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Surface Diversity in *Mycoplasma agalactiae* Is Driven by Site-Specific DNA Inversions within the *vpma* Multigene Locus

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The ruminant pathogen *Mycoplasma agalactiae* possesses a family of abundantly expressed variable surface lipoproteins called Vpmas. Phenotypic switches between Vpma members have previously been correlated with DNA rearrangements within a locus of *vpma* genes and are proposed to play an important role in disease pathogenesis. In this study, six *vpma* genes were characterized in the *M. agalactiae* type strain PG2. All *vpma* genes clustered within an 8-kb region and shared highly conserved 5′ untranslated regions, lipoprotein signal sequences, and short N-terminal sequences. Analyses of the *vpma* loci from consecutive clonal isolates showed that *vpma* DNA rearrangements were site specific and that cleavage and strand exchange occurred within a minimal region of 21 bp located within the 5′ untranslated region of all *vpma* genes. This process controlled expression of *vpma* genes by effectively linking the open reading frame (ORF) of a silent gene to a unique active promoter sequence within the locus. An ORF (*xer1*) immediately adjacent to one end of the *vpma* locus did not undergo rearrangement and had significant homology to a distinct subset of genes belonging to the λ integrase family of site-specific *xer* recombinases. It is proposed that *xer1* codes for a site-specific recombinase that is not involved in chromosome dimer resolution but rather is responsible for the observed *vpma*-specific recombination in *M. agalactiae*.

Mycoplasmas belong to a group of cell wall-less pathogens that are widespread in nature and cause diseases that are generally chronic and difficult to eradicate in humans, animals, plants, and insects. Among free-living organisms, they possess one of the smallest genomes, which is mainly responsible for their parasitic lifestyle. Yet, despite having a limited coding capacity relative to other bacteria, mycoplasmas have developed quite an extensive repertoire of molecular mechanisms to generate surface protein variability at a high frequency either by phase or size variation (10, 28, 42). Many of these surface proteins belong to multigene families of lipoproteins that have the ability to switch the expression of individual components on and off and are suspected of playing a role in host cell adhesion. The ability of these proteins to switch expression between different forms is believed to be important in immune evasion and rapid adaptation in the host.

Mycoplasma agalactiae is the major etiological agent of the syndrome contagious agalactia, which primarily causes mastitis, arthritis, and keratoconjunctivitis in sheep and goats (4). Recently, a family of related but distinct surface lipoproteins, designated Vpmas, has been identified in this pathogen, and Vpma expression has been shown to undergo high-frequency variation (16). Vpmas are abundantly expressed in *M. agalactiae* and display an unusual processing of lipoproteins in which a peptidase II-like enzyme cleaves 2 amino acids (aa) (position −2) N terminal to the cysteine residue rather than at the cysteine to which the lipid anchor is attached. This atypical processing is also being increasingly observed with other mycoplasma lipoproteins such as P48 (position −2) from *M. agalactiae* (31) and MAA2 (position −1) from *M. arthritidis* (41). Detailed genetic analyses have suggested that the *vpma* system is composed of several genes, two of which have been sequenced (16). The 5′ untranslated regions and those encoding the signal peptide were found to be conserved within the *vpma* family and revealed a high identity to the equivalent regions in genes encoding a family of lipoproteins, called Vsps, in the phylogenetically related species *M. bovis* (3, 20, 23). Based on these data, the Vpmas of *M. agalactiae* were proposed to represent a system homologous to the Vsps in *M. bovis* (16). In addition, the two sequenced *vpma* genes were found to encode some of the epitopes that have been shown to be involved in Vsp host binding (32), suggesting that Vpmas could share a similar function. This, together with the fact that Vpmas are abundant and are released from their cell surfaces at levels as high as 59% relative to that present in the cells themselves per volume of medium (16), may emphasize the importance of Vpmas and Vpma switching in relation to disease progression. However, this issue has not yet been extensively examined as such studies require a precise characterization of the *vpma* system.

Control of *vpma* gene expression has been hypothesized to be linked to DNA rearrangements within the *vpma* locus, as *vpma* DNA restriction fragment length polymorphisms were found to correlate with switches in Vpma expression among clonal variants (16). DNA rearrangements associated with changes in gene expression have also been found for Vsps (20, 22). Two other well-described systems involving DNA rearrangements in mycoplasmas are the *vsa* and *hsd* systems in *M. pulmonis* (5, 35, 37, 38). Both systems generate variation in expression of a given product by exchanging the C-terminal region of the active gene with the open reading frame (ORF)

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of a silent gene by site-specific recombination. Each expressed gene possesses the same N-terminal sequence and active promoter. A third system, the *vlh* multigene family encoding surface lipoproteins in *M. synoviae*, involves gene conversion and the replacement of the C-terminal region of the expressed gene within an expression locus with another C-terminal sequence from a pool of *vlh* ORFs (25). The nature of the enzyme(s) responsible for this type of gene conversion is not known, but an as-yet-uncharacterized site-specific recombinase has been proposed to be involved in the DNA rearrangements observed for the *vsa*, *hsd*, *vpma*, and *vsp* systems.

This study describes the genetic organization of the entire *vpma* multigene locus in the PG2 type strain of *M. agalactiae* and the molecular characterization of the mechanism controlling Vpma expression. By isolation of PG2 clonal variants and characterization of their Vpma expression and *vpma*-specific DNA rearrangements, we show that a unique sequence containing the putative promoter for *vpma* expression is rearranged by site-specific recombination and linked to *vpma* ORFs to control their expression. An ORF unrelated to the *vpma* genes at one end of the locus and having homology to site-specific recombinases was found and is proposed to be responsible for the observed *vpma*-specific recombination in *M. agalactiae*. In addition, a pathogenicity island-like locus is described in a pathogenic mycoplasma species for the first time.

MATERIALS AND METHODS

Mycoplasma culture and derivation of clonal lineages. Mycoplasmas were grown in standard medium, as described previously by Aluotto et al. (1), at 37°C. The mycoplasma clones used in this study were obtained from *M. agalactiae* type strain PG2 (39). The PG2 clones, 55-5 and 55-7, identified by using monoclonal antibody (MAb) 3B3 and the colony immunoblot technique, have been described previously (16). The same procedure was used to obtain the 3B3-negative clone, 55-5-10, from 55-5, and both 55-5-10-4 (3B3-positive) and 55-5-10-7 (3B3-negative) were derived from 55-5-10.

Oligonucleotides and plasmids. All oligonucleotides listed in Table 1 were purchased from GibcoBRL, except A4F, which was modified at the 5′ end with digoxigenin (DIG) and synthesized by VBC-Genomics BIOSCIENCE Research-GmbH, Vienna, Austria.

The following plasmids contain DNA fragments from 55-5 (Fig. 1) in pUC18: p5H4.7 contains a 4.7-kbp *Hin*dIII fragment possessing ORF2′, *xer1*, *vpmaZ*, and *vpmaU*; p5H1.8 contains a 1.8-kbp *Hin*dIII fragment possessing the 5′ region of *vpmaY* and *vpmaX*; p5H3.0 contains a 2.9-kbp *Hin*dIII fragment possessing *vpmaW* and *vpmaV*; p5E2.2 contains a 2.2-kbp *Hin*dIII-*Eco*RI fragment from p5H4.7; p5E1.0 contains a 1.0-kbp *Eco*RI fragment from p5H4.7; p5E1.2 contains a 1.2-kbp *Eco*RI-*Hin*dIII fragment from p5H4.7; p5X0.26 contains a 262-bp *Xba*I fragment from p5H2.9; and p5X0.4 contains a 408-bp *Xba*I-*Hin*dIII fragment from p5H2.9. The 2.2-kbp *Xba*I fragment from p5H2.9, containing *vpmaV* and the 5′ region of *vpmaW*, was cloned into pBluescript II pKS. Plasmids pPCR9, pPCR75, and pPCR91 (16) contain PCR products (PCR9, PCR75, and PCR91, respectively) generated from A3F and 55-5 DNA (PCR9, 1.6 kbp) or 55-7 DNA (PCR75, 1.6 kbp; PCR91, 2.7 kbp).

