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Antigenic Topology of the P29 Surface Lipoprotein of *Mycoplasma fermentans*: Differential Display of Epitopes Results in High-Frequency Phase Variation

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Antibodies to P29, a major lipid-modified surface protein of *Mycoplasma fermentans*, reveal phase variation of surface epitopes occurring with high frequency in clonal lineages of the organism. This occurs despite continuous expression of the entire epitope-bearing P29 product (detected by Western immunoblotting) and contrasts with phase variation of other surface antigens mediated by differential expression of proteins. To understand the structure and antigenic topology of P29, the single-copy *p29* gene from strain PG18 was cloned and sequenced. The gene encodes a prolipoprotein containing a signal sequence predicted to be modified with lipid and cleaved at the N-terminal Cys-1 residue of the mature P29 lipoprotein. The remaining 218-residue hydrophilic sequence of P29 is predicted to be located external to the single plasma membrane. Additional Cys residues at positions 91 and 128 in the mature protein were shown to form a 36-residue disulfide loop by selectively labeling sulfhydryl groups that were liberated only after chemical reduction of monomeric P29. Two nearly identical charged amino acid sequences occurred in P29, within the disulfide loop and upstream of this structure. Two distinct epitopes binding different monoclonal antibodies were associated with opposite ends of the P29 protein, by mapping products expressed in *Escherichia coli* from PCR-generated 3′ deletion mutations of the *p29* gene. Each monoclonal antibody detected high-frequency and noncoordinate changes in accessibility of the corresponding epitopes in colony immunoblots of clonal variants, yet sequencing of the *p29* gene from these variants and analysis of disulfide bonds revealed no associated changes in the primary sequence or disulfide loop structure of P29. These results suggest that P29 surface epitope variation may involve masking of selected regions of P29, possibly by other surface components undergoing phase variation by differential expression. Differential masking may be an important mechanism for altering the antigenic or functional surface topology of this mycoplasma and other wall-less mycoplasmas.

Mycoplasma fermentans is an infectious agent of the human urogenital and pulmonary systems (12, 22, 27, 32a, 35). Because it has been shown to be associated with lesions in the kidneys of human immunodeficiency virus-positive patients (3) and causes a fulminating disease in monkeys (18), this agent is under investigation as a potential human pathogen. Whether this organism is a cofactor in the progression of AIDS, as has been postulated (17), or represents an opportunistic infection in immunocompromised patients, investigation of the surface proteins of this organism is needed to understand its role in infection, pathogenicity, virulence, and host-cell interactions. Recently, several distinct surface lipoproteins in *M. fermentans* PG18 were identified by using specific antibodies (Abs) (38). Screening of nitrocellulose lifts of colonies (colony immunoblots) with these Abs revealed a system of high-frequency antigenic variation involving most of these lipoproteins (33). By using this type of analysis to define surface epitope variation, clonal lineages oscillating between phenotypically negative and positive populations were established (33).

Increasingly, complex systems of antigenic variability of mycoplasma surface proteins are being described (11, 37, 40), and their roles in host-cell interactions are being defined. These variable antigens, often surface lipoproteins, may be key to the organism's ability to avoid or alter the host immune response. Since variant surface lipoproteins of mycoplasmas may be key immunological determinants and mediators of cellular interactions within the host, analysis of the mechanisms of variation and the antigenic topology of lipoproteins is currently a critical issue for several mycoplasma species, including *M. fermentans.*

Two distinct phenomena have been observed to generate surface antigenic variation in *M. fermentans* (33). The first occurs through differences in the expression of individual lipoproteins, which determines the presence or absence of the epitope-bearing products detected on the cell surface. A second form of phase variation of surface epitopes in *M. fermentans* appears to involve differential accessibility of epitopes present on constitutively expressed surface lipoproteins. This newly described phenomenon appears to be responsible for phenotypic variation of P29, a major surface lipoprotein of *M. fermentans* (33). Western immunoblots stained with the C19A monoclonal antibody (MAb) to P29 showed this protein to be consistently expressed, even in switched clonal isolates failing to bind the C19A MAb as determined by colony immunoblotting (33). In this report, we additionally show that selected regions of P29 can be differentially displayed on the mycoplasmal surface, by using a second MAb to detect P29 on colony immunoblots of clonal variants that do not bind the C19A MAb. The basis of this type of variation was not previously understood, but it underscored the range of potential surface structural mosaics on *M. fermentans* and the complexity of this organism's surface membrane topology. Since P29 is an abundant surface protein contributing to antigenic diversity, a more detailed molecular study was undertaken to characterize its unusual role in generating high-frequency phenotypic variation in *M. fermentans.*

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In this study, we have determined the sequence and deduced structural features of P29, localized two distinct epitopes on P29 that are differentially and independently displayed on the cell surface during phase variation, and assessed possible mechanisms underlying differential surface display as a form of antigenic variation in mycoplasmas. In the course of this work, P29 was shown to contain an invariant intrachain disulfide loop which is unusual for surface lipoproteins in mycoplasmas or in phylogenetically related gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. *M. fermentans* type strain PG18 has been described previously (12, 22, 27) and characterized elsewhere (33, 38). The *Escherichia coli* strains used were as follows: KW251 (Promega Biotech, Madison, Wis.) for chromosomal library construction and propagation of bacteriophage isolates, competent DH5αF′ MCR cells (BRL Life Technologies, Inc., Gaithersburg, Md.) and BMH 71-18 mut S cells (Promega) for site-directed mutagenesis, and competent DH5α MCR cells (BRL) for expression of all plasmid-encoded, recombinant proteins. Plasmids pMFPZ-1 and pMFPZ-2 were derived from pGEM-7Z (Promega). pMFPSEL-1 and pMFPSEL-1.2M were derived from pSELECT (Promega). pMFPZ-21, pMFPZ-24, and pMFPZ-34 were derived from pGEM-3Z (Promega). All *M. fermentans* DNA constructs placed sequence inserts under control of the vector-encoded T7 promoter. Recombinant protein expression was regulated by cotransformation with plasmid pGP1-2, encoding the temperature-inducible T7 RNA polymerase (32).

Media, chemicals, and enzymes. *M. fermentans* PG18 was grown in modified Hayflick medium as previously described (38, 39). KW251 cells were grown with shaking at 37°C in NZCYM broth (28) supplemented with 0.2% maltose. DH5α MCR, DH5αF′ MCR, and BMH 71-18 mut S cells were grown with shaking at 37°C in 2× yeast-tryptone medium supplemented with ampicillin at 100 μg/ml, tetracycline at 15 μg/ml, or kanamycin at 40 μg/ml, as appropriate for plasmid selection. Competent *E. coli* DH5α MCR or DH5αF′ MCR cells were transformed according to the manufacturer's instructions (BRL Life Technologies). Plasmid DNA was extracted from *E. coli* by the Wizard Minipreps DNA purification system (Promega). Restriction enzymes, T4 ligase, and Klenow fragment were obtained from Promega, Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or BRL Life Technologies and were used as recommended by the manufacturers. *Amplitaq* (Perkin-Elmer, Branchburg, N.J.) or Vent (New England Biolabs, Beverly, Mass.) DNA polymerase was used for PCR amplifications. Oligonucleotide primers were synthesized at the University of Missouri DNA Core Facility, using an ABI model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).

