Lipid-Modified Surface Protein Antigens Expressing Size Variation within the Species *Mycoplasma hyorhinis*

MICHAEL J. BOYER AND KIM S. WISE*

Department of Microbiology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

Received 27 July 1988/Accepted 6 October 1988

Monoclonal antibodies (MAbs) previously shown to recognize distinct epitopes selectively expressed on the surface of some *Mycoplasma hyorhinis* strains were used to define two discrete sets of lipid-modified membrane surface proteins showing marked size variation within this species. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of Triton X-114 phase-fractionated proteins from six isolates of *M. hyorhinis* defined a set of amphiphilic integral membrane proteins of 23, 50, and 55 kilodaltons (kDa) recognized on respective isolates by one MAb and a second set of integral proteins of 88, 120, and 100 to 150 kDa recognized by another MAb. The first group of proteins all contained a common, amphiphilic 18-kDa limit tryptic polypeptide bearing the epitope. The size- and strain-variable surface antigens identified by the MAbs were shown to be lipid-modified proteins. Phase fractionation of $[^3H]$palmitate-labeled organisms revealed numerous $[^3H]$-labeled proteins in all isolates, which partitioned exclusively into the hydrophobic phase. These proteins generally showed pronounced size variation among isolates and included the antigen variants recognized by the two MAbs, as demonstrated directly by immunoprecipitation of correspondingly sized $[^3H]$-labeled proteins from each isolate. A third MAb recognized an invariant, lipid-associated surface protein of 70 kDa on all *M. hyorhinis* isolates. Covalent modification of lipid-associated proteins was confirmed by identifying $[^3H]$-labeled methyl palmitate after acid methanolysis of Triton X-114 phase proteins derived from $[^3H]$palmitate-labeled organisms. However, removal of covalently bound lipid from chloroform-methanol-extracted proteins by alkaline hydroxylamine was selective; complete removal was observed with only a few proteins, possibly including the 120-kDa form of one antigen variant. This suggested potential differences in the nature of covalent linkage among lipid-modified *M. hyorhinis* surface antigens. Intrasppecies antigen variants described here in *M. hyorhinis* share some characteristics with size-variant antigens reported in phylogenetically related gram-positive eubacteria and may contribute to phenotypic diversification and differences in pathogenicity of mycoplasmas.

*Corresponding author.

*Mycoplasma hyorhinis* shares many features with other species in the genus *Mycoplasma*, including a small genome size, the presence of a single limiting plasma membrane, and a propensity to colonize the surface of host cells both in vitro and during infection in vivo (3, 13, 25, 36). However, considerable variation in a number of phenotypic characteristics has been documented within the species *M. hyorhinis*, including the ability to cytadsorb to mammalian cells (3) and the potential to induce a chronic, degenerative arthritis in the natural swine host (25, 36). Structural and antigenic differences expressed on the single limiting membrane of this pathogenic organism may be particularly important in dictating phenotypically variable characteristics because of the direct involvement of this surface with host cells and with the host immune system (6). Evidence of antigenic surface variation in *M. hyorhinis* has been suggested on the basis of earlier studies (14) in which antibodies with metabolic inhibition activity (thought to be directed to surface components) were shown to recognize intrasppecies serogroups. More recent studies from our laboratory with two monoclonal antibodies (MAbs) have further defined specific surface epitopes differentially expressed on some strains of this species (39, 40). We have shown that proteins bearing strain-restricted surface epitopes on one prototype *M. hyorhinis* isolate (GDL) are among a large group of amphiphilic integral membrane proteins tightly associated with lipids (5). Killing of the organism by a complement-dependent mycoplasmal immunoglobulin M (IgM) MAb is mediated by one of these proteins (24).

For this report, we used a set of MAbs to examine the structural features of membrane surface antigens selectively expressed on certain isolates of *M. hyorhinis*, and we describe a striking variation among isolates in the size of proteins bearing the surface epitopes recognized by these MAbs. Covalent lipid modification of the size-variable surface proteins is demonstrated. Mechanisms for generating alternative forms of antigens are compared with motifs of size variation in membrane proteins of phylogenetically related gram-positive eubacteria, and possible immunologic and evolutionary consequences of this structural diversification at the mycoplasma surface are considered.

