

Occurrence, Plasticity, and Evolution of the *vpma* Gene Family, a Genetic System Devoted to High-Frequency Surface Variation in *Mycoplasma agalactiae*^{∇†}

Laurent-Xavier Nouvel,^{1,2} Marc Marena,^{1,2,‡} Pascal Sirand-Pugnet,^{3,4} Eveline Sagné,^{1,2} Michelle Glew,^{1,2,§} Sophie Mangenot,⁵ Valérie Barbe,⁵ Aurélien Barré,⁶ Stéphane Claverol,⁷ and Christine Citti^{1,2*}

Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France¹; INRA, UMR 1225, F-31076 Toulouse, France²; Université de Bordeaux, UB2, UMR1090, F-33076 Bordeaux, France³; INRA, UMR 1090, F-33883 Villenave d'Ornon, France⁴; CEA-IG, Genoscope, F-91057 Evry Cedex, France⁵; Centre de Bioinformatique de Bordeaux, UB2, F-33076 Bordeaux, France⁶; and Pole Protéomique, Plateforme Génomique Fonctionnelle Bordeaux, UB2, 33076 Bordeaux, France⁷

Received 25 February 2009/Accepted 10 April 2009

Mycoplasma agalactiae, an important pathogen of small ruminants, exhibits a very versatile surface architecture by switching multiple, related lipoproteins (Vpmas) on and off. In the type strain, PG2, Vpma phase variation is generated by a cluster of six *vpma* genes that undergo frequent DNA rearrangements via site-specific recombination. To further comprehend the degree of diversity that can be generated at the *M. agalactiae* surface, the *vpma* gene repertoire of a field strain, 5632, was analyzed and shown to contain an extended repertoire of 23 *vpma* genes distributed between two loci located 250 kbp apart. Loci I and II include 16 and 7 *vpma* genes, respectively, with all *vpma* genes of locus II being duplicated at locus I. Several Vpmas displayed a chimeric structure suggestive of homologous recombination, and a global proteomic analysis further indicated that at least 13 of the 16 Vpmas can be expressed by the 5632 strain. Because a single promoter is present in each *vpma* locus, concomitant Vpma expression can occur in a strain with duplicated loci. Consequently, the number of possible surface combinations is much higher for strain 5632 than for the type strain. Finally, our data suggested that insertion sequences are likely to be involved in 5632 *vpma* locus duplication at a remote chromosomal position. The role of such mobile genetic elements in chromosomal shuffling of genes encoding major surface components may have important evolutionary and epidemiological consequences for pathogens, such as mycoplasmas, that have a reduced genome and no cell wall.

Bacteria of the *Mycoplasma* genus belong to the class *Mollicutes* and represent a remarkable group of organisms that derived from the *Firmicutes* lineage by massive genome reduction (41, 51). Consequent to this regressive evolution, modern mycoplasmas have been left with small genomes (580 to 1,400 kb), a limited number of metabolic pathways, and no cell wall. Due to these particularities, members of the *Mycoplasma* genus have often been portrayed as “minimal self-replicating organisms.” Despite this apparent simplicity, a large number of mycoplasma species are successful pathogens of humans and a wide range of animals, in which they are known to cause diseases that are often chronic and debilitating (1, 33). The surface of their single membrane is considered a key interface in mediating adaptation and survival in the context of a complex, immunocompetent host (10, 13,

34, 40). Indeed, mycoplasmas possess a highly versatile surface architecture due to a number of sophisticated genetic systems that promote intracolonial variation in the expression and structure of abundant surface lipoproteins (9, 50). Usually, these systems combine a set of contingency genes with a molecular switch for turning expression on or off that is based on either (i) spontaneous mutation (slipped-strand mispairing), (ii) gene conversion, or (iii) specific DNA rearrangements (9). While high-frequency phenotypic variation using the two first mechanisms has been described thoroughly for other bacteria (47), switching of surface components by shuffling of silent genes at a particular single expression locus has been studied mainly in mycoplasmas (3, 8, 14, 16, 23, 39, 43).

Mycoplasma agalactiae, an important pathogen responsible for contagious agalactia in small ruminants (listed by the World Organisation for Animal Health), possesses a family of lipoproteins encoded by the *vpma* genes for which phase variation in expression is driven by a “cut-and-paste” mechanism involving a tyrosine site-specific recombinase designated Xer1 (16). Data previously gathered with the PG2 type strain identified a single *vpma* cluster (42) composed of six *vpma* genes adjacent to one *xer1* gene (Fig. 1A). Based on fine genetic analyses, Xer1 was further shown to mediate frequent site-specific DNA rearrangements by targeting short DNA sequences located upstream of each *vpma* gene (8, 16). While some *vpma* rearrangements can be phenotypic-

* Corresponding author. Mailing address: UMR 1225 INRA, ENVT, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles, BP 87614, 31076 Toulouse Cedex 3, France. Phone: 33.(0)5.61.19.38.56. Fax: 33.(0)5.61.19.32.73. E-mail: c.citti@envt.fr.

† Supplemental material for this article may be found at <http://jb.asm.org/>.

‡ Present address: School of Veterinary Science, 250 Princes Highway, Werribee, Victoria 3030, Australia.

§ Present address: Bio21 Institute, Melbourne Dental School, 30 Flemington Rd., Parkville 3010, Australia.

∇ Published ahead of print on 17 April 2009.