Construction and screening of the *M. fermentans* PG18 chromosomal expression library. LambdaGEM-11 vector arms which had been digested with *Xho*I and partially filled in were used for library construction as described by the manufacturer (Promega). *M. fermentans* PG18 chromosomal DNA was partially digested with *Sau*3AI under conditions resulting in fragments of approximately 8 to 12 kb. These fragments were partially filled with dGTP and dATP to create incompatible half-sites that prevent formation of concatamers. Approximately 5 μg of insert DNA and 0.5 μg of lambda vector arms were ligated and then packaged with the Packagene in vitro packaging system (Promega). Phage plaques were generated in KW251 cells and immunoscreened for epitope expression. Briefly, 60-mm-diameter plates containing 1×10^3 to 2×10^3 PFU were overlaid with nitrocellulose filters (BA85, 0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) for 10 min. The filters were removed, blocked with TS buffer (150 mM NaCl, 10 mM Tris; pH 7.4) containing 3% (wt/vol) bovine serum albumin (BSA) (Fisher Scientific Co., Fairlawn, N.J.), and then incubated overnight at 4°C with a combination of polyclonal antibodies (PAbs) generated (38) either against whole organisms of *M. fermentans* PG18 or against proteins obtained in the detergent phase (Triton X-114 [TX-114]-phase proteins) after TX-114 phase fractionation of the organisms. PAbs were diluted 1:300 in phosphate-buffered-saline (PBS) (2.7 mM KCl, 1.5 mM H_2PO_4, 137 mM NaCl, 8.0 mM Na_2HPO_4; pH 7.2) containing 10% (vol/vol) fetal bovine serum (FBS) (Hazelton Dutchland, Inc., Denver, Pa.). The PAb mixture was preabsorbed with sonically disrupted *E. coli* KW251 cells prior to library screening (28). After being washed with TS buffer, nitrocellulose lifts were incubated for 1 h in peroxidase-conjugated secondary goat Ab against mouse immunoglobulin G (Cappel, Organon Teknika, West Chester, Pa.) diluted 1:1,000 in PBS containing 10% FBS and were then developed with *o*-dianisidine substrate (34). Phage isolates able to bind Ab were isolated and stored over chloroform in SM buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM $MgSO_4$, 0.02% gelatin; pH 7.5) for future analysis.

Elution of specific Abs from phage plaques. Nitrocellulose blots of purified phage isolates expressing *M. fermentans* antigens were prepared from 60-mm-diameter plates containing approximately 5,000 PFU per plate. The filters were blocked and incubated with the combination of PAbs described above. The blots were rinsed extensively with TS buffer, and a small portion of each filter was removed for subsequent immunostaining. The remaining filter was treated immediately with 3 ml of elution buffer (50 mM glycine, 0.1% [wt/vol] BSA, 500 mM NaCl; pH 2.2) for 10 min at room temperature with gentle agitation. The eluate was collected and immediately adjusted to pH 7.5 with 3 M Tris (pH 8). FBS was added to a final concentration of 10% (vol/vol), and the eluted Ab solution was filter sterilized and used as a primary Ab to stain Western blots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated *M. fermentans* TX-114-phase proteins. In order to monitor the effectiveness of elution, eluted filters were rinsed in TS buffer, incubated with secondary Ab and then substrate, and compared with identically treated portions of filters set aside prior to Ab elution.

Generation of 5′ exonuclease III deletions. 5′ exonuclease III deletions of the 12-kb *M. fermentans* chromosomal DNA fragment encoding P29 were generated by using the Erase-a-Base system (Promega). Approximately 20 μg of pMFPZ-1 was digested with *Sma*I and *Aat*II, resulting in a 5′ blunt end sensitive to exonuclease III and a 3′ overhang resistant to this enzyme. DNA was precipitated and resuspended in exonuclease III buffer containing approximately 277 U of the enzyme, and the reaction mixture was placed at 37°C. Samples were removed every 4.5 min six times, mixed with 2.5 U of S1 nuclease (stopping the reaction and removing the resulting single-stranded tails), and placed on ice. Samples were placed at room temperature for 30 min. S1 stop buffer (300 mM Tris, 50 mM EDTA; pH 8) was added, and the samples were heated at 70°C for 10 min. DNA from each time point was analyzed on a 1% agarose gel, and the sizes ranged from 11 to 5 kb. Samples were transferred to 37°C, and 5 U of Klenow fragment, enzyme buffer, and 0.125 mM (each) deoxynucleoside triphosphates were added. After 5 min, 0.5 U of T4 DNA ligase and the appropriate buffer were added to samples, which were then shifted to room temperature for 4 h. The religated plasmids and pGP1-2 were cotransformed into competent DH5α MCR cells as described by the manufacturer.

DNA hybridizations. *Hin*dIII- and *Nsp*V-digested *M. fermentans* PG18 chromosomal DNA or the *p29* gene PCR amplified from various *M. fermentans* strains (see below) was transferred by Southern blot from an agarose gel onto a positively charged nylon membrane filter (Boehringer Mannheim, catalog no. 1209299) with a TransVac vacuum blotter (Hoefer Scientific Instruments, San Francisco, Calif.) as specified by the manufacturer. The Genius System (Boehringer Mannheim) was used for labeling and detection of the DNA probe. Briefly, the *Hin*dIII fragment containing the entire *p29* gene (see Fig. 2) was excised from an agarose gel by using the GeneClean system (Bio 101, Inc., Vista, Calif.), randomly primed, and labeled with digoxigenin-11-dUTP. Hybridization at 65°C overnight was performed, and the membranes were washed at 65°C in 2× wash solution (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] [19]–0.1% SDS) and then in 0.5× wash solution (0.5× SSC–0.1% SDS) and developed by colorimetric detection as indicated by the manufacturer.

PCR amplification of the *p29* gene from *M. fermentans* strains. Chromosomal DNA from *M. fermentans* PG18 or strains Incognitus, K-12, SK 5, and MT-2 and isolates cultivated from human immunodeficiency virus-positive individuals were provided by Shyh-Ching Lo, at the Armed Forces Institute of Pathology, and used as templates for PCRs. The oligonucleotide primers used flanked the *Hin*dIII fragment containing the entire *p29* gene. The forward primer (F1; see Fig. 2) was 5′-GGATCAGAGCACGAGC-3′, and the reverse primer (R1; see Fig. 2) was 5′-GCCCCTTTTAGTAAGCTA-3′. The PCR mixture contained approximately 50 ng of chromosomal DNA, 10× *Amplitaq* buffer, 5 U of *Amplitaq* DNA polymerase, 20 mM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, and 1 nmol of each primer in a total volume of 100 μl. The cycling profile consisted of denaturing at 95°C for 1.5 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min for 25 cycles in a model 480 DNA thermal cycler (Perkin-Elmer). PCR products were analyzed by size on 0.8% agarose gels and by Southern hybridization as described above.

PCR amplification and sequencing of the *p29* gene from *M. fermentans* PG18 clonal isolates. Samples (200 μl) of mid-logarithmic-phase *M. fermentans* cultures were centrifuged and rinsed with PBS. Cells were resuspended in 75 μl of water and heated at 100°C for 10 min. The same PCR mixture, cycling profile, and primers described above were used to amplify the *p29* gene. The PCR products were purified with the GeneClean system (Bio 101, Inc.) and cloned into the pNoTA/T7 shuttle vector with the Prime PCR Cloner Cloning System as described by the manufacturer (5 Prime→3 Prime, Inc., Boulder, Colo.). Automated sequencing of the cloned PCR products was performed with a vector-encoded universal M-13 forward primer, a *p29* gene-specific internal primer (5′-GCTGAACTTAAAAAGGAAG-3′) (nucleotides [nt] 373 to 391 in Fig. 2), and a vector-encoded universal M-13 reverse primer.

