 1 and 2). Restriction mapping of the 11.2-kb DNA insert localized the 1.5-kb *Hin*dIII fragment carrying the *vspA* gene

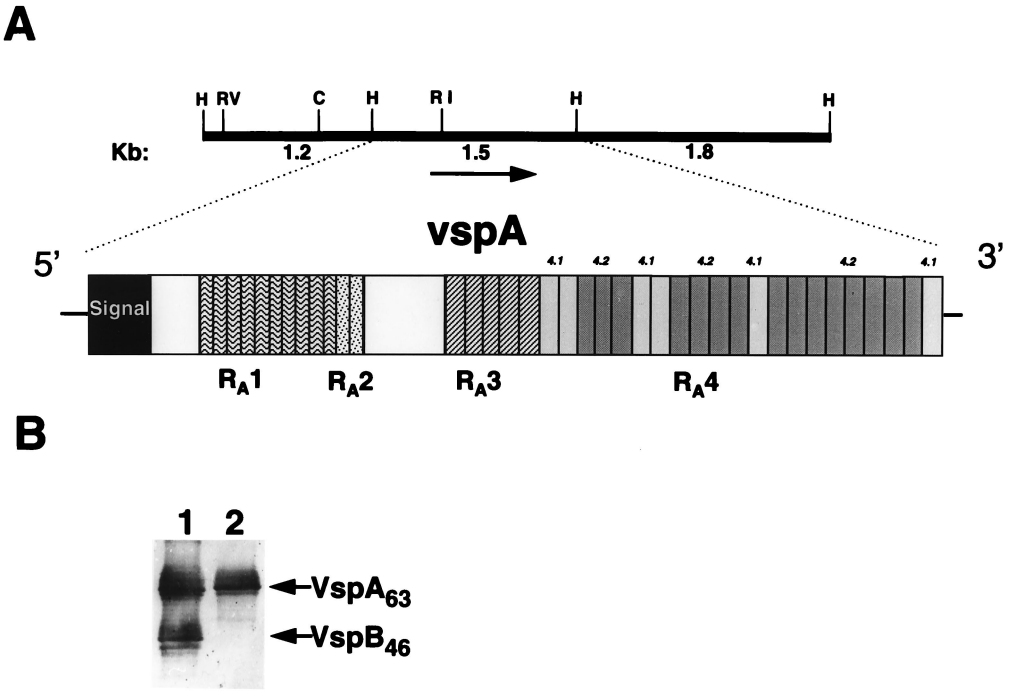

FIG. 1. (A) Schematic representation and structural features of the VspA protein. The solid line represents a partial restriction map indicating positions of *Cla*I (C), *Eco*RI (RI), *Eco*RV (RV), and *Hin*dIII (H). The location and the orientation of the *vspA* gene are shown by an arrow. The VspA ORF is depicted as a rectangle consisting of internal blocks delineating various features of the VspA protein. The solid block labeled signal contains 25 aa of a putative lipoprotein signal peptide. Different hatched or shaded blocks designated R_A1, R_A2, R_A3, and R_A4 represent four in-frame repetitive regions encoding distinctive periodic amino acid sequences of 6, 6, 8, and 8 aa, respectively. Subrepetitive units within the R_A4 region designated $R_A4.1$ and $R_A4.2$ (see also Fig. 2) are indicated. (B) Expression of mycoplasma-encoded VspA protein in *E. coli* control. *E. coli* cells expressing under selective induction of the T7 promoter, the recombinant plasmid pKA_{63} carrying the *vspA* gene in the orientation shown in panel A, were separated by SDS-PAGE and immunoblotted with MAb 1E5. Total proteins of a clonal isolate of *M. bovis* PG45 exhibiting the phenotype $VspA^+$ $VspB^+$ $VspC^-$ were used as a positive control (lane 1). A 63-kDa protein of VspA and a 46-kDa protein of VspB representing authentic products expressed in the mycoplasma (lane 1) and a 63-kDa recombinant polypeptide expressed in *E. coli* (lane 2) are indicated by arrows.

adjacent to 1.2- and 1.8-kb *Hin*dIII fragments as shown in Fig. 1A.

