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Molecular Basis of Size and Antigenic Variation of a *Mycoplasma hominis* Adhesin Encoded by Divergent *vaa* Genes

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The molecular basis for the size and antigenic diversity of the variable adherence-associated (Vaa) antigen, a major surface protein and a putative adhesin of *Mycoplasma hominis*, is described. Size-variant alleles of the single-copy *vaa* gene encode abundant surface lipoproteins containing one to four nearly identical, tandem repetitive units of 121 amino acids in the central region of the mature Vaa product. Gain or loss of central repeats in *vaa* genes gives rise to distinct size-variant Vaa antigens in clonal populations of this organism. The N-terminal and repeat regions of Vaa contain highly conserved sequences, while the C-terminal region, implicated as the adherence-mediating module, is highly variable and divergent among different strains of this pathogen. Sequence variation in this region may underlie the strain-dependent binding of some monoclonal antibodies to Vaa products. The Vaa antigen is expressed in vivo during chronic, active arthritis associated with *M. hominis* infection and is highly immunogenic in the human host. Size variation and C-terminal antigenic divergence of Vaa could affect the adherence of *M. hominis* and evasion of antibody-mediated immunity, thereby contributing to the organism's adaptive capability in the human host. Variation in *vaa* genes reveals a distinct pattern of mutations generating mycoplasma surface variation.

Despite a limited genome size (17, 49), mycoplasmas have successfully adapted to a wide range of hosts and typically cause persistent infections in humans and animals (23, 39). Pronounced diversity within mycoplasma species and phenotypic variation in propagating clonal populations have recently been shown to affect the surface architecture of mycoplasmas, predominantly (but not exclusively) involving surface lipoproteins, as reviewed elsewhere (14, 52, 54). In the absence of cell walls in mycoplasmas, versatility in surface protein structure may be crucial for these organisms to adapt to host niches and to evade host defense mechanisms. Directly or indirectly, high-frequency mutations affecting the expression or structure of genes encoding surface lipoproteins appear to be a major source of this diversification (52, 54). The molecular basis for surface lipoprotein variation was first elucidated in Vlp proteins of *Mycoplasma hyorhinis* (8, 57, 58), and more recently in the pMGA protein of *Mycoplasma gallisepticum* (30, 31), the MB antigen of *Ureaplasma urealyticum* (59), surface lipoproteins of *Mycoplasma fermentans* (46–48), and the V-1 (Vsa) antigen of *Mycoplasma pulmonis* (2, 40). These examples illustrate the complexity of mechanisms in diversification of mycoplasma surface proteins, including (i) size variation caused by gain or loss of intragenic repetitive sequences, (ii) phase switching by deletion/insertion mutations or DNA inversion affecting promoter activity, (iii) differential presentation of expressed surface proteins, and (iv) presence of multigene families or multiple copies of partial genes in the mycoplasmal chromosome (17), furnishing genetic reservoirs for alternate sequences. Determining the molecular genetic mechanisms of mycoplasma surface variation and adaptation is essential for understanding the mycoplasma-host interaction.

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Mycoplasma hominis is associated with human urogenital diseases, pneumonia, and septic arthritis (23). This species shows extensive population heterogeneity in vitro (4) and possibly in vivo (34). Previous studies have indicated that surface antigenic profiles of *M. hominis* are highly heterogeneous among different strains (4, 6, 7, 26, 35) or even among clinical isolates derived longitudinally from a single patient (34). Although several surface proteins, including P120 (7), P135 (26), and P50 (19), have been cloned and characterized, the molecular basis of variation in *M. hominis* has not been directly established.

In a previous study, a putative adhesin defined by a monoclonal antibody (MAb), H3, that inhibits the metabolism of *M. hominis* 1620 and blocks attachment of the organism to human fibroblast cells has been described (35). The component is expressed on the surface of *M. hominis* and was identified as a lipoprotein on the basis of partitioning characteristics in detergent and metabolic labeling with [^{14}C]palmitate (35). The antigen recognized by MAb H3 showed variation in size and expression among various *M. hominis* strains (35) and among isolates derived longitudinally from a single patient (34). Therefore, the antigen defined by MAb H3 is designated in this paper the variable adherence-associated (Vaa) antigen. Features of Vaa function and variation suggest a possible role in the interaction of *M. hominis* with the human host. The molecular basis for structural and antigenic variation of the Vaa lipoprotein has not been defined and yet may be critical to our understanding of Vaa in mycoplasmal infection. In this study, alleles of the *vaa* gene from clonal variants of pathogenic *M. hominis* 1620 were molecularly cloned and characterized and the basis for two aspects of Vaa variation were defined: (i) size variation caused by gain or loss of central repetitive sequences in the *vaa* lipoprotein gene and (ii) antigenic variation associated with marked sequence divergence in the distal C-terminal portion of Vaa, compared among additional strains of *M. hominis*. The role of variation in modulating adherence and/or evading the immune system is discussed.

MATERIALS AND METHODS

Mycoplasmas and MAbs. *M. hominis* 1620 and MAbs H3, G5, and A8, defining surface lipoproteins of *M. hominis* (35), were supplied by Lyn D. Olson, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md. Strain PG21 (M-711-002-084) was obtained from the National Institute of Allergy and Infectious Diseases, Research Resources Branch, Bethesda, Md. Mycoplasmas were grown in modified Hayflick medium (53) supplemented with 20% horse serum (Hazelton Dutchland) and 0.25% arginine. MAbs 30.3.1, 43.2, 552, 26.7D, and 667, also recognizing surface antigens of *M. hominis* (6, 7, 25, 26), were provided by Gunna Christiansen, University of Aarhus, Aarhus, Denmark. As shown in the current study, MAbs H3, A8, and 30.3.1 define the same set of size variants of the Vaa antigen (see Fig. 1). Three clonal lineages (Vaa-2, Vaa-3, and Vaa-4) of *M. hominis* expressing distinct size variants of the Vaa antigen were derived from strain 1620 by procedures previously described for other mycoplasmas (37, 38).