Site-directed mutagenesis. Site-directed mutagenesis of the *p29* gene was carried out with the Altered Sites System (Promega). The *Hin*dIII fragment containing the *p29* gene was cloned into the pSELECT vector by standard techniques, yielding the construct pMFPSEL-1. Site-directed mutagenesis was performed as described by the manufacturer with the mutagenic oligonucleotide 5′-GGAAGAATTAATTGGAGCAATGGAGACAA-3′. The oligonucleotide was designed to change a UGA (Trp) codon to a UGG (Trp) codon at amino acid position 135 of the mature P29 protein (see Fig. 2). Briefly, pMFPSEL-1 was transformed into DH5αF′ MCR cells and single-stranded DNA was obtained. Approximately 1.2 pmol of the mutagenic oligonucleotide was used to generate a plasmid containing the mutant strand. The final construct, pMFPSEL-1.2M, containing the point mutation, was transformed into DH5α MCR cells, and the mutation was confirmed by DNA sequencing of the insert.

PCR-generated 3′ deletions. pMFPSEL-1 digested with *Eco*RV was used as a template for PCR reaction mixtures, consisting of 10× Vent polymerase buffer, 2 U of Vent polymerase, approximately 0.1 μg of template DNA, 20 mM each deoxynucleoside triphosphate, and 1 nmol of each primer in a total volume of 100 μl. Twenty-five cycles were performed, using a profile consisting of denaturing at 95°C for 1.5 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min. The forward primer (F2) was designed from the vector-encoded T7 promoter sequence, 5′-TAATACGACTCACTATAGGG-3′. Reverse primers were R2 (5′-GCTTCTGCTTCCTTTTTAAG-3′), R3 (5′-CTGTTTTAGCAG GATCCAAATC-3′), and R4 (5′-CTCCTTTTTTGCTATTATATG-3′). See Figure 4 for locations of primers. PCR products were digested with *Eco*RI (this restriction enzyme site was amplified from vector sequence; see Fig. 4B), resulting in an *Eco*RI-blunt-ended fragment. Products of the correct sizes were excised from agarose gels with the GeneClean kit (Bio 101, Inc.), directionally cloned into pGEM-3Z digested with *Eco*RI and *Sma*I, and cotransformed with pGP1-2 into competent DH5α MCR cells. Deletion constructs were confirmed by DNA sequencing.

Expression of recombinant proteins, SDS-PAGE analysis, and immunoblotting with MAbs. The T7 expression system (32) was used to express all recombinant proteins. *E. coli* DH5α MCR cells containing pGP1-2 and individual plasmids containing the deletions described above were grown at 30°C with shaking. The temperature was shifted to 42°C for 20 min, rifampin (200 μg/ml) was added, and the cultures were placed at 37°C for 2 h. Cells were then harvested and resuspended in SDS-PAGE sample buffer. SDS-PAGE was performed by the method of Laemmli (15) using either 14 or 9% polyacrylamide gels containing 3% (wt/vol) urea. Samples were heated at 100°C for 10 min under reducing conditions (5% [vol/vol] β-mercaptoethanol) prior to SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose filters (BA85, 0.45-μm pore size; Schleicher & Schuell, Inc.) and immunostained with C19A or 4437E12.C MAb to P29. MAb C19A was described previously (38), and MAb 4437E12.C was generated by fusion of the myeloma line SP2/O with splenocytes from BALB/c mice immunized with freeze-thaw-disrupted whole organisms of *M. fermentans* PG18. Western blots were treated with peroxidase-conjugated secondary goat Ab to mouse immunoglobulin (Cappel Laboratories), diluted 1:1,000 in PBS containing 10% FBS, and developed with *o*-dianisidine substrate. Screening of colony nitrocellulose lifts with MAbs to determine phenotypes has been described elsewhere (33).

Detection of disulfide bonds. Sulfhydryl groups were detected by using a digoxigenin protein labeling kit (Boehringer Mannheim). Briefly, organisms from passaged *M. fermentans* PG18 cultures, or clonal isolates of this strain selected for negative or positive phenotypes of P29 epitope expression (determined by screening colony immunoblots with the C19A MAb to P29 [33]), were detergent fractionated with TX-114 (38), and the TX-114-phase proteins were precipitated with methanol. Proteins were separated by SDS-PAGE under nonreducing conditions, and samples on identical gels were electrophoretically transferred to duplicate nitrocellulose filters. Filter 1, used subsequently to detect free sulfhydryl groups on immobilized proteins, was placed in buffer (50 mM potassium phosphate; pH 7.0) overnight at room temperature. The second filter (filter 2), used to detect sulfhydryl groups generated after chemical reduction of disulfide bonds, was first incubated overnight at room temperature in buffer containing 20 mM *N*-ethylmaleimide to block any free sulfhydryl groups. This filter was then washed three times (15 min each) with the buffer described above. Potential disulfide bonds were then reduced by incubating filter 2 in buffer containing 2% (vol/vol) β-mercaptoethanol for 1 h and washing the filter as before. Both filters were treated for 2 h in 10 ml of buffer containing 5 μl of digoxigenin-3-*O*-succinyl-[2-(*N-maleimido*)]ethylamide and 0.01% Nonidet P-40. This labels free sulfhydryl groups initially present in the proteins (filter 1) or liberated from disulfide bonds in chemically reduced proteins (filter 2). The filters were then washed for 15 min with Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl; pH 7.5) containing 0.1% Tween 20 and then washed twice for 15 min each in TBS alone. Finally, the filters were incubated with an alkaline phosphatase-conjugated Ab to digoxigenin and developed as described by the manufacturer.

DNA sequencing. DNA sequencing reactions were performed at the University of Missouri Molecular Biology Program DNA Core Facility using *Taq* DyeDeoxy terminators and an automated DNA sequencer (model 373A; Applied Biosystems, Inc.).

Computer analysis. DNA and protein sequences were analyzed with the Genetics Computer Group sequence analysis software package from the University of Wisconsin, Madison (9).

Nucleotide sequence accession number. The DNA sequence of the *p29* gene (accession no. U50827) and the putative lysyl-tRNA synthetase gene (accession no. U50826) have been submitted to GenBank.