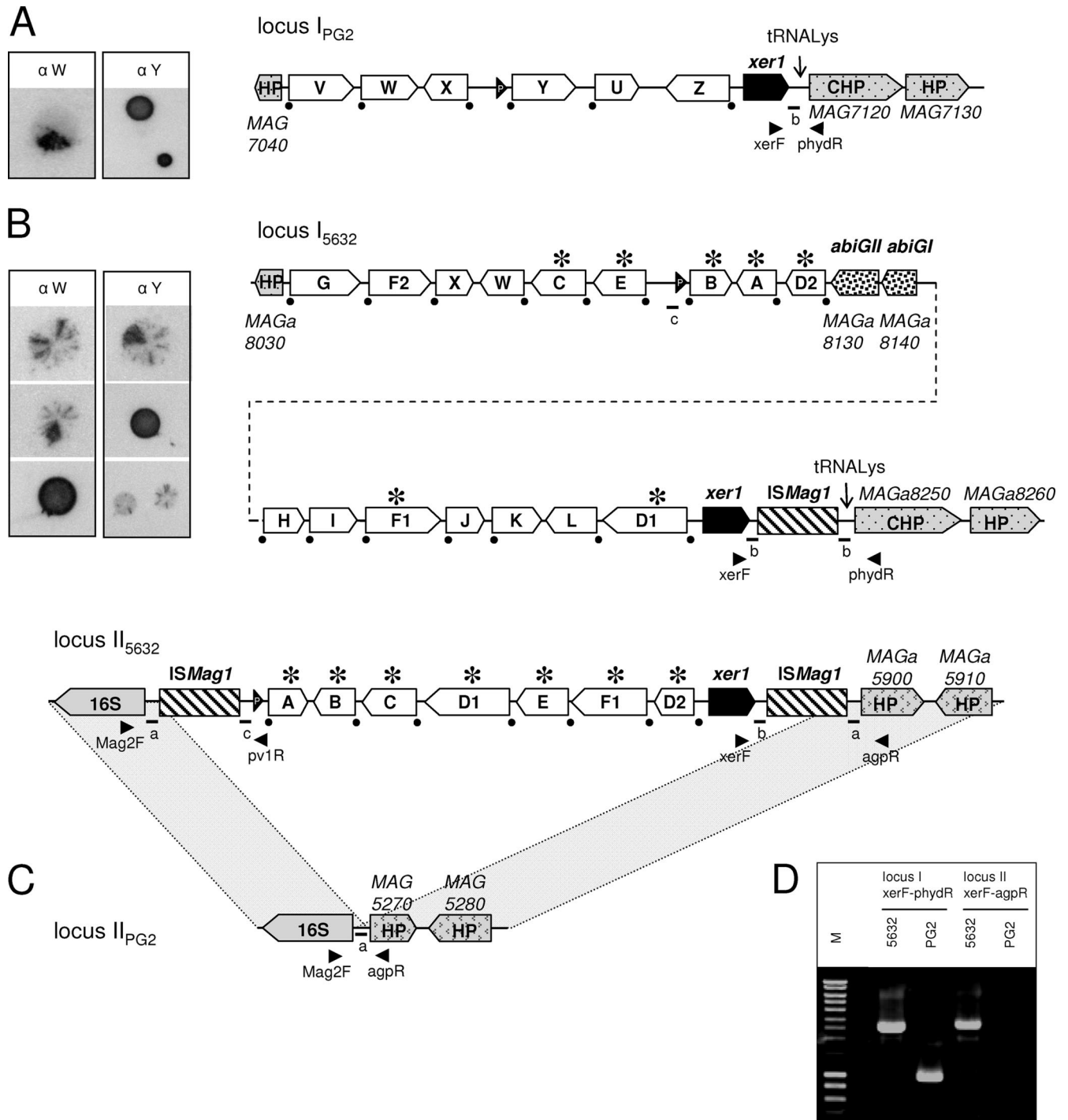


FIG. 1. Comparison of *M. agalactiae* *vpma* loci between the PG2 type strain and strain 5632. Schematics represent the organization of the *vpma* loci in clonal variant 55.5 derived from PG2 (16, 42) (A) and in clonal variant c1 derived from strain 5632 (B). (C) Counterpart of locus II₅₆₃₂ in PG2 showing the absence of *vpma* genes in this region. (D) The presence of two distinct loci in 5632 was confirmed by PCR, using the primer pair xerF-phydR or xerF-agpR, and the resulting amplicons are shown. The locations of the primers are indicated by arrowheads in panels A, B, and C. Large white arrows labeled with letters represent Vpma CDSs. The positions of the promoters are represented by black arrowheads labeled "P." The two non-Vpma-related CDSs (*abiG*_I and *abiG*_{II}) are indicated by large arrows filled with a dotted pattern. ISMag1 elements are indicated by hatched boxes. Recombination sites downstream of each *vpma* gene are indicated by black dots. An asterisk indicates that the corresponding *vpma* gene is present at two distinct loci. Schematics were drawn approximately to scale. HP, hypothetical protein; CHP, conserved hypothetical protein. Small letters and bars indicate the positions of short particular sequences mentioned in the text and in Fig. 3 and 4. The pictures on the left side of panels A and B illustrate the variable surface expression of Vpma, as previously described (8, 17). These correspond to colony immunoblots using Vpma-specific polyclonal antibodies recognizing PG2 VpmaW (α W) and VpmaY (α Y) epitopes.

ically silent, others result in Vpma on-off switching by linking a silent *vpma* gene sequence immediately downstream of the unique *vpma* promoter. Because site-specific recombination can be reciprocal, the initial *vpma* configuration can be restored without a loss of genetic information.

The *vs*a family of the murine pathogen *M. pulmonis* (3, 39), the *vsp* family of the bovine pathogen *M. bovis* (2, 24), and the *vpma* family of *M. agalactiae* all generate intraclonal surface diversity by using very similar molecular switches (23), although their overall coding sequences seem to be specific to the *Mycoplasma* species. DNA rearrangements also govern phase variation of the 38 *mpl* genes of the human pathogen *M. penetrans* (27, 35, 38). However, in this mycoplasma species the molecular switch is slightly different, since each *mpl* gene possesses its own invertible promoter (19). In *M. penetrans*, the individual expression of each *mpl* gene can then be switched on and off in a combinatorial manner, resulting in a large number of possible Mpl surface configurations. Since *M. pulmonis*, *M. bovis*, and *M. agalactiae* all belong to the *Mycoplasma hominis* phylogenetic cluster (48) and are relatively closely related, while *M. penetrans* belongs to the distinct *Mycoplasma pneumoniae* phylogenetic cluster (30, 48), it is tempting to speculate that the *vs*a, *vsp*, and *vpma* systems were all inherited from a common ancestor and that the bulk of their coding sequences evolved independently in their respective hosts while the molecular switch mechanism was retained.

In so-called “minimal” bacteria, the occurrence of relatively large genomic portions dedicated to multigene families, with genes encoding phase-variable, related surface proteins, suggests that they serve an important function(s). Data accumulated over the years for several mycoplasma species tend to indicate that one general purpose of these systems is to provide the mycoplasma with a variable shield that modulates surface accessibility in order to escape the host response and to adapt to rapidly changing environments (10, 11, 13, 40, 50). On the other hand, the sequences of phase-variable proteins are relatively conserved within one species but divergent between species, suggesting a more specific role for these molecules.

The role of the Vpma family of *M. agalactiae* has yet to be elucidated, but it was recently shown that Vpma switches in expression occur at a remarkably high rate in vitro (10^{-2} to 10^{-3} per cell per generation) (8, 17). The *vpma* systems described for PG2 (16) and another *M. agalactiae* strain, isolated in Israel (in which Vpmas were designated Avg proteins [14]), both revealed a repertoire of six *vpma* genes and only one promoter, suggesting that in *M. agalactiae* the number of Vpma configurations is limited to six. This contrasts with the situation commonly found in other *Mycoplasma* variable systems, which can offer a larger mosaic of surface architecture because of the concomitant switches of several related surface proteins and/or because of a larger number of phase-variable genes.

To further understand the degree of diversity that can be generated at the surface of *M. agalactiae*, we analyzed the *vpma* gene content of a field strain, 5632, whose genome was recently sequenced by our group (unpublished data). The present study shows that 5632 contains a total of 23 *vpma* genes distributed in two distinct loci that both contain a recombinase gene. Further genomic and proteomic analyses indicated that the capacity of 5632 to vary its Vpma surface architecture is far more complex than that described for the

type strain. Unlike the case for PG2, both 5632 *vpma* loci are associated with several mobile genetic insertion elements (IS) that could play an evolutionary role in the dynamics of *vpma* repertoires, as suggested by data presented here. One 5632 *vpma* locus contains open reading frames (ORFs) that are highly conserved in both *M. bovis*, a closely related bovine mycoplasma, and the phylogenetically distant mycoplasmas of the *M. mycoides* cluster, which are also important ruminant pathogens. Whether these were acquired through evolution or through horizontal transfer is discussed. The present study reveals an additional degree of complexity for the Vpma system and further suggests that some field strains might have more dynamic genomes and a more variable surface than was first estimated (42).

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA isolation. *M. agalactiae* type strain PG2, clone 55.5 (16), and strain 5632, clonal variant C1 (26), have been described previously. These strains were isolated independently from goats in Spain. The experiments reported here were all performed with these clonal variants, but for simplicity, we refer to them as strains PG2 and 5632 only. *M. agalactiae* field isolates were kindly provided by F. Poumarat (AFSSA, Lyon, France) (see Table S1 in the supplemental material). Mycoplasmas were propagated in SP4 liquid medium (46) at 37°C, and genomic DNAs were extracted as described elsewhere (7, 37).