DNA manipulations, Southern blot analysis, and library construction. Plasmid DNA was purified using AX100 columns from Macherey-Nagel. The genomic DNA isolation, DNA sequencing, and Southern blot technique have been described previously (16). Restriction endonucleases *Alw*I and *Ase*I were obtained from New England BioLabs, and *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I, and *Xba*I were obtained from Promega. Restriction endonuclease digestions were performed as recommended by the manufacturers. Probes were hybridized with membranes overnight at the same temperatures that were used for the washing step. PCR-generated *vpma* gene-specific probes were washed from the membranes in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) for 1 h at 60°C (*vpmaU*, *vpmaW*, *vpmaX*, *vpmaY*, and *vpmaZ*) or 55°C (*vpmaV*). After hybridization, DIG-labeled A4F was washed from the membrane two times in 6× SSC-0.1% SDS for 10 min at 46°C. A library of *Hin*dIII-digested genomic DNA from *M. agalactiae* clone 55-5 was cloned into pUC18 (Pharmacia) and screened as previously described (16). DNA fragments were further cloned after restriction digestion either into pBluescript II pKS (Stratagene) or pUC18. PCR products were cloned into pGEM-Teasy (Promega). DNA inserts were sequenced as previously described (16)

TABLE 1. Oligonucleotide sequences used in this study

Name	Sequence (5′ to 3′)[a]
conF2	cgcggatccAAGCTTAGTAAAATCAGTATAGAT
conF3	CTGAACCATTAGGAGTAGTAAC
MAX2R	TTCTACCATATTGACTCCTATGT
Z3R	GACCTCCGCCCTGTGTGTCT
Z1F	cgcggatccCAAACAGATTCAACTCCGTCAAC
E1F	CCAAACTATTCAAGCCCAATTGAA
E1R	GTCAGATTCTGATTGTGCCGA
Z1R	aaactgcagTTATTCGTATTTAGGTAATAGTCTTC
E2F	GTCTGAAAATGATAAGAAATAAC
E2R	GCTCTAGTAAAAGGATTTGAACG
U1R	aaactgcagTCAACCTTAGATAAACCACCTAAC
U1F	cgcggatccGATAAAGAAGATAAGACAGGTGGTAG
UV2R	CTTGTGCCATTCTTTCAGGGG
UV1R	GGTTTTCCTGGTTGTTCTGTG
Y1R	gctctagaGATTAAACTTTTTTTACAGTAAATG
Y1F	ccggaattcAATGCAAACGCTGCAGAAAATG
YX1R	CTTYAAATTYTGGAGCTAAGCCTC
Y4R	TGACTGCCTTCTGCTGGAGT
X2R	TATTTTGACCTCTACCTTGTGTA
X1F	cgcggatccAAAGTAATGAAGGTCAATTACC
H4F	GAAACCCCAGAAGAAAGGGATG
X1R	aaactgcagGCTTAAGGATTTTTTAAAATGATG
W1R	aaactgcagAATAACTTTATCTAGTTCTATACC
H4R	CCTTAAAAATAGTGGTATAGAACTAGA
W3R	GTTGTTGCACTGTTACCATTACT
W1F	cgcggatccAATGGCGGAAATAGTAATGGTAAC
V2R	ATCTCAACGTATTATCGTTTTAC
V1R	gctctagattaTCCAGATGATGTTTCAACTTC
V1F	ccggaattcGTTGAGGAAGCAATTAAAACAGC
conR3	GCAAATTAGTTGGTTTTTCAGC
conR2	cgcggatccAAGCTTAATGATTTGTGCTATAAAA
A3F	AARTGYGGWGGWACWAMWRA
A4F-DIG	AARTGTGGTGGYACTAAAGA
5′ leader1	GGATAAATTTATGAAAAAATC
leader2	ATGAAAAAATCAAADTTTKT

[a] Uppercase letters indicate target sequences, and lowercase letters indicate bases introduced to form restriction sites to aid in the cloning of subsequent PCR products.

PCR. Unless otherwise stated, PCR amplifications were performed using *Taq* DNA polymerase (Promega) as previously described (16). PCR or DIG labeling of *vpma* gene-specific probes by PCR was performed using the following primer sets and templates: U1F-U1R and p5E1.2 (for the *vpmaU*-specific probe); V1F-V1R and p5X2.2 (*vpmaV*); W1F-W1R and p5H3.0 (*vpmaW*); X1F-X1R and p5H1.8 (*vpmaX*); Y1F-Y1R and 55-5 genomic DNA (*vpmaY*); and Z1F-Z1R and p5H4.7 (*vpmaZ*). The PCR cycling conditions for these probes were as follows: 1 cycle of 94°C for 1 min and 28 cycles of 94°C for 20 s, 55°C (*vpmaY*), 60°C (*vpmaW* and *vpmaX*), 65°C (*vpmaV*), or 70°C (*vpmaU*) for 30 s, and 72°C for 10 s (*vpmaV*), 18 s (*vpmaU*, *vpmaX*, and *vpmaY*), or 54 s (*vpmaW* and *vpmaZ*), followed by 1 cycle at 72°C (performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler). The PCR cycling conditions for the primer pairs with 55-5 DNA as a template were 1 cycle of 94°C for 1 min and 28 cycles of 94°C for 20 s, 55°C (E2F-E2R) or 60°C (E1F-E1R and H4F-H4R) for 30 s, and 72°C for 18 s, followed by 1 cycle at 72°C for 7 min.

Long-range (LR) PCR was conducted using the primer set conF2-conR2 at an annealing temperature of 60°C and the Expand Long Template PCR system from Roche according to the manufacturer's instructions.

The names of the PCR fragments and the primer sets and templates that were used for their generation, respectively, (see Fig. 4) are as follows: A2, Z3R-MAX2, and 55-7 LR PCR product (LRP); C2, UV2R, and 55-7 LRP; D2, V1F-W3R, and 55-7 LRP; F2, A3F, and 55-7 DNA; G2, conR2-Y4R, and 55-7 LRP; A3, YX1R-MAX2R, and 55-5-10 LRP; B3, Y1F-Z3R, and 55-5-10 LRP; D3, UV1R-X2R, and 55-5-10 LRP; F3, W3R-V2R, and 55-5-10 LRP; G3, UV1R-conR3, and 55-5-10 LRP; A4, MAX2R-UV1R, and 55-5-10-4 LRP; C4,