Construction of a genomic library. A plasmid library of *M. hominis* was constructed as described previously (10) with genomic DNA prepared from Vaa-4. An *Xho*I half-site arm cloning strategy (Promega) was adopted for construction of the genomic library. Briefly, genomic DNA fragments (1 to 4 kb) generated by partial digestion with *Mbo*I were partially end filled with dATP and dGTP and were ligated with plasmid p7ZCW (10) that had been digested with *Xho*I and then filled with dTTP and dCTP. Inserts and vector were cleaned with a gel extraction kit (QIAGEN) prior to ligation. The ligation mixture was used to transform *Escherichia coli* CC118 by electroporation, and the transformants were plated on Luria-Bertani plates containing chloramphenicol (25 μg/ml).

PCR, probe labeling, and DNA hybridization. All of the oligonucleotide primers for PCR were synthesized by the Molecular Biology Program DNA Core Facility, University of Missouri—Columbia. The sequences of these primers are as follows: F1, 5′-CCCCGGAGATTATTAAGTCT-3′; R1, 5′-GTGCCCATTAGTAGCACTAT-3′; F2, 5′-CAGCAGCAGTAGAAAATGC-3′; R2, 5′-CGGTTTTTGAAAGTTCAAAGG-3′; F3, 5′-CAAATAAAAAAATTGCTGATG-3′; R3, 5′-CTTCTTCTCATTCTGATACG-3′; F4, 5′-TGTAATGATGATAAACTAGCAGG-3′; R4, 5′-GATCAAGCTTTTTTAACTTCTTC-3′; F5, 5′-CGTATCAGAATGAGAAGAAGT-3′ (see Fig. 2 for the positions of these primers). PCRs were performed in a DNA thermal cycler (Perkin Elmer model 480) in a volume of 100 μl, under the following conditions: 200 nM primers, 10 ng of template, 2.5 mM $MgSO_4$, and 5 U of *Taq* DNA polymerase. A 330-bp DNA fragment that encodes the epitope for MAb 30.3.1 (6) was amplified with primers F3 and R3 from the genome of Vaa-4 and was labeled with digoxigenin-11-dUTP (DIG; Boehringer Mannheim) as described previously (15). This DIG-labeled PCR product was designated probe 30.3.1 and was used for Southern hybridization of *M. hominis* genomic DNA and for colony blotting during screening of the genomic library. For Southern hybridization, genomic DNA was completely digested with *Nci*I or *Taq*I, transferred to nylon membranes, hybridized with probe 30.3.1, and detected with the chemiluminescent substrate Lumi-phos 530 (Boehringer Mannheim). Colony blotting with DIG-labeled probe 30.3.1 was performed as described in the guide provided by the manufacturer. The colorimetric substrates nitroblue tetrazolium and X-phosphate (Boehringer Mannheim) were used for detection.

Cloning of PCR products. The entire tandem repeat regions of the Vaa genes in Vaa-2, Vaa-3, and Vaa-4 were amplified by PCR with primers F2 and R2. Each PCR product was separated on an agarose gel, cleaned with the QIAEX gel extraction kit (QIAGEN), and directly cloned into pT7blue vector (Novagen) as specified by the procedures supplied with the vector. The construct containing the repetitive region of Vaa-4 was designated pCL3 and sequenced by nested deletion (see below). The repetitive regions of Vaa-2 and Vaa-3 were partially sequenced with primers F2 and R2. For each variant, two clones from two separate PCRs were sequenced. The 3′ region of *vaa* in strain PG21 was amplified with primers F5 and R1 (see Fig. 2) from strain PG21 genomic DNA and was directly sequenced as described below.

DNA sequencing. For the sequencing strategy, see Fig. 2. All template plasmids for sequencing were prepared with a QIAwell 8 Plus miniprep kit (QIAGEN). For direct sequencing of PCR products, amplicons were excised from agarose gels and purified with the QIAquick gel extraction kit (QIAGEN) prior to reaction. The sequence was obtained with an automated fluorescent-DNA sequencer (model 370A; Applied Biosystems) and the *Taq* DyeDeoxy terminator cycle-sequencing kit (Applied Biosystems). Sequencing and primer synthesis were performed by the Molecular Biology Program DNA core facility, University of Missouri—Columbia. Both strands of the *vaa* gene were sequenced. To sequence repetitive regions of insert DNA, nested sets of deletions were constructed in recombinant plasmids with exonuclease III by using the Erase-a-Base System (Promega). Sequence analysis was performed with the Genetics Computer Group sequence analysis software package (12).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (27), with a 10% separating gel containing 3% (wt/vol) urea. Samples were heated at 100°C for 5 min under reducing conditions prior to electrophoresis. Proteins separated on the gels were visualized by staining with Coomassie brilliant blue R-250. For immunoblotting, proteins on the gel were transferred to nitrocellulose membranes and immunostained with antibodies as described previously (38).

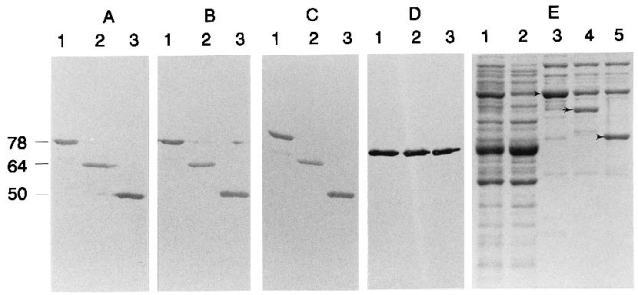

FIG. 1. Identification of amphiphilic size variant Vaa antigen by MAbs. (A to D) Clonal isolates of *M. hominis* 1620 expressing Vaa-4 (lane 1), Vaa-3 (lane 2), or Vaa-2 (lane 3) were partitioned with TX-114, and the detergent phase was separated by SDS-PAGE and immunostained with MAb H3 (A), A8 (B), 30.3.1 (C), or G5 (D). (E) Coomassie brilliant blue R-250 staining shows total protein (lane 1), aqueous-phase proteins (lane 2), and TX-114 phase proteins (lane 3) of the isolate expressing Vaa-4, as well as TX-114 phase proteins derived from isolates expressing Vaa-3 (lane 4) and Vaa-2 (lane 5). The sizes of the Vaa antigens (indicated by arrowheads in panel E) are shown on the left in kilodaltons.

