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Multiple Promoter Inversions Generate Surface Antigenic Variation in *Mycoplasma penetrans*

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***Mycoplasma penetrans* is a newly identified species of the genus *Mycoplasma*. It was first isolated from a urine sample from a human immunodeficiency virus (HIV)-infected patient. *M. penetrans* changes its surface antigen profile with high frequency. The changes originate from ON↔OFF phase variations of the P35 family of surface membrane lipoproteins. The P35 family lipoproteins are major antigens recognized by the human immune system during *M. penetrans* infection and are encoded by the *mpl* genes. Phase variations of P35 family lipoproteins occur at the transcriptional level of *mpl* genes; however, the precise genetic mechanisms are unknown. In this study, the molecular mechanisms of surface antigen profile change in *M. penetrans* were investigated. The focus was on the 46-kDa protein that is present in *M. penetrans* strain HF-2 but not in the type strain, GTU. The 46-kDa protein was the product of a previously reported *mpl* gene, pepIMP13, with an amino-terminal sequence identical to that of the P35 family lipoproteins. Nucleotide sequencing analysis of the pepIMP13 gene region revealed that the promoter-containing 135-bp DNA of this gene had the structure of an invertible element that functioned as a switch for gene expression. In addition, all of the *mpl* genes of *M. penetrans* HF-2 were identified using the whole-genome sequence data that has recently become available for this bacterium. There are at least 38 *mpl* genes in the *M. penetrans* HF-2 genome. Interestingly, most of these *mpl* genes possess invertible promoter-like sequences, similar to those of the pepIMP13 gene promoter. A model for the generation of surface antigenic variation by multiple promoter inversions is proposed.**

Mycoplasmas are bacteria with no cell wall and the minimum range of genome sizes necessary for self-replication. They lack most of the genes required for nutrient metabolism and adopt a parasitic lifestyle in host organisms. Over 100 mycoplasma species have been isolated from a wide range of host organisms. Several of these species are well recognized as pathogens (29, 30). As parasitic bacteria, mycoplasmas can continue to colonize the host even in the presence of a specific immune response. This property of mycoplasmas may explain the slowly progressive chronic manifestations of mycoplasma-associated diseases. The mechanisms for evasion of host immune responses in mycoplasmas are poorly understood. However, a number of recent studies have demonstrated that many mycoplasma species can modify their surface antigenic molecules with high frequency (31, 32), which might play a key role in circumventing the host immune system. The rapid change of surface antigenic molecules may generate phenotypic heterogeneity in the propagating mycoplasma population and provide advantages not only for evasion of host immune responses but also for other aspects of mycoplasma survival, such as adaptation to environmental changes.

Most of the variable surface antigenic molecules of mycoplasmas are lipoproteins (5, 45). These lipoproteins, depending upon the species, are encoded by single or multiple genes and undergo frequent phase and size variation during mycoplasma growth (31, 32, 46). A variety of genetic mechanisms are used to modulate the expression of these lipoprotein genes, including DNA rearrangements, nucleotide insertions and deletions, gene conversions, and site-specific recombination (3, 7, 12, 26, 37). The characterization of these mechanisms may provide a detailed understanding not only of mycoplasma antigenic variation but also of bacterial gene regulation systems.

Mycoplasma penetrans is a newly identified species of mycoplasma that infects humans. It was first isolated from a urine sample from a human immunodeficiency virus (HIV)-infected patient (21). Epidemiological studies have demonstrated that *M. penetrans* detection is mainly associated with HIV infection (14, 42, 43); however, *M. penetrans* has also been isolated from a patient with a case of primary antiphospholipid syndrome without HIV infection, suggesting that *M. penetrans* may be pathogenic for humans without HIV (47). The morphology of this mycoplasma is that of an elongated flask with a tip-like structure at one pole of the cell (10, 20).

M. penetrans also has the ability to change its surface antigenicity (24, 33). The surface-exposed lipid-associated membrane proteins (LAMPs) of *M. penetrans* frequently change their profiles. The most abundant LAMP is the P35 lipoprotein, a major antigen recognized by the human immune system during *M. penetrans* infection (25). P35 undergoes high-frequency ON↔OFF phase variation, causing the change of LAMP profile (24). In addition to P35, LAMPs contain a considerable number of the P35 family lipoproteins that are encoded by the *mpl* genes (for *M. penetrans* lipoprotein). These lipoproteins also independently undergo ON↔OFF phase variation (24, 33). Although the phase variation of the P35 family lipoproteins seems to occur at the transcriptional level of *mpl* genes, the precise mechanism remains unclear.

In this study, we investigated the molecular mechanisms of LAMP profile change in *M. penetrans*, focusing on the anti-

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genic variation of the 46-kDa protein, which is present in the *M. penetrans* isolate HF-2 but not in the GTU type strain. We established that the 46-kDa protein is the product of the previously reported *mpl* gene pepIMP13 and demonstrated that the antigenic variation was caused by promoter inversion.

Furthermore, the whole genome sequence of *M. penetrans* strain HF-2 has been determined recently. This information has enabled us to identify all of the *mpl* genes and their localization in the genome. The *M. penetrans* HF-2 genome contains at least 38 *mpl* genes, and most of them possess independent invertible promoter-like sequences. We propose a novel system for generating antigenic variations by multiple promoter inversions.

MATERIALS AND METHODS

Mycoplasma strains and culture conditions. *M. penetrans* GTU-54 was the original isolate from a urine sample of an HIV-infected patient (21) and was kindly provided by S.-C. Lo (Armed Forces Institute of Pathology, Bethesda, Md.). *M. penetrans* HF-2 was isolated from an HIV-negative patient with primary antiphospholipid syndrome (47) and was kindly provided by L. Cedillo and A. Yáñez (Centro de Investigación Biomédica de oriente-IMSS and Benemérita Universidad Autónoma de Puebla, Puebla City, Mexico). *M. penetrans* strains were cultured in PPLO medium (2.1% PPLO broth [Difco Laboratories, Detroit, Mich.], 0.25% glucose, 0.002% phenol red, 5% yeast extract [Difco Laboratories], 10% horse serum [Gibco BRL, Rockville, Md.], 50 μg of ampicillin per ml) at 37°C.

Antibodies. A murine monoclonal antibody (MAb) specific to P35 (MAb 7) was established by T. Sasaki and was previously shown to react specifically with the P35 protein (24, 33). MAb 7 was used in this study at a 1:5,000 dilution for immunoblot analysis. Serum 6 (anti-HF-2) was produced by immunization of mice with total cell lysate of *M. penetrans* HF-2 and was used at a 1:500 dilution for immunoblot analysis.

Protein analysis. *M. penetrans* proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under the conditions described by Laemmli (19). In most cases, 12% gels were used. Fractionation of LAMPs was done by the Triton X-114 (TX-114) phase-partitioning method (9). Membrane and cytosolic protein fractionation and TX-100 partitioning were also performed using published methods (28). For immunoblot analysis, proteins were transferred to nitrocellulose membranes after electrophoresis (38) and were detected by antibodies.

Peptide sequencing of the 46-kDa protein. The 46-kDa protein was extracted from strain HF-2 by TX-114 phase partitioning and was purified by SDS-PAGE. The 46-kDa protein band was excised from the gel and treated with *Staphylococcus aureus* V8 protease (Sigma-Aldrich, Steinheim, Germany). The digested peptide fragments were separated using SDS–15% PAGE gels, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.), and stained with Coomassie blue. Two major peptide fragment bands (approximately 15 and 8 kDa) were excised from the membrane, and the N-terminal sequences were analyzed by Edman degradation. Peptide sequencing was performed at APRO Life Science Institute (Tokushima, Japan) using the Procise 494 HT protein-sequencing system (Applied Biosystems, Foster City, Calif.).