RESULTS

Cloning of the *p29* gene and detection in *M. fermentans* strains. To identify genes encoding antigenic proteins, an expression library was constructed in LambdaGEM-11 using chromosomal DNA of *M. fermentans* PG18 partially digested with *Sau*3AI, as described in Materials and Methods. Because UGA (Trp) codons in mycoplasma genes (42) can result in inappropriately translated protein products in the *E. coli* host (23), the library was screened with hyperimmune PAbs generated against whole organisms or TX-114-phase proteins of *M. fermentans* PG18 (38) to increase the range of epitopes recognized on expressed products. Positive plaques were isolated and used to generate high-density plaque immunoblots. After treatment of blots with PAbs, Abs specifically binding to epitopes expressed in the phage were eluted (14) and used to identify the corresponding TX-114-phase proteins of *M. fermentans* PG18 (33, 38) by Western immunoblotting (data not shown). One phage, found to express epitopes shared by the P29 lipoprotein, was analyzed further. This phage contained a 12-kb insert which was excised and cloned in both orientations into pGEM-7Z (the cloning strategy is shown in Fig. 1A). When expressed in *E. coli* under T7 promoter control, one of these constructs, pMFPZ-1, expressed two major protein products of 30 and 16 kDa, which were identified by Western immunoblot using the C19A MAb (38), defining a phase-variant surface epitope on P29 (33). 5′ exonuclease III deletions of the insert in pMFPZ-1 yielded a construct containing a truncated 6-kb insert, which expressed the same two epitope-bearing products. This construct (pMFPZ-2) was partially sequenced by using primers to the vector-encoded T7 promoter sequence and primers derived from insert DNA sequences. An open reading frame (ORF) of 256 amino acids containing a start Met residue and a characteristic prokaryotic lipoprotein signal sequence (5, 7, 31) was identified. The ORF was flanked by two *Hin*dIII sites, creating an 893-bp fragment that was subcloned into the pSELECT vector to generate pMFPSEL-1. This construct expressed both the 30- and the 16-kDa protein products detected by the C19A MAb to P29 (Fig. 1B, lane pMFPSEL-1), thereby localizing the *p29* gene to the 893-bp *Hin*dIII fragment, which was subsequently sequenced. DNA sequence flanking the *p29* gene was obtained from pMFPZ-2. As detailed below (see "Localization of P29 phase-variant epitopes differentially displayed on the cell surface"), the 16-kDa product was shown to occur because of a UGA (Trp) codon resulting in premature termination of the P29 product expressed in *E. coli*, while the 30-kDa product resulted from a translational read-through of this UGA codon.

Results from Southern blot analysis using the 893-bp *Hin*dIII fragment as a labeled probe were consistent with a single copy of *p29* in *M. fermentans* PG18. Single bands with similar intensities and predicted sizes (based on flanking sequence data) were detected in chromosomal DNAs digested with *Hin*dIII (approximately 0.9 kb) and *Nsp*V (approximately 1.9 kb) (data not shown). Additionally, the *p29* gene was identified in other *M. fermentans* strains by using PCR amplification from the forward (F1) and reverse (R1) primers indicated in Fig. 2. For all strains tested, a product of approximately 0.9 kb which hybridized to the labeled *Hin*dIII cloned fragment containing the *p29* gene was amplified from chromosomal DNA. Control reactions without a template or using *Mycoplasma hominis* 1620 chromosomal DNA failed to amplify a product (data not shown). The gene encoding the major P29 surface lipoprotein was detected in several strains of *M. fermentans*, including Incognitus, K-2, SK 5, and MT-2 and five individual isolates cultured from human immunodeficiency virus-positive patients (17).

P29 sequence and predicted structural features. Figure 2 shows the coding and flanking DNA sequence of the *p29* gene with the deduced amino acid sequence of the P29 prolipoprotein. The G+C content within the *p29* coding region is 30%, consistent with the overall G+C content of *M. fermentans* chromosomal DNA of 27.5 to 28.7% (13). A putative ATG

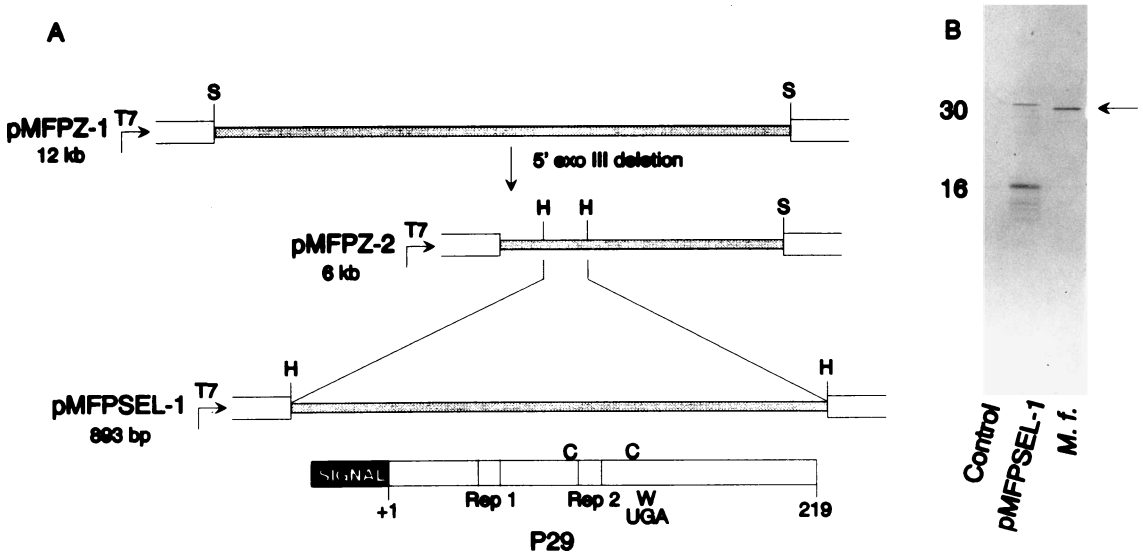


FIG. 1. Strategy for cloning the *p29* gene. (A) Constructs are designated on the left, with the sizes of the DNA inserts indicated underneath. A 12-kb *Sac*I-*Sac*I DNA fragment encoding the phase variant epitope recognized by MAb C19A to P29 was isolated from a lambda phage *M. fermentans* chromosomal expression library (as described in Materials and Methods) and subcloned into the pGEM-7Z vector to generate pMFPZ-1. 5′ exonuclease III deletions of this insert created pMFPZ-2, containing a 6-kb fragment also encoding the epitope. An 893-bp *Hin*dIII fragment containing an ORF of 256 amino acids was cloned from pMFPZ-2 into pSELECT to generate pMFPSEL-1. Features of the P29 protein sequence located on the *Hin*dIII fragment are indicated: the characteristic prokaryotic lipoprotein signal sequence (black box), two amino acid sequence repeats (Rep 1 and Rep 2), two Cys residues (C), and an in-frame UGA Trp codon (W). Numbers indicate amino acid positions in the mature protein, assuming cleavage of the prolipoprotein on the N-terminal side of a Cys residue in the signal sequence (see Fig. 2). (B) SDS-PAGE and immunoblot analysis of protein products expressed under T7 in DH5α MCR cells from vector without insert (lane Control), vector containing the *Hin*dIII insert encoding P29 (lane pMFPSEL-1), or proteins from whole organisms of *M. fermentans* PG18 (lane *M. f.*). Immunoblots were stained with the C19A MAb to *M. fermentans* P29. The two recombinant, epitope-bearing protein products of 30 and 16 kDa expressed from pMFSEL-1 and P29 in *M. fermentans* (arrow) are shown.

start codon at nt 178 is located 6 nt downstream of a consensus ribosome binding site (29), and a TAA stop codon occurs at nt 910. Subsequent sequencing revealed no strong stem-loop structures and no ORFs for 1 kb downstream of this stop codon. No ORFs (over 63 codons long or with recognizable ribosome binding sites or start sites) occurred in either orientation upstream of P29 for a distance of approximately 1 kb. Further upstream, an ORF of 501 amino acids was found in the opposite orientation to the *p29* gene. This ORF (GenBank accession no. U50826) has a high degree of protein sequence similarity with the lysyl-tRNA synthetase protein family, showing 49.6% identity with the *Bacillus subtilis* homolog (data not shown). No ORFs were encoded upstream of this synthetase gene on either strand for 350 nt.