Structural features of the *vspA* gene and its deduced protein. The nucleotide sequence of the 1.5-kb *Hin*dIII fragment bearing the *vspA* gene was determined. Within the sequenced fragment, an open reading frame (ORF) containing 1,050 nucleotides, starting with an ATG invitation codon and terminating at a TAA stop codon, was identified. The nucleotide and the deduced amino acid sequences of this ORF and its immediate flanking regions are shown in Fig. 2. Since this ORF expressed in *E. coli* a 63-kDa polypeptide reacting with MAb 1E5 that was similar to the authentic VspA product in the mycoplasma (Fig. 1B), this gene was designated the *vspA* gene. Examination of the deduced VspA amino acid sequence revealed an unusual structure. About 80% of the VspA molecule is composed of reiterated sequences extending from the N terminus to the C terminus of the VspA protein (Fig. 1A and 2). Four distinct internal regions of repetitive sequences as tandem in-frame blocks were identified. These regions, designated R_A1, R_A2, R_A3, and R_A4, create a periodic polypeptide structure spanning the entire VspA molecule. The R_A1 region contains blocks of 6 amino acids (aa) directly repeated 10 times; the R_A2 region contains blocks of 6 aa repeated twice; the R_A3 region contains blocks of 8 aa repeated five times; and the R_A4 region contains blocks of 8 aa repeated 21 times (Fig. 1A and 2). Distinctive signatures distributed within the R_A4 region that generated two kinds of subrepeats designated $R_A4.1$ and $R_A4.2$ (Fig. 1 and 2) were used during sequencing as markers to confirm their number. The hydropathy profile (Fig. 3) and deduced amino acid sequence (Fig. 2) predicted that VspA is hydrophilic except for the N-terminal portion. The N-terminal end of the VspA contained a positively charged amino-terminal region and a central hydrophobic region. It ends with a cysteine residue in the predicted acylation site and point of membrane anchorage of a mature processed prokaryote lipoprotein (10). However, the presence of a Lys residue in front of the Cys residue is not consistent with a prototypical prokaryotic lipoprotein signal sequence.

High-frequency rearrangements in the chromosome of *M. bovis* are associated with Vsp phenotypic switching. To examine molecular events regulating the high-frequency Vsp phenotypic switching, a lineage of clonal isolates of *M. bovis* PG45 displaying well-defined expression states of the VspA, VspB, and VspC proteins was generated. This was done by immunostaining *M. bovis* PG45 colonies with MAb 1E5 to monitor changes in the expression of Vsps on the cell surface. Individual *M. bovis* PG45 colonies exhibiting variation in expression of Vsps were isolated, and their progenies were plated and in turn subjected to immunostaining. Continued switching (ON to OFF and vice versa) in the expression of VspA, VspB, or VspC within these lineages was confirmed by Western blot (immunoblot) analysis, and the precise distinction between VspA, VspB, and VspC was determined as described previously (1). An example of such analysis is illustrated in Fig. 4A, where selected clonal variants of strain PG45 representing different expression states of VspA, VspB, and VspC products are shown. Genomic DNA from these clonal isolates was extracted, digested with *Hin*dIII, and subjected to Southern blot hybridization with plasmid pKA_{63} bearing the *vspA* gene as a probe. A complex pattern of multiple *vspA*-related restriction