DNA-sequencing analysis of pepIMP13 gene region of strain GTU. Genomic DNA was isolated from a 5-ml culture of *M. penetrans* strain GTU (QIAmp DNA Mini Kit; Qiagen, Hilden, Germany) and was used as a template for PCR amplification. The pepIMP13 gene region was amplified by PCR with the oligonucleotide primers IMP13-CF (GCAACTGCAGATGGCAACAA) and IMP13-CR (ATGGCACCGCCTGATAACAT) using a high-fidelity DNA polymerase, Pyrobest (Takara, Tokyo, Japan). The amplified fragments were ligated into the *Sma*I site of the pUC19 plasmid. Sequencing of cloned PCR fragments was performed by a primer-walking method with the Big Dye terminator cycle-sequencing kit and the DNA sequencer PRISM 310 (Applied Biosystems). To avoid the artificial factor of mutations generated in the course of PCR, three independent plasmid clones were sequenced, and the data were integrated.

RNA isolation and slot blot analysis. Total cellular RNA was isolated from mid-logarithmic-phase cultures of both *M. penetrans* GTU and HF-2 (RNeasy Mini Kit; Qiagen). Twenty micrograms of total RNA were treated with 10 U of RNase-free DNase (Takara) for 1 h at 37°C. One microgram of RNA was diluted in RNase-free denaturation solution containing formamide (66%), formaldehyde (8%), and MOPS (morpholinepropanesulfonic acid) buffer and heated at 65°C for 5 min. The RNAs were blotted onto a GeneScreen Plus membrane (NEN, Boston, Mass.) by vacuum, using the Convertible Filtration Manifold System (Invitrogen, Carlsbad, Calif.). The oligonucleotide probes P35-P (CCC TTAATTGCAGCAGAATCACC) for the *p35* gene transcript and P42-P (TTA AATCTGTTTCAGCTGTAATTT) for the *p42* gene transcript were enzymatically labeled with digoxigenin (DIG)-labeled ddUTP by using the DIG oligonucleotide 3′-end labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The blots were hybridized with the probes at 37°C for 16 h in DIG Easy Hyb solution (Roche Diagnostics). After incubation, the hybridization signals were detected with the DIG luminescence detection kit (Roche Diagnostics) and visualized by exposure to medical X-ray film (Fuji Film, Tokyo, Japan).

Primer extension analysis. Two oligonucleotide primers, P42-EXT3 (GCAA CAATCCCAAAAGCT) and P42-EXT4 (TCCATTTCCATTATTGTTAT), were used for primer extension analysis to identify the 5′ end of the *p42* gene transcript. The primer extension reaction was performed using the primer extension system-avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, Wis.). Briefly, the primers were end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Ten micrograms of total RNA prepared from *M. penetrans* HF-2 was mixed with each of the labeled primers in the AMV primer extension buffer and heated at 58°C for 20 min. The mixtures were cooled at room temperature for 10 min, and the AMV reverse transcriptase mix solution, containing 1 U of enzyme, was added. The extension reaction mixtures were incubated at 42°C for 30 min, 20 μl of loading dye was added, and the extension was terminated by heat inactivation at 90°C for 10 min.

The *p42* gene region was cloned from *M. penetrans* HF-2 genomic DNA by PCR with primers IMP13-CF and IMP13-CR. The amplified *p42* gene fragment was purified with the QIAquick PCR purification kit (Qiagen) for use as a template for DNA-sequencing reactions. The same primers that were used for primer extension were also used for sequencing. The *fmol* DNA-sequencing system (Promega) was used for DNA-sequencing reactions. The products of primer extension and sequencing reactions were analyzed by electrophoresis, using a 5% polyacrylamide sequencing gel. After electrophoresis, the gel was dried and exposed to X-ray film overnight to visualize the products.

Nucleotide sequence accession numbers. The complete genome sequence of *M. penetrans* strain HF-2 was recently determined by our group in collaboration with Kitasato University. The detail of the genome sequence analysis will be described in another paper (33a). The whole-genome sequence data for *M. penetrans* HF-2 will appear in the DDBJ, EMBL, and GenBank databases under accession numbers AP004170, AP004171, AP004172, AP004173, and AP004174. The nucleotide sequence data for the *p42* gene region of *M. penetrans* GTU will also appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB084070.

RESULTS

Comparison of protein profiles between *M. penetrans* strains. The protein electrophoresis patterns of the *M. penetrans* type strain, GTU, and isolate HF-2 were compared to identify molecular polymorphisms between *M. penetrans* strains (Fig. 1A). The major surface lipoprotein P35 of *M. penetrans* was present in both strains. However, a 34-kDa protein was present only in GTU, and a 46-kDa protein was found only in HF-2. The electrophoresis patterns were reproducible when the protein samples were prepared from cultures of other single colonies of each strain. Immunoblotting analysis gave a similar result (Fig. 1B and C). MAb 7 detected the P35 protein in both strains (Fig. 1B), while serum 6 (anti-HF-2) reacted with many proteins of both strains (Fig. 1C). Although the strongest reaction was observed against the P35 protein, serum 6 clearly detected a 46-kDa protein only in HF-2.

Characterization of 34- and 46-kDa proteins. We thought that the 34- and 46-kDa proteins were variable surface lipoproteins of the P35 family (products of *mpl* genes) because of their different expression patterns among *M. penetrans* strains. To confirm this, we characterized the properties of the 34- and 46-kDa proteins. First, we fractionated the proteins of *M. penetrans* strains GTU and HF-2 by a TX-114 extraction proce-

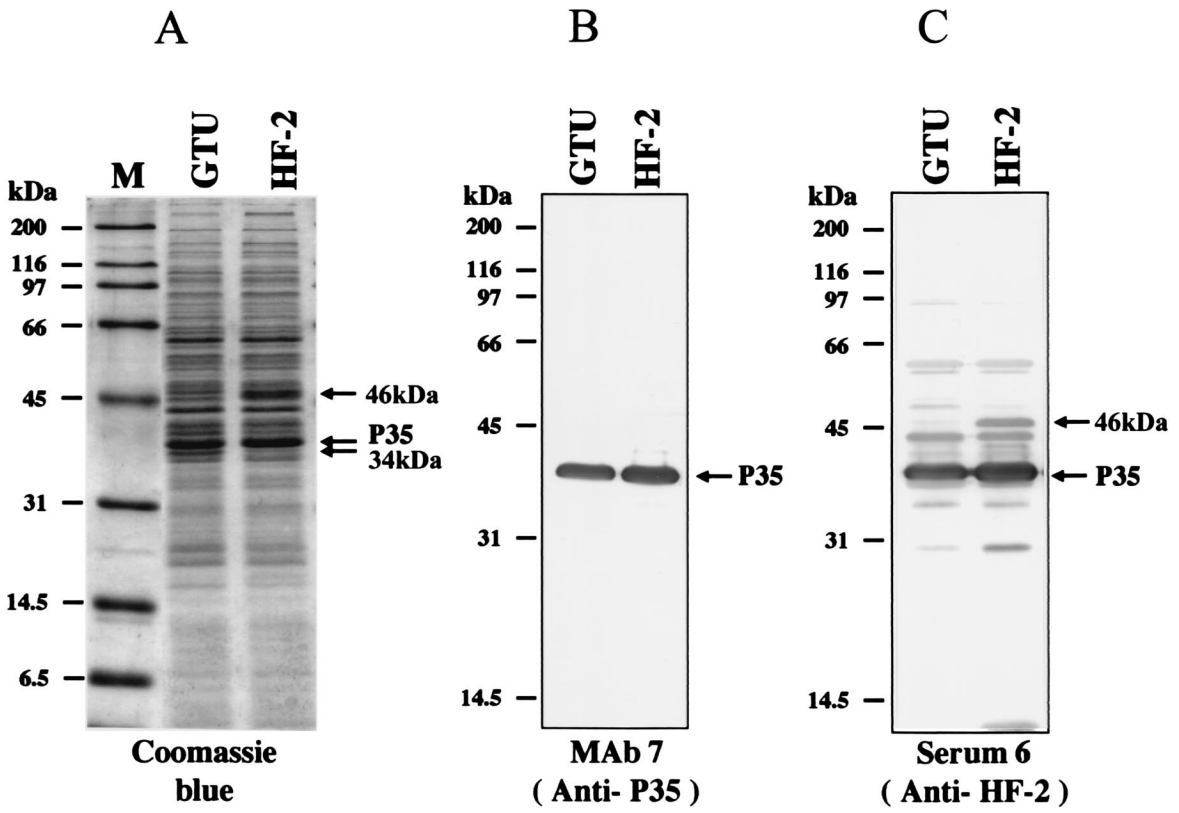

FIG. 1. Analysis of protein profiles of *M. penetrans* strains GTU and HF-2. (A) Coomassie blue-stained SDS–12% PAGE of total proteins of *M. penetrans*. A protein molecular mass marker (Bio-Rad, Hercules, Calif.) is in lane M, and the masses are shown on the left. The P35 and 34- and 46-kDa proteins are indicated. (B) Immunoblot analysis of *M. penetrans* proteins. Anti-P35 MAb (MAb 7) was used for detection. The P35 protein is indicated. (C) Immunoblot analysis with the polyclonal serum 6 (anti-HF-2). The positions of the P35 and 46-kDa proteins are indicated.