The predicted P29 sequence (Fig. 2) contains a characteristic prokaryotic prolipoprotein signal peptide (5) of 26 amino acids, consisting of a positively charged N terminus, a hydrophobic core, and a tetrapeptide recognition cleavage site immediately preceding a Cys residue at the C-terminal end of the signal. This +1 Cys residue is the predicted site of lipid modification and anchorage in the membrane after cleavage of the signal peptide (8). A single UGA (Trp) codon is located at amino acid position 135 of the mature protein. Two nearly identical, repeated amino acid sequences, K E A E A I K K D (repeat 1 [Rep 1]) and K E A I E A I K K D (Rep 2), occur in the P29 sequence, beginning at positions 45 and 102, respectively. Two Cys residues, in addition to the +1 Cys, occur at positions 91 and 128, respectively. Analysis of the mature P29 amino acid sequence revealed a hydrophilic, basic protein (pI = 9.8) with alpha-helix or beta-sheet structure predicted throughout. No significant sequence similarities to other proteins were found in the current Genbank databases.

Structural characteristics of P29 from phase-variant isolates. Since expression of the P29 product does not differ in phase-variant isolates differentially displaying P29 epitopes (33), determination of the secondary structure of this lipoprotein becomes important in understanding possible mechanisms underlying its surface antigenic variation. The presence of the two Cys residues (Cys-91 and Cys-128) raised the possibility of a disulfide loop occurring in P29. The lack of interchain disulfide linkage involving P29 (38) and the relative rarity of Cys residues in external portions of surface lipoproteins (5, 31) further prompted examination of their possible role in the secondary structure of P29.

To determine the oxidative state of the Cys-91 and Cys-128 residues in P29, free sulfhydryl groups, or sulfhydryl groups chemically liberated from disulfide bonds, were selectively labeled with digoxigenin-3-*O*-succinyl-[2-(*N-maleimido*)]ethylamide (see Materials and Methods). Whole organisms of *M. fermentans* PG18 were first detergent fractionated with TX-114, and the TX-114-phase proteins containing P29 (38) were then separated by SDS-PAGE under nonreducing conditions and electrophoretically transferred to nitrocellulose filters. In the first assay, filter 1 (Fig. 3) was treated directly with digoxigenin-3-*O*-succinyl-[2-(*N-maleimido*)]ethylamide to label existing sulfhydryl groups. P29 was not labeled under these conditions (Fig. 3, lane 1). This result was consistent with the absence of reduced Cys residues in P29. To ensure that experimental manipulation of P29 (e.g., with TX-114) did not oxidize free sulfhydryl groups and thereby prevent labeling, a recombinant P29 product containing Cys-91 and Cys-128, but not the +1 Cys residue, was produced in *E. coli* as a cytoplasmic fusion protein from the pMAL-c2 vector (New England Biolabs), affinity purified, and released from the maltose-binding fusion protein by being cleaved with Factor X protease (16). The recombinant P29 protein was TX-114 fractionated (partitioning as an aqueous protein) and subjected to SDS-PAGE analysis under nonreducing conditions identical to

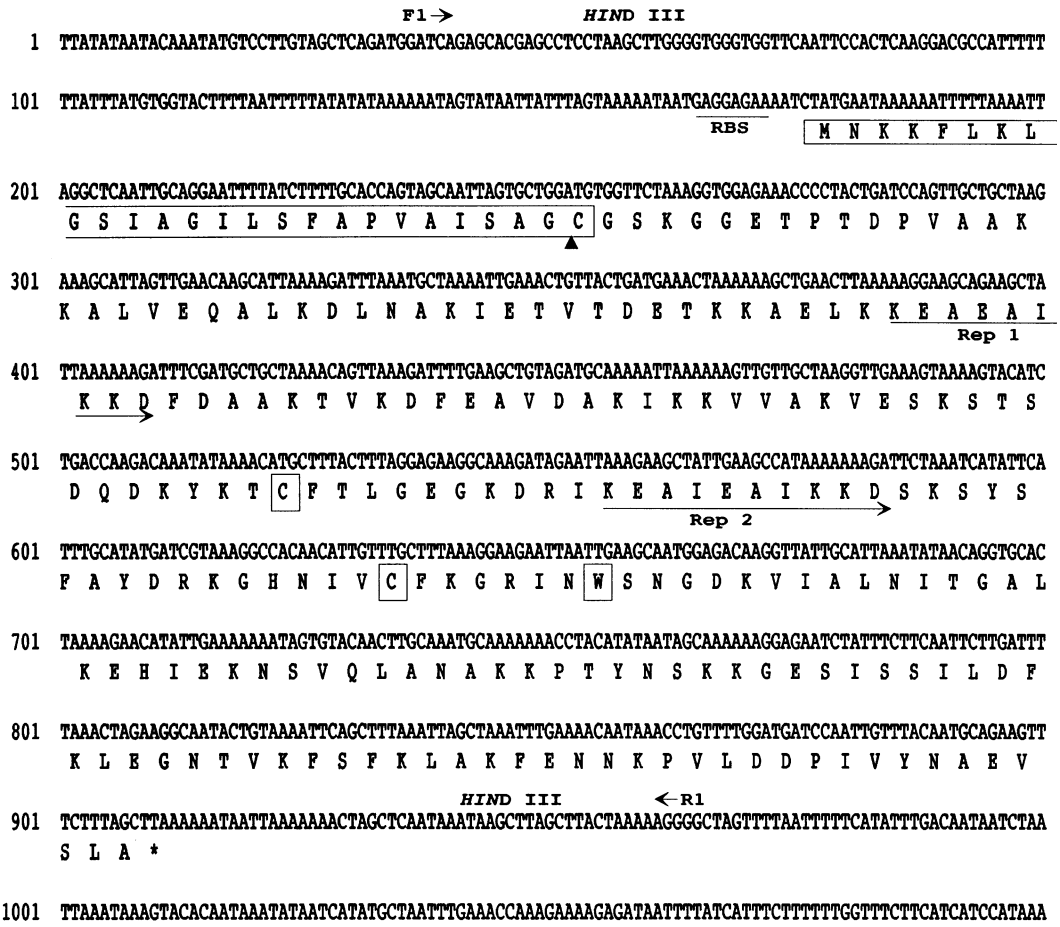


FIG. 2. Nucleotide sequence of the *p29* gene and its flanking region (coding strand), with the deduced amino acid sequence (single-letter code) shown below. The *Hin*dIII sites used for cloning are indicated. A putative ribosome binding site (RBS) is underlined. The positions of the F1 and R1 primers used for PCR amplification of the *p29* gene are shown. The sequences of the primers are given in Materials and Methods. A putative prokaryotic lipoprotein signal sequence with a predicted start methionine (boxed), a typical recognition cleavage site for the signal peptide (arrowhead), a TAA stop codon (*), amino acid sequence repeats (Rep 1 and Rep 2) (underlined by arrows), and two internal Cys (C) residues and a UGA (Trp) codon (W) (boxed) are indicated.

those used for the treatment of mycoplasmal protein preparations. As predicted from P29 synthesis in the cytoplasm (a reducing environment), free sulfhydryl groups on the recombinant P29 protein were labeled without further chemical reduction (data not shown).