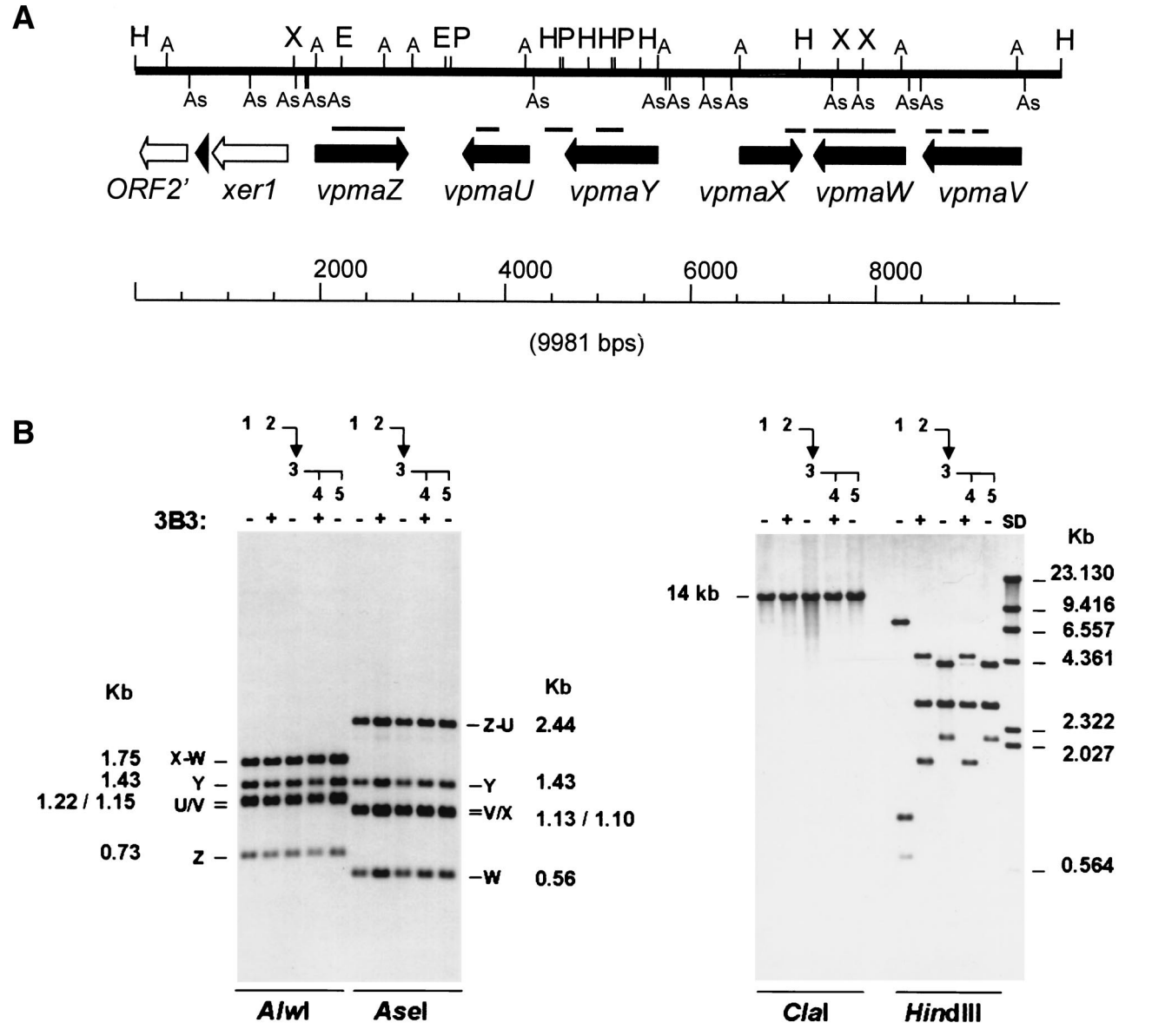

FIG. 1. (A) Restriction map and arrangement of the ORFs of *vpma* genes within the *vpma* locus of 55-5. Black arrows represent *vpma* genes and white arrows indicate ORFs unrelated to *vpma* genes. Lines above *vpma* ORFs indicate the locations of the *vpma*-specific PCR probes. A, *Alw*I; As, *Ase*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I. The location of a tRNA-lys gene is indicated by a black arrowhead. (B) Southern blot analysis of restricted DNA (1 μg) from PG2 clones hybridized with A4F. The tree schematic above each lane outlines the derivation of PG2 clones; each number represents a different clone which was either MAb 3B3-positive (+) or 3B3-negative (−). Lanes 1, 55-7; lanes 2, 55-5; lanes 3, 55-5-10; lanes 4, 55-5-10-4; lanes 5, 55-5-10-7. The restriction enzyme used is indicated below each panel. SD, λ*Hin*dIII DNA standard.

Y1F-Z3R, and 55-5-10-4 LRP; D4, YX1R, and 55-5-10-4 LRP; F4, W3R-V2R, and 55-5-10-4 LRP; G4, UV1R-conR3, and 55-5-10-4 LRP. The LRP PCR templates were used at a final concentration of 0.1 ng/μl. The cycling conditions for these primer sets and templates were as follows: 1 cycle of 94°C for 3 min and 28 cycles of 94°C for 30 s, 55°C (G2), 57°C (A2, B3, D3, F3, G3, A4, C4, F4, and G4), 60°C (C2, D2, A3), or 63°C (D4) for 40 s, 72°C for 30 s (A2), 32 s (B3, F3, G3, A4, C4, F4, and G4), 33 s (D2), 38 s (G2), 44 s (A3), 1 min 30 s (C2 and D4), or 2 min (D3) (performed with a model PTC-100 thermocycler from MJ Research, Inc., and *Taq* DNA polymerase from Promega, except for A2, G2, and D4, for which enzyme and 1× buffer from GibcoBRL and a 1.5 mM final concentration of $MgCl_2$ were used.) The PCR conditions for primer A3F have been described previously (16).

The PCR products (see Fig. 4) were cloned into the T-tailed pGEM-Teasy plasmid vector (Promega) and sequenced as previously described (16).

Northern blot analysis. Total RNA was prepared and subjected to Northern blot analysis as previously described (15). The hybridization and washing conditions for the *vpma* gene-specific probes were the same as those for the Southern blot analysis. The control DNA consisted of unlabeled PCR products identical to the DIG-labeled *vpma* gene-specific PCR probes.

DNA analysis. Blast and Advanced Blast (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/blast/blast.cgi]) were used to query amino acid or translated DNA sequences against all nonredundant sequences in the following databases: GenBank CDS translation, PDB, SwissProt, PIR, and PRF for protein and GenBank, EMBL, DDBJ, and PDB for DNA query sequences. DNA alignments were performed using Clustal W version 1.81 (European Bioinformatics Institute [http://www2.ebi.ac.uk/clustalw]). Identity and similarity values were calculated from the maximum length of the alignment, and the similarity values for protein alignments included the number of identical and

conserved substitutions but excluded semiconserved substitutions. Translations and calculations of the molecular mass and pI of proteins were performed using the programs available at the Expert Protein Analysis System proteomics server (http://www.expasy.ch/).

Nucleotide sequence accession number. The sequence data described here have been submitted to the GenBank database under accession no. AF411984.

RESULTS AND DISCUSSION

***vpma* multigene locus.** In a previous study, a 1.8-kbp fragment and three adjacent *Hin*dIII fragments of approximately 0.3 kbp were sequenced from an *M. agalactiae* clonal variant (55-5) expressing a Vpma product of 39 kDa, revealing the presence of two *vpma* genes, *vpmaX* and *vpmaY* (16). However, Southern blot analysis suggested that the *vpma* multigene family is composed of at least four genes sharing a common 5′ region. The sequence of the entire *vpma* locus in clone 55-5 was obtained after the cloning of two additional *Hin*dIII fragments of 2.9 and 4.7 kbp (Fig. 1B) from the previously generated library, as described in Materials and Methods. The six *Hin*dIII fragments that carried the entire *vpma* gene repertoire all hybridized with either the common amino-terminal oligonucleotide probe, A4F, that recognizes all six *vpma* genes and/or to *vpma*-specific PCR probes (Fig. 1). The orientation of the *Hin*dIII fragments relative to each other was determined by amplification using primers located in adjacent fragments and was confirmed by sequencing.

Analysis of the entire contiguous sequence revealed that the locus contained a total of six *vpma* genes in both orientations, which were arbitrarily designated *vpmaU* to *vpmaZ* (Fig. 1A). As previously described for *vpmaX* and *vpmaY* (16), all *vpma* genes share a highly conserved 5′ untranslated region (92% identity over 71 nucleotides between all genes, with the sequences of *vpmaV*, *vpmaW*, and *vpmaY* being identical to each other) followed by a conserved sequence encoding a 25-aa lipoprotein leader ending in an identical acylation/peptidase II cleavage motif of AAKC. The leader sequences possess an overall nucleotide and amino acid identity of 76% (92% aa similarity), and this high identity continues for a short distance into the mature polypeptide, with the next 4 aa being identical between all genes, but drops to about 50% for the following 19 aa, after which no overall significant identity could be found. These same *vpma* 5′ untranslated and N-terminal-coding regions are also conserved in the equivalent regions of the *vsp* genes from *M. bovis* (3, 20, 23); however, beyond this, *vsp* genes have no significant similarity to any *vpma* genes in *M. agalactiae*.