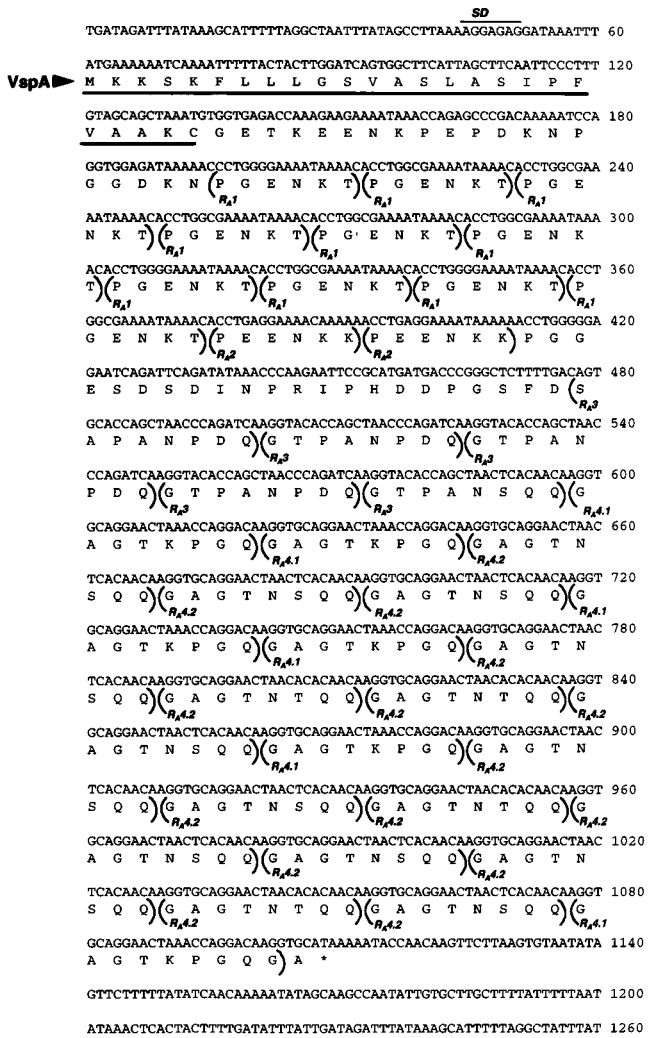

FIG. 2. Nucleotide sequence and deduced amino acid sequences of the *M. bovis* PG45 *vspA* gene. Nucleotide positions are numbered 5′ to 3′ and are indicated on the right. The single-letter code for each amino acid residue is indicated below the nucleotide positions on the first letter of each codon. The arrowhead labeled VspA (at nucleotide 61) indicates the start and the direction of the VspA ORF. Amino acids constituting the putative VspA lipoprotein signal peptide are underlined. Nucleotides representing a putative ribosome-binding site (SD) are overlined. In-frame repetitive amino acid sequences corresponding to the four distinct repetitive regions, R_A1, R_A2, R_A3, and R_A4 (see Fig. 1A), are indicated by labeled parentheses. Two subrepetitive units within the R_A4 region, $R_A4.1$ and $R_A4.2$, are also labeled (see also Fig. 1A). The asterisk in the amino acid sequence denotes a termination codon.

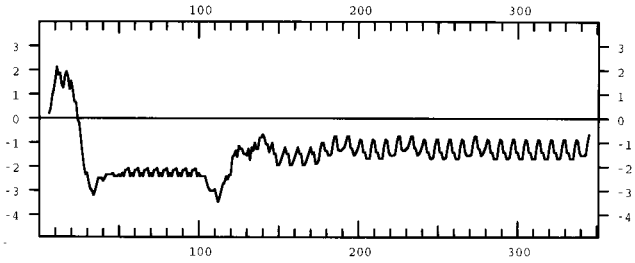

FIG. 3. Hydropathy plot of the deduced amino acid sequencing of *M. bovis* PG45 VspA. Positive numbers on the left indicate hydrophobic regions, negative numbers indicate hydrophilic regions, and amino acid residues are numbered along the bottom of the panel.

fragments was observed (Fig. 4B). Direct switches of particular Vsp products were accompanied by detectable rearrangements of the corresponding genomic fragments (Fig. 4A and B, note the transitions from lanes 1 to 2 and from lane 1 to lanes 3, 4, and 5).

Thus far, the data have suggested that Vsp phenotypic switching is associated with chromosomal rearrangements occurring at a high frequency within a single strain. At this point, we were interested in identifying restriction fragments corresponding to the *vspA* gene and in assessing whether these fragments undergo high-frequency rearrangements during oscillating phase transition of VspA. The R_A4 synthetic oligonucleotide representing a sequence complementary to the repetitive region R_A4 of the *vspA* gene (Fig. 2) was used as a probe in Southern blot hybridization against *Hin*dIII-digested genomic DNA of the clonal isolates depicted in Fig. 4A, lanes 1 to 5. Two R_A4-related restriction fragments exhibiting size variation among the various clonal isolates were detected (Fig. 4C). These two fragments were part of the complex restriction pattern detected by the intact *vspA* gene probe shown in Fig. 4B.