MATERIALS AND METHODS

*Mycoplasma* isolates and culture conditions. Six isolates of *M. hyorhinis* and the mycoplasma species *Mycoplasma hyopneumoniae*, *Mycoplasma fermentans*, *Mycoplasma pulmonis*, and *Mycoplasma flocculare* were obtained from the following sources. *M. hyorhinis* GDL (GDL-1) and the arthritogenic isolate SK76 (25) were obtained from R. F. Ross, Iowa State University, Ames; *M. hyorhinis* GDL (ATCC 23839) (GDL-11) and *M. hyopneumoniae* (Mycoplasma suispneumoniae) I (ATCC 25934) were obtained from the American Type Culture Collection, Rockville, Md.; *M. hyorhinis* D10 (GDL-111) and *M. hyorhinis* PG29 were obtained from M. F. Barile, Food and Drug Administration, Bethesda, Md.; *M. hyorhinis* BTS-7 (M718-002-084), *M.
fermentans PG-18 (M713-002-084), and M. pulmonis (ASH) (M717-002-084) were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Mycoplasma stocks were obtained as filter-cloned cultures (33) or were purified as described previously (30). All mycoplasma species were grown in broth culture at 37°C in spinner flasks (Bellco Glass, Inc., Vineland, N.J.). A modified Hayflick broth medium containing 20% fetal bovine serum (40) was used to grow all species except M. hyopneumoniae and M. flocculare, which were grown in FF medium containing 20% porcine serum as described previously (38). For some experiments indicated, M. hyorhinis GDL-1 was also grown in FF medium supplemented with 20% porcine serum. Mycoplasmas were harvested from logarithmic-phase broth cultures by centrifugation and stored at -70°C in phosphate-buffered saline as described previously (40).

MAbs. MAbs used in this study included the following. F20C17F (anti-p23/17), F24C4Fg (anti-p38), F81C39R (anti-p51), and F146C11B (anti-p120) have been described elsewhere (39, 40). F192C17a (anti-p23) is an IgG2a(κ) MAb directed toward the 23-kilodalton (kDa) protein of GDL-1, which is also recognized by MAb F20C17F (5, 24). AB3C (anti-p70) is an IgG1(κ) MAb recognizing a 70-kDa surface protein of M. hyorhinis (5). All MAbs were generated by immunization with antigens of M. hyorhinis GDL-1 except the MAb to p70, which was raised against a protein from M. hyorhinis derived from a contaminated tissue culture (5). The production and propagation of hybridoma cells have been previously described (38, 40). Supernatants from hybridoma cultures were used for immunofluorescence and radioimmunoprecipitation procedures.

Metabolic labeling of mycoplasmas. Mycoplasmas from logarithmic-phase broth cultures were labeled with 500 μCi of [9,10,5-H]palmitic acid (specific activity, 30 Ci/mmole; Dupont, NEN Research Products, Boston, Mass.) per ml or with 100 μCi of L-[35S]methionine (specific activity, 1,127 Ci/mmole; Dupont, NEN) per ml as described in detail elsewhere (5, 38).

TX-114 phase partitioning. Triton X-114 (TX-114; Sigma Chemical Co., St. Louis, Mo.) phase partitioning was performed by the procedure of Bordier (4), as adapted to mycoplasmas and described in detail elsewhere (5, 24, 38). Briefly, mycoplasmas from logarithmic-phase cultures were solubilized (2 mg of protein per ml) at 4°C for 30 min in TS buffer (10 mM Tris, 150 mM NaCl [pH 7.4]) containing 1% (wt/vol) TX-114 and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 4°C for 3 min at 13,000 × g to remove insoluble material, the supernatant was transferred to a new tube and subjected to three cycles of phase fractionation. The condensed detergent phase was adjusted to 1% (wt/vol) TX-114 and used in parallel with the aqueous phase for subsequent electrophoresis and immunoblot analysis.

Electrophoresis and immunoblot techniques. Samples were prepared under reducing conditions and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 18) in 9 to 14% linear gradient gels containing 0.5 M urea, as previously described (40). After electrophoresis, gels were stained with Coomassie blue (Bio-Rad Laboratories, Richmond, Calif.) and autoradiography or fluorography was performed as described elsewhere (5, 38).

Nitrocellulose filter blots were prepared by the procedure of Towbin et al. (32) with slight modifications reported previously (40). Filter blots were treated for 1 h at 37°C or overnight at 4°C with TS buffer containing 5% (wt/vol) nonfat dry milk and were then incubated with hybridoma culture fluid containing MAbs, followed by peroxidase-conjugated antibody (goat) against mouse IgG (Organon Teknika, Malvern, Pa.) as described elsewhere (38). Bound antibody was visualized by using the enzyme substrate o-dianisidine. Relative molecular weights were calculated from blots of standards run simultaneously and stained with amido black after electrophoretic transfer.

Radioimmunoprecipitation. Radioimmunoprecipitation analysis of mycoplasma proteins used organisms metabolically labeled with [3H]palmitate and subjected to TX-114 phase fractionation as described in detail elsewhere (5, 38). Briefly, MAbs were immobilized on Sepharose 4B beads covalently coupled to affinity-purified goat anti-mouse IgG (heavy and light chains) (Affibeads; Organon Teknika) and beads with bound MAbs were added to detergent fractions (adjusted to 0.1% TX-114) containing [3H]palmitate-labeled proteins. After incubation for 18 h at 4°C, beads were rinsed four to six times in 0.1% TX-114 and prepared for SDS-PAGE as described previously (5, 38). Gels were fluorographed as described above.