dure that is used to separate LAMPs of *M. penetrans* (9, 33). The 34- and 46-kDa proteins were found in the TX-114 detergent phase together with the P35 protein (Fig. 2). Differences between the TX-114 extraction profiles of strains GTU and HF-2, in addition to the 34- and 46-kDa proteins, were also observed (Fig. 2). The GTU strain had at least two distinct bands, between 36 and 40 kDa, that were missing in the HF-2 profiles. One of these bands might be the P38 lipoprotein previously reported to be present in strain GTU (24, 33). We then analyzed the GTU and HF-2 proteins by membrane and cytosolic separation or by the TX-100 extraction method (28). In these analyses, the 34- and 46-kDa proteins were separated into membrane or TX-100-soluble fractions, along with the P35 protein (data not shown). These results suggested that the 34- and 46-kDa proteins are hydrophobic proteins with properties similar to those of the P35 lipoprotein.

The 46-kDa protein was chosen for amino acid-sequencing analysis. Initial attempts to analyze the intact 46-kDa protein by Edman degradation were unsuccessful, probably because of N-terminal blockage. Therefore, the 46-kDa protein was digested with protease V8 and peptide fragments of ~15 and 8 kDa were sequenced by Edman degradation. The sequence LNDKVSLAGS was obtained from the N terminus of the 15-kDa fragment. Two sequences, TDLKITVDGG and FNFNIG IDST, were obtained from the analysis of the 8-kDa fragment, suggesting that it was a mixture of two peptides. These partial amino acid sequences of the 46-kDa protein matched the de-

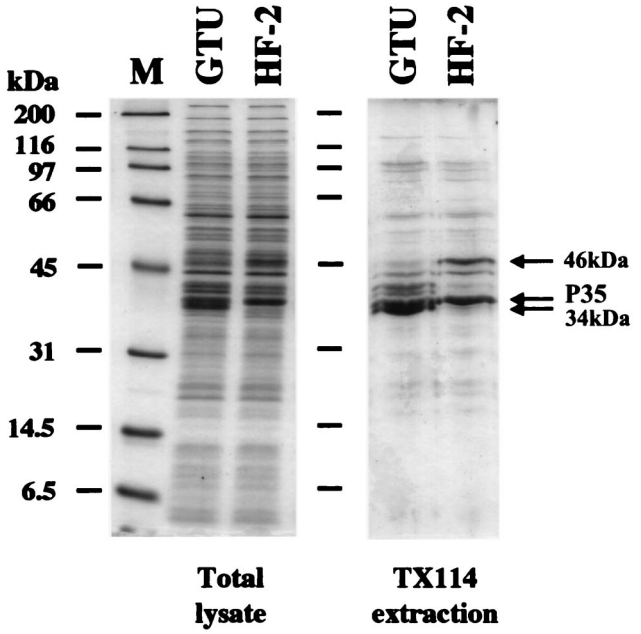

FIG. 2. Analysis of LAMP profiles of *M. penetrans*. The total cell lysate and TX-114 phase-fractionated proteins of *M. penetrans* strains GTU and HF-2 were analyzed by SDS–12% PAGE. The proteins were stained with Coomassie blue. The positions of the P35 and 34- and 46-kDa proteins are indicated. A protein molecular mass marker is in lane M, and molecular masses are on the left.

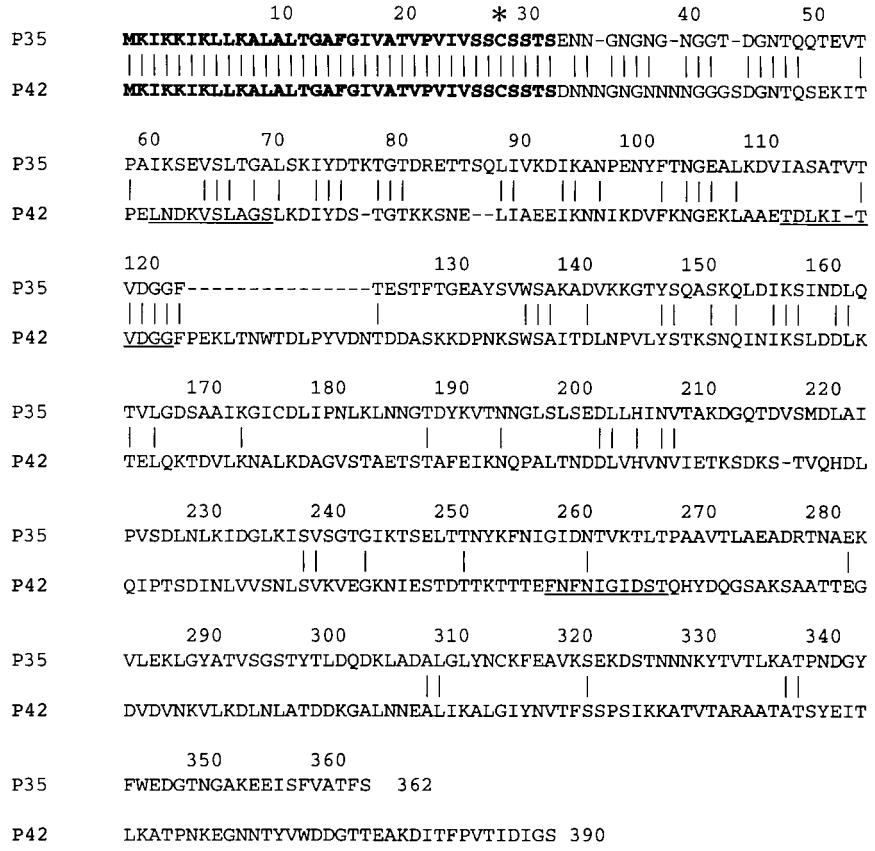

FIG. 3. Alignment of amino acid sequences of P35 and 46-kDa (pepIMP13) proteins. The 46-kDa protein was designated P42 based on the calculated molecular mass. The three partial amino acid sequences of P42 determined by Edman degradation of digested peptide are underlined. The signal sequence of the P35 family lipoprotein is shown in boldface characters. The cysteine residue marked with an asterisk is the potential binding site of fatty acid chains.

duced amino acid sequence of a previously reported putative *mpl* gene, pepIMP13 (24). The previously reported pepIMP13 sequence is derived from strain GTU (GenBank accession no. AJ006698) and is truncated at the N terminus. To obtain the full-length pepIMP13 gene sequence, the complete genome sequence of strain HF-2 (see Materials and Methods) was searched. The pepIMP13 gene was found in the *M. penetrans* HF-2 genome as an open reading frame (ORF), designated MYPE6630 in accordance with the nomenclature system of the genome project. The ORF MYPE6630 was located at nucleotide positions 850970 to 852142 of the genome. The amino acid sequence of the 46-kDa protein was deduced from the MYPE6630 sequence and was compared to that of the P35 protein (Fig. 3). The full-length 46-kDa protein (the pepIMP13 protein) consists of 390 amino acids (aa) and possesses an N-terminal sequence identical to that of the P35 protein. This 30-aa N-terminal sequence is thought to be the signal peptide of the P35 family lipoproteins (24). The calculated molecular mass of the 46-kDa protein was 41,814 Da, somewhat smaller than that estimated by SDS-PAGE. We designated the 46-kDa protein P42, based on the calculated molecular mass.