The number of *vpma* genes in PG2 is quite low considering that there are at least 13 *vsp* genes in the homologous system in *M. bovis* (23). A number of efforts were made to find evidence of any additional *vpma* genes within the PG2 genome. First, an additional 2 kb of sequence was obtained at the 3′ end of the locus, which led to the identification of two ORFs (ORF3 and ORF4) with no relationship to *vpma* genes. Second, a 5-kbp *Xba*I fragment from 55-5 which hybridized to the *vsp* oligonucleotide probe, vspS-2 (identical in sequence to a region of the *vspA* leader from *M. bovis*), but not to the *vpma* common amino-terminal probe, A3F (16), was cloned and the appropriate region was sequenced. Analysis revealed the presence of a lipoprotein gene that showed a leader sequence similar to those of the *vpma* and *vsp* genes but with a different mature N-terminal sequence. Finally, two oligonucleotides, 5′leader1 and leader2 (Table 1), which would hybridize to all known *vpma* and *vsp* genes, revealed in Southern blot analysis a pattern identical to that obtained with A4F (data not shown). Thus, only six *vpma* genes are present in PG2 and all are grouped within a single cluster.

Based on their N-terminal sequences and other shared sequences, the *vpma* genes could be divided into two homology groups (Fig. 2). The first group (VpmaW, VpmaX, and VpmaY) shares a short region (between 27 and 33 aa) of high identity (Fig. 2). VpmaW is predominantly composed of 2.7 repeats of 91 aa. The *vpmaY* gene encodes two large repeats of 186 aa and is unusual as the second repeat is prematurely terminated due to the insertion of two nucleotides that introduce a TAA stop codon so that only 104 aa are translated. VpmaX also contains a truncated version of the *vpmaY* repeat, while the remainder of the ORF consists of a unique sequence. The second group (VpmaV, VpmaU, and VpmaZ) also shares various regions. VpmaV consists of 2.9 repeats of 86 aa and shares 76 of its N-terminal amino acids with VpmaU. VpmaU also contains a truncated version of the V repeat (31%) and shares 79 C-terminal amino acids with VpmaZ (Fig. 2).

The truncation in the second repeat of the *vpmaY* gene appears to be at an evolutionarily early stage since the remainder of the second repeat-coding sequence is still intact. Truncated repeats in other *vpma* genes (*vpmaU*, *vpmaV*, *vpmaW*, and *vpmaX*) may have simply arisen by deletion, since there was no evidence in all three reading frames that the repeat continued past the stop codon. The truncation of repeats in the C-terminal region may be a means by which unnecessary sequences are eliminated, while variability between genes is optimized by the creation of new carboxyl-terminal sequences such as those for the *vpmaX* and *vpmaU* gene products. The *vpma* genes encoding common amino-terminal sequences but different C-terminal sequences may also have derived from gene duplication followed by recombination to exchange the C-terminal portion of only one of the genes with that of an unrelated sequence. This phenomenon has been observed for the single *vaa* gene of *M. hominis*, in which subpopulations within the same strain possess different *vaa* alleles consisting of different numbers of a central repeat followed by different C-terminal sequences. This C-terminal variability has been proposed to confer cytadherence function depending on which sequence is present (44).

Regions of common sequence between *vpma* genes may also represent functionally important domains. Interestingly, *vpma* repeats are, in general, more conserved within a gene than between genes, particularly for the repeats of *vpmaW* and *vpmaV*, which each have 100% DNA identity within a gene. This may indicate that the number of these repeats periodically increases or decreases, a phenomenon which has been previously described for other variable surface lipoproteins belonging to gene families in mycoplasmas (2, 5, 43).

Four *vpma* genes, *vpmaX*, *vpmaY*, *vpmaU*, and *vpmaZ*, were found to be identical to four *avg* genes, *avgA*, *avgB*, *avgC*, and *avgD*, previously sequenced from the PG2 type strain of *M. agalactiae* by another group of researchers (12), with the exception of *avgB*, which only contains one repeat and an additional 4 aa at the C terminus. It is unclear from the available sequence (accession no. AF112467) whether *avgB* is prema-

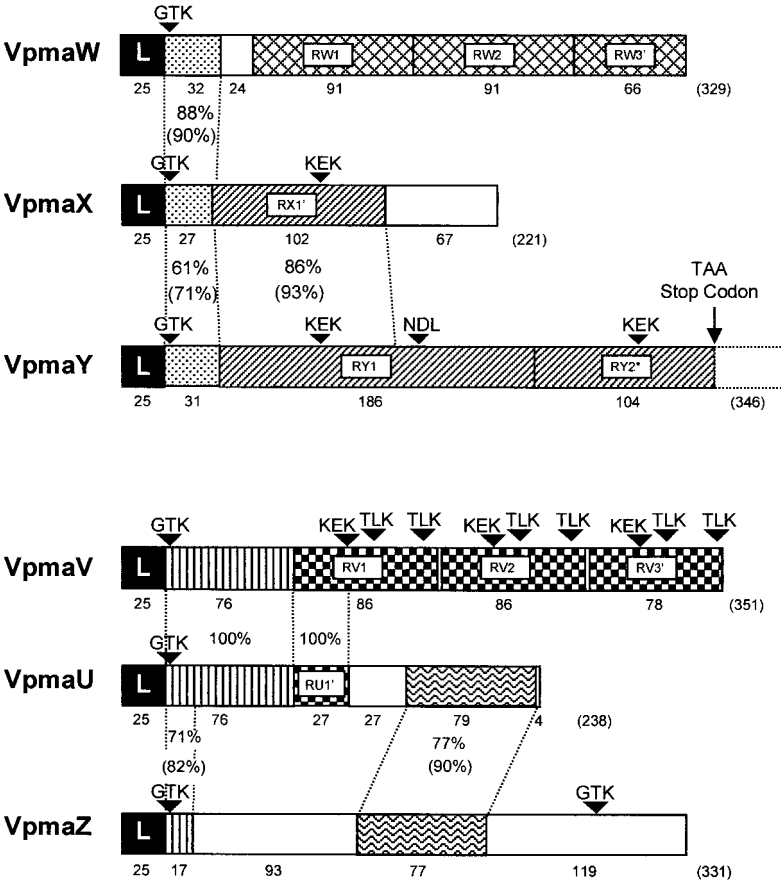

FIG. 2. Schematic of *vpma* ORFs. The ORFs are represented by boxes and begin with a homologous 25-aa leader sequence (L) followed by regions that have homology between genes or regions that are repeated (R, followed by gene designation, or R' for truncated repeats) within a gene. Homologous regions or repeated sequences are indicated with the same pattern, and unique sequences are not shaded. The percentages of amino acid identity between homologous regions between genes are indicated, and the percentages of similarity are in parentheses. The theoretical molecular mass (kDa), pI, and percentage of charged amino acids, respectively, calculated from the mature polypeptides for VpmaU, VpmaV, VpmaW, VpmaX, VpmaY, and VpmaZ are 23.2, 6.90, and 37%; 35.0, 9.09, and 34%; 33.1, 9.43, and 31%; 22.4, 6.33, and 42%; 35.2, 8.27, and 34%; and 34.2, 7.01, and 32%. Arrowheads indicate possible epitopes involved in host cytadhesion based on those determined for *vsps* from *M. bovis* (32).

turely truncated by mutation and still contains the coding sequence for a second repeat, as for *vpmaY*, or whether this second repeat has been completely deleted.

Only one pseudogene, which lacks a start codon and shares N-terminal coding sequence from the sixth amino acid of the leader sequence to 5 aa after the leader cysteine residue, was found downstream from, and in the same orientation to, *vpmaZ*. This homologous sequence shows an overall DNA identity of 69% (64% amino acid identity) with the other *vpma* genes. The leader sequence possesses an altered lipoprotein motif of ASKC and continues for an additional 5 aa (GDTKE) before terminating.