The next step was to assess whether the restriction fragments corresponding to the *vspA* gene observed in Fig. 4B and C are subjected to rearrangement during VspA phase variation. Three *M. bovis* PG45 clonal isolates representing three successive generations and exhibiting oscillating phase transitions of VspA, i.e., ON→OFF→ON, while VspB and VspC are not expressed were chosen for this experiment and are shown in Fig. 5A, lanes 1 to 3, respectively. Genomic DNA from these three clonal variants was extracted, restricted by *Hin*dIII, and subjected to Southern blot hybridization with the plasmid pKA_{63} bearing the *vspA* gene as a probe (Fig. 5B). The resulting hybridization pattern showed that reversible rearrangements of *vspA*-associated restriction fragments have occurred during phase transition of VspA. Two invariant *vspA*-associated restriction fragments were observed for all isolates tested, despite the expression state of VspA (Fig. 5B). However, two additional restriction fragments were detected only when VspA was expressed. One is the 1.5-kb *Hin*dIII fragment carrying the *vspA* gene, and the other is a fragment 8.0 kb in size (Fig. 5B, lanes 1 and 3). Genomic rearrangements during VspA phase variation (ON→OFF) apparently caused the disappearance of the 1.5-kb *Hin*dIII fragment and the generation of a new 2.3-kb restriction fragment shown only in the clonal variant in which the *vspA* gene has turned off (Fig. 5B, lane 2). Notably, an additional 8.5-kb *Hin*dIII fragment was shown to be present only in the variant in Fig. 5 (lane 1).

Genomic localization of the *Hin*dIII fragment undergoing rearrangement during VspA phase variation. The finding that the 1.5-kb *Hin*dIII fragment carrying the *vspA* gene (Fig. 1), rearranged during phase transition of VspA (ON→OFF) to generate a 2.3-kb *Hin*dIII fragment and vice versa (Fig. 5B), has led us to examine the genomic stability of the regions flanking the *vspA* gene during phase transition of VspA. We extended our sequence analysis to the 1.2- and 1.8-kb *Hin*dIII fragments localized upstream and downstream, respectively, of the 1.5-kb *Hin*dIII fragment carrying the *vspA* gene (Fig. 1A). Two additional ORFs displaying features characteristic of the *vspA* gene were identified. These features include a highly conserved 5′ noncoding region, a highly conserved N-terminal end, and distinct repetitive regions within the structural genes (data not shown). Expression products of these two ORFs in *E. coli* failed to react with MAb 1E5, and the corresponding genes