Identification of *M. agalactiae* *vpma* and associated loci. Whole-genome sequencing of strain 5632 (clonal variant C1) was performed as follows. A library of 3-kb inserts (library A) was generated by mechanical shearing of the DNA followed by cloning of the blunt-ended fragments into the pCDNA2.1 (Invitrogen) *Escherichia coli* vector. Two libraries of 25-kb (library B) and 80-kb (library C) inserts were generated by HindIII partial digestion and cloning into the pBeloBAC11 (Caltech) modified *E. coli* vector. The plasmid inserts of 10,752, 3,072, and 768 clones picked from libraries A, B, and C, respectively, were end sequenced by dye terminator chemistry on ABI3730 sequencers. The PHRED/PHRAP/CONSED software package was used for sequence assemblies. Gap closure and quality assessment were performed according to the Bermuda rules, with 10,307 additional sequences. The *vpma* loci of strain 5632 were detected by DNA homology to those previously described for PG2 (positions of the loci on the 5632 genome are given with reference to the first nucleotide of the *dnaA* gene as nucleotide [nt] 1) and were annotated using the CAAT-Box platform (15), with the aid of Artemis software (36) and ACT software (5). The BLAST program suite was used for sequence homology searches of nonredundant databases (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). In order to determine the extent of sequence similarity, alignments between sequences were performed using Needle (Needleman-Wunsch global alignment algorithm) and Water (Smith-Waterman local alignment algorithm) (<http://www.ebi.ac.uk/Tools/emboss/align/>) software. Designations of 5632 *vpma* genes were based on local and global alignment scores, using all *vpma* gene sequences, including those previously described for PG2. The same name was attributed to a *vpma* gene presenting an amino acid sequence identity of >50% in the global alignment (Needle) and an amino acid sequence identity of >70% in the local alignment (Water). Using these rules, strain 5632 was found to contain the *vpmaW* and *vpmaX* genes also present in PG2. Also, distinct names were given to *vpmaY* and *vpmaF*, although they displayed global identities of >50%, because their local identities were <70%. *vpma* products with highly similar sequences that differed only in the number of C-terminal repeats were considered allelic versions and were designated with the same letter followed by different numbers (i.e., VpmaD1 and VpmaD2 or VpmaF1 and VpmaF2). Finally, *vpmaI* was not considered an allelic version of *vpmaD* because the C-terminal repeated region of its corresponding product displayed a local identity of 64.7% (<70%) with the VpmaD1 and -D2 counterparts.

Direct sequencing of PCR products and of genomic DNA (18, 20) was performed using specific primers (see Table S2 in the supplemental material) at the sequencing facility of UMR 5165 (CNRS, UPS, CHU Purpan, Toulouse, France).

Phylogenetic analyses were performed using MEGA 3.1 (21) and the neighbor-joining tree method. The reliability of the tree nodes was tested by performing 500 bootstrap replicates.

PCR assays. PCR assays were performed on an Eppendorf Mastercycler ep-Gradient thermocycler, using 5 ng *M. agalactiae* DNA as a template and using specific primers (see Table S2 in the supplemental material). PCR assays were performed with 25- μ l reaction mixtures containing a 0.4 mM concentration of each primer, PCR buffer (with MgSO₄; New England Biolabs) at a 1 \times final concentration, a 200 mM concentration of each deoxynucleoside triphosphate, and 2.5 U *Taq* DNA polymerase (New England Biolabs). Reaction mixtures were subjected to 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final elongation step of 5 min at 72°C. All PCR assays were performed at the unique annealing temperature of 55°C, using primer pair *xerF*-*phydR* for specific amplification of locus I, primer pair *xerF*-*aggR* or *Mag2F*-*aggR* for locus II, primer pair *aip1F*-*aip1R* for the *abiG1* gene, and primer pair *aip2F*-*aip2R* for the *abiGII* gene. PCR products were analyzed by gel electrophoresis on 1% agarose gels.

Colony immunoblotting and proteomic analyses. Colony immunoblotting was performed as previously described (8). Briefly, nitrocellulose membranes were placed on mycoplasma colonies freshly grown on agar medium and then removed and rinsed three times in TS buffer (10 mM Tris, 154 mM NaCl, pH 7.4). Membranes were then incubated overnight at 4°C with previously described rabbit Vpma-specific antibodies (8), washed three times in TS buffer containing 0.05% Tween 20 (Roth), and then incubated for 1 h at 25°C in a 1:2,000 dilution of swine anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Dako). After three washes, the colony blots were developed for 15 to 30 min in 4-chloro-1-naphthol and 0.02% hydrogen peroxide. The reaction was stopped by washing the blots in sterile distilled water.

Proteins that partitioned into Triton X-114 were extracted from strain 5632 as previously described (4), precipitated overnight at -70°C after the addition of 9 volumes of cold methanol, centrifuged for 10 min at 12,000 \times g, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was sliced into 16 sections which were subjected to trypsin digestion. Peptides were further analyzed by nano-liquid chromatography coupled to ion-trap tandem mass spectrometry (LC-MS/MS).

Peptides were identified with SEQUEST through the Bioworks 3.3.1 interface (Thermo-Finnigan, Torrance, CA), using a database consisting of both direct and reverse-sense *Mycoplasma agalactiae* strain 5632 entries (1,652 entries). Using the following criteria as validation filters, the false-positive rate was null: Δ CN \geq 0.1; Xcorr versus charge state, \geq 1.5 (+1), 2.0 (+2), or 2.5 (+3); peptide probability, \leq 0.001; and number of different peptides, \geq 2.

Nucleotide sequence accession numbers. The GenBank accession numbers for locus I₅₆₃₂ and locus II₅₆₃₂ are FP245515 and FP245514, respectively.

RESULTS AND DISCUSSION

Genome sequencing of strain 5632 reveals an extended *vpma* repertoire. The fully sequenced genome of *M. agalactiae* strain 5632 revealed a total of 23 *vpma* genes (Fig. 1). In contrast to the case for the PG2 type strain, this extended repertoire is distributed on two distinct chromosomal loci, loci I and II, that contain 16 and 7 *vpma* genes, respectively. BLAST analyses identified only two genes having significant similarity with those previously described for PG2 (16, 42), namely, *vpmaW* and *vpmaX*, with the others (*vpmaA* to *vpmaG*) only sharing blocks of high similarity with PG2 *vpma* genes (Fig. 2).

More precisely, locus I₅₆₃₂ is 19,453 bp long and is the counterpart of the PG2 *vpma* locus (Fig. 1A) because of their flanking coding sequences (CDSs) being nearly identical. In addition to 16 *vpma* genes, locus I₅₆₃₂ contains two CDSs (MAGa8140 and MAGa8130) that have no homology with the Vpma family but were found to have high amino acid similarity with *Streptococcus agalactiae* *abiG1* and *abiGII* gene products (45). The two corresponding genes are lacking in PG2 and were designated *abiG1* and *abiGII*, respectively. Locus I₅₆₃₂ further differs from its PG2 counterpart by the presence of an IS element corresponding to the previously described IS*Mag1* (31). This IS element is immediately adjacent to the 3' end of the integrase-recombinase *xer1* gene, whose product is identical to that in PG2.