Analysis of the nucleotide sequence of the *p42* gene and flanking region. To investigate the mechanism that underlies the antigenic variation of the 46-kDa protein (P42), we analyzed the nucleotide sequences of the *p42* gene of strains HF-2 and GTU. The nucleotide sequence containing the *p42* gene and flanking regions of HF-2 was obtained from complete genome sequence data. Using this sequence, we designed PCR primers to amplify the corresponding region from strain GTU. The PCR-cloned *p42* gene region from GTU was sequenced by the primer-walking method and was compared to the sequence from HF-2. There were no differences in either the *p42* structural gene itself or the downstream region between the two strains. However, sequence differences were found in the upstream region of the *p42* gene (Fig. 4). The difference began 238 bp upstream from the ATG start codon of the *p42* gene and was 135 bp in length (Fig. 4A). It was found that the 135-bp DNA sequence was inverted between the GTU and HF-2 strains and was flanked by 12-bp inverted-repeat sequences (Fig. 4A), suggesting that the inversion might be produced by site-specific recombination between 12-bp inverted-repeat sequences. We hypothesized that this inversion was the cause of the antigenic variation of the P42 protein and would affect the structure of promoter or other regulatory sequences. As expected, inspection of the 135-bp inverted DNA sequences revealed the presence of a promoter-like sequence in this region. The promoter-like sequence was very similar to the experimentally characterized promoter of the *p35* gene (24). In the region containing a −10-like consensus sequence and a +1 transcription start site, the *p42* promoter-like sequence and the *p35* promoter were identical (Fig. 4B). In strain HF-2, the

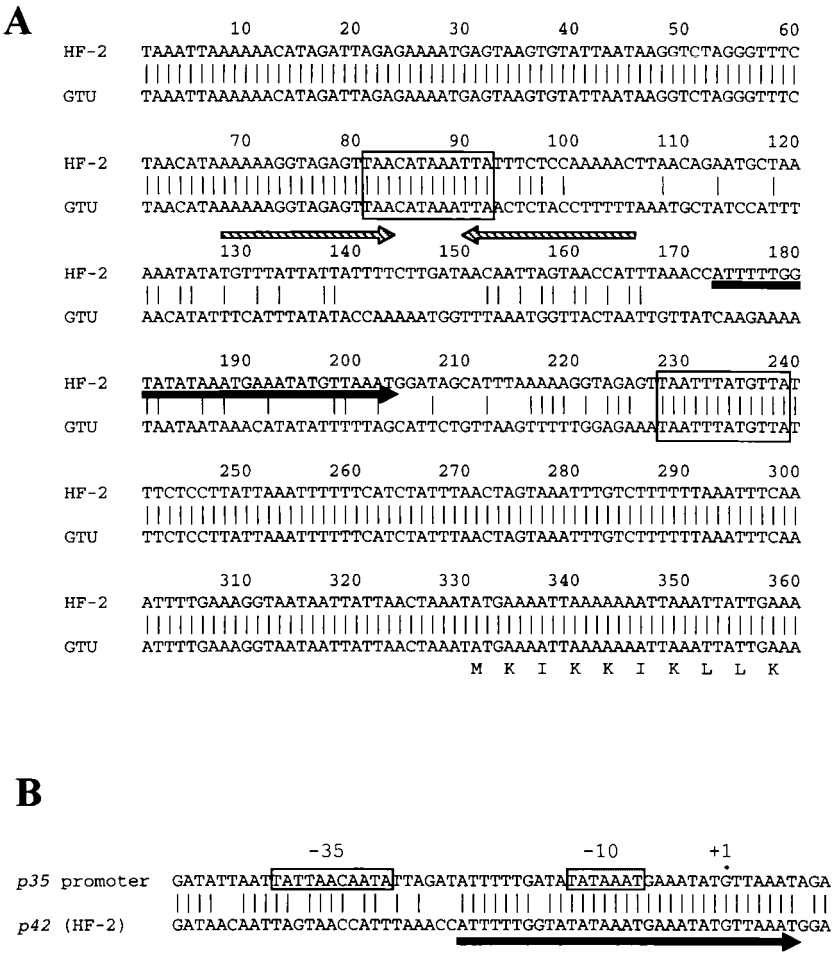

FIG. 4. Nucleotide sequence of *p42* gene upstream region. (A) Comparison of *p42* gene upstream regions from strains GTU and HF-2. The 12-bp inverted-repeat sequences adjacent to the 135-bp inverted DNA region (see the text) are boxed. The solid arrow indicates the core of the promoter sequence in the HF-2 sequence (panel B). The hatched arrows indicate a 16-bp inverted repeat in the GTU sequence. The deduced amino acid sequence of the P42 protein (the first 10 aa) is shown. The nucleotides are counted from 330 bp upstream from the ATG start codon. (B) Comparison of *p42* gene upstream sequence with promoter from *p35* gene. The −10 and − 35 consensus sequences of the *p35* promoter are boxed (24). The probable start site of *p35* transcription is also marked +1. The highly conserved region between the *p35* and *p42* promoter sequences is underlined with an arrow.

promoter-like sequence was oriented to transcribe the *p42* gene, but this orientation was inverted in strain GTU (Fig. 4A). The directions of the promoter-like sequence (toward the *p42* structural gene or not) were consistent with the expression patterns of the P42 protein in strains GTU and HF-2 (Fig. 1 and 2).

The existence of the 12-bp inverted repeat that flanked the promoter-like sequence raised the possibility that inversion of the promoter-like sequence would be reversible. Therefore, we analyzed the shotgun clones that were used in the whole-genome sequencing of strain HF-2 and found a clone that carried the *p42* promoter region in the inverted orientation that was identical to the promoter region found in strain GTU (data not shown). This finding strongly suggests that the *p42* promoter region is inverted in the strain HF-2 population during culture.

A characteristic 16-bp inverted repeat that partly overlapped one of the 12-bp inverted-repeat sequences adjacent to the 135-bp DNA was also found (Fig. 4A and 5A). This 16-bp inverted repeat seems to form a hairpin structure that resembles a terminator sequence (Fig. 5B). The calculated ΔG of this structure is −22.5 kcal/mol. The 16-bp inverted repeat can be formed only in the promoter orientation that is characteristic of GTU. In HF-2, DNA inversion between 12-bp inverted-repeat sequences disrupts the formation of the 16-bp inverted repeat and the hairpin structure (Fig. 5).

Transcriptional analysis of *p42* gene. To confirm whether the inversion of promoter-like sequence actually affects the expression of the *p42* gene, we analyzed the transcription of the *p42* gene. Total RNAs were isolated from strains GTU and HF-2 and analyzed by slot blot hybridization (Fig. 6). Using the oligonucleotide probe for the *p35* gene transcript, the hybridization signals from both strains were observed. In contrast, a hybridization signal was obtained only from HF-2 with the probe for the *p42* transcript. These results indicate that *p42* transcription occurs in HF-2 but not in GTU. To further characterize the *p42* promoter-like region, we performed primer extension analysis. Using the oligonucleotide primer P42-