While authentic mycoplasma P29 showed no free sulfhydryl groups after detergent fractionation, sulfhydryl groups could be specifically labeled after reduction of this lipoprotein with β-mercaptoethanol. This was demonstrated by using a second, identical blot (filter 2) of TX-114-phase proteins separated by nonreducing SDS-PAGE and transferred as described above. However, after a blocking with *N*-ethylmaleimide to bind any possible free sulfhydryl groups (which were not anticipated on the basis of the results from the first experiment), filter 2 was then incubated with β-mercaptoethanol to reduce disulfide bonds, allowing specific labeling of newly generated free sulfhydryl groups by subsequent treatment with digoxigenin-3-*O*-succinyl-[2-(*N-maleimido*)]ethylamide. After chemical reduction, Cys residues in P29 were accessible as free sulfhydryl groups (Fig. 3, lane 4). Since the predicted diacylglyceryl modification of the +1 Cys residue (5) would render it inaccessible to reduction, this result suggested that Cys-91 and Cys-128 residues form an intramolecular disulfide bond, creating a 36-residue disulfide loop in the surface-exposed P29 lipoprotein. No labeling occurred when the sulfhydryl groups on the control recombinant P29 described above were first blocked with *N*-ethylmaleimide (data not shown).

Since the disulfide loop may be an important secondary structure involved in presentation of P29 epitopes, events which alter the oxidative state of Cys-91 and Cys-128 could result in the observed differences in epitope presentation on the mycoplasmal cell surface. To determine whether the P29 disulfide loop is present in clonal populations undergoing phase variation in epitope presentation, the oxidative state of the Cys residues from clonal isolates was determined. Regardless of the P29 surface phenotype (negative or positive as assayed by colony immunoblots with the C19A MAb), P29 showed similar patterns of reactivity with the compounds detecting sulfhydryl groups (Fig. 3, lanes 2, 3, 5, and 6), indicating that P29 contains a stable disulfide loop in isolates undergoing phase variation in the presentation of P29 surface epitopes.

A second mechanism which could render epitopes inaccessible to MAbs involves changes in the P29 primary structure due to mutational events. Mutations directly affecting epitope structure are unlikely, since Western blots of phenotypically negative isolates still show binding of the C19A MAb to P29 (33). However, structural alterations of P29 could occur through mutations in the coding region affecting P29 surface

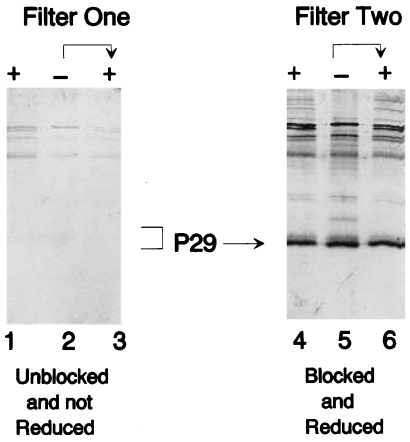


FIG. 3. Identification of intramolecular disulfide bonds in P29. SDS-PAGE and nitrocellulose blots of TX-114-phase proteins from passaged stock culture of *M. fermentans* PG18 (lanes 1 and 4) clonal isolates phenotypically negative (lanes 2 and 5) or phenotypically positive (lanes 3 and 6) for P29 surface epitope expression are shown. Arrows above lanes 2 and 3 and lanes 5 and 6 indicate progenitor and progeny populations. Phenotypes of P29 epitopes determined by colony immunoblots with the MAb C19A are indicated (+ or −). Free sulfhydryl groups on proteins were selectively labeled with digoxigenin-3-*O*-succinyl-[2-*N*-*maleimido*)]ethylamide. Filter 1 was treated without blocking of free sulfhydryl groups. Filter 2 was first treated with *N*-ethylmaleimide to block free sulfhydryl groups and then treated with β-mercaptoethanol to chemically reduce disulfide bonds prior to the same labeling procedure. The location of P29, determined by subsequent immunostaining with the C19A MAb (data not shown), is indicated (arrow and bracket).

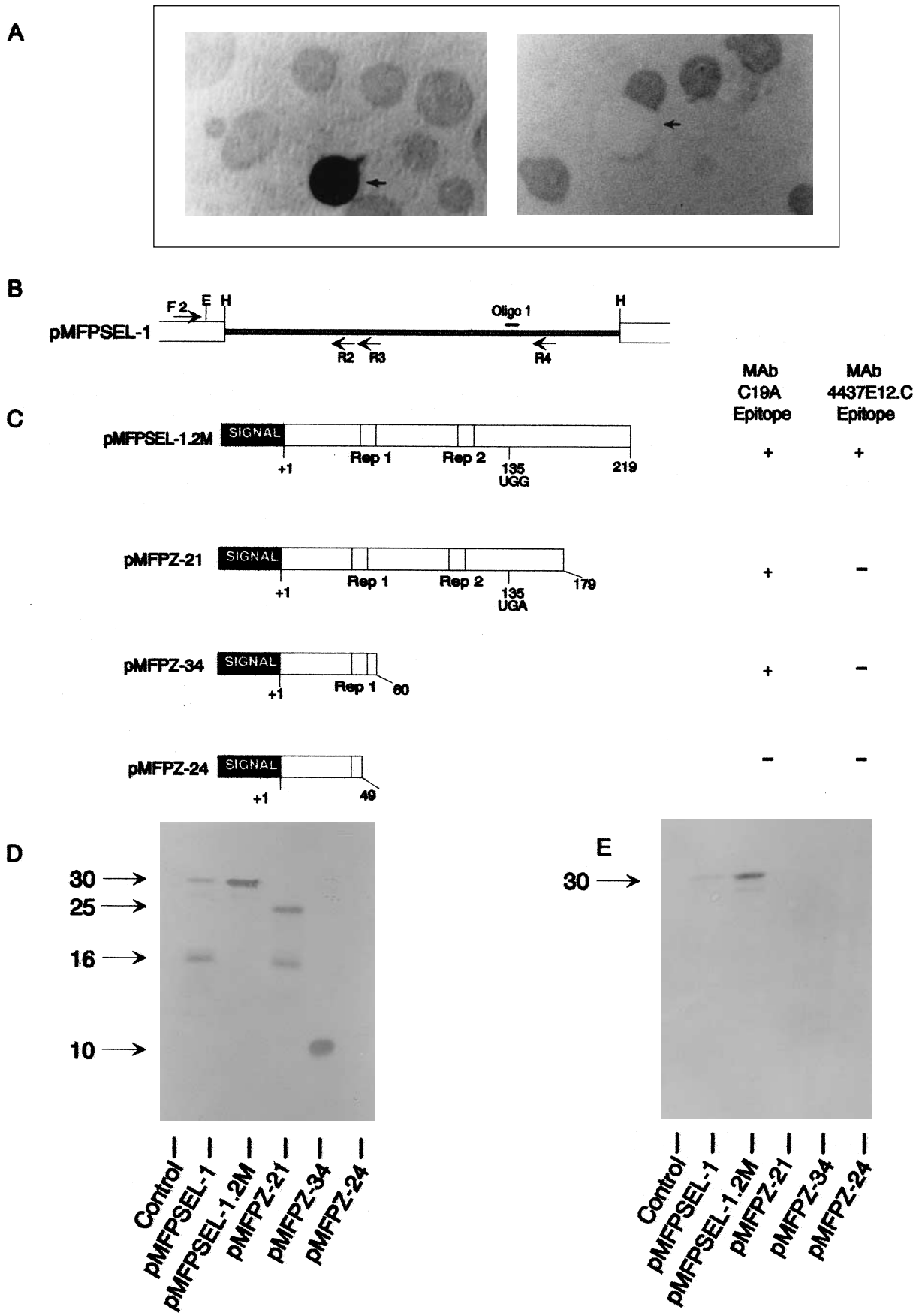
topology. To examine this possibility, the DNA sequences of the *p29* gene obtained by PCR (Fig. 2, primers F1 and R1) were determined for two isolates displaying a negative phenotype and one isolate displaying a positive phenotype (assayed as described previously [33] by colony immunoblotting with MAb C19A). Each sequence was identical to that shown in Fig. 2, which had been derived from a cloned chromosomal fragment of *M. fermentans* PG18. Mutations within the *p29* coding region could therefore be ruled out as a means of generating structural changes that might affect the binding of MAbs to their respective surface epitopes on P29 and thereby cause variation in epitope accessibility.