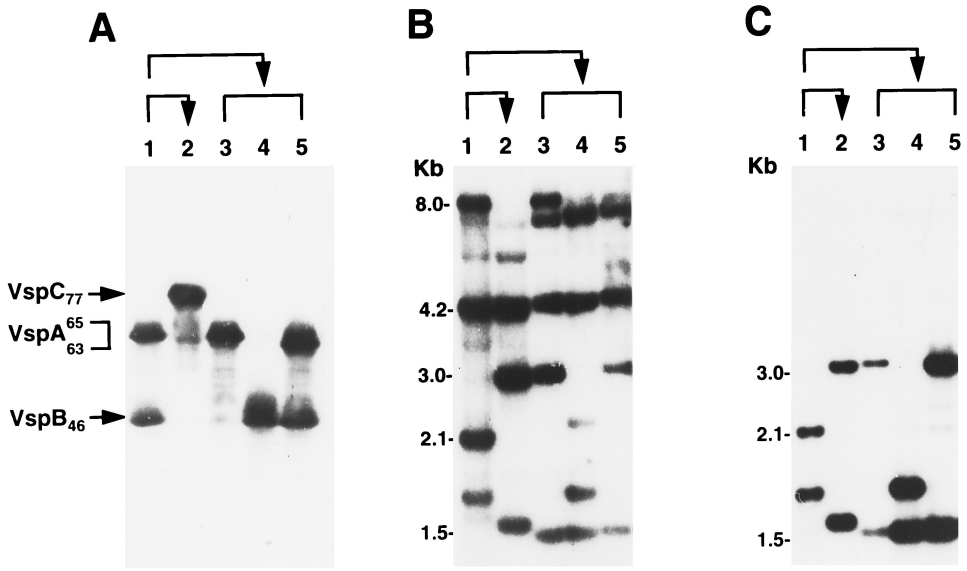

FIG. 4. Identification of *vspA*-associated restriction fragments undergoing high-frequency rearrangements linked with Vsp phenotypic switching. (A) Western blot analysis of sequential clonal isolates of *M. bovis* PG45 representing different expression states of VspA, VspB, and VspC products. Whole organisms were subjected to SDS-PAGE and immunoblotted with MAb 1E5. Phenotypic transitions are indicated at the top of the panels by arrows. VspA (two size variants, $VspA_{65}$ and $VspA_{63}$), $VspB_{46}$, and $VspC_{77}$ are indicated. (B and C) Southern blot hybridization of *M. bovis* PG45 clonal isolates. Four micrograms of chromosomal DNA from the clonal isolates depicted in panel A (lanes 1 to 5) was digested to completion with *Hin*dIII and probed with the ^{32}P-labeled plasmid pKA_{63} carrying the *vspA* gene (B) or with the ^{32}P-labeled *vspA* R_A4 oligonucleotide (C) which represents a sequence complementary to the repetitive R_A4 region of the *vspA* gene. Molecular size markers are indicated on the left in panels B and C.

were therefore designated *vspE* and *vspF*. Their location and orientation are shown in Fig. 6A.

Two oligonucleotides representing distinct sequences complementary to the repetitive regions of the *vspE* (R_E1) and *vspF* (R_F2) genes and oligonucleotide R_A4 of the *vspA* gene were used as probes in Southern blot hybridization against *Hin*dIII-digested genomic DNA of the three VspA phase variants depicted in Fig. 5A. By use of these three Vsp oligonucleotides, a precise identification of the *Hin*dIII genomic fragment undergoing high-frequency reversible rearrangements during phase transition of VspA could be made (Fig. 6). The R_A4 probe clearly identified the *vspA* gene-bearing *Hin*dIII fragment as the region undergoing reversible size variation (1.5 to 2.3 to 1.5 kb) in correlation with the change in VspA expression state (ON→OFF→ON [Fig. 6C, lanes 1 to 3]). Interestingly, this oligonucleotide also recognized an additional and invariant fragment about 3 kb in size that is present in all three VspA variants (Fig. 6C). On the other hand, the 1.2- and 1.8-kb *Hin*dIII fragments flanking the *vspA* gene were detected by the corresponding oligonucleotides (R_E1 and R_F2, respectively) as invariant fragments despite the VspA expression state (Fig. 6B, D, and E, lanes 1 to 3). This localized the 1.5-kb *Hin*dIII fragment carrying the *vspA* gene as the region subjected to reversible rearrangement occurring during VspA ON-OFF switching.

DISCUSSION

Accumulating evidence in the last few years shows that mycoplasmas possess an ability to rapidly alter surface antigens, generating surface diversity measured at a high frequency of 10^{-2} to 10^{-3} per cell per generation (1, 8, 20, 25–27, 36, 39–43). In a few examples, surface antigens, usually as a complex of lipoproteins containing repetitive domains, have been identified (27, 32, 43). Members of the recently described Vsp lipoprotein family were shown to be antigenically and structurally related (1, 25), suggesting that the corresponding *vsp* genes may share regions of significant homology.

Two valuable tools prepared in this study allowed us to monitor DNA rearrangement events occurring at high frequency within a single strain and to link them with Vsp expression. The first one was a clonal lineage representing several sequential isolates derived from a single strain and exhibiting variation in expression of distinct Vsps (Fig. 4A and 5A). The second one was a set of synthetic oligonucleotides representing sequences complementary to a distinct repetitive region (R_A4) of the *vspA* gene and adjacent *vsp* genes, *vspE* (R_E1) and *vspF* (R_F2). Southern blot analysis with the *vspA* gene or the *vspA* R_A4 oligonucleotide as a probe against genomic DNA of *M. bovis* clonal isolates displaying well-defined expression states of VspA, VspB, and VspC showed that any change in Vsp expression profile was associated with detectable changes at the DNA level (Fig. 4B). Moreover, examination of the hybridization patterns obtained with a lineage of VspA phase variants, in which only VspA is switching, could identify reversible rearrangements of *vspA*-associated restriction fragment occurring during VspA phase transition (1.5 kb [ON] → 2.3 kb [OFF] → 1.5 kb [ON]) (Fig. 5 and 6). These results suggest that Vsp phenotypic switching involves high-frequency reversible rearrangements in the chromosome of *M. bovis*. This genetic variation appears to be a result of random events independent of environmental regulation. The data do not support the possibility that the observed DNA rearrangements of the *vsp* region arose as a result of DNA methylation or point mutations effecting restriction enzyme recognition sites.