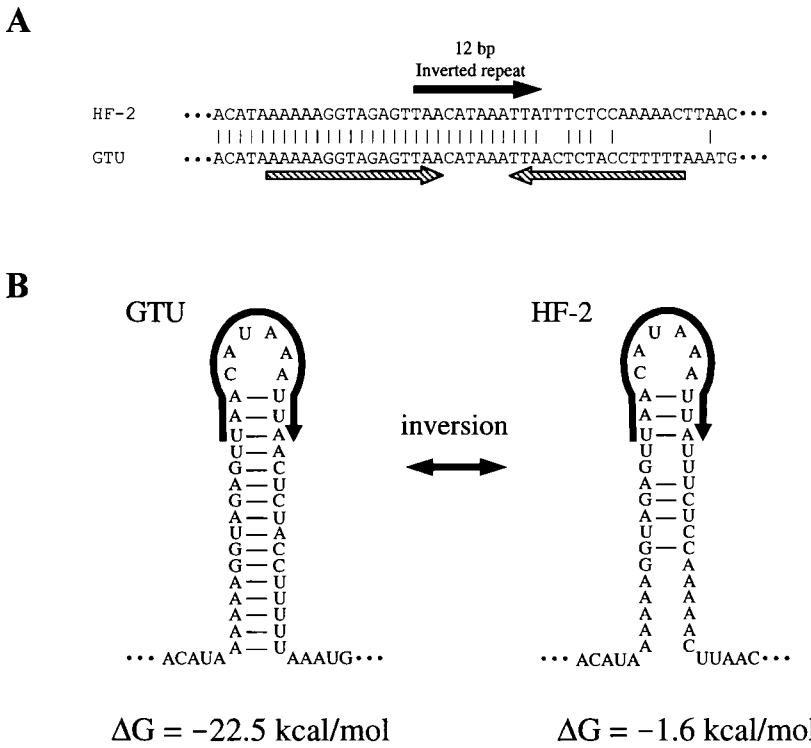

FIG. 5. Structure of 16-bp inverted-repeat sequence located upstream of *p42* gene. (A) Nucleotide sequences of 16-bp inverted-repeat regions from strains HF-2 and GTU. The nucleotide sequence corresponds to nucleotides 63 to 110 in Fig. 4A. The solid arrow indicates a 12-bp inverted-repeat sequence adjacent to a 135-bp inverted DNA sequence. The hatched arrows indicate the 16-bp inverted repeat that is formed only in the GTU sequence. (B) Hairpin structures of the 16-bp inverted repeat. Formation of the hairpin structure is disrupted in the HF-2 sequence by DNA inversion between 12-bp inverted-repeat sequences. The hairpin structures are shown as RNA sequences. The calculated Δ*G*s of these hairpin structures are shown at the bottom.

EXT3, the extension product was obtained with RNA from the HF-2 strain (Fig. 7A). The probable transcriptional start site was identified 137 bp upstream from the ATG start codon. This position corresponds to 3 bp upstream of the previously reported transcriptional start site of the *p35* gene (24) (Fig. 7B). The primer extension product was also obtained with the other oligonucleotide primer, P42-EXT4, and the same start site was identified (data not shown). These results indicated that the *p42* promoter-like sequence is an active promoter in *M. penetrans* cells.

Organization of *mpl* genes in *M. penetrans* HF-2 genome. It was demonstrated that the promoter inversion was the cause of antigenic variation of P42 protein. The involvement of a promoter inversion mechanism in *p42* gene expression raised the question of whether other *mpl* gene expression is regulated by a similar mechanism. To answer this question, we searched for all of the *mpl* genes in the whole-genome sequence of *M. penetrans* HF-2, using the *p35* gene sequence as a query. Homology search revealed that the *M. penetrans* HF-2 genome contains at least 38 *mpl* genes, including *p35* and *p42*. The deduced amino acid sequences of these *mpl* genes showed homology to that of the P35 protein (34 to 70% identity) and had almost identical *mpl* signal sequences in the N termini (data not shown). The signal sequences contain one cysteine residue (Fig. 3) that is thought to be the site which is modified with fatty acids, as are other known surface lipoproteins (5, 45). In contrast to the signal peptide sequences, the rest of the amino acid sequences of the *mpl* genes were of low homology. The homology search also detected six other genes (ORFs MYPE7020, -7030, -7040, -7050, -7060, and -7070) that were similar to the *p35* gene, with homology ranging from 28 to 44% identity (data not shown). However, the deduced amino acid

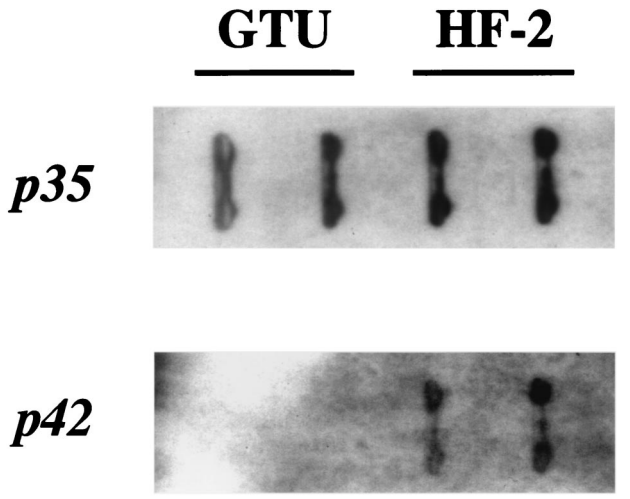

FIG. 6. RNA slot blot analysis of *p35* and *p42* gene transcription in *M. penetrans* strains GTU and HF-2. RNA samples from *M. penetrans* strains GTU and HF-2 were blotted in duplicate. The blots were hybridized with the oligonucleotide probes for *p35* and *p42* transcripts as indicated on the left.

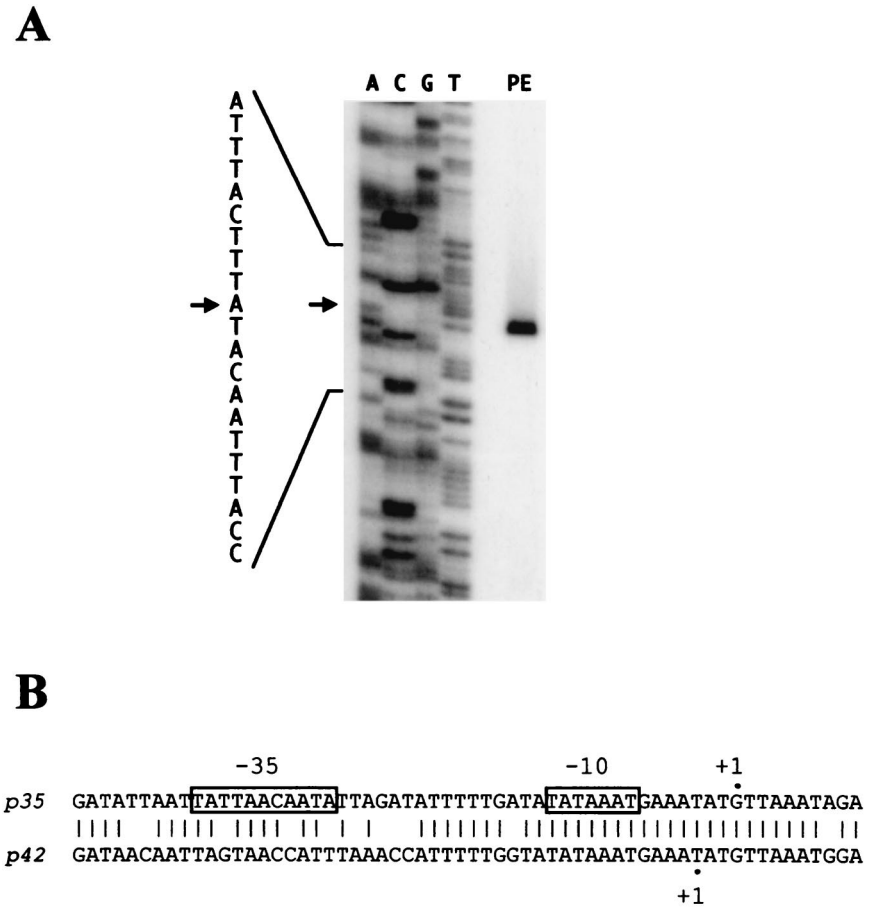

FIG. 7. Primer extension analysis of *p42* gene transcription. (A) Autoradiogram of sequencing gel used to analyze the primer extension product. PE indicates the primer extension product obtained with *M. penetrans* HF-2 RNA as a template. The corresponding nucleotide sequence is shown on the left, and the probable start site is indicated by arrows. (B) Comparison of the transcriptional start sites in *p35* and *p42* promoters. Probable start sites determined by primer extension of the *p35* promoter (24) and the *p42* promoter (panel A) are indicated as +1. The putative −10 and −35 consensus regions are boxed.

sequences of these six ORFs did not have the P35 signal peptide sequence in their N termini, so we did not include these genes among the members of the *mpl* gene family.