Identification of individual *vpma* gene expression within clones. In a previous study, two clonal variants, 55-5 and 55-7, were derived from the same PG2 strain and were shown to express Vpma39 and Vpma34, respectively (16). These two related but distinct Vpma products were not previously assigned to any one particular *vpma* gene, as all *vpma* genes had not been identified. After the entire *vpma* locus had been sequenced, gene assignments were still difficult to make because the N-terminal sequence of Vpma39 (14 aa) was identical to the predicted N-termini of VpmaY, VpmaX, and VpmaW, and the N-terminal sequence of Vpma34 (14 aa) was identical to those of VpmaU, and VpmaV. To resolve this problem, Northern blot analyses were performed (Fig. 3) using *vpma* gene-specific PCR probes (the probe positions relative to each *vpma* gene are shown in Fig. 1). The results established that the Vpma39 protein, expressed by 55-5, was encoded by the *vpmaY* gene. If *vpma* genes used a promoter located similarly to that of the expressed *vsp* genes of *M. bovis* (see below and Fig. 5), then the 1.60-kb *vpmaY* transcript would include the untranslated remainder of the second *vpmaY* repeat and would terminate very close to the start of the downstream gene, *vpmaU*. If this termination were to occur within the 5′ region of the downstream gene, *vpmaU* in this case, then this may affect the transcript size or even stability of *vpmaY* in other backgrounds with different configurations of *vpma* genes directly downstream from expressed *vpmaY* genes. The Vpma34 protein expressed by 55-7 was found to be encoded by a 1.05-kb *vpmaU* gene transcript (Fig. 3). This transcript has the potential to encode the entire *vpmaU* ORF, with termination occurring between *vpmaU* and *vpmaZ*, approximately 100

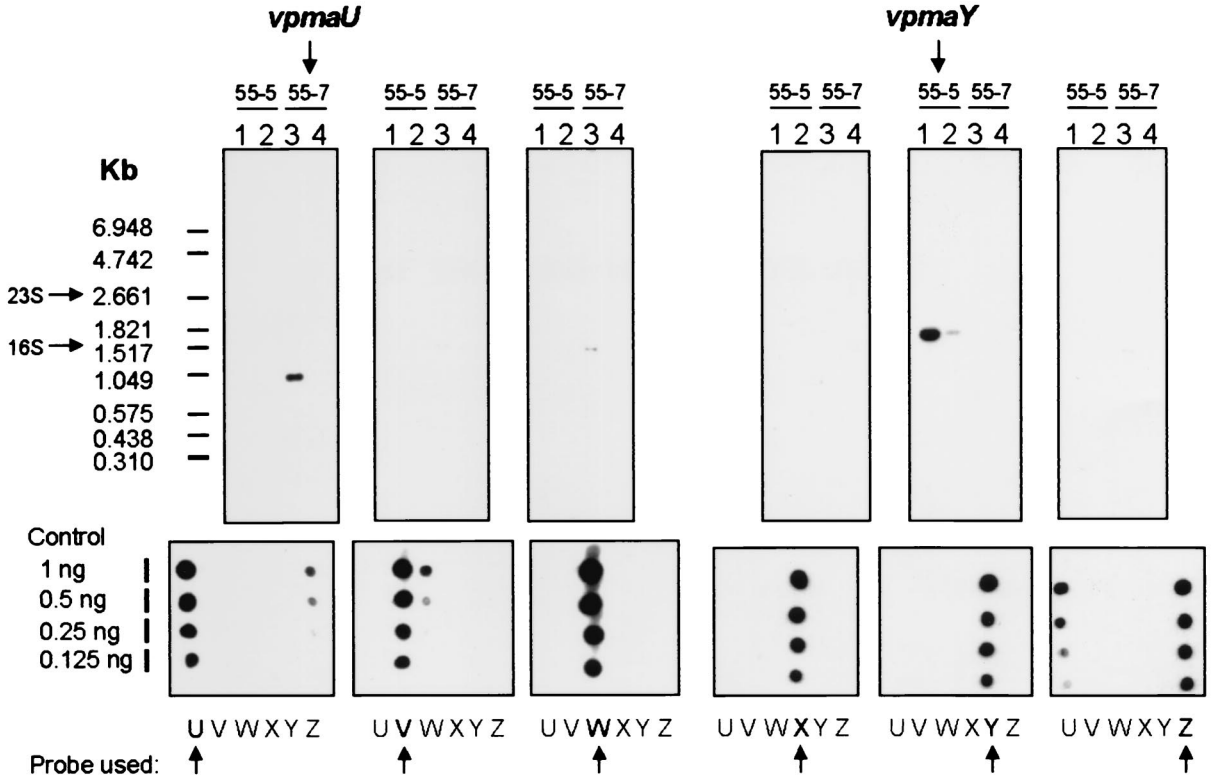

FIG. 3. Northern blot analysis of replica loadings of total RNA from clones 55-5 and 55-7 (lanes 1 and 3, 2 μg; lanes 2 and 4, 0.5 μg) hybridized with each *vpma* gene-specific probe. The *vpma* probe used for each replica is indicated by an arrow below each panel showing dot blotted unlabeled PCR probe DNA (underneath the panels showing the results of Northern blot analysis) diluted in a twofold series.

bases immediately downstream from the *vpmaU* stop codon. These results indicate that *vpma* gene transcripts are monocistronic, with only one *vpma* gene being expressed at any one time in each clonal isolate. Therefore, we conclude that control in *vpma* gene expression is at the transcriptional level and suggest that a single active promoter is present within the locus.

Since MAbs were used to obtain the *M. agalactiae* clones investigated, it is not surprising that the collection expressed only a limited range of *vpma* genes from the locus, namely, *vpmaU* and *vpmaY*. Preliminary data obtained by using polyclonal antibodies specific to each *vpma* gene product in both colony and Western blot analyses have also provided evidence for the expression of VpmaV, VpmaW, VpmaX, and VpmaZ in PG2.

Recombination within the *vpma* multigene locus is site specific and links a single promoter-like element to individual *vpma* ORFs to control *vpma* gene expression. As hypothesized in an earlier paper (16), switches in Vpma expression are due to the occurrence of specific DNA rearrangements within the *vpma* locus. In order to locate the exact sequences involved in these events, a detailed genetic analysis of the *vpma* locus was performed using the two sibling clones, 55-5 and 55-7, and three clonal variants derived from 55-5 (55-5-10, 55-5-10-4, and 55-5-10-7), each expressing either Vpma39 (MAb-positive) or Vpma34 (MAb-negative) (Fig. 1B). For this purpose, a strategy based on the sequence of clone 55-5 was devised that combined genomic DNA restriction digestion with *Alw*I or *Ase*I and Southern blot analysis to identify DNA fragments containing single *vpma* genes or up to two *vpma* genes orientated convergently (Fig. 1). This was possible because the enzymes cleaved within the conserved 5′ regions of each *vpma* gene. Southern blot analysis could then identify the *Alw*I or *Ase*I fragment that hybridized with the common N-terminal probe, A4F. Interestingly, an identical fragment banding pattern for both restriction enzymes was observed for all five clones tested (55-7, 55-5, 55-5-10, 55-5-10-4, and 55-5-10-7 [Fig. 1B]). In contrast, different restriction fragment length polymorphisms were observed when *Hin*dIII was used, while in all clones, the *vpma* genes were clustered on the same *Cla*I fragment (Fig. 1B). This indicated that the intergenic regions, rather than the *vpma* coding sequences themselves, were involved in the apparent *vpma* DNA rearrangements between clones.