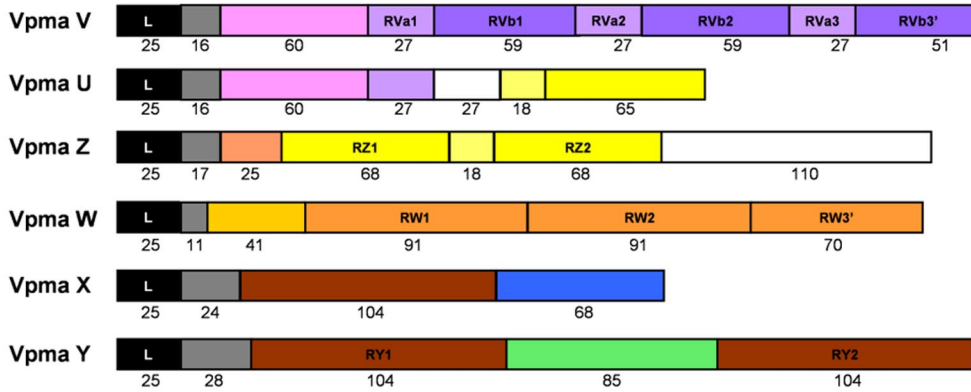
The second *vpma* locus of 5632, locus II₅₆₃₂, is 8,462 kb long and contains only seven *vpma* genes clustered between two IS*Mag1* copies, themselves flanked by a 16S rRNA gene at the 5' end and by a CDS annotated as a hypothetical protein (HP) gene (MAGa5900) at the 3' end. In PG2, this region is highly conserved, except that it does not contain any IS element or *vpma* or any other gene (locus II_{PG2}) (Fig. 1C and below). DNA BLAST analyses of locus II₅₆₃₂ demonstrated that all seven *vpma* coding sequences are identical to the seven *vpma* genes of locus I₅₆₃₂ (Fig. 1B). Interestingly, an identical *xer1* gene also occurs at both *vpma* loci of strain 5632. Since a single functional gene would be sufficient to generate DNA rearrangements in each locus, the duplication was further confirmed by PCR to rule out any possible artifact of sequence assembling. This was performed using a *xer1*-specific primer in combination with a primer specific to the 3' region of locus I (*phydR*) or locus II (*aggR*) (Fig. 1D).

The Vpma repertoire is composed of common and distinct structures. Detailed analyses of the 23 *vpma* coding regions of strain 5632 showed that (i) 2 are allelic versions of PG2 *vpma* genes (*vpmaW* and *vpmaX*), (ii) 7 are duplicated (*vpmaA*, -B, -C, -D1, -D2, -E, and -F1) and distributed in two *vpma* loci that are 250 kbp apart, and (iii) 2 are allelic copies of *vpmaD* (*vpmaD1* and -D2) and *vpmaF* (*vpmaF1* and -F2) that vary mainly in the number of C-terminal repeat motifs. Consequently, strain 5632 contains 12 new, distinct Vpma products compared to PG2 (Fig. 2). All *vpma* gene products contain a conserved amino acid signal sequence at the N terminus, followed by a lipobox and an 11-amino-acid conserved sequence (41). Within 5632, some mature *vpma* gene products are unique (VpmaA, -G, and -W), while others share blocks of amino acids between them (VpmaB, -C, -D1, -D2, -E, -F1, -F2, -H, -I, -J, -K, -L, and -X). Also, most Vpmas contain amino acid blocks that are directly repeated (VpmaA, -B, -D1, -D2, -I, -F1, -F2, -G, and -W). These data indicate that the diversity in the Vpma family is represented by a mosaic of structures that is suggestive of homologous recombination events between *vpma* genes, gene duplication, and/or insertion-deletion of repeated motifs. For instance, VpmaF1 and VpmaF2 share a common block of 104 amino acids with VpmaK and VpmaX and a common block of 85 amino acids with VpmaY that is not found in VpmaK or VpmaX. Some Vpmas, such as VpmaD1 and -D2 or VpmaF1 and -F2, represent size variants of the same Vpma with a variable number of repeated motifs at the C terminus (e.g., VpmaD1 and -D2 contain 20 and 2 repeated motifs of 15 amino acids, respectively). Additionally, VpmaI may have a common ancestor with VpmaD1 and -D2 and can be seen as the result of combined events, such as gene duplication followed by sequence drift and/or repeat expansion at the 3' end.

Anti-VpmaW and anti-VpmaY rabbit antibodies previously produced against Vpma products of strain PG2 (8) also reacted with 5632 surface-exposed epitopes in colony immunoblots (Fig. 1) and revealed the presence of sectorized colonies characteristic of high-frequency variation in expression. Most likely, 5632 Vpmas recognized by these antibodies correspond to VpmaW and to VpmaF1 and/or -F2, which share blocks of amino acids with PG2 VpmaY (Fig. 2).

To better define the Vpma products expressed by strain 5632, proteins that partitioned in Triton X-114 detergent were

Vpma PG2



Identified as

expressed by MS/MS

Vpma 5632

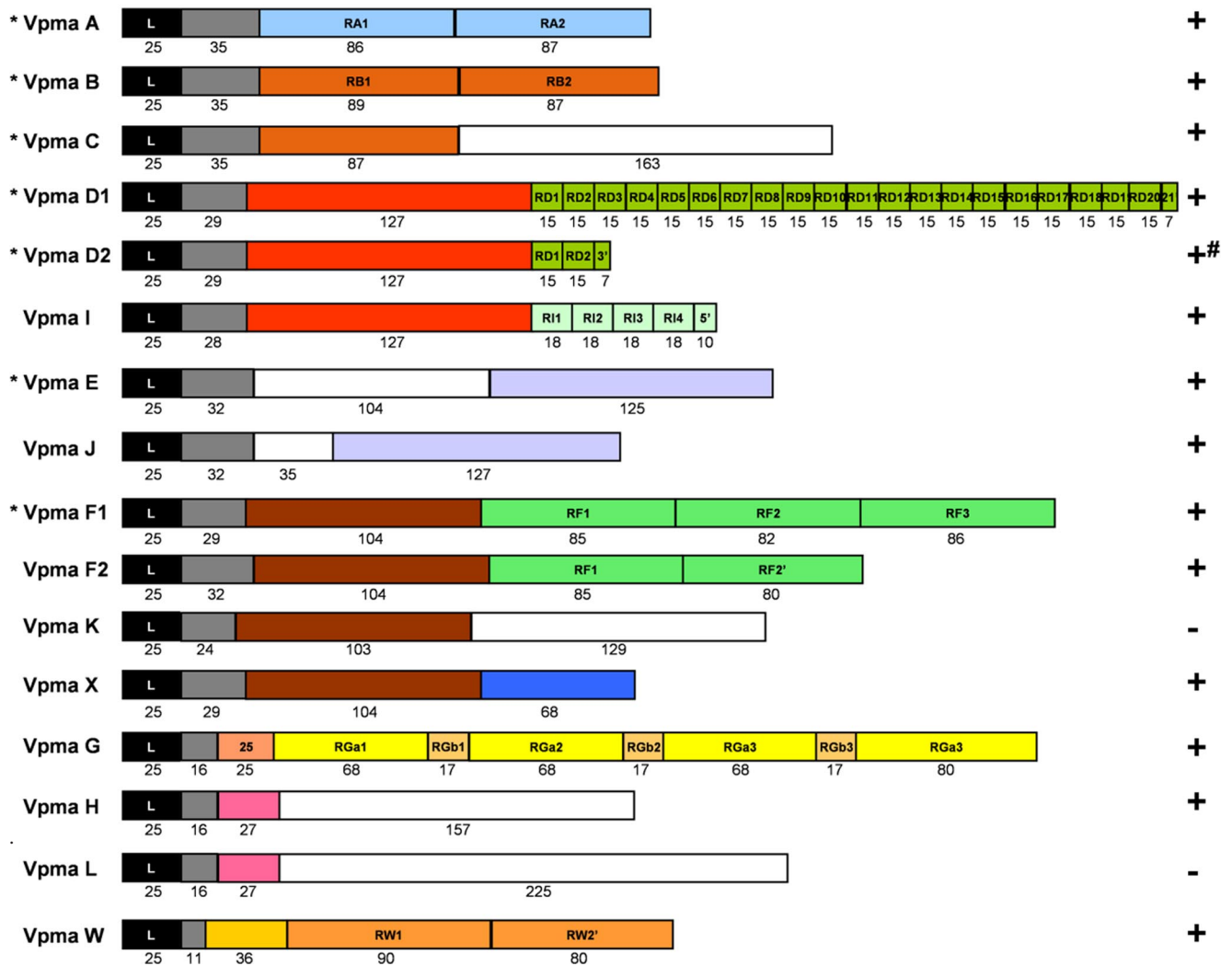


FIG. 2. Structural features and comparison of *vpma* gene products in *M. agalactiae* strains PG2 and 5632. Predicted Vpma proteins are represented schematically by boxes and begin with a homologous 25-amino-acid leader sequence (black boxes) followed by regions that have homology between *vpma* gene products or that are repeated within the same product (colored boxes). Two boxes of the same color display an amino acid identity of >30%. White boxes represent unique sequences. Numbers below boxes indicate the numbers of amino acids. For strain 5632, detection by MS/MS of expressed specific Vpma peptides is noted (+). -, no specific peptides were detected for the corresponding Vpma; +#, VpmaD2 peptides detected are not specific because all are shared with VpmaD1 or -I (see the list of detected peptides in Table S3 in the supplemental material). An asterisk indicates that the corresponding *vpma* gene is present in 5632 at both *vpma* loci.