The 38 *mpl* genes clustered at three positions of the genome (Fig. 8). The largest cluster was located at nucleotide positions 830000 to 882000 of the genome (Fig. 8A). This 50-kb region contained 30 *mpl* genes and 6 non-*mpl* genes. The *p35* and *p42* genes were found in this cluster (MYPE6810 and -6630). The second cluster was found in a 20-kb DNA region at nucleotide positions 335500 to 355500 (Fig. 8B). In this region, two pairs of *mpl* genes are separated by five non-*mpl* genes. The last *mpl* gene cluster was located at nucleotide positions 966000 to 975000 (Fig. 8C). In this region, four *mpl* genes were found within 9 kb of DNA. In all three clusters, all the identified *mpl* genes were oriented in the same direction. The previously identified *mpl* genes, *p34A* (pepIMP14), *p30* (*p33*), and *p38* from strain GTU (24, 33), were also found in the largest cluster (Fig. 8). However, the previously reported pepIMP12 gene of GTU (24) (GenBank accession no. AJ006697) was not found in the HF-2 genome sequence. To determine whether the missing pepIMP12 gene in the HF-2 genome was the result of genomic polymorphism between strains GTU and HF-2, we used PCR to amplify the pepIMP12 gene sequence with primers IMP12-F (TAATATTAAATCTTTAGATG) and IMP12-R (AATTAAATGATAAAGTTAGC). Unexpectedly, the pepIMP12 sequence was not amplified from our GTU and HF-2 strains (data not shown). The reason is as yet unknown. It was also found that three *mpl* genes (MYPE6520, -6500, and -7380) possessed frameshift mutations in their sequences and were disrupted by internal stop codons. Of 14 non-*mpl* genes that exist in the three *mpl* gene clusters, MYPE6600, -6610, and -6620 showed relatively high homology to the transport system permease protein P69 (*Mycoplasma hyorhinis*), the ABC transporter ATP-binding protein (*Mycoplasma pulmonis*), and the high-affinity transport system protein P37 (*M. hyorhinis*), respectively. However, the other non-*mpl* genes did not show any significant homology to other known proteins (data not shown).

To investigate the mechanism involved in the expression of these *mpl* genes, we analyzed the intergenic sequences of these clusters. This showed that the intergenic sequences are well conserved and have structures similar to that of the *p42* promoter region, namely, 133- to 138-bp DNA sequences were flanked by 12- to 14-bp inverted-repeat sequences, suggesting that these regions are also invertible DNA (data not shown). Specifically, it was also noted that the 133- to 138-bp DNA

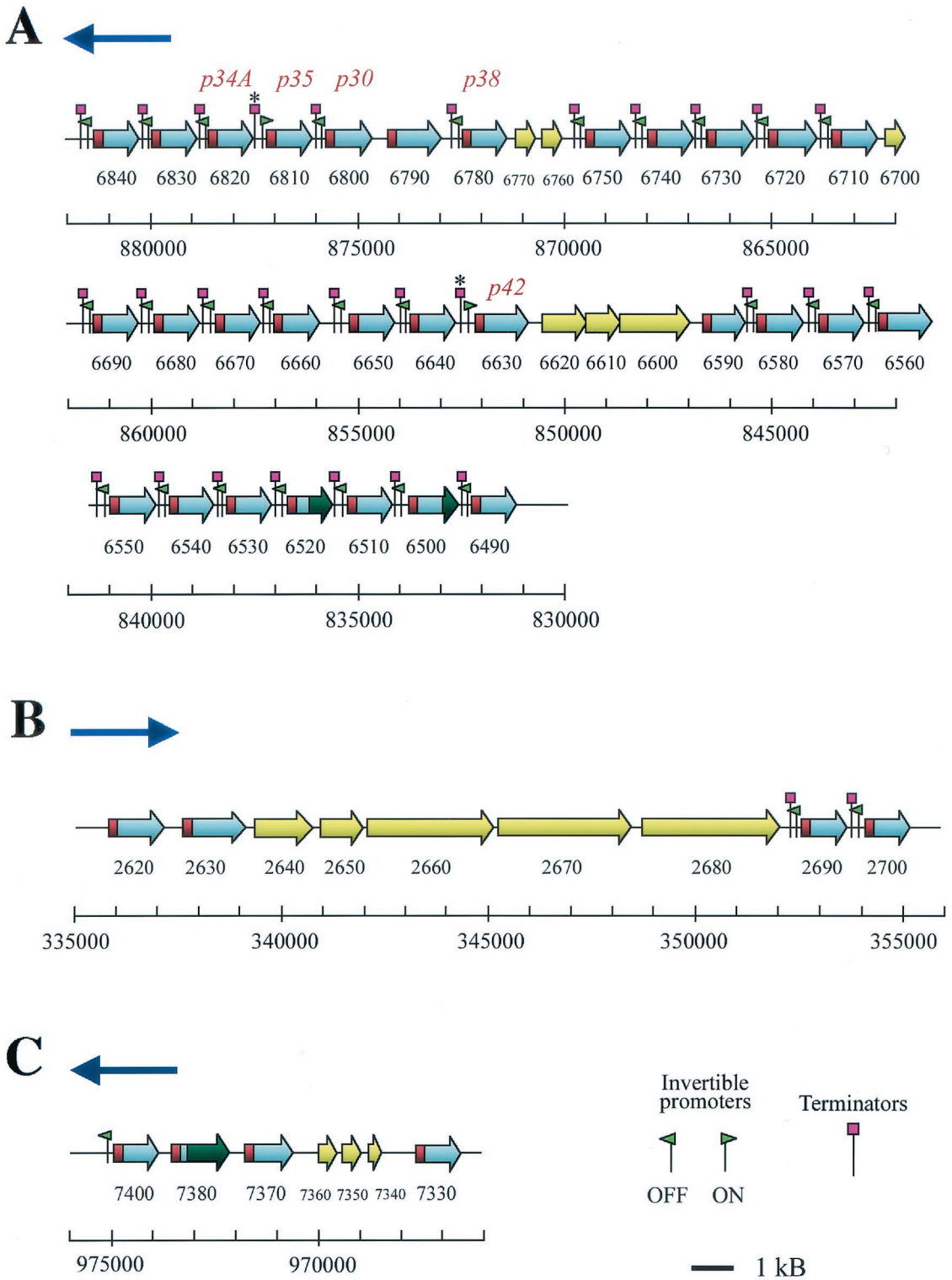

FIG. 8. Genomic organization of *mpl* genes in *M. penetrans* HF-2. Three *mpl* gene clusters are illustrated. The gray and red boxes in the arrows represent *mpl* genes. The red regions indicate signal sequence regions of *mpl* genes. The yellow arrows represent non-*mpl* genes. The numbers under the genes are MYPE serial numbers that are used to describe *M. penetrans* ORFs in the whole-genome sequencing analysis. The approximate nucleotide positions of *mpl* genes in the genome are also shown with the scale. The genes MYPE6500, -6520, and -7380 have frameshift mutations and are disrupted by internal stop codons. In these genes, the regions after the stop codons are shown as dark green. The small green arrowheads indicate invertible promoter-like sequences and their directions. The small purple squares represent 16-bp inverted-repeat terminator-like sequences. Two terminator-like sequences (upstream of the *p35* and *p42* genes that are marked with asterisks) are inactivated because of promoter inversions (see the text). The genes for P35, P42, and previously reported lipoproteins (P30, P38, and P34A) are labeled. The blue arrows above each cluster indicate relative directions in the genome.

sequences were fairly well conserved but the sequences of the 12- to 14-bp inverted repeats were not conserved between intergenic sequences (data not shown). In most cases, the 133- to 138-bp DNA regions contained a highly conserved promoter-like sequence that was similar to those of the *p35* and *p42* promoters. Although the −35-like consensus regions of these promoter-like sequences were not conserved, the −10-like consensus and transcriptional-start-site regions were almost identical to those of the *p35* and *p42* promoters (data not shown). As the lack of a −35-like consensus sequence in mycoplasmal promoters has been reported in *Mycoplasma pneumoniae* (44), these promoter-like sequences may be active. These results suggested that most of the *mpl* genes possessed independent invertible promoter-like sequences in their upstream regions (Fig. 8). (See also the genome sequence data for *M. penetrans* HF-2 and their annotation. This will appear in databases.)