To further define the sequences involved in *vpma* recombination, a strategy was devised to fast-track the analysis of only the *vpma* intergenic regions rather than sequencing the entire *vpma* locus from each chosen *M. agalactiae* clone. Clones 55-7, 55-5, 55-5-10, 55-5-10-4, and 55-5-10-7 were subjected to LR PCR using primers at each end of the *vpma* locus shown in Fig. 1A, including the two extreme *Hin*dIII sites. An approximately 10-kbp LR PCR fragment was obtained for each clone and was used to map the location and orientation of the *vpma* genes by using Southern blot analysis with gene-specific probes and single and double digestions of a combination of the following enzymes: *Eco*RI, *Hin*dIII, *Pst*I, and *Xba*I (Fig. 4). For the LR

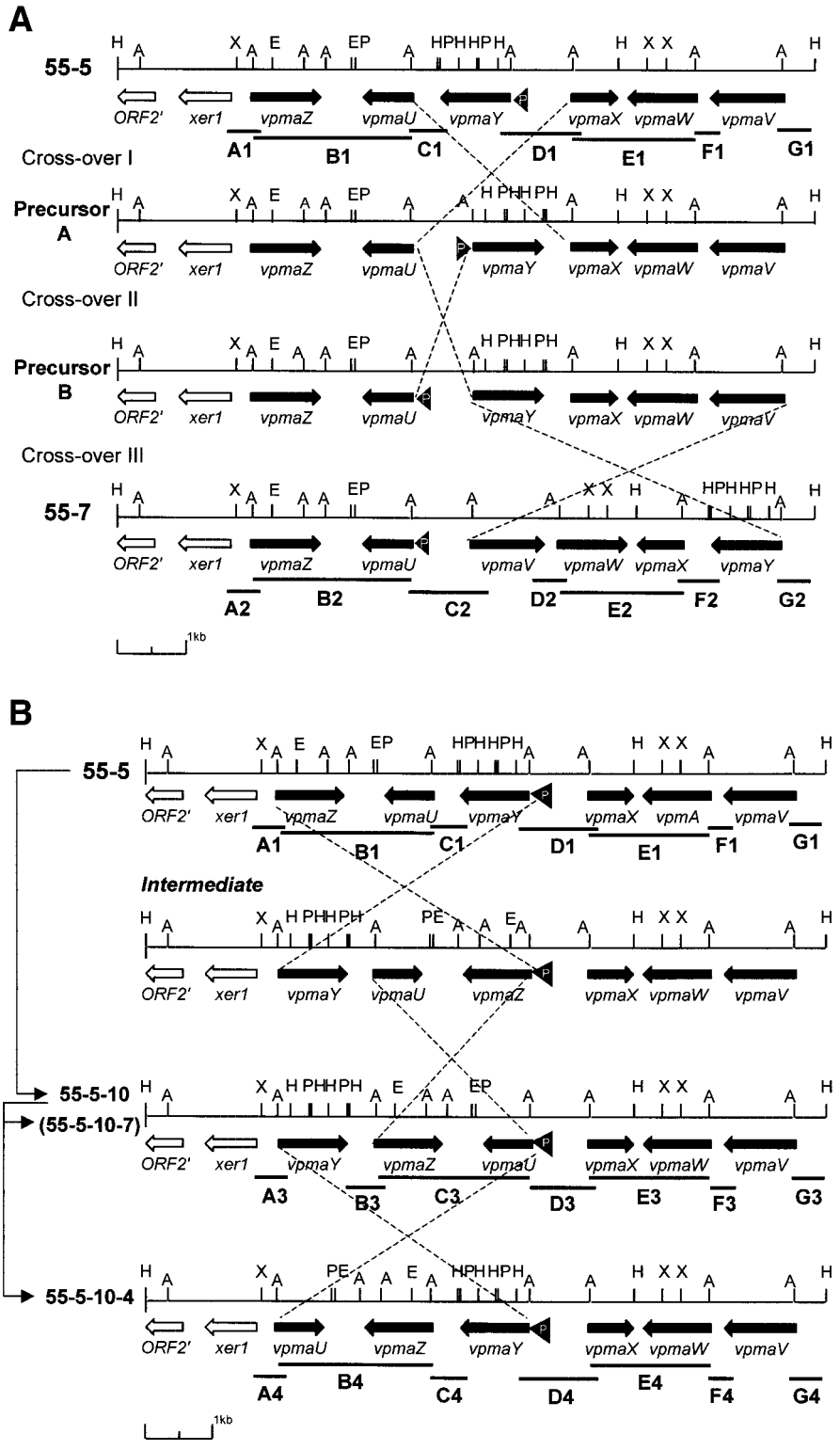

FIG. 4. Transition states of *vpma* multigene loci of related *M. agalactiae* clones due to DNA inversions. (A) Transition states between 55-5 and 55-7 (the restriction map symbols are like those shown in Fig. 1A). The ends of the crossed broken lines indicate the locations of DNA recombinational crossover events (I, II, and III) between inverted identical sequences producing DNA inversions. Genotypes labeled Precursor A and Precursor B represent potential ancestors from which 55-5 and 55-7 could have arisen using a minimum of three crossover events. (B) Transitions between 55-5, 55-5-10, and 55-5-10-4. Arrowed lines at left show the lineage order of the clones. Clone 55-5-10-7 has a restriction map identical to that of 55-5-10. Lines labeled with a letter and number combination represent sequenced PCR products, except B1, E1, B2, E2, C3, E3, B4, and E4, which are *Alw*I-digested genomic DNA fragments identified by Southern blot using A3F or A4F. Each number following a letter represents a particular clone (1, 55-5; 2, 55-7; 3, 55-5-10; 4, 55-5-10-4). All remaining symbols are defined in the legend to Fig. 1A.

PCR fragment from 55-7, partial digestion with *Ase*I and Southern blot using two separate internal oligonucleotides immediately downstream from each LR primer was used as a second method for ordering the *vpma* genes and for restriction map confirmation. The *vpma* intergenic regions for each locus were then amplified by gene-specific primers designed at each end of every *vpma* gene, and the PCR product was cloned and sequenced. The location of each intergenic PCR fragment is indicated in Fig. 4, except for B1, B2, C3, B4, E1, E2, E3, and E4, whose locations were determined by Southern blot analysis. This analysis showed that the *vpma* gene organization of clone 55-7 differs from that of its sibling, 55-5. Interestingly, the 5′ untranslated sequence of the expressed *vpmaY* gene of 55-5, which contains the promoter element discussed below, is located upstream from the expressed *vpmaU* gene of 55-7 rather than the upstream sequence of the silent *vpmaU* gene of 55-5. This suggests that recombinational crossover events must have occurred in the parental population so that a minimum of three crossover events (I, II, and III [Fig. 4A]) are theoretically required to generate both *vpma* genotypes of 55-7 and 55-5 from a common ancestor. It is possible that crossover III, shown in Fig. 4A, may have taken place before crossover II, which would then have resulted in an intermediate, with the *vpmaV* gene possessing the promoter element discussed below. Likewise, two recombinational crossover events must have taken place to produce the necessary DNA inversions to obtain the 55-5-10 *vpma* genotype from 55-5, while only one would be required to produce 55-5-10-4 from 55-5-10 (Fig. 4B). The sibling clone to 55-5-10-4, 55-5-10-7, retained the genotype of the parent, 55-5-10 (Fig. 4B). All *vpma* genes, except *vpmaW*, were documented to have been involved in *vpma* rearrangement. The results indicate that the strand exchange must have taken place within the conserved 5′ untranslated regions of the genes. The exchange between *vpmaY* and *vpmaV* extends this possible region halfway into the leader sequence, since their sequences are identical up to this point. Based on the recombination events involving the *vpmaZ* gene, the minimal region in which the recombinational strand exchange could have taken place was identified as a 21-bp region within the 5′ untranslated region between nucleotides 51 and 71 upstream from *vpma* start codons (Fig. 5). It is possible, but unlikely, that this minimal sequence could be extended by 6 bases at the 3′ end, where *vpmaZ* possesses three nucleotide differences relative to all other *vpma* genes, if the cleavage of the DNA were to occur on either side of these 6 bases. Cleavage involving heterogeneous sites for recombination between *vsaA* and *vsaF* in *M. pulmonis*, where there are two base differences within the proposed 6-base cleavage site in *vsaF* relative to the same region in *vsaA*, has been documented. After recombination between *vsaA* and *vsaF*, two forms of each gene were observed with one or the other 6-base sequence (35). No evidence was observed of the three base differences in *vpmaZ*, immediately 3′ to the proposed minimal region involved in *vpma* DNA recombination, being transferred to either of the two genes, *vpmaY* or *vpmaU*, after crossover with *vpmaZ* (Fig. 4B), although direct sequencing of the A3, A4, B3, C4, D3, and D4 PCR products was not done. Also, no sequence similarity was observed between the *vpma* sequences undergoing recombination and those of *vsa* and *hsd* of *M. pulmonis* (35, 37, 38).