Chromosomal rearrangements have been shown to be associated with phenotypic switching in many bacterial systems. Homologous recombination, gene conversions, DNA inversions, gene duplications, additions, or deletions of tandem repetitive units, and movement of transposable elements are frequently employed mechanisms regulating genes encoding surface antigens in other bacterial systems (3, 7, 9, 11, 19, 24,

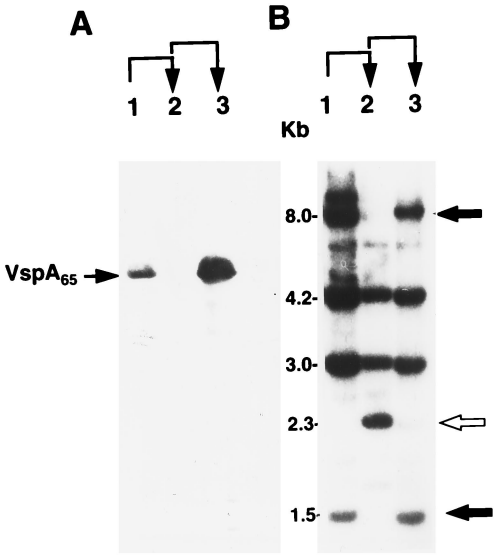

FIG. 5. Identification of restriction fragments undergoing rearrangements during VspA phase variation. (A) Western blot analysis of an *M. bovis* PG45 sequential lineage showing oscillating phase transitions of VspA. Whole organisms were subjected to SDS-PAGE and immunoblotted with MAb 1E5. The 63-kDa VspA protein band is indicated by an arrow. Phenotypic transition of VspA (ON→OFF→ON through lanes 1 to 3, respectively) is indicated at the top of the panels by arrows. (B) Southern blot hybridization of VspA phase variants depicted in panel A. Four micrograms of chromosomal DNA was digested with *Hin*dIII and probed with the ^{32}P-labeled recombinant plasmid pKA_{63} carrying the *vspA* gene. Two *Hin*dIII restriction fragments present only when VspA was expressed (lanes 1 and 3) are indicated by arrows. A 2.3-kb *Hin*dIII fragment shown only in the clonal variant in which VspA has turned off (lane 2) is indicated by an open arrow. Molecular size markers are indicated on the left of panel B.

29, 30, 33, 34). One or more of these mechanisms are likely to be involved in the regulation of the *vsp* genes. Regions of homology shared by the *vsp* genes may serve as recombination sites. Tandem repetitive units within the *vsp* genes are a favorite target for a recombinative mechanism (15, 21, 33) capable of duplicating or deleting these domains.

The only reported example of a mycoplasma in which high-frequency rearrangements correlate with phenotypic switching is *M. pulmonis*. Bhugra and Dybvig (2) reported that changes in the susceptibility of *M. pulmonis* cells to mycoplasma virus P1, in which the V-1 antigen was involved, were correlated with phenotypic switching. Recently, sequence analysis of the V-1-encoding gene of *M. pulmonis* showed that DNA inversion mechanisms regulate the ON-OFF switching of the V-1 antigen (32).

A notable feature that delineates the unique characteristic of the VspA protein emerged from sequence analysis of the corresponding gene. Approximately 80% of the VspA molecule is made up of four distinct regions, each of which consists of tandemly repeated sequences. This unusual structure motif creates a periodic polypeptide extending from the N-terminal to the C-terminal end of the VspA molecule. Interestingly, this structure is quite analogous to that found in the size-variant M protein surface antigen of group A streptococci in which 80% of the M protein is made up of four blocks (A to D) of repetitive sequences (12, 13).