In summary, we have identified the invertible promoter-like sequences for 31 *mpl* genes (including *p35* and *p42*) but not those for 7 other *mpl* genes (MYPE 6790, -6590, -2620, -2630, -7380, -7370, and -7330) (Fig. 8). Interestingly, of these 31 invertible promoter-like sequences, only two (for the *p35* and *p42* genes) were in the ON orientation. The other promoter-like sequences were in the OFF orientation (Fig. 8). These patterns of the promoter-like sequences of *mpl* genes are consistent with the expression patterns of P35 family lipoproteins of *M. penetrans* HF-2 (i.e., P35 and P42 are major lipoproteins expressed in strain HF-2) (Fig. 2).

We have also identified 16-bp inverted-repeat sequences upstream of all invertible promoter-like sequences, with the exception of MYPE7400 (Fig. 8). These 16-bp inverted-repeat sequences were homologous to those found upstream of the *p42* gene of GTU (Fig. 4 and 5).

DISCUSSION

In this report, we have confirmed that the LAMP profiles of *M. penetrans* are different in strains GTU and HF-2. The 46-kDa protein (P42 lipoprotein) is present only in strain HF-2, and the 34-kDa protein is found only in strain GTU. Röske et al. also recently reported the difference between the LAMP profiles of GTU and HF-2 (33). They reported that the P35 lipoprotein is found in both strains, as observed in our study. However, the 34-kDa protein found in our GTU is not present in their GTU. In addition, a 40-kDa protein is present in their HF-2 strain, but this is smaller than the P42 of our HF-2. Although we cannot fully explain the reason for these differences, the presence and absence of a 34-kDa protein in GTU may be due to antigenic variation between two laboratory strains caused by phase-variable expression of *mpl* genes during the culture passages. On the other hand, in HF-2, the electrophoresis patterns of LAMPs are essentially identical in both studies. Judging from the fact that the calculated molecular mass of P42 is 41.8 kDa, it is possible that the 40-kDa protein in Röske's HF-2 is the P42 protein in our HF-2. The size discrepancy may be caused by different gel conditions and size marker systems.

We have shown that the P42 protein in our HF-2 strain is the product of the putative *mpl* gene pepIMP13 and found that promoter inversion was the cause of the antigenic variation of this protein between GTU and HF-2. Although the reversible inversion event of this promoter region has not yet been demonstrated directly, the data presented in this study strongly suggest that the promoter-containing 135-bp DNA region is an invertible element that functions as a genetic switch. DNA inversion is a mechanism commonly used by bacteria to regulate gene expression and generate antigenic variation (8, 15). There are many examples of DNA inversion systems, including the Hin system for flagellar gene expression of *Salmonella enterica* serovar Typhimurium and the type 1 fimbrial gene (*fim*) expression by *Escherichia coli* (1, 52). DNA inversion systems have also been reported in some mycoplasma species (22, 34, 36). The characteristic feature of the promoter inversion of the *p42* gene is the length of the invertible DNA region. To our knowledge, the 135-bp region is the shortest invertible element discovered so far, although an invertible promoter region consisting of 192 bp of DNA was recently identified in *Bacteroides fragilis* (17). One hundred thirty-five base pairs seems quite short for an invertible element that functions as a frequent genetic switch, because an inversion reaction requires the looping of DNA and close contact between two recombination sites. Looping of the 135-bp DNA results in tight DNA curvature and might not occur frequently. One solution to this problem is a participation of the bacterial histone-like protein HU that mediates tight DNA curvature (16, 41). It has been reported that DNA ring closure by self-ligation does not occur in 126-bp linear DNA, but it occurs in the presence of the HU protein (16). It is possible that the HU-like protein stimulates 135-bp DNA inversion of the *p42* promoter region. The gene for the HU-like protein was found in the *M. penetrans* genome (MYPE2490). Another possible factor in inversion stimulation is DNA bending. Since the *p42* promoter region is highly AT rich (Fig. 4), DNA bending is suggested in this region (40). The DNA bending may directly enhance the formation of DNA loops or binding of some regulatory proteins that support DNA looping (27, 39). In the inversion of the *p42* promoter, the main enzymes that directly mediate the recombination reaction are not yet known. However, we could identify two candidate ORFs, MYPE2900 and -8180, for invertase genes in the *M. penetrans* HF-2 genome. These ORFs have homology to the integrase-recombinase family of genes that has been identified in the *Ureaplasma urealyticum*, *M. pulmonis*, and *Bacillus subtilis* genomes (4, 11, 18).

The analysis of the genomic organization of the P35 family lipoprotein genes revealed that there were 38 *mpl* genes in the *M. penetrans* HF-2 genome. These genes were clustered at three positions in the genome (Fig. 8). The surprising finding was that most of the *mpl* genes possess the promoter-like sequences that seem to be invertible (Fig. 8). These promoter-like sequences may be active because of their high sequence similarity to the functional *p35* and *p42* promoters and may express downstream genes when they are turned on by inversion. The presence of independent invertible promoters may allow every *mpl* gene to be switched ON↔OFF simply. This conclusion, based on the analysis of *mpl* gene structure, accounts well for the phenotypic observations of lipoprotein variation that show frequent, independent phase variation of the *mpl* gene products (24, 33).

The lipoprotein gene families that mediate surface antigenic variations have been reported in several mycoplasma species.

These are the *vlp*, *vsp*, *vsa*, *pMGA*, *vlh*, and *vpma* families of *M. hyorhinis*, *Mycoplasma bovis*, *M. pulmonis*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae,* and *Mycoplasma agalactiae*, respectively (4, 6, 12, 13, 23, 26). These gene families employ different molecular mechanisms to change the expression patterns of lipoprotein genes. The *vlp* and *pMGA* families employ nucleotide insertion and deletion mechanism in their promoter regions (7, 12). The *vlh* family changes gene expression by gene conversion (26). The *vsp* and the *vsa* families utilize DNA inversions for modulation of lipoprotein gene expression (22, 34, 35). Although the *vsp* and *vsa* families developed the sophisticated genetic-switch systems with DNA inversions, these systems are not like the *mpl* gene system of *M. penetrans*. In the *vsp* and *vsa* systems, the multiple lipoprotein genes are also clustered in the genome, but these genes are not oriented in the same direction and there is only one active promoter in the cluster. Only a selected lipoprotein gene that is properly connected to an active promoter can be expressed in this locus. DNA inversions exchange the lipoprotein genes that connect to active promoters and generate antigenic variations (22, 34). In contrast to the *vsp* and *vsa* systems, the *mpl* gene system of *M. penetrans* is a system that employs multiple invertible promoters. This system is a novel mechanism for the generation of antigenic variation of mycoplasmal lipoproteins.