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by in-gel trypsin digestion and by LC-MS/MS. The results showed that at least two specific peptides were detected for 13 Vpmas (VpmaA, -B, -C, -D1, -E, -F1, -F2, -G, -H, -I, -J, -X, and -W), indicating that these were expressed by 5632 during propagation (see Table S3 in the supplemental material). While VpmaD1-specific peptides were detected, the expression of VpmaD2 could not be assessed because all VpmaD2 trypsin peptides were also carried in VpmaD1 or VpmaI. No peptides specific to VpmaL and VpmaK were detected, although they possess long specific sequences of 225 and 129 amino acids, respectively (Fig. 2). Whether VpmaL and VpmaK are expressed at low levels or under different conditions cannot be determined. Finally, all Vpmas encoded by locus II₅₆₃₂ are duplicated at locus I₅₆₃₂, and consequently, their expression cannot be monitored by this method or by any other method, such as Northern blot analysis, because their sequences are also identical at the DNA level.

The global proteomic approach taken above is qualitative and does not reflect the level of expression of each Vpma, two of which are predicted to be expressed at high levels by the clonal population, while others should reflect only a minority of back-switchers. In PG2, the *vpma* locus contains a unique promoter which drives the expression of the *vpma* gene localized immediately downstream, while the other *vpma* genes remain silent (16). This promoter region was more precisely defined by primer extension by Flitman-Tene et al. (14), and a similar sequence was detected in each locus of strain 5632. Therefore, Vpma expression in 5632 is most likely driven at each locus by a single promoter sequence which is highly conserved between the two loci (99% nucleotide identity). In locus I₅₆₃₂, the promoter sequence is located upstream of the *vpmaB* gene, while in locus II₅₆₃₂ it is upstream of *vpmaA*, suggesting that these two Vpmas would be coexpressed predominantly by the clonal population. However, in the absence of specific antibodies, this hypothesis cannot be tested. One interesting observation is that the length of a poly(T) tract located between the putative -35 and -10 boxes of the *vpma* promoters varies among the three *vpma* promoters sequenced and is 14, 15, and 12 nt long for loci I of PG2 and 5632 and for locus II of 5632, respectively (see Fig. S1 in the supplemental material). In other variable mycoplasma systems, i.e., the Vlp system of *M. hyorhina*, fluctuation in the length of the polynucleotide tract between the -35 and -10 boxes has been shown to affect transcription (12). Whether this is also the case for the *M. agalactiae* species, and for strain 5632 in particular, cannot currently be tested because of the complexity of the 5632 *vpma* system. In PG2, DNA rearrangements are mediated by XerI, which recognizes a specific recombination site (RS) sequence located upstream of each *vpma* gene. Indeed, analysis of the 5632 and PG2 *vpma* loci revealed that a rigorously identical RS sequence is located upstream of each of the 29 *vpma* genes (23 in 5632 and 6 in PG2). This suggests that each *vpma* locus of strain 5632 can independently undergo DNA rearrangements and that the concomitant expression of two distinct Vpmas is most likely to occur frequently in this strain. If so, 5632 could theoretically display 91 different Vpma surface configurations, compared to only 6 for the type strain PG2. In parallel, additional Vpma size variations by insertion or deletion of direct repeats can further participate in altering surface architecture.

TABLE 1. *abiG*-related gene products in *Mollicutes* and other bacteria

Strain	Presence of sequence (% identity, % similarity) related to <i>Mycoplasma agalactiae</i> strain 5632 sequence ^a	
	AbiGI-like (MAGa8140)	AbiGII-like (MAGa8130)
<i>M. agalactiae</i> PG2	–	–
<i>M. bovis</i> PG45	+ (84.8, 93.6)	+ (81.1, 91.3)
<i>M. mycoides mycoides</i> SC PG1	+ (40.8, 59.2)	+ (41.8, 64.6)
<i>M. mycoides mycoides</i> LC GM12	+ (41.7, 59.2)	+ (31.9, 51.6)
	(Mmycm_04185)	(Mmycm_04180)
	+ (40.1, 58.5)	+ (32.6, 50.9)
	(Mmycm_00130)	(Mmycm_00135)
Other <i>Mollicutes</i> strains	–	–
<i>Streptococcus agalactiae</i> H36B	+ (40.2, 58.3)	+ (41.2, 65.3)
<i>Streptococcus suis</i> 05ZYH33	+ (41.2, 58.3)	+ (40.4, 66.3)
Non- <i>Streptococcus</i> spp.	+ (<40, not available)	+ (<40)

^a +, presence of homologous sequence; –, absence of homologous sequence in public databases (as of June 2008). Identity and similarity percentages between amino acid sequences were obtained using the Needle alignment program (<http://mobyle.pasteur.fr/cgi-bin/portal.py>).

Evidence of horizontal gene transfer at the *vpma* locus of 5632. As mentioned earlier, *M. bovis* is a close relative of *M. agalactiae* and generates surface variation by using a very similar genetic system, designated the *vsp* gene family. Both the *vsp* and *vpma* systems have several common features, including (i) *vsp* or *vpma* related genes are clustered in close proximity to a conserved recombinase gene (32); (ii) the recombination site involved in DNA rearrangements is identical, except for a single nucleotide polymorphism that is conserved in each species; and (iii) *vpma* or *vsp* genes code for proteins having a signal peptide that is highly conserved between the two systems. Unlike the PG2 *vpma* loci, the *vpma* loci of 5632 are associated with IS elements, as in *M. bovis* (23), and one, locus I₅₆₃₂, contains two non-*vpma* genes, *abiG_I* and *abiG_{II}*, that have 86.97 and 85.15% overall DNA identity with two *M. bovis* ORFs, ORF4 and ORF5 (23), respectively, located in the *vsp* locus. A search of the databases revealed that *abiG_I* and *abiG_{II}* from strain 5632 also match with gene products annotated as conserved hypothetical products in *Mycoplasma mycoides* subsp. *mycoides* biotype LC strain GM12 (22) and *M. mycoides* subsp. *mycoides* SC strain PG1 (49), with >50% amino acid similarity in the global alignment (Table 1). While *abiG* homologs are present in several gram-positive bacteria, such as *Streptococcus agalactiae* and *Streptococcus suis* (6, 45), none was found in other *Mollicutes* sequences available in the databases, which included 21 complete genomes.

The *abiG_I* and *abiG_{II}* genes were first described for *Lactococcus lactis* subsp. *cremoris* UC653 (28), where they occur as an operon carried by a conjugative plasmid and confer resistance to phage infection (29). Detection by LC/MS-MS of AbiGII-like specific peptides in the Triton X-114 detergent phase of 5632 indicates that the corresponding *abiG_{II}* gene is expressed, although there is no hydrophobic domain that could account for this partitioning. Previous attempts to define the functions of *abiG_I* and *abiG_{II}* of *Streptococcus* species failed (44), and whether *abiG* homologues found in mycoplasmas have a role in protecting them against phage attack remains to be addressed. Nevertheless, comparison of AbiGI and AbiGII amino acid sequences by constructing

neighbor-joining trees (500 bootstraps) indicated a strong relationship between *Mycoplasma* and *Streptococcus* sequences, suggesting that their corresponding genes might have undergone horizontal gene transfer (see Fig. S2 in the supplemental material).