Construction and immunoblot analysis of recombinant Vaa fusion protein. To generate a recombinant form of the Vaa antigen, a fragment of the *vaa* gene representing the N-terminal protein sequence from the Cys residue at position 27 to the Ser residue at position 224 (see Fig. 3) was amplified by PCR from pCL1 with primers F4 and R4 (see Fig. 2), using Vent polymerase (New England Biolabs) to generate a blunt-ended, high-fidelity PCR product. The PCR product was digested with *Hin*dIII to generate a blunt-*Hin*dIII fragment, which was gel cleaned with the QIAEX gel extraction kit (QIAGEN) and ligated with *Xmn*I- and *Hin*dIII-digested pMAL-c2 (New England Biolabs). This created an in-frame fusion of the *vaa* gene to the 3′ end of the *malE* gene (confirmed by DNA sequencing), resulting in the expression of a translational fusion with maltose-binding protein (MBP). Since three UGA codons were present near the 3′ end of this mycoplasmal insert, translation in *E. coli* would terminate at the first UGA codon encountered (see the boxed Trp at position 207 in Fig. 3). Procedures for expression of fusion proteins from the pMAL-c2 vector were performed as specified by the manufacturer. Expressed fusion proteins were affinity purified and analyzed by SDS-PAGE and immunoblotting, with various MAbs or synovial fluids derived from a patient harboring *M. hominis* 1620 (34) or a patient with rheumatoid arthritis (provided by R. Hoffman, University of Missouri). Immunoblots of human synovial fluids were developed as described for mouse MAbs but with secondary antibody to human immunoglobulin G (Accurate Chemical and Scientific Corp.).

Nucleotide sequence accession numbers. The full sequence of the *vaa* gene from strain 1620 described in this report (see Fig. 3) was deposited in GenBank with accession number U56827. The partial sequence of a *vaa* gene homolog in strain PG21 was deposited with accession number U56828.

RESULTS

Size variation of the Vaa antigen. By using MAb H3 to examine clonal lineages generated from *M. hominis* 1620, three size variants of the Vaa antigen were identified and designated Vaa-2, Vaa-3, and Vaa-4. In the original population examined, over 90% of the population of strain 1620 were Vaa-2 variants, expressing a 50-kDa lipoprotein. This was demonstrated by SDS-PAGE analysis of individual colonies. However, a minority of clonal variants expressed larger versions of the antigen, of 78 kDa (Vaa-4) or 64 kDa (Vaa-3) (Fig. 1A to C). The three Vaa variants partitioned during Triton X-114 (TX-114) phase fractionation into the detergent phase and were specifically recognized by MAb H3 (Fig. 1A). Vaa antigens were one of the most abundant membrane proteins in *M. hominis* (Fig. 1E). Another MAb (A8), previously shown to identify size-variable surface antigens of *M. hominis* (35), selectively stained the same size-variant proteins as detected by MAb H3 (Fig. 1B), strongly suggesting that these two MAbs recognized the same antigen (i.e., lipoprotein) of *M. hominis*. This finding clarified a previous ambiguity regarding the identity of antigens defined by MAbs H3 and A8 (35). A third MAb (30.3.1), previously

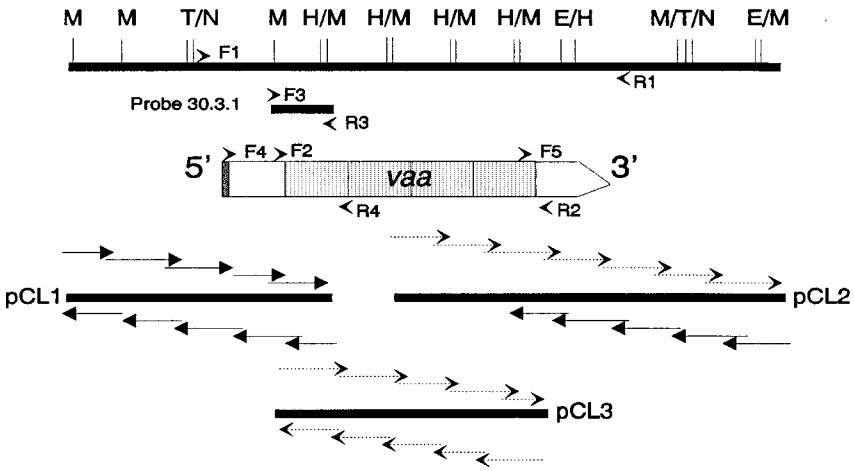

FIG. 2. Cloning and sequencing strategy, partial restriction maps, and features of the *vaa* gene and its flanking regions. Restriction sites are represented by single letters: M (*Mbo*I), T (*Taq*I), H (*Hin*dIII), N (*Nci*I), and E (*Eco*RI). The orientation (5′ → 3′) and location of the *vaa* gene are indicated by the large boxed arrow (*vaa*). The shaded central areas within the *vaa* gene indicate tandemly repeated sequences (also see Fig. 3). pCL1, pCL2, and pCL3 indicate inserts in individual recombinant plasmids used for sequencing (indicated by solid bars). The location of DNA probe 30.3.1 is also depicted by a solid bar. Sequencing strategies are indicated by broken-line arrows (regions sequenced by nested deletions) or solid-line arrows (regions sequenced by primer walking). Additional arrowheads indicate the locations of various oligonucleotide primers used for PCR (F1, R1, F2, R2, F3, R3, F4, R4, and F5).

shown to define an *M. hominis* surface epitope encoded within a cloned DNA fragment (6), also was shown to specifically recognize the size-variant Vaa antigens (Fig. 1C). In contrast to these three MAbs, MAbs G5 (Fig. 1D) and 667, 43.2, 552, and 26.7D (data not shown), also directed to *M. hominis* surface antigens (6, 7, 26), all stained *M. hominis* membrane proteins distinct from Vaa.