The predicted VspA structure confirmed our earlier observations: the nonequidistant Vsp ladder pattern obtained by a carboxy peptidase Y argues for the occurrence of multiple sets of similar or nonsimilar repeat blocks (1). The presence of repeated blocks throughout the *vspA* gene may explain a unique feature of the Vsp system, i.e., its ability to generate size variants as a result of deletions or additions of repetitive blocks throughout the *vsp* gene. This feature distinguishes the Vsp system from the reported Vlp system of *M. hyorhinis* and of the V-1 antigen of *M. pulmonis*. Size variation in these two systems is generated by structural changes only at the repetitive C-terminal portion.

Examination of the hybridization pattern obtained with the R_A4 oligonucleotide showed that, in addition to the restriction fragment subjected to a reversible rearrangement event during *vspA* gene switching, another and invariant fragment about 3 kb in size was observed (Fig. 6C). This argues that the invariant 3-kb fragment contained at least one *vsp* gene copy that is remarkably similar to the *vspA* gene. This similarity is consistent with our recently reported data showing that VspA and VspC exhibit a remarkable structural similarity, differing only in a deletion of an N-terminal region (1).

It is intriguing to speculate that the striking surface diversity found in the mycoplasmas allows these wall-less organisms to resist the immune defense system. Alternatively, it equips the mycoplasmas with adaptive ability to survive in a diverse range of natural habitats. Moreover, the high rate of DNA recombination observed in the chromosome of *M. bovis* as well as in *M. pulmonis* places the mycoplasma chromosome as one of the most dynamic and variable genomes known. This may serve as a significant source for creating new coding sequences (5) and compensates for the limited mycoplasma genome size. Elucidating the molecular basis of the Vsp switching system will provide insight into the rearrangement process occurring in the small genome of these organisms and enable us to better un-

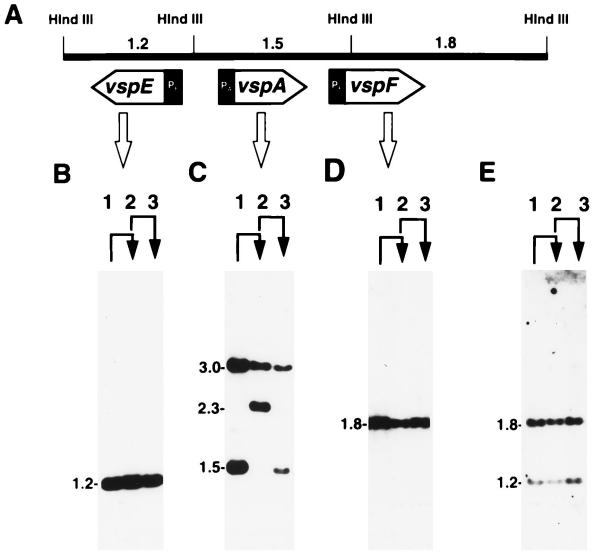

FIG. 6. Rearrangement of the *vspA*-bearing fragment during VspA phase variation. (A) The solid line shows the position of the 1.5-kb *Hin*dIII fragment carrying the *vspA* gene relative to the two adjacent *Hin*dIII fragments of 1.2 and 1.8 kb. The location and direction of VspA, VspE, and VspF ORFs are indicated by large open labeled arrows. Highly homologous regions 5′ of each Vsp ORF are indicated by black labeled boxes. (B to E) Southern blot hybridization of the *M. bovis* PG45 VspA phase variant depicted in panel A. Phenotypic switching of VspA (ON→OFF→ON through lanes 1 to 3, respectively) are indicated at the top of the panels by arrows. Four micrograms of chromosomal DNA was digested with *Hin*dIII and probed with (i) the oligonucleotide R_E1, which represents repetitive sequences of the *vspE* gene (B), (ii) the oligonucleotide R_A4, which represents repetitive sequences of the *vspA* gene (C), (iii) the oligonucleotide R_F2, which represents repetitive sequences of the *vspF* gene (D), and (iv) a mixture of R_E1 and R_F2 oligonucleotides (E). Molecular size markers of the detected restriction fragments are indicated on the left of each panel.

derstand the pathogenesis of *M. bovis* infections and therefore improve our ability to prevent them in the future.

ACKNOWLEDGMENTS

This work was supported in part by the Israel Science Foundation, the German-Israeli Foundation (G.I.F.), and a grant (Ro739/2-1) from the Deutsche Forschungsgemeinschaft. R.R. was supported by a fellowship (Ro739/5-1) from the Deutsche Forschungsgemeinschaft.

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