Localization of P29 phase-variant epitopes differentially displayed on the cell surface. It is possible that the entire P29 protein might be made accessible or inaccessible to Abs, resulting in high-frequency phase variation in Ab binding. However, some clonal isolates failing to react with the C19A MAb were shown to be positive with a PAb against P29, suggesting that selected regions of P29 may be differentially exposed on the cell surface in any given population (33). To further explore this possibility and to localize P29 surface epitopes which may be independently accessible to their respective MAbs, *M. fermentans* clonal isolates were screened with an additional MAb to P29. One isolate which was negative by colony immunoblot screening with MAb C19A (the majority population in Fig. 4A, left panel) was positive when screened with a second MAb to P29, 4437E12.C (majority population in Fig. 4A, right panel). Like the epitope recognized by MAb C19A (Fig. 4A, left panel), the epitope recognized by 4437E12.C displayed high-frequency variation in Ab binding within this population (a negative colony is indicated in Fig. 4A, right panel). The two MAbs differed in their staining of the majority population of the same clonal isolate (Fig. 4). This indicates that presentation of the two epitopes varies independently. Notably, colonies negative by immunoblot staining with MAb 4437E12.C expressed full-length P29, as shown in Western blots using this MAb or other Abs to P29 (data not shown). These results indicate, first, that distinct epitopes on the P29 lipoprotein show independently oscillating accessibility to their respective MAbs and, second, that individual clonal populations can display different epitopic regions of P29.

To localize the two phase-variant epitopes defined by MAbs C19A and 4437E12.C, mutated constructs of the *p29* gene were made and the protein products were expressed in *E. coli*. First, the UGA codon creating a translational stop in *E. coli* was mutated to a UGG (Trp); see Fig. 4B and C for construction of specific mutations. The single A→G point mutation was confirmed by DNA sequencing, and the resulting construct, pMFPSEL-1.2M, was placed under T7 expression in *E. coli*. A major 30-kDa protein product was detected in Western immunoblots by the C19A MAb (Fig. 4D, lane pMFPSEL-1.2M), consistent with the expression of a full-length product. The 16-kDa product shown in Fig. 4D, lane pMFPSEL-1, was absent in pMFPSEL-1.2M, suggesting that the UGA codon functions as a stop codon, resulting in a truncated, epitope-bearing protein in *E. coli*. This also argued against the smaller component as a breakdown product of the full-length protein. The 30-kDa product expressed from the wild-type (unmutated) *p29* gene was further shown to result from a translational read-through of the UGA codon. This was determined by constructing a PCR-generated (see Fig. 4B for location of primers) 3′ deletion (using pMFSEL-1 as a template) to create pMFPZ-21, which truncated the P29 sequence downstream of the UGA codon. When expressed in *E. coli*, this construct generated the 16-kDa product as well as a truncated 25-kDa product (Fig. 4D, lane pMFPZ-21), which is consistent with a read-through of the UGA codon. These initial constructs indicated that the variant P29 epitope recognized by the C19A MAb was localized to a region upstream of the UGA (Trp) codon.

Additional PCR-generated 3′ deletions of the *p29* gene were constructed to further localize phase-variant P29 surface epitopes. As shown in Fig. 4C, pMFPZ-34 represents a 3′ deletion of the P29 sequence that eliminated Rep 2 and contained only the first 60 residues of the mature protein. pMFPZ-24 removed Rep 2 and the C-terminal portion of Rep 1. The C19A MAb detected a protein product of 10 kDa expressed from pMFPZ-34 (Fig. 4D, lane pMFPZ-34), consistent with the expected size of the deletion mutation. This epitope was not detected on the product expressed from pMFPZ-24 (Fig. 4D, lane pMFPZ-24), even though a truncated protein product of the predicted size was specifically expressed from this shorter construct, as detected by Coomassie blue staining (data not shown). Thus, sequences in the N-terminal portion of the mature P29 lipoprotein are required to form the phase-variant epitope defined by the C19A MAb.

A screening of the same set of mutational constructs with the 4437E12.C MAb revealed a full-length P29 product in pMFPSEL-1.2M (Fig. 4E, lane pMFPSEL-1.2M). The epitope was not detected on the truncated protein products expressed from pMFPZ-21, pMFPZ-34, or pMFPZ-24 (Fig. 4E), nor was the 16-kDa protein product detected with this MAb in the constructs containing the wild-type *p29* sequence containing the unmutated UGA codon (Fig. 4E, lanes pMFPSEL-1 and pMFPZ-21). Although not defining the complete epitope structure, these results indicate that sequences required for presenting the phase-variant epitope detected by 4437E12.C MAb (either directly or by conformational effects) are located downstream of the UGA codon, in the C-terminal portion of P29. Therefore, at least two discrete regions on opposite ends of the P29 product contribute to the phase-variant presentation of distinct surface epitopes on the organism.


A
B
pMFPSEL-1
F2
E
H
Oligo 1
H
R2
R3
R4
C
MAb C19A Epitope
MAb 4437E12.C Epitope
pMFPSEL-1.2M
SIGNAL
+1
Rep 1
Rep 2
135 UGG
219
+
+
pMFPZ-21
SIGNAL
+1
Rep 1
Rep 2
135 UGA
179
+
–
pMFPZ-34
SIGNAL
+1
Rep 1
60
+
–
pMFPZ-24
SIGNAL
+1
49
–
–
D
30
25
16
10
Control
pMFPSEL-1
pMFPSEL-1.2M
pMFPZ-21
pMFPZ-34
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Control
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pMFPSEL-1.2M
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pMFPZ-34
pMFPZ-24

FIG. 4. Mutation constructs to localize P29 phase variant epitopes. (A) Colony nitrocellulose lifts of an *M. fermentans* clonal isolate stained with either the C19A MAb, showing a negative P29 phenotype (left), or the 4437E12.C MAb, showing a positive P29 phenotype (right). Arrows indicate colonies with an opposite phenotype. (Differences in intensities between negative and positive colonies in each population reflect differences in MAb staining properties.) (B) pMFPSEL-1, described in the legend to Fig. 1, containing the 893-bp *Hin*dIII (H) insert containing the *p29* gene was used as a template for site-directed mutagenesis. Location of the oligonucleotide (oligo 1) used to mutate the UGA (Trp) codon to a UGG (Trp) codon is shown. pMFPSEL-1 was also used as a template to generate a series of 3′ deletion mutations of the *p29* gene by PCR techniques. Locations of the forward primer (F2) used for each construct and three unique reverse primers (R2, R3, and R4) are indicated (sequences of primers and oligonucleotide are given in Materials and Methods). The vector-encoded *Eco*RI (E) site was used for directional cloning of the PCR products into pGEM-3Z. (C) Protein features of products expressed from mutational constructs. The prokaryotic lipoprotein signal sequence (black box) and amino acid sequence repeats (Rep 1 and Rep 2) are shown. Numbers indicate amino acid positions in the mature P29 protein. pMFPSEL-1.2M contains the point mutation changing the UGA codon to a UGG Trp (W) codon. Deletion construct pMFPZ-21 contains both amino acid repeats, Rep 1 and Rep 2. pMFPZ-34 contains only the first repeat, Rep 1. pMFPZ-24 contains only the first 5 amino acids of Rep 1. Vector-encoded amino acids 3′ of truncated P29 sequences are not indicated. Detection of the epitopes on recombinant products expressed from each construct is indicated (+ or −) on the right. (D) SDS-PAGE and immunoblot analyses of protein products expressed from the construct containing the wild-type *p29* gene (lane pMFPSEL-1) and the above mutational constructs (lanes pMFPSEL-1.2M, pMFPZ-21, pMFPZ-34, and pMFPZ-24). The control lane contains protein expressed from pSELECT without an insert. Immunoblots were stained with C19A MAb to *M. fermentans* P29. The sizes (in kilodaltons) of the epitope-bearing products are indicated on the left. (E) Western immunoblot (as described for panel D) stained with 4437E12.C MAb against *M. fermentans* PG18.