Cloning and sequencing *vaa* genes. The plasmid library containing genomic DNA fragments of *M. hominis* 1620 (Vaa-4) was screened with probe 30.3.1. Two positive recombinant plasmids, designated pCL1 and pCL2 (Fig. 2), were identified. These two constructs contained mycoplasma DNA inserts of 1.4 kb (pCL1) and 2.5 kb (pCL2), respectively, as determined by restriction analysis. The insert in pCL1 was sequenced by primer walking. The insert in pCL2 (shown below to contain repetitive sequences) was completely sequenced by a strategy of nested deletion (Fig. 2; see Materials and Methods). An intervening genomic region not represented in pCL1 or pCL2 was determined by sequencing cloned PCR products (pCL3) amplified by primer F2 (residing in pCL1) and primer R2 (residing in pCL2) from genomic template DNA of the mycoplasmal isolate expressing Vaa-4.

Features of the *vaa* gene and deduced Vaa surface lipoprotein sequences. The putative *vaa* gene in the variant expressing Vaa-4 contained an open reading frame (ORF) of 2,148 bp encoding 716 amino acids (GenBank accession no. U56827). The G+C content of *vaa* is 25%, comparable to the overall 29% G+C in the *M. hominis* genome (32). Four nearly identical tandem repeats, each containing 363 bp, occurred in the central region of the *vaa-4* gene (also see Fig. 5). Each repeat contains one *Mbo*I and one *Hin*dIII restriction site (Fig. 2). While all repeats are identical in length, the 3′ repeat sequence differs at three positions, two of which are not synonymous with those in the other repeats. Fifteen UGA (Trp) codons (56) occur in the *vaa-4* gene. A putative Rho-independent terminator ($\Delta G = -12$ kcal [−50.2 kJ]) occurs 18 nucleotides downstream of the TAA stop codon. A potential ribosome-binding site occurs 12 nucleotides upstream of the proposed ATG start codon.

The deduced 716-amino-acid sequence of Vaa-4 reveals an N-terminal signal peptide, consisting of 27 amino acids terminating with the prolipoprotein signal and cleavage site T-I-S-C (Fig. 3) (3, 10, 44). This result is consistent with previous biochemical evidence (35), based on metabolic labeling with palmitate, that Vaa is a lipoprotein. Partitioning of Vaa in the TX-114 detergent phase as an amphiphilic protein is also consistent with a lipoprotein structure, particularly since the entire mature sequence is otherwise predicted to be hydrophilic (24) with predominant alpha-helical characteristics. The calculated molecular mass of the mature Vaa-4 is 79.5 kDa, comparable to the 78 kDa estimated by SDS-PAGE (Fig. 1). The four tandem repetitive DNA sequences encode periodic peptide structure, accounting for 68% of the Vaa protein sequence from this isolate (Fig. 3). Each repeat contains 121 amino acids, with a calculated mass of 14 kDa that corresponds to the size difference among the Vaa variants measured by SDS-PAGE (Fig. 1). Repeats contain a high proportion of charged residues; however, the net charge of a repeat is close to neutral. No other ORFs were present on the Vaa coding strand. However, an ORF of 643 amino acids was detected on the opposite strand, spanning most of the Vaa coding region, including repetitive sequences. This ORF contained a potential start codon and ribosome-binding site and was very hydrophobic, with extensive beta-sheet structure.

Gain or loss of intragenic repeats in *vaa* alleles causes size variation of Vaa. Sequence analysis indicated the presence of *Taq*I and *Nci*I sites, each closely flanking the ends of the *vaa* gene (Fig. 2). To determine the length and copy number of *vaa* alleles, genomic DNA was extracted from the three clonal lineages (Vaa-2, Vaa-3, and Vaa-4) of strain 1620, completely digested with *Taq*I or with *Nci*I, and then hybridized with DIG-labeled probe 30.3.1 (Fig. 2). This probe identified a single *Nci*I-*Nci*I or *Taq*I-*Taq*I fragment in the genome of each of these three *M. hominis* isolates (Fig. 4A). The sizes of the hybridizing restriction fragments from the clonal lineages expressing size-variant Vaa antigens were approximately 2.1 kb (Vaa-2), 2.5 kb (Vaa-3), and 2.9 kb (Vaa-4), consistent with the fragment sizes predicted from restriction sites identified by sequencing. In addition, the difference in size among the single genomic restriction fragments in each of the three variant lineages was proportional to the predicted size differences (approximately 14 kDa) among the corresponding Vaa proteins in