Recombinational crossover events for all PG2 clones analyzed were also only observed to occur between two divergently or convergently oriented 21-bp minimal regions. This would be necessary, as recombination between two sites in the same orientation would lead to deletion of the interspanning DNA between them. It is possible that this process does occur at a low frequency, since some clones, upon amplification of their *vpma* loci, produced smaller-sized PCR fragments in a lower proportion to the expected full-length fragment (data not shown).

Recently, it has been documented that the region involved in DNA inversions between two *vsp* genes in *M. bovis* (*vspA* and *vspO*) includes a 34-bp region between (and including) nucleotides 38 and 71 upstream from the *vspA* start codon (20). This region includes the equivalent 21-bp region we have observed to be involved in DNA inversions between *vpma* genes (Fig. 5), indicating that these two gene families share a highly conserved mechanism for site-specific recombination in the two related mycoplasma species.

To understand which 5′ sequences are necessary for transcribing *vpma* genes, it was necessary to identify which *vpma* gene was expressed in each related clone that was analyzed at the molecular level. It was already known that clone 55-5 expressed *vpmaY* and that clone 55-7 expressed *vpmaU*. Since specific rabbit antisera had previously been raised to both VpmaY and VpmaU (16), they were used to confirm that the MAb-negative clones 55-5-10 and 55-5-10-7 expressed VpmaU and that 55-5-10-4 (MAb-positive) expressed VpmaY (data not shown). All *vpmaU* and *vpmaY* genes that were actively expressed in these clones were also found to possess a 5′ untranslated sequence identical to that of the *vpmaY* gene in 55-5, located upstream from the minimal region involved in *vpma* DNA recombination. This region should contain the active promoter element that drives *vpma* gene expression, and this sequence is highly conserved with the equivalent region of the expressed *vspA* gene of *M. bovis* (Fig. 5) but it is not present in silent *vpma* genes and published *vsp* genes (3, 20, 21, 23). Very recently, the start point for transcription of the expressed gene, *vspA*, was found to be located 192 bp upstream from the initiation codon (Fig. 5), and it was proposed that this start point possesses a σ^{70}-like -10 consensus sequence promoter element (21). Since this promoter region of expressed *vsp* genes is highly conserved with that of expressed *vpma* genes, it is most likely that *vpmaY* and other expressed *vpma* genes use the same start point for transcription. Overall, these data imply that the 5′ untranslated region, specific to expressed genes only, provides the promoter required to initiate transcription of *vpma* genes and is rearranged and linked with a silent gene via site-specific DNA inversion during switches of expression from one *vpma* gene to another within the locus.

A possible recombinase candidate in *M. agalactiae* is related to the Xer site-specific recombinases. In the course of cloning and sequencing the *vpma* locus, two ORFs unrelated to *vpma* genes were found immediately adjacent and in the opposite orientation to *vpmaZ* (Fig. 1A). ORF2′ is a partial ORF and has homology to a conserved hypothetical protein with various proposed functions in bacteria. More importantly, the complete ORF immediately adjacent to *vpmaZ* has homology to Xer recombinases included in the databanks mentioned above (*xer1* [Fig. 1A]). The highest-scoring alignments were obtained with the XerC homologues in *Ureaplasma urealyticum* (acces-

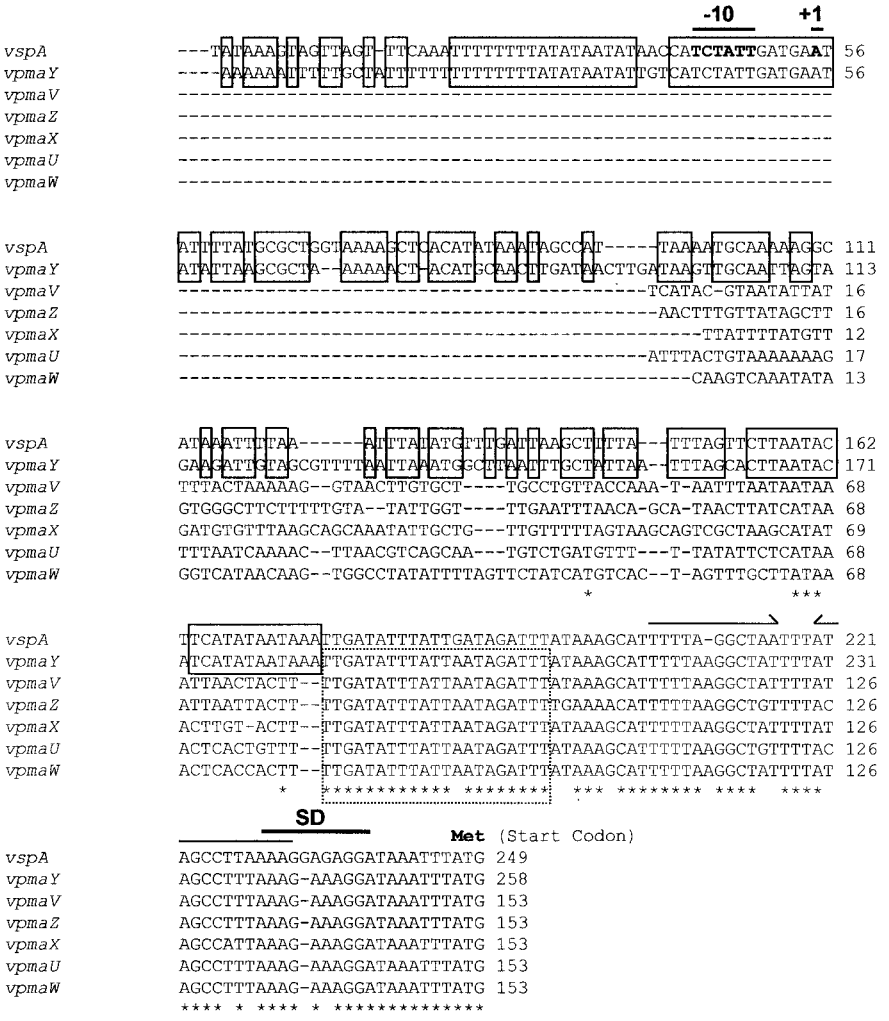

FIG. 5. DNA alignment of 5′ untranslated regions ending at the start codon of six *vpma* genes and *vspA* from *M. bovis* (21). A putative Shine-Dalgarno (SD) sequence, the start point of transcription (+1), and the promoter element (−10) are indicated by lines above the sequence based on *vspA*. An inverted repeat is identified by half arrows. The box indicated with broken lines shows the minimum region where DNA cleavage must occur during recombination between *vpma* genes. The boxes indicated with solid lines show regions of complete identity between *vspA* and *vpmaY* sequences upstream from the minimum region of recombination. Asterisks represent identity between all sequences, and dashes indicate gaps introduced to optimize the alignment.

sion no. AAF30630 [14]) and *M. pulmonis* CAC13704 (8), which have overall identities of 38% (68% similarity) and 42% (69% similarity), respectively, to *xer1*. The Xer recombinases belong to a large tyrosine recombinase family called the λ integrase family that shares an invariant tetrad of amino acid residues involved in catalysis, R-H-R-Y (11, 26). Within this group, the Xer proteins form a distinct subfamily based on their C-terminal regions (11). Xer1 in 55-5 also possesses the R-H-R-Y tetrad (Fig. 6) and numerous other distinctive residues in common with Xer proteins (26). The Xer site-specific recombination system has been best studied in *Escherichia coli* and consists of two homologous recombinases, XerC and XerD, which act on specific chromosomal sequences called *dif* sites to initiate DNA strand exchange to resolve chromosome dimers that have arisen from homologous recombination before chromosome segregation and cell division (7, 9, 36).

Homologues of XerC and XerD have been identified in a number of gram-negative and gram-positive bacteria (11, 26) but are absent in the circular genomes of *M. genitalium* and *M. pneumoniae* (18), which is hypothesized to be due to a deficiency in homologous recombination in these organisms which would render the Xer system obsolete (29). Although *dif*, XerC, and XerD are dispensable, their removal from *E. coli* (6, 19), *B. subtilis* (except XerC) (33), or *Haemophilus influenzae* (24) produces a subpopulation of filamentous cells that contain aberrant nucleoids. The absence of an Xer system may also explain why some *Mycoplasma* species have been observed to form filamentous branching forms (13, 34). Since the Xer system is unnecessary in mycoplasmas, it is proposed that the Xer homologue found in *M. agalactiae* and located immediately adjacent to the *vpma* multigene locus is involved in the observed *vpma*-specific DNA recombination that controls *vpma* gene expression rather than chromosome segregation.