To further define the occurrence of the two *abi* genes in *M. agalactiae*, a collection of 92 strains representative of (i) various geographical areas and (ii) various histories (see Table S1 in the supplemental material) was screened by PCR for the presence of *abiG* genes, using the specific primer pairs *aip1F*-*aip1R* and *aip2F*-*aip2R*. PCR products corresponding to each gene were obtained for 11 strains of 92 total strains. Further PCR assays showed that like the case for *Streptococcus agalactiae*, the two *abiG* genes always occur next to each other. Interestingly, direct genome sequencing of six *abiG*-positive strains, using *aip2F2* as a primer, showed that the *abiG*_{II} gene is always associated with *vpma* genes. Because strains displaying *abiG* genes in their *vpma* loci have very different geographical origins (Ivory Coast, Spain, and France), this result raised the question of whether these genes were inherited vertically from an *M. agalactiae*/*M. bovis* common ancestor or whether the *vpma* locus is a hot spot for their insertion. Since the *vpma* locus is frequently subjected to DNA rearrangements, deletion of *abiG* genes by intrarecombination involving, for instance, *vpma*-RS sequences, may easily have occurred, resulting in *abiG*-negative strains.

Plasticity of *M. agalactiae* *vpma* loci. To define whether the occurrence of the two *vpma* loci observed in strain 5632 is common among *M. agalactiae* strains, a PCR assay was performed with primer pairs *xerF*-*phydR*, *xerF*-*agpR*, and *Mag2F*-*agpR*, which yield amplicons corresponding to either locus I or II of PG2 and/or 5632. More specifically, the primer pairs *xerF*-*phydR* and *xerF*-*agpR* target the 3' ends of the loci, which are not affected by *vpma* rearrangements, while *Mag2F* and *agpR* were designed to amplify a more or less empty locus II under our PCR conditions. The assay was conducted with the panel of 92 strains (see above). The results showed that 89 strains produced an amplification profile identical to that obtained with PG2, while 3 showed new profiles, none of which was identical to that obtained with 5632 (Fig. 3). Sequencing of PCR products or selected genomic regions showed that (i) strain 13628, like 5632, contains an IS element at locus I while displaying a locus II similar to that of PG2; and (ii) strains 13375 and 4025, like PG2, display no IS at locus I while carrying a locus II containing one or more IS but no *vpma* genes, with strain 4025 having an IS30-like insert in an *ISMag1* element. These data suggest that the occurrence of a *vpma* cluster at locus II is a rare event and has so far been observed only in strain 5632, while the occurrence of *vpma* genes at locus I is shared by all *M. agalactiae* strains tested so far.

The presence of an *ISMag1* element in close proximity to *vpma* locus I in 5632 is not an isolated event, since it is shared by at least one other strain, 13628, which unlike 5632, was isolated in France in 2003 from a goat ear canal. *ISMag1* belongs to the IS30 family (31), which generates, when inserting itself into a host genome, a direct repeat that flanks the IS (25). Indeed, *ISMag1* of locus I is flanked in 5632 and in 13628 by an identical 14-nt directly repeated sequence (Fig. 3), suggesting that its insertion most likely occurred in an ancestor common to the two strains. Interestingly, the

14-nt element found on each side of the IS of locus II₁₃₃₇₅ (Fig. 3) is also repeated in locus II₅₆₃₂ as if flanking the whole locus, including the IS. Because no other 14-nt directed repeat is found in the vicinity of the two IS, this suggested the introduction of the *vpma* cluster at locus II₅₆₃₂ via a duplication-insertion mechanism involving a circular intermediate carrying a set of *vpma* genes together with an *ISMag1* element (Fig. 4). In this scenario, the duplicated *vpma* genes would have first clustered at one end of locus I via a series of DNA recombination events. In a second step, part of locus I, including *xer1* and *ISMag1*, would have been duplicated and circularized to finally recombine at locus II via a single crossover between the IS elements. One argument in favor of the occurrence of the circular form is that a PCR product was obtained with strain 5632, using outwardly oriented primers *pv1R* and *xerF*, and its partial sequencing revealed the presence of an *ISMag1* element next to a sequence "c," usually found next to the promoter (Fig. 4). Whether this result really reflects a duplication-excision event of part of *vpma* locus I and whether the circular form can reinsert itself elsewhere in the genome have still to be demonstrated formally. Nevertheless, the 100% nucleotide identity observed between loci I and II of 5632 indicates that this duplication event occurred relatively recently.

Conclusions. A number of sophisticated genetic systems devoted to high-frequency surface variation have been described for mycoplasma species. Many of these systems appear as small sets of species-specific genes, which are nearly identical in all of the isolates and are clustered at one locus. The clustering of these genes has been proposed to facilitate recombination events which generate diversity, and theoretically, a wide range of surface diversity can be produced with only a few genes, particularly as they are prone to internal rearrangements of their sequences. Our characterization of a new set of *vpma* genes in an *M. agalactiae* field isolate (5632) illustrates this point. This new repertoire is far more complex than that first described for the PG2 type strain and represents a mosaic of structures that is suggestive of homologous recombination events between *vpma* genes, gene duplication, and/or insertion-deletion of repeated motifs (see Fig. S3 in the supplemental material). This opens the question of the true diversity in equivalent variable systems from mycoplasmas with apparently few variable genes, most of which have been sequenced from type strains. Field or clinical isolates have the potential to reveal greater-than-suspected diversity in variable gene repertoires, which could be useful to design molecular probes for epidemiology studies.

One of the most interesting findings of our study was the occurrence of duplicated *vpma* loci in strain 5632. Because one *vpma* locus can express only a single *vpma* gene per cell, the consequences of such an event are tremendous in terms of variability because it allows concomitant expression and, in turn, multiplies the number of possible surface combinations. Our data suggest that this situation most likely resulted from the duplication of one 5632 *vpma* region at a remote chromosomal position (250 kbp away) and that it involves IS elements. The role of such mobile genetic elements in chromosomal shuffling of genes encoding major surface components may have important evolutionary and epidemiological