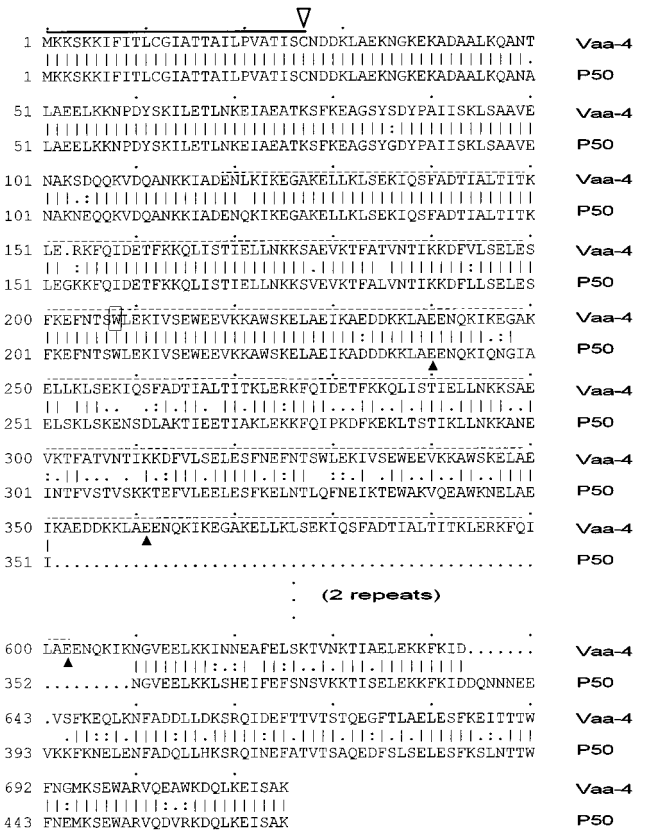

FIG. 3. Amino acid sequence of the Vaa-4 protein of strain 1620 and comparison with the Vaa homolog P50 of strain FBG (GenBank accession no. X73834). Amino acids are represented by the universal single-letter code and are numbered from the N terminus to the C terminus as indicated on the left. UGA codons are designated Trp (W). The UGA (Trp) codon (residue 207) nearest the N terminus of Vaa-4 is boxed. The region of tandemly repeated amino acid sequence of Vaa-4 is overlined (broken line), and the ends of repeats are indicated by solid arrowheads. A prolipoprotein signal peptide (residues 1 to 27) is overlined, and the proposed cleavage site is indicated by an open arrowhead. Comparison was conducted with the Genetics Computer Group GAP program. Dots in the sequences indicate gaps. Periods and colons between sequences depict similarity. Vertical lines between sequences indicate identity.

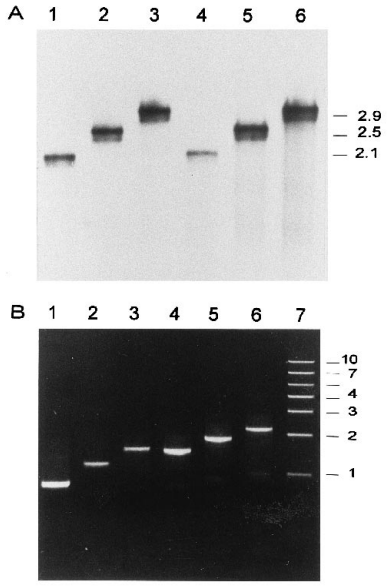

FIG. 4. Southern blot and PCR analysis of size-variant *vaa* alleles in genomic DNA of *M. hominis* 1620. (A) Genomic DNA extracted from clonal isolates expressing Vaa-2 (lanes 1 and 4), Vaa-3 (lanes 2 and 5), or Vaa-4 (lanes 3 and 6) was digested with *Nci*I (lanes 1 through 3) or *Taq*I (lanes 4 through 6), transferred to a nylon membrane, and hybridized with DIG-labeled probe 30.3.1, as described in Materials and Methods. Size markers are indicated on the right in kilobase pairs. (B) Genomic DNA from the same isolates expressing Vaa-2 (lanes 1 and 4), Vaa-3 (lanes 2 and 5), or Vaa-4 (lanes 3 and 6) was used as a template to generate PCR products, with primers F2 and R2 (lanes 1 through 3) flanking the internal repeat region of the *vaa* genes, or primers F1 and R1 (lanes 4 through 6) flanking the entire ORF encoded by the *vaa* gene (see Fig. 2 for the locations of primers). Lane 7 contains DNA markers, indicated in kilobase pairs.

each variant (Fig. 1). Since the fragment in each variant was not large enough to contain multiple copies of the *vaa* gene, these results indicated (i) that only one copy of the *vaa* gene occurs in *M. hominis* 1620 and (ii) that no reservoir of *vaa* repeats, other than intragenic copies, occurs in the genome.

These results predicted that the size of expressed Vaa lipoprotein should correlate with the length of ORFs encoded by *vaa* alleles in mycoplasma variants. To confirm this, the entire ORFs encoding size-variant Vaa proteins in the three variants were amplified from genomic DNA by PCR with primers F1 and R1 (Fig. 2). The PCR products obtained from the three variants were 2.3 kb (Vaa-4), 1.9 kb (Vaa-3), and 1.5 kb (Vaa-2), respectively (Fig. 4B), consistent with the expression of size-variant proteins. Digestion of the PCR products with *Hin*dIII and *Eco*RI yielded the expected fragments (data not shown), indicating that the PCR products contained specific *vaa* sequences. The length of a *vaa* gene corresponded well to the size of the respective Vaa protein expressed by these clonal variants (Fig. 1 and 4B).

The precise location of regions responsible for size variation in *vaa* genes was further confirmed by a PCR strategy with two primers (F2 and R2) closely flanking the internal repetitive region of *vaa* genes. As shown in Fig. 4B, the sizes of the repeat region in the three clonal lineages were proportional to the sizes of the *vaa* genes and to the corresponding Vaa proteins. The size differences among the Vaa proteins and among the corresponding *vaa* alleles were fully accounted for by the size differences contributed by the repeat regions, indicating that the internal repeats alone were responsible for the size variation of the Vaa antigen. PCR products containing the repeat sequences of Vaa-2 and Vaa-3 were also cloned into the pT7blue vector (Novagen) and sequenced. On the basis of sequencing and the sizes of the cloned repeat regions, the three variants were determined to have two (Vaa-2), three (Vaa-3), or four (Vaa-4) internal repeats, respectively.

Structural modules and C-terminal sequence divergence of Vaa antigens. A database search indicated that the Vaa antigen from *M. hominis* 1620 had regions of significant DNA and protein sequence similarity to P50 (Genbank accession no. X73834), a 50-kDa adhesin previously found in *M. hominis* FBG (18, 19). On the basis of this comparison (Fig. 3), we propose that the P50 product is a variant Vaa antigen expressed in *M. hominis* FBG. Regions of similarity between these proteins were localized, suggesting the presence of modular features of Vaa antigens, possibly subject to different mechanisms of diversification in the corresponding *vaa* genes. To simplify comparisons, *vaa* genes were operationally divided into six regions, as shown in Fig. 5. The P50 variant of Vaa had an identical signal peptide (region I) and a highly homologous region II compared with Vaa-4 from strain 1620. However, only one unit of the repeat (region III) occurred in P50. This single repeat unit in P50 is similar to that in Vaa-4 but differs by one additional amino acid (residue 153 in Fig. 3 and arrow-