Quite strikingly, the annotated integrase/recombinase from

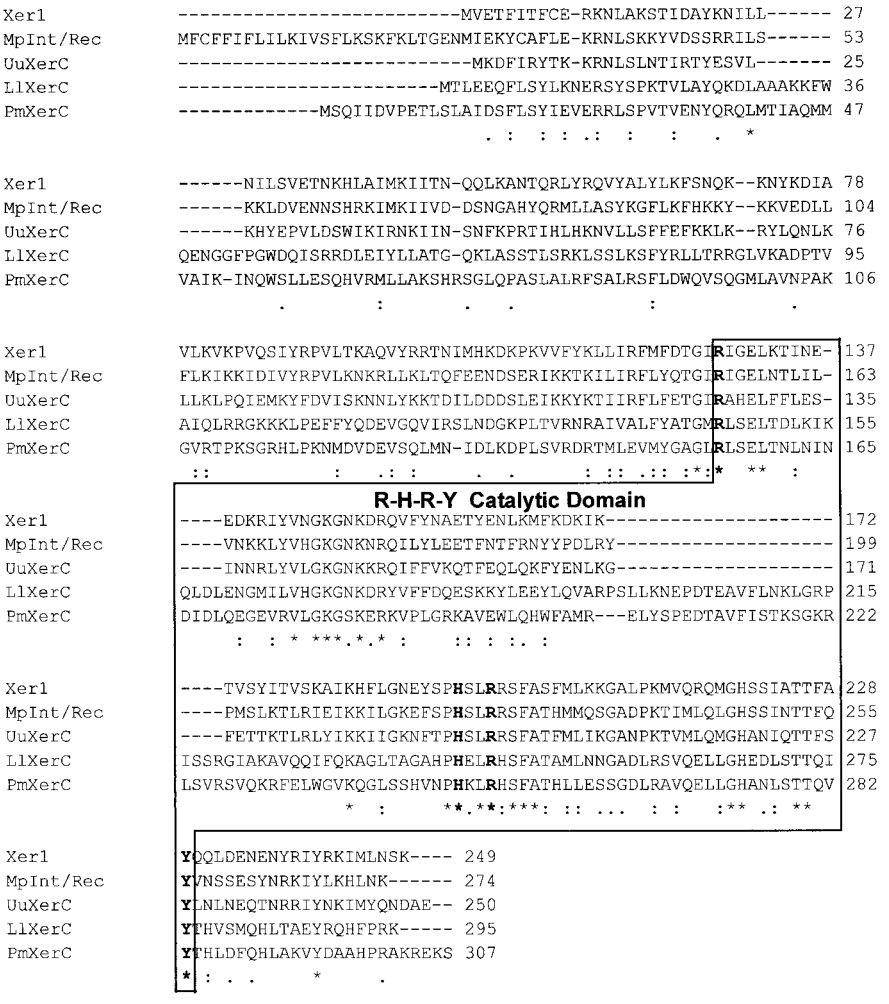

FIG. 6. Amino acid alignment of Xer1 from *M. agalactiae* type strain PG2 with XerC recombinases from *M. pulmonis* (MpInt/Rec), *U. urealyticum* (UuXerC), *Proteus mirabilis* (PmXerC), and *Lactobacillus leichmannii* (LlXerC). The boxed C-terminal sequences form the catalytic domain that includes the four amino acids (R, H, R, and Y, shown in boldface type) that are invariant for the λ integrase family of recombinases. Dashes indicate gaps introduced to optimize alignment, and asterisks indicate positions which have a single fully conserved residue. Colons and dots indicate that one residue of the strong and weak groups (as defined by ClustalW, version 1.8) is fully conserved.

M. pulmonis mentioned above (accession no. CAC13704), which is the only recombinase found in this organism, is located adjacent (3.216 kb) to the *vsa* multigene locus. Therefore, it is also possible that this recombinase functions to control Vsa expression by DNA inversions generated by site-specific recombination.

It would be expected that the Xer-like recombinases of *M. agalactiae* and *M. pulmonis* would function in a fashion similar to that of the Xer site-specific recombinases of *E. coli* but that they would be different in one very important aspect: in mycoplasmas, the reaction would require only one recombinase rather than two different recombinases. Interestingly, the region that provides DNA-binding specificity to XerC and XerD in *E. coli* and has sequence conservation among Xer proteins (26, 40) is not conserved in either Xer1 or the integrase/recombinase from *M. pulmonis*, which may explain why *vpma* and *vsa* genes do not use the same *dif* sequence. Additionally, the gene for the conserved hypothetical protein in *M. pulmonis*, which is the homologue to ORF2′ in *M. agalactiae*, is situated at a distance of approximately 200 kbp from the integrase/recombinase gene. Thus, the organization of these two genes in *M. pulmonis* is not conserved relative to ORF2′ and *xer1* in *M. agalactiae*.

The presence of the *vpma* pseudogene mentioned above suggests that the *vpma* locus is likely to be prone to instability. Whether this is the result of aberrant recombinase activity at nonpermissive recombination sites or simply of homologous recombination is not clear. Also, analysis of 32 other *M. agalactiae* strains and field isolates for their *vpma* gene contents, using the probes specific for *vpmaU* to *vpmaZ* from PG2, revealed that the *vpma* repertoires varied among strains. Approximately 50% of the strains were missing certain *vpma* genes and/or had additional new genes (unpublished data). Again, the question arises concerning whether an active recombinase aids and abets this evolutionary process for *vpma* loci or whether it is due to gene duplication, RecA-dependent

homologous recombination, and/or lateral transfer of *vpma* (or even *vsp*) genes.

The *vpma* multigene family, the *xer1* recombinase gene, and an adjacent tRNA-lys gene are typical elements of a pathogenicity island. Adjacent to the *vpma* locus is a tRNA-lys (anticodon CTT) gene (Fig. 1A) located between *xer1* and ORF2′ (Fig. 1A) which appears to be related more to genes from *Streptomyces lividans* and *Brucella abortus* than those from mycoplasmas (*M. genitalium*, *M. pneumoniae*, and *M. capricolum*). The tRNA-lys (CTT) gene is missing from *M. pulmonis*, but its absence might be explained by the fact that tRNA-lys (TTT) can read both AAA and AAG codons. It is important to note that *vpma* genes use an above-average percentage of AAA (12.7%) and AAG (3.7%) lysine codons compared with the average percentages used by all translated genes for the genomes of *M. genitalium*, *M. pneumoniae*, and *M. pulmonis* (averages of 7.01, 4.73, and 9.79%, respectively, for AAA and 2.44, 3.83, and 1.20%, respectively, for AAG), except for the average percentage of AAG codon usage in *M. pneumoniae*. The *xer1* gene also exhibits high usage of the AAG lysine codon (4.4%). Keeping in mind that the characterized gene products of two *vpma* genes, *vpmaY* and *vpmaU*, are each abundantly expressed in their respective clones and represent the most abundant proteins in their membrane fractions (16), the presence of the adjacent tRNA-lys (CTT) gene and the codon bias may play an important regulatory role in *vpma* and *xer1* gene expression, similar to that played by *leuX* in the expression of the FimB recombinase (30) and numerous other proteins, including type 1 fimbriae (27), in *E. coli*.

Several loci, each consisting of genes encoding a recombinase and factors involved in virulence, are usually associated with a tRNA gene(s) and form mobility units in bacteria that have been termed pathogenicity islands (17). It is interesting that these same elements are present within the *vpma* locus (Fig. 1A). This poses the question of whether *vpma* loci represent mobile elements important for intraspecies, or even interspecies (*vsps* of *M. bovis*), evolution and whether Vpmas per se or switches in Vpma phenotype contribute to virulence in *M. agalactiae*.

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