DNA isolation and Southern hybridization. Mycoplasma genomic DNA was isolated as described previously (30) and was digested to completion with restriction endonuclease EcoRI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) under conditions suggested by the supplier. Samples were electrophoresed through horizontal 0.7% agarose slab gels in Tris acetate buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.2]), and DNA fragments were transferred to nitrocellulose filter paper (HA type; Millipore Corp., Bedford, Mass.) by the method of Southern (28). Filters were hybridized and autoradiographed as described elsewhere (30) with nick-translated DNA from plasmid pMC5, which contains a 4.8-kilobase-pair (kb) insert including the 23S, 5S, and part of the 16S rRNA genes from Mycoplasma capricolum (2).

Treatment of mycoplasma proteins with trypsin. TX-114 fractions (50 μl; 1% [wt/vol] TX-114) from various M. hyorhinis isolates were adjusted to a final concentration of 1 mg of acetylated trypsin (Sigma) per ml and were incubated for 1 h at room temperature along with control fractions receiving no enzyme. Samples were immediately prepared for SDS-PAGE, and subsequent filter blots were prepared and immunologically stained with MAbs as described above.

Removal of noncovalently bound lipid from amphiphilic proteins. Noncovalently bound lipid was removed from TX-114-fractionated proteins as described in detail elsewhere (38). Briefly, 0.5 ml of a TX-114 phase containing proteins from [3H]palmitate-labeled mycoplasmas was precipitated by adding 4.5 ml of methanol (high-performance liquid chromatography [HPLC] grade; Fisher Scientific Co., Pittsburgh, Pa.) and incubating for 18 h at -70°C. The precipitate was dried under nitrogen, dissolved in 50 μl of 0.1% SDS, and transferred to a Reacti-vial (Pierce Chemical Co., Rockford, Ill.). After being precipitated again with methanol, the pellet was extracted four times with 0.5 ml of chloroform-methanol (2:1, vol/vol). Twenty microliters of each sequential extract was assayed for tritium by liquid scintillation counting to ensure that extraction was complete. After the final extraction, excess solvent was removed by evaporation under a stream of nitrogen and delipidated protein was subjected to methanolysis or was applied to SDS-PAGE and visualized by fluorography.

Analysis of methanolysis and hydrolysis products from fatty acid-labeled proteins. Acid methanolysis of [3H]palmitate-labeled TX-114 phase proteins of M. hyorhinis was performed by the method of Olson et al. (23), as described in detail elsewhere (38). Briefly, delipidated proteins (described
above) were incubated in evacuated vials at 95°C for 16 h with 83% methanol containing 2 N HCl. Samples were then extracted four times with HPLC-grade petroleum ether (Fisher). The combined ether extracts received 2 μl of methanol containing 40 μg each of the following fatty acid methyl esters: caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), and palmitic (C16:0) (Sigma), and 4 μl of methanol containing 40 μg of palmitic acid. Samples containing internal standards were dried under nitrogen, dissolved in 250 μl of HPLC-grade methanol, and applied to isocratic reverse-phase HPLC by using a 10 μlBondapak C18 column (3.9 mm by 30 cm; Waters Associates, Inc., Milford, Mass.) with 80% acetonitrile as the mobile phase at a flow rate of 1.0 ml/min. Fractions were collected at one-half-minute intervals, and 3H was quantitated by liquid scintillation counting. Labeled methanalysis products were identified by comparison with internal standards, as described elsewhere (38).

The effect of hydroxylamine treatment on 3H-labeled M. hyorhinis proteins was examined by variations (38) of the method described by Simonis and Cullen (26). Briefly, TX-114 phase proteins from [3H]palmitate-labeled organisms were subjected to SDS-PAGE. Gels were stained with Coomassie blue, washed extensively with water, and treated for 18 h at room temperature with freshly prepared 1 M hydroxylamine (Sigma) at pH 10.0. Untreated control gels were incubated in water or 1 M Tris (pH 7). Gels were rinsed in water and processed for fluorography as described above.

RESULTS

Identification of M. hyorhinis isolates by rRNA probe of genomic restriction digests. As an independent, nonimmunologic criterion to ensure that various M. hyorhinis isolates were indeed characteristic of this species and were not contaminated with other mycoplasmas, the plasmid pMC5 containing a 4.8-kb insert representing rRNA genes of M. capricolum (1, 2) was hybridized to EcoRI genomic digests from various strains of M. hyorhinis as well as from M. hyopneumoniae and M. flocculare. Amikam and co-workers (1) have shown that these rRNA sequences are highly conserved among mycoplasmas and are present in a low copy number. Moreover, hybridization patterns have been shown by these workers and by ourselves (31) to be highly distinctive for particular mycoplasma species, including M. hyorhinis. Hybridization analysis with this probe was therefore particularly useful for species identification and for detection of any contamination of stock cultures with other mycoplasma species.

Figure 1 shows that for all M. hyorhinis isolates, the pMC5 probe hybridized with an EcoRI genomic restriction fragment of approximately 12 kb, characteristic of M. hyorhinis and distinct from hybridization patterns obtained with other mycoplasma species (31), including two other swine mycoplasma species shown. All isolates of M. hyorhinis were therefore shown by this criterion to be characteristic of the species and free from contaminating organisms