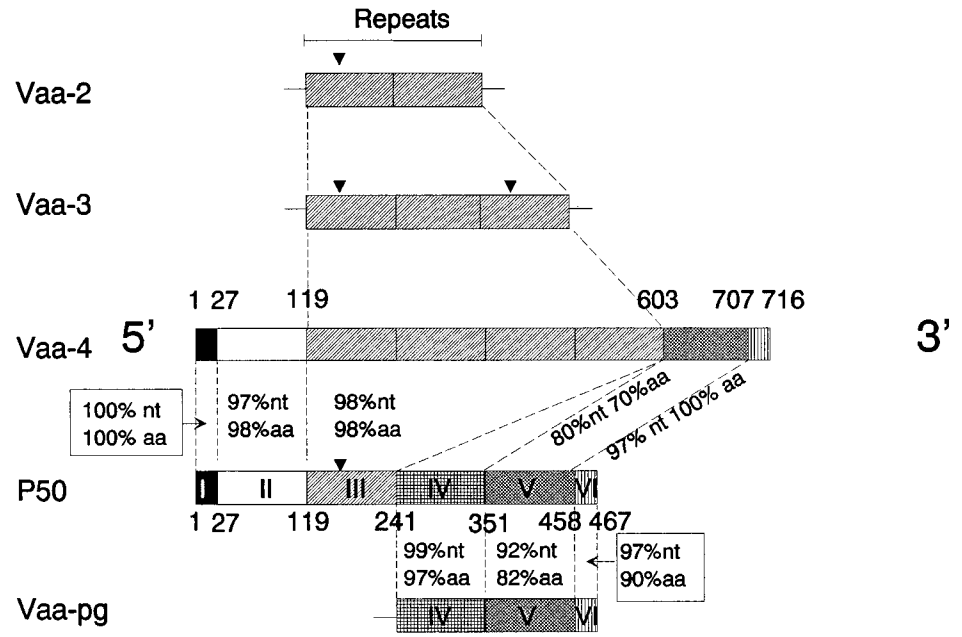

FIG. 5. Structural features and comparison of Vaa antigens. The ORFs encoded by various known alleles of the *vaa* gene are arbitrarily divided into six regions, which are shaded to indicate similarities in sequence. Amino acids are numbered from the N terminus to the C terminus along the ORFs of Vaa-4 of *M. hominis* 1620 and P50 of strain FBG. The C-terminal region of Vaa-pg, the Vaa homolog in strain PG21, is also shown. Region I represents the putative prolipoprotein signal peptide consisting of 27 amino acids. Region II contains the sequence from the predicted N terminus of the mature lipoprotein (Cys-27) to the beginning of the first tandem repeat. Region III contains variable numbers of highly homologous internal-repeat sequences. Some repeats in region III contain an additional nucleotide triplet (AGG), depicted as arrowheads. Region IV of P50 is absent from Vaa-4. Region V represents the variable C-terminal region of sequence divergence. Region VI contains the C-terminal tip sequence that is conserved among the three strains. The percent homology between regions of variant Vaa proteins is indicated by the numbers between ORFs. nt, nucleotide; aa, amino acid.

heads in Fig. 5), which occurred as a result of an additional nucleotide triplet (AGG) in the *vaa* gene creating a conservative substitution (K → R) and an additional in-frame codon. Some repeats in Vaa-2 and Vaa-3 also contained this extra codon (Fig. 5), showing diversity for this feature even among intragenic repeats in a single variant. Interestingly, the 3′ repeats in region III of Vaa-2, Vaa-3, and Vaa-4 share a fingerprint of three distinctive nucleotide substitutions (data not shown). In addition to similarity in the N-terminal regions (I, II, and III), the last 10 amino acids at the C-terminal tip (region VI) are identical between the Vaa variants P50 and Vaa-4 (Fig. 3). In contrast, striking differences between these two variant proteins occurred within regions IV and V, shown in Fig. 3 and 5. Sequence divergence in region V, near the C terminus, was manifest as nucleotide substitutions and by the presence or absence of short, in-frame oligonucleotide sequences. One region of P50 (indicated as region IV) shares significant but lower homology (69% nucleotide and 53% amino acid identity) with the region III repeat unit, possibly suggesting divergence from a common sequence. These results identified the 3′ portion of *vaa* genes as a region of sequence diversity.

To further evaluate the divergence of Vaa sequence among additional *M. hominis* strains, genomic DNA from *M. hominis* PG21 (the type strain) was used to generate PCR products representing different portions of the *vaa* gene. Oligonucleotide primers (Fig. 2) derived from sequences in regions I, II, and III of *vaa-4* successfully amplified predicted DNA fragments from strain PG21, whereas primers designed from regions V and VI of *vaa-4* failed to yield any PCR products from this strain (data not shown). In addition, a DNA probe containing sequences of regions V and VI of *vaa-4* did not hybridize to any genomic DNA fragments of strain PG21 digested with *Nci*I, although probe 30.3.1 identified a single DNA fragment in this strain. To compare the positions of suspected divergence, the 3′ region of *vaa* in PG21 was amplified with primers F5 and R1 (Fig. 2) and directly sequenced. It was found that the PG21 version of *vaa* contained a region IV nearly identical to that of P50 (99% nucleotide and 97% amino acid identity). However, comparison of region V from the three strains revealed significant divergence. For example, sequences in this region showed only 80% nucleotide and 70% amino acid identity between strains 1620 and FBG (Fig. 5; see also Fig. 3); 92% nucleotide and 82% amino acid identity between strains PG21 and FBG (Fig. 5); and 70% nucleotide and 68% amino acid identity between strains 1620 and PG21 (not indicated in Fig. 5).