DISCUSSION

This study details several key structural features of P29, a surface lipoprotein of *M. fermentans* involved in variant recognition of epitopes by MAbs. It has previously been shown by the accessibility of P29 to proteolytic enzyme digestion that the protein is on the mycoplasma surface (38). The P29 amino acid sequence contains no transmembrane domains and thus predicts a surface localization with the entire sequence external to the single plasma membrane. This is consistent with our data indicating that P29 can be stained on colony immunoblots by MAbs to N-terminal or C-terminal regions of the mature protein.

It has also been shown that P29 can be labeled with palmitate and is membrane associated (38). Since its amino acid sequence contains a prokaryotic prolipoprotein signal sequence and predicts no transmembrane domains in the mature protein, P29 is likely processed and anchored in the single limiting membrane of *M. fermentans* in a manner similar to those of other bacterial (5, 31) and mycoplasmal (7, 8, 36) lipoproteins. The slightly greater size of the recombinant P29 protein expressed in *E. coli* (30 kDa) compared with that of the mycoplasmal product (29 kDa; Fig. 1B and 4D and E) may result from a failure to cleave the heterologous signal peptide, as seen in other mycoplasmal lipoproteins expressed in this host (8). Because P29 is surface exposed and membrane anchored only through lipids, direct communication with the cytoplasmic environment cannot occur by transmembrane protein sequence. This most likely rules out intracellular posttranslational modifications (24) as a means of generating structural alterations of the P29 surface topology.

A third structural feature defined in this study is the presence of a 36-amino-acid disulfide loop in P29. We believe this report documents the first intramolecular disulfide bridge occurring in a rapidly growing list of mycoplasmal surface lipoproteins (7). More generally, this type of secondary structure is not commonly found in lipoproteins of eubacteria, including gram-positive organisms (31), to which mycoplasmas are phylogenetically related (41). Since intramolecular disulfide bonds play an important role in the folding, structure, and stability of many proteins (2, 10), this key secondary structure may be critical for P29 function and becomes an important issue in studies involving recombinant P29 protein synthesized in *E. coli.*

P29 was shown to contain two nearly identical amino acid sequences. Despite similarity at the amino acid level, the DNA sequences encoding the repeats are quite divergent. This argues for a conserved role of the repeats in the function of P29, which is currently unknown. The more N-terminal repeat was found to be necessary for epitope recognition by the C19A MAb. However, additional data (not shown) indicated that a synthetic peptide comprising the sequence K E A E A I K K D failed to bind the C19A MAb, suggesting that this linear sequence is not in itself sufficient for MAb recognition. It is possible that the epitope is dependent on structure contributed both by the repeat and by adjacent sequences (e.g., upstream of this motif). The second repeat is located within the disulfide loop. A proposed hypothetical model of P29 is presented in Fig. 5, showing the collective features deduced from these studies.

In addition to characterizing the structure of P29, this work suggests a mechanism of high-frequency phase variation in recognition of P29 epitopes expressed on the mycoplasma cell surface. Numerous bacteria, such as *Neisseria gonorrhoeae* (21) and *Borrelia* species (1), employ systems of antigenic variation in order to promote survival by evading the host immune system (25). Recently, the molecular basis of antigenic variation in various mycoplasmas has been described (11, 37), including Vlp proteins in *M. hyorhinis* (6, 43, 44), the V-1 protein of *M. pulmonis* (4, 30), and the pMGA protein of *M. gallisepticum* (20). All of these systems appear to create differences in the expression of the variable proteins. In contrast, P29 is a useful model with which to demonstrate antigenic variation arising from differences in epitope accessibility on the cell surface. The subtlety of variation in the topology of mycoplasma surface antigens is underscored by these results, show-

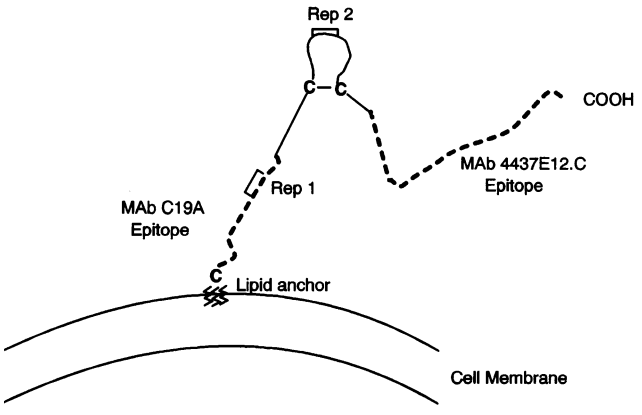

FIG. 5. Hypothetical model of the lipoprotein P29, which differentially displays two distinct epitopes on the surface of *M. fermentans*. Surface topology and features of P29 include the +1 Cys residue anchored by lipid in the membrane; the locations of Rep 1 and Rep 2; the sequences involved in epitope recognition by MAbs C19A and 4437E12.C (dashed lines); and the 36-amino-acid disulfide loop formed by Cys-91 and Cys-128.

ing marked switching in the accessibility of surface epitopes without changes in expression or structure of the protein target. High-frequency variations in display of epitopes associated with the C-terminal or N-terminal regions of this surface lipoprotein were shown to occur independently of each other. This altered recognition of surface epitopes did not result from changes in the primary structure of P29 or secondary structure involving the external disulfide loop. This mechanism therefore differs from alterations generated by high-frequency phase variation of several other *M. fermentans* surface proteins which undergo changes in their expression (33). Altered patterns of expression of these other surface proteins may in fact affect access to selected portions of P29, providing the basis for differential recognition of P29 surface epitopes. The precise portions of P29 that are accessible may depend on which other membrane proteins are expressed in a particular clonal population (33).

The P29 protein studied here serves as a model to demonstrate the wide range of alterations in antigenic surface topology available in propagating populations of mycoplasma. The ability to differentially mask cell surface structure may contribute to the survival of *M. fermentans* in different host environments, particularly those in which host recognition of specific regions of surface proteins could mediate damaging immune reactions. Whether specific proteins showing phase variation in surface expression (33) might mask particular sites on P29 remains to be determined. An important consequence of this proposed system is that definition of the precise accessible surface epitopes of a particular population may be experimentally impossible because of the complex factors determining the actual surface antigenic structures on individual organisms. Finally, from a practical standpoint, typing of primary mycoplasma isolates, even by PAbs, can be difficult to interpret, as recently illustrated by studies showing masking of antigens expressed on colonies from several mycoplasmal species, including *M. fermentans* (26).

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