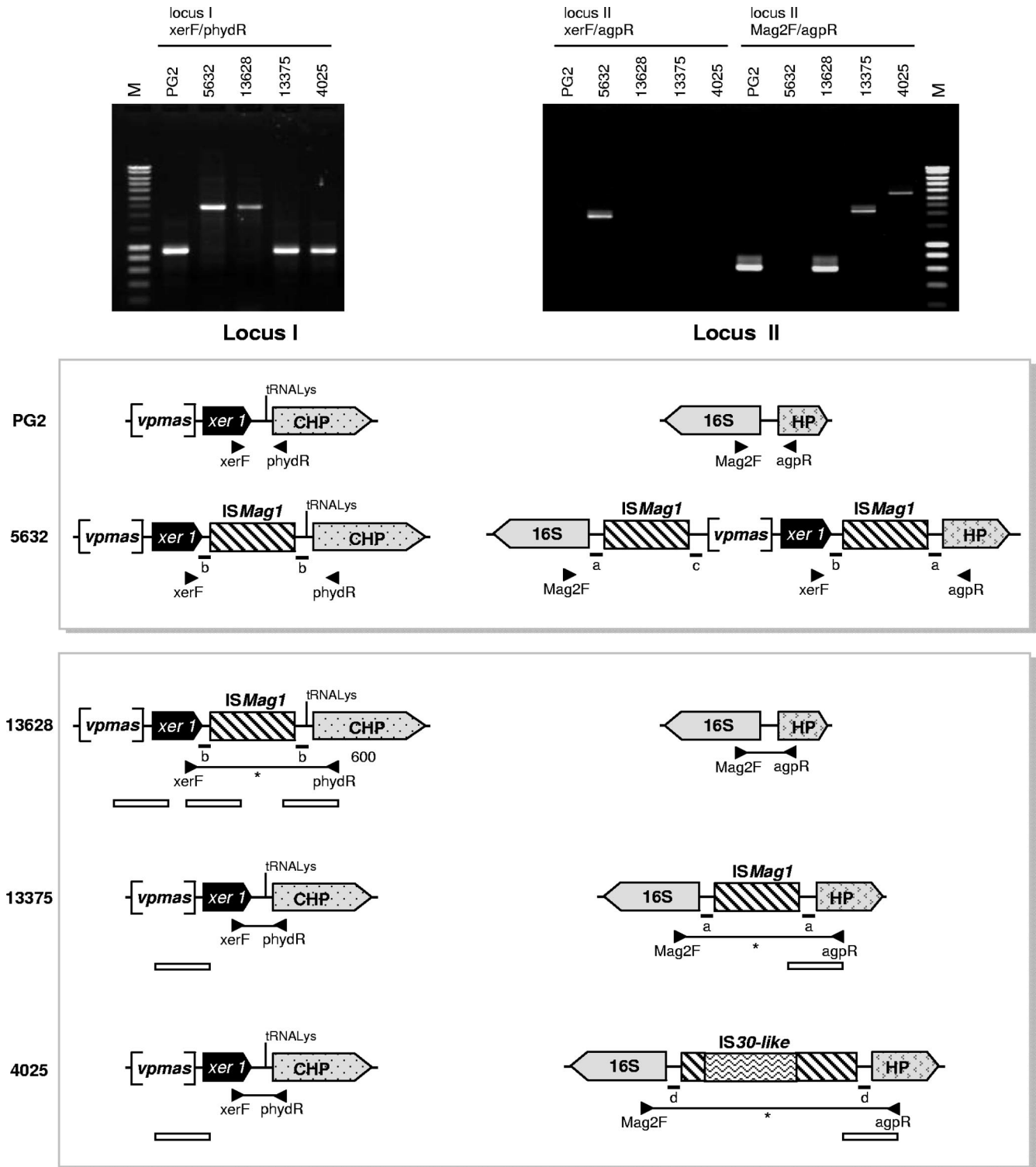


FIG. 3. Comparison of *vpma* loci or their counterparts in *M. agalactiae* strains PG2, 5632 13628, 13375, and 4025. Pictures represent the amplification of locus I and/or II by use of primers xerF-phydR, xerF-agpR, and Mag2F-agpR, whose positions are indicated in the schematics by arrowheads. Schematics represent *vpma* locus I and *vpma* locus II or its counterpart. Brackets indicate *vpma* gene cluster localization next to the *xer1* gene (filled black arrow). IS elements (*ISMag1* or *IS30-like*) are indicated by hatched boxes or boxes with wavy lines. Lines traced between primers symbolize the PCR amplicons obtained, with asterisks indicating those which were directly sequenced. White bars below the loci indicate regions that were directly sequenced using genomic DNA. Small letters (a, b, c, and d) represent the 14-nt sequences that flanked the IS.

France) for providing the strains. We also thank A. Blanchard for helpful discussions.