Antigenic variation among Vaa allelic products. A recombinant fusion protein expressed from a *malE*::*vaa* fusion (described in Materials and Methods) was used to analyze the locations of specific epitopes by immunoblotting (Fig. 6A1). This construct contained the N-terminal mature Vaa-4 sequence spanning residues 27 (Cys) to 206 (Ser), including most of the first repeat (Fig. 3). As expected, MAb 30.3.1 specifically recognized the fusion protein (Fig. 6A1), because this construct included the sequence of probe 30.3.1 (Fig. 2), known to encode the epitope recognized by this MAb (6). MAb H3 also reacted with the fusion protein (Fig. 6A1), suggesting that the epitope defined by MAb H3 was located in either region II or region III. In contrast, MAb A8 (Fig. 1) and three other MAbs, shown to react with the authentic Vaa protein of strain 1620, did not recognize the truncated recombinant Vaa, suggesting that these MAbs might recognize an epitope involving the more C-terminal portion of Vaa (such as region V beyond the last repeat structure of region III [Fig. 5]). Notably, MAb A8 and the three others did not react with any proteins in strain

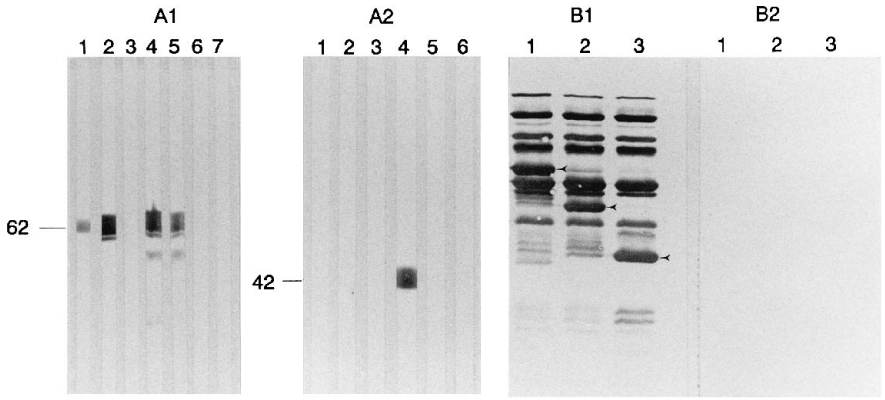

FIG. 6. Epitope mapping and demonstration of the host immune response to Vaa. (A) Blots of SDS-PAGE separated MBP-Vaa fusion protein (A1) or MBP alone (A2) were immunostained with MAb H3 (lane 1), 30.3.1 (lane 2), A8 (lane 3), or anti-MBP (lane 4) or synovial fluid SF 2117 (lane 5) or SF 9031 (lane 6). The positions of the 42-kDa MBP protein and the 62-kDa Vaa-MBP fusion protein are indicated at the left of panels A1 and A2. The G5 MAb to an unrelated antigen (Fig. 1) is included as a negative control (lane 7 in panel A1). (B) *M. hominis* antigens were immunostained with SF 2127 (B1) or SF 9031 (B2). TX-114 detergent phase proteins derived (as in Fig. 1) from clonal variants of strain 1620 expressing Vaa-4 (lane 1), Vaa-3 (lane 2), or Vaa-2 (lane 3) were stained with the respective synovial fluids, and immunoblots were developed to detect human immunoglobulin G. Arrowheads indicate the size-variable Vaa antigens.

PG21, although this strain expressed a 50-kDa Vaa identified by MAb H3 (35) and MAb 30.3.1 (data not shown). These results indicate that some MAbs directed to strain 1620 Vaa protein are unable to recognize the C-terminal epitopes of Vaa in PG21, possibly because of C-terminal sequence divergence between strains 1620 and PG21.

In vivo antibody response to Vaa. *M. hominis* 1620 was originally isolated from a joint of a patient with septic arthritis (42) and was shown to cause septic arthritis in primates (1). Synovial fluid samples were used to evaluate the in vivo antibody response to the Vaa antigen. One sample (SF 2127) was obtained from the same patient and joint from which *M. hominis* 1620 was retrieved (34), and a control sample (SF 9031) was obtained from a patient with rheumatoid arthritis not associated with *M. hominis* infection (provided by R. Hoffman, University of Missouri). Immunoblotting with SF 2127 revealed a strong immunoglobulin G antibody response to multiple antigens of *M. hominis*, including the variable Vaa antigen (Fig. 6B1). Indeed, the Vaa antigen was one of the predominant immunogens of the mycoplasma in this individual. In addition, immunoglobulin G antibodies in SF 2127 recognized the recombinant Vaa (Fig. 6A1), further confirming that a specific antibody response to Vaa occurred in vivo and was directed, at least in part, to N-terminal or repetitive regions of the lipoprotein. Control SF 9031 showed no antibody to *M. hominis* antigens or to the Vaa fusion proteins. These results indicate that Vaa is expressed and is highly immunogenic in vivo in an individual with septic *M. hominis*-associated arthritis.

DISCUSSION

Genetic analysis in this study indicates that Vaa is a lipoprotein with multiple sequence modules subject to mutational variation (Fig. 5). Immunolabeling with MAb 30.3.1 and H3 previously showed that Vaa is located on the surface of *M. hominis* (6, 35). The sequence data reported here, combined with biochemical properties cited, suggest a model for Vaa as a surface lipoprotein anchored to the plasma membrane by lipid covalently bound to an N-terminal cysteine, with alpha-helical central repeats and C-terminal regions external of the plasma membrane. This model provides a structural basis to speculate on possible functions associated with domains of the variable Vaa protein. The central repeats could function as a spacer for surface presentation of the C-terminal module, which is a likely adherence-mediating structure of Vaa. In P50 of strain FBG, the adherence-mediating structure has been provisionally mapped to amino acids 390 to 405 (19), which is located in region V (Fig. 3 and 5). Since P50 is considered a variant Vaa protein, it is possible that region V generally contains the adherence module of Vaa. This possibility is consistent with the previous finding that MAb A8, now suspected from our current study to define an epitope in the C-terminal region of Vaa from strain 1620, inhibited adherence of this *M. hominis* strain (35). In any case, sequence variability in region V suggests that this portion of the lipoprotein may be under selective pressure in the host. This is consistent both with its location as an external module exposed on the membrane surface and with the putative biological function of Vaa, mediating the interaction of *M. hominis* with host cells. It should be noted that other means of MAb inhibition might exist. Inhibition of adherence by MAb H3 (35), recognizing an epitope in region II or III of Vaa, might involve steric hindrance rather than direct binding to the adherence structure. Recombinant constructs representing specific regions or variant Vaa sequences may clarify the role of specific domains in adherence.