The use of multiple promoters seems to be an effective way of generating antigenic variation. The multiple promoters can express lipoprotein genes in a large number of combinations, generating additional antigenic diversity. However, in this system, some problems are predicted to exist. One problem is the inversion frequency of promoters. Although 31 invertible promoters were predicted in *mpl* gene clusters, only two promoters (*p35* and *p42*) are in the ON orientation in the HF-2 genome (Fig. 8). If all of the promoters invert randomly and at the same probability forward and backward, the number of ON promoters may be nearly half of the total promoters. So it is likely that there are some biases in promoter inversion reactions, such as selective inversion of some promoters or directional bias in ON↔ OFF reactions. Therefore, the frequency of each promoter inversion must be assessed by further study. The other problems come from the structure of the *mpl* gene clusters. In the *mpl* gene clusters, all genes are oriented in the same direction and every *mpl* gene is about 1 kb in length (Fig. 8). When one of the promoters turns on in these clusters, it is likely that the transcription from the ON promoter may reach downstream genes, reading through the intergenic sequences, and produce polycistronic mRNA containing several *mpl* gene sequences. This may cause the expression of some downstream genes even if the promoter of downstream genes is in the OFF orientation. If this situation really occurred, it would not make sense for *mpl* genes to have independent invertible promoters. Moreover, if the transcription was initiated from promoters in the OFF orientation to upstream genes, it might produce a large number of useless mRNAs that would not be translated because the *mpl* genes are not bi-directional. The production of excess untranslated mRNA (antisense *mpl* RNAs) is not advantageous for optimum use of cell metabolic energy. However, the *mpl* gene system seems to possess the mechanisms to overcome these serious problems. We could identify conserved 16-bp inverted-repeat sequences in the regions just upstream of most invertible promoters (Fig. 8) that were consistent with the pattern of terminator sequences (Fig. 5), although their activity has not been demonstrated experimentally. In addition, it was found that the formation of these 16-bp inverted repeats depended upon the promoter inversions (Fig. 5). If these 16-bp inverted-repeat sequences really function as terminators, the *mpl* gene system will work effectively. An explanation of the function of the 16-bp inverted-repeat sequences, using the *p42* promoter region as an example, is shown in Fig. 9. When the promoter is in the OFF orientation (in strain GTU), the active form of the terminator sequence (the 16-bp inverted repeat) exists just upstream of the promoter. This active terminator blocks transcription both from the *p42* gene promoter and from upstream if there are promoters in the ON orientation. In this configuration, the *p42* gene is kept OFF, regardless of the ON↔ OFF orientation of the upstream promoters. Alternatively, when the promoter turns on by inversion (in strain HF-2), the terminator structure is divided into two parts and inactivated. In this configuration, the transcription of the *p42* gene can initiate from the *p42* promoter. Also, if there was transcription from the upstream ON promoter, this might read through the inactive terminators and transcribe the *p42* gene, resulting in efficient expression of the *p42* gene. Although our model requires experimental proof, the coupling of the promoter inversion and the terminator activation is a compelling mechanism for the regulation of gene expression.

Our results show that the *p35* promoter also seems to be invertible, because it has inverted-repeat sequence for inversion. However, Neyrolles et al. (24) previously analyzed the *p35* gene regions from $P35^+$ and $P35^-$ phenotype strains and reported that the P35 phase variation was not associated with DNA sequence change, although the phase variation occurred practically at the transcriptional level. This observation does not seem to agree with our promoter inversion model. However, in Neyrolles' study, the *p35* promoter region was cloned by PCR and sequenced. Because the upstream primer sequence for this PCR was located within the invertible DNA region (24), the *p35* promoter region of $P35^-$ strains might have been amplified from $P35^+$ revertants arising in the $P35^-$ strain population, and thus, no inversion could have been detected by sequencing. The frequency of phase variation of P35 family lipoproteins was reported to be about 10^{-2} to 10^{-4} per cell per generation (24, 33). This frequency is sufficiently high for DNA amplification by PCR from revertants.

In addition to ON↔ OFF phase variation, variable surface lipoproteins of mycoplasma species frequently exhibit size variation (2, 48). These size variations are mainly created by the increase or decrease of repetitive sequences within the lipoprotein genes (23, 49, 50, 51). Unlike other variable surface lipoprotein genes of mycoplasmas, the *mpl* genes do not contain certain repeated regions in their sequences. There is no evidence of size variation in *mpl* gene products. However, we found that the coding lengths of one of the *mpl* genes, *p30* (MYPE6800), in GTU and HF-2 are different because of a frameshift mutation. In GTU, the P30 protein consists of 318 aa (9), while it is 376 aa in HF-2 (data not shown). Although the promoter of the *p30* gene is in the OFF orientation in both strains (Fig. 8A), P30 proteins with different sizes must be expressed when the promoters of these genes are turned on. In addition, we identified frameshift mutations in three other *mpl* genes of the HF-2 genome (Fig. 8). Thus, the frequency of

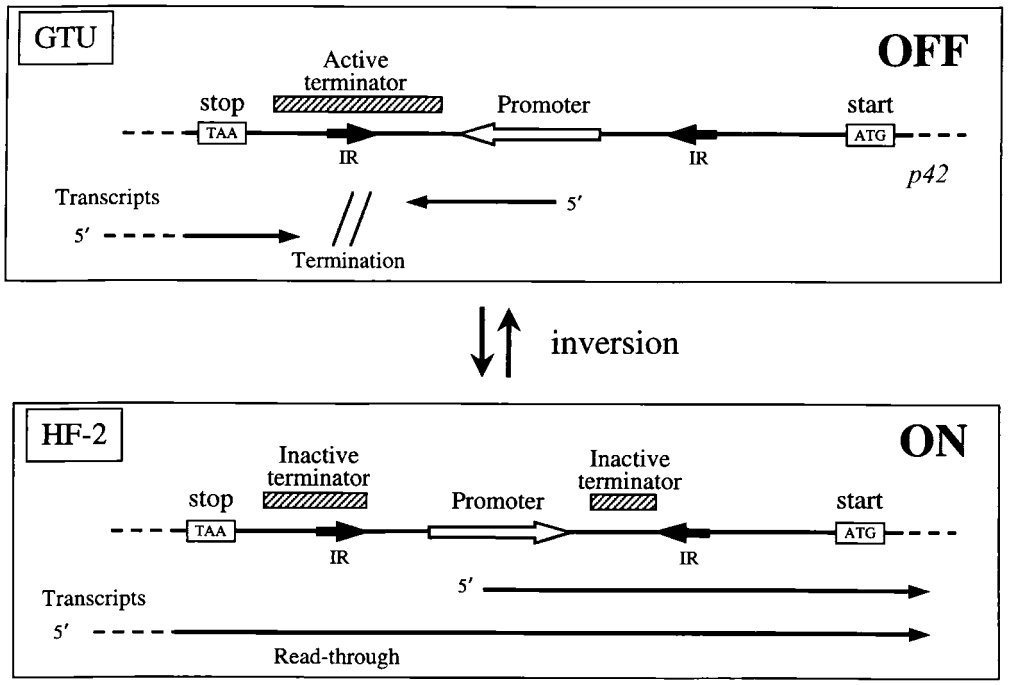

FIG. 9. Model for genetic switch of *mpl* genes by promoter inversion. The upstream intergenic regions of the *p42* genes are illustrated. The open arrows represent promoter sequences and their directions. The short solid arrows represent 12-bp inverted repeats (IR). The hatched boxes represent terminator-like sequences (16-bp inverted repeats). When the promoter is in the OFF orientation (GTU), an active terminator is formed. The inversion between 12-bp inverted-repeat sequences changes the promoter direction from OFF to ON and splits the terminator sequence. RNA transcripts from the promoter and from upstream are represented by thin solid arrows.

frameshift mutation seems to be high in the *mpl* gene locus. It is possible that frequent frameshift mutation generates size variations in *mpl* gene products.

In this study, we have investigated the molecular mechanism of antigenic profile changes of *M. penetrans* P35 family lipoproteins and reported the presence of novel systems for the regulation of lipoprotein gene expression. Although the actual biological functions of P35 family surface lipoproteins remain unclear, they are abundant, major surface antigens recognized by the human immune system during infection (14, 25, 42). The generation of extensive variations of these surface antigens by multiple promoter inversions may contribute to the survival of *M. penetrans* in the host. The *M. penetrans mpl* gene system may be a useful model for expanding our understanding of bacterial host adaptation and mechanisms for the evasion of immune responses.

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