REFERENCES

- Baseman, J. B., and J. G. Tully. 1997. Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety. *Emerg. Infect. Dis.* **3**:21–32.
- Behrens, A., M. Heller, H. Kirchhoff, D. Yogeve, and R. Rosengarten. 1994. A family of phase- and size-variant membrane surface lipoprotein antigens (Vsp) of *Mycoplasma bovis*. *Infect. Immun.* **62**:5075–5084.
- Bhugra, B., L. L. Voelker, N. Zou, H. Yu, and K. Dybvig. 1995. Mechanism of antigenic variation in *Mycoplasma pulmonis*: interwoven, site-specific DNA inversions. *Mol. Microbiol.* **18**:703–714.
- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**:1604–1607.
- Carver, T. J., K. M. Rutherford, M. Berriman, M. A. Rajandream, B. G. Barrell, and J. Parkhill. 2005. ACT: the Artemis comparison tool. *Bioinformatics* **21**:3422–3423.
- Chen, C., J. Tang, W. Dong, C. Wang, Y. Feng, J. Wang, F. Zheng, X. Pan, D. Liu, M. Li, Y. Song, X. Zhu, H. Sun, T. Feng, Z. Guo, A. Ju, J. Ge, Y. Dong, W. Sun, Y. Jiang, J. Yan, H. Yang, X. Wang, G. F. Gao, R. Yang, and J. Yu. 2007. A glimpse of streptococcal toxic shock syndrome from comparative genomics of *S. suis* 2 Chinese isolates. *PLoS ONE* **2**:e315.
- Chen, W. P., and T. T. Kuo. 1993. A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res.* **21**:2260.
- Chopra-Dewasthaly, R., C. Citti, M. D. Glew, M. Zimmermann, R. Rosengarten, and W. Jechlinger. 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. *Mol. Microbiol.* **67**:1196–1210.
- Citti, C., G. F. Browning, and R. Rosengarten. 2005. Phenotypic diversity and cell invasion in host subversion by pathogenic mycoplasmas, p. 439–484. In A. Blanchard and G. F. Browning (ed.), *Mycoplasmas: molecular biology, pathogenicity and strategies for control*. Horizon Bioscience, Norfolk, United Kingdom.
- Citti, C., M. F. Kim, and K. S. Wise. 1997. Elongated versions of Vlp surface lipoproteins protect *Mycoplasma hyorhinis* escape variants from growth-inhibiting host antibodies. *Infect. Immun.* **65**:1773–1785.
- Citti, C., and R. Rosengarten. 1997. Mycoplasma genetic variation and its implication for pathogenesis. *Wien Klin. Wochenschr.* **109**:562–568.
- Citti, C., and K. S. Wise. 1995. Mycoplasma hyorhinis vlp gene transcription: critical role in phase variation and expression of surface lipoproteins. *Mol. Microbiol.* **18**:649–660.
- Denison, A. M., B. Clapper, and K. Dybvig. 2005. Avoidance of the host immune system through phase variation in *Mycoplasma pulmonis*. *Infect. Immun.* **73**:2033–2039.
- Flitman-Tene, R., S. Mudahi-Orenstein, S. Levisohn, and D. Yogeve. 2003. Variable lipoprotein genes of *Mycoplasma agalactiae* are activated in vivo by promoter addition via site-specific DNA inversions. *Infect. Immun.* **71**:3821–3830.
- Frangoul, L., P. Glaser, C. Rusniok, C. Buchrieser, E. Duchaud, P. Dehoux, and F. Kunst. 2004. CAAT-box, contigs-assembly and annotation tool-box for genome sequencing projects. *Bioinformatics* **20**:790–797.
- Glew, M. D., M. Marena, R. Rosengarten, and C. Citti. 2002. Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the *vpma* multigene locus. *J. Bacteriol.* **184**:5987–5998.
- Glew, M. D., L. Papazizi, F. Poumarat, D. Bergonier, R. Rosengarten, and C. Citti. 2000. Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. *Infect. Immun.* **68**:4539–4548.
- Heiner, C. R., K. L. Hunkapiller, S. M. Chen, J. I. Glass, and E. Y. Chen. 1998. Sequencing multimegabase-template DNA with BigDye terminator chemistry. *Genome Res.* **8**:557–561.
- Horino, A., Y. Sasaki, T. Sasaki, and T. Kenri. 2003. Multiple promoter inversions generate surface antigenic variation in *Mycoplasma penetrans*. *J. Bacteriol.* **185**:231–242.
- Hudson, P., T. S. Gorton, L. Papazizi, K. Cecchini, S. Frasca, Jr., and S. J. Geary. 2006. Identification of a virulence-associated determinant, dihydro-lipoamide dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through in vivo screening of transposon mutants. *Infect. Immun.* **74**:931–939.
- Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5**:150–163.
- Lartigue, C., J. I. Glass, N. Alperovich, R. Pieper, P. P. Parmar, C. A. Hutchison, III, H. O. Smith, and J. C. Venter. 2007. Genome transplantation in bacteria: changing one species to another. *Science* **317**:632–638.
- Lysnyansky, I., Y. Ron, and D. Yogeve. 2001. Juxtaposition of an active promoter to *vsp* genes via site-specific DNA inversions generates antigenic variation in *Mycoplasma bovis*. *J. Bacteriol.* **183**:5698–5708.
- Lysnyansky, I., R. Rosengarten, and D. Yogeve. 1996. Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovis* involves high-frequency chromosomal rearrangements. *J. Bacteriol.* **178**:5395–5401.
- Mahillon, J., C. Leonard, and M. Chandler. 1999. IS elements as constituents of bacterial genomes. *Res. Microbiol.* **150**:675–687.
- Marena, M. S., E. M. Vilei, F. Poumarat, J. Frey, and X. Berthelot. 2004. Validation of the suppressive subtractive hybridization method in *Mycoplasma agalactiae* species by the comparison of a field strain with the type strain PG2. *Vet. Res.* **35**:199–212.
- Neyrolles, O., I. Chambaud, S. Ferris, M. C. Prevost, T. Sasaki, L. Montagnier, and A. Blanchard. 1999. Phase variations of the *Mycoplasma penetrans* main surface lipoprotein increase antigenic diversity. *Infect. Immun.* **67**:1569–1578.
- O'Connor, L., A. Coffey, C. Daly, and G. F. Fitzgerald. 1996. AbiG, a genetically novel abortive infection mechanism encoded by plasmid pCI750 of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl. Environ. Microbiol.* **62**:3075–3082.
- O'Connor, L., M. Tangney, and G. F. Fitzgerald. 1999. Expression, regulation, and mode of action of the AbiG abortive infection system of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl. Environ. Microbiol.* **65**:330–335.
- Petersson, B., M. Uhlen, and K. E. Johansson. 1996. Phylogeny of some mycoplasmas from ruminants based on 16S rRNA sequences and definition of a new cluster within the hominis group. *Int. J. Syst. Bacteriol.* **46**:1093–1098.
- Pilo, P., B. Fleury, M. Marena, J. Frey, and E. M. Vilei. 2003. Prevalence and distribution of the insertion element ISMag1 in *Mycoplasma agalactiae*. *Vet. Microbiol.* **92**:37–48.
- Ron, Y., R. Flitman-Tene, K. Dybvig, and D. Yogeve. 2002. Identification and characterization of a site-specific tyrosine recombinase within the variable loci of *Mycoplasma bovis*, *Mycoplasma pulmonis* and *Mycoplasma agalactiae*. *Gene* **292**:205–211.
- Rosengarten, R., C. Citti, P. Much, J. Spersger, M. Drosesse, and M. Hewicker-Trautwein. 2001. The changing image of mycoplasmas: from innocent bystanders to emerging and reemerging pathogens in human and animal diseases. *Contrib. Microbiol.* **8**:166–185.
- Rosengarten, R., and K. S. Wise. 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. *Science* **247**:315–318.
- Roske, K., A. Blanchard, I. Chambaud, C. Citti, J. H. Helbig, M. C. Prevost, R. Rosengarten, and E. Jacobs. 2001. Phase variation among major surface antigens of *Mycoplasma penetrans*. *Infect. Immun.* **69**:7642–7651.
- Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream, and B. Barrell. 2000. Artemis: sequence visualization and annotation. *Bioinformatics* **16**:944–945.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sasaki, Y., J. Ishikawa, A. Yamashita, K. Oshima, T. Kenri, K. Furuya, C. Yoshino, A. Horino, T. Shiba, T. Sasaki, and M. Hattori. 2002. The complete genomic sequence of *Mycoplasma penetrans*, an intracellular bacterial pathogen in humans. *Nucleic Acids Res.* **30**:5293–5300.
- Shen, X., J. Gumulak, H. Yu, C. T. French, N. Zou, and K. Dybvig. 2000. Gene rearrangements in the *vs*a locus of *Mycoplasma pulmonis*. *J. Bacteriol.* **182**:2900–2908.
- Simmons, W. L., A. M. Denison, and K. Dybvig. 2004. Resistance of *Mycoplasma pulmonis* to complement lysis is dependent on the number of Vsa tandem repeats: shield hypothesis. *Infect. Immun.* **72**:6846–6851.
- Sirand-Pugnet, P., C. Citti, A. Barre, and A. Blanchard. 2007. Evolution of molluscites: down a bumpy road with twists and turns. *Res. Microbiol.* **158**:754–766.
- Sirand-Pugnet, P., C. Lartigue, M. Marena, D. Jacob, A. Barre, V. Barbe, C. Schenowitz, S. Mangelot, A. Couloux, B. Segurens, A. de Daruvar, A. Blanchard, and C. Citti. 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. *PLoS Genet.* **3**:e75.
- Sitaraman, R., A. M. Denison, and K. Dybvig. 2002. A unique, bifunctional site-specific DNA recombinase from *Mycoplasma pulmonis*. *Mol. Microbiol.* **46**:1033–1040.
- Tangney, M., and G. F. Fitzgerald. 2002. AbiA, a lactococcal abortive infection mechanism functioning in *Streptococcus thermophilus*. *Appl. Environ. Microbiol.* **68**:6388–6391.
- Tettelin, H., V. Masignani, M. J. Cieslewicz, C. Donati, D. Medini, N. L. Ward, S. V. Angiuoli, J. Crabtree, A. L. Jones, A. S. Durkin, R. T. Deboy, T. M. Davidsen, M. Mora, M. Scarselli, I. Margarit y Ros, J. D. Peterson, C. R. Hauser, J. P. Sundaram, W. C. Nelson, R. Madupu, L. M. Brinkac, R. J. Dodson, M. J. Rosovitz, S. A. Sullivan, S. C. Daugherty, D. H. Haft, J. Selengut, M. L. Gwinn, L. Zhou, N. Zafar, H. Khouri, D. Radune, G. Dimitrov, K. Watkins, K. J. O'Connor, S. Smith, T. R. Utterback, O. White, C. E. Rubens, G. Grandi, L. C. Madoff, D. L. Kasper, J. L. Telford, M. R. Wessels, R. Rappuoli, and C. M. Fraser. 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial “pan-genome.” *Proc. Natl. Acad. Sci. USA* **102**:13950–13955.
- Tully, J. G. 1995. Culture medium formulation for primary isolation and maintenance of molluscites, p. 33–39. In J. G. Tully (ed.), *Molecular and diagnostic procedures in mycoplasmaology: molecular characterization*. Academic Press, San Diego, CA.

47. **van der Woude, M. W.** 2006. Re-examining the role and random nature of phase variation. *FEMS Microbiol. Lett.* **254**:190–197.
48. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol.* **171**:6455–6467.
49. **Westberg, J., A. Persson, A. Holmberg, A. Goesmann, J. Lundeberg, K. E. Johansson, B. Pettersson, and M. Uhlen.** 2004. The genome sequence of *Mycoplasma mycoides* subsp. *mycoides* SC type strain PG1T, the causative agent of contagious bovine pleuropneumonia (CBPP). *Genome Res.* **14**:221–227.
50. **Wise, K. S.** 1993. Adaptive surface variation in mycoplasmas. *Trends Microbiol.* **1**:59–63.
51. **Woese, C. R.** 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.