The divergent Vaa antigen is a major surface protein of *M. hominis* (Fig. 1E) and is highly immunogenic in the human host (Fig. 6B1). The role of Vaa variation in vivo remains to be determined in future studies. It is postulated that variation in region V of Vaa could affect the specificity or affinity of adherence and provide *M. hominis* with the flexibility to adapt to different niches, such as the urogenital tract, the respiratory tract, or the joints, where distinctive receptors may be required for optimal colonization. There is only limited information on the actual receptor bound by *M. hominis*. One report suggests that sulfated glycolipids might play this role in the urogenital tract (33). Any role for Vaa in this interaction has yet to be determined. In addition to modulation of function, C-terminal sequence variation could reflect antigenic drift of Vaa, as illustrated by the lack of MAb A8 binding to the Vaa antigen of strain PG21, in which the C-terminal sequence of Vaa appears to be substantially divergent. Such variation could play a possible protective role, but this has yet to be demonstrated.

Size variation of Vaa caused by a gain or loss of intragenic repetitive sequence could also contribute to the adaptation of

M. hominis. Contraction or expansion of the alpha-helical repeat region of Vaa could alter the accessibility of the C-terminal module to host receptors or antibodies, thereby modulating the adherence of *M. hominis* or its susceptibility to antibody-mediated damage. Differential exposure of selected regions of mycoplasma surface lipoproteins has also been documented in *M. fermentans*, in which a masking mechanism contributes to high-frequency phenotypic variation in surface epitope expression on some lipoproteins (46, 48). Although additional roles for size variation in *M. hominis* are speculative, other mycoplasma systems offer possible functions associated with this phenomenon. In the Vlp system of *M. hyorhinis* for example, convalescent-phase host serum appears to selectively inhibit the growth of organisms expressing smaller Vlp size variants (containing fewer internal repetitive sequences), whereas this serum had no effect on organisms expressing larger Vlp size variants (9).

Repetitive amino acid sequences are present in a number of toxins and surface antigens of pathogenic bacteria (13, 22). Many of these are ligand-binding proteins and are known or proposed virulence factors, such as M proteins (16, 20) and the fibronectin-binding adhesin of streptococci (45), RTX toxins of general gram-negative bacteria (50), lipoprotein H.8 of *Neisseria* species (21), and several toxins from clostridia (55). Repeat structures in these molecules are either directly related to ligand binding, are associated with antigenic variation, or contribute to the overall protein conformation that facilitate pathogen-host interactions (13, 20, 22). Repetitive sequences are also found in many mycoplasma surface antigens (2, 14, 40, 54, 57, 59). Homologous recombination and slipped-strand mispairing have been invoked as mechanisms to explain the duplication or exchange of repetitive sequences (28, 36, 41). Whichever mechanism underlies the contraction or expansion of Vaa repeats, at least two copies of the repeated unit are required. Because only one copy of the *vaa* gene occurs in the chromosome of *M. hominis*, the intragenic repeats are the only sources for duplication. This suggests that *vaa* alleles with only a single repeat unit may fail to undergo further size variation. For example, there is no evidence that P50, with only one repeat unit, shows size heterogeneity (19). Unlike *vaa*, several other known mycoplasma adhesin genes, such as the genes encoding the P1 adhesin of *Mycoplasma pneumoniae* (43), the MgPa adhesin of *Mycoplasma genitalium* (11, 17, 29) and the pMGA adhesin of *M. gallisepticum* (30), have been shown to occur either as multiple copies of entire genes or as portions of the genes dispersed at different loci on the mycoplasma chromosome. The significance of these redundant repetitive sequences in the diversification of mycoplasmal adhesins has not been clarified, although they could provide genetic reservoirs for DNA rearrangement. It is noteworthy in this context that region V of the Vaa sequence shows diversity through apparent insertion/deletion of small in-frame oligomeric sequences. It is possible that reservoirs of these limited segments are located in other chromosomal locations.

Vaa antigens belong to an important and expanding group of mycoplasmal surface lipoproteins. Prokaryotic lipoproteins are abundant in the periplasmic space and on the surface of gram-negative and gram-positive eubacteria, respectively (3, 44). They also occur as major membrane surface proteins of mycoplasmas (10, 51, 54). An increasing number of diverse biological functions, including substrate binding, protein secretion, signal transduction, and adherence, have been attributed to lipoproteins. Theoretically, membrane-anchored mycoplasma lipoproteins are ideal candidates for executing these biological roles in mycoplasmas, which lack cell walls or other common surface appendages observed in most eubacteria. Other surface lipoproteins of *M. hominis*, in addition to Vaa, have been identified (7, 25, 26). One of these is a 120-kDa protein (P120) defined by MAb 26.7D (7). P120 has a typical prokaryotic lipoprotein signal peptide and does not have repetitive sequences but possesses hypervariable and semivariable regions, which undergo spontaneous mutations leading to antigenic variation of P120 (5). The hypervariable region of P120 is near the N terminus of the protein, unlike that in Vaa. The biological function of P120 remains to be determined. Another interesting surface protein is the Lmp1 antigen, a 135-kDa hydrophilic basic surface antigen of *M. hominis* (26). Lmp1 appears not to be a lipoprotein, as determined by sequence and biochemical analysis. Although multiple repeats are present in Lmp1, they have not been implicated in the size variation of this protein. Identification and comparison of the genes encoding Vaa lipoprotein adhesins provide new information on the mechanisms of *M. hominis* diversification. Since the molecular structure and basis of variation of Vaa antigens are known, the possible role of Vaa variation in vivo may now be more directly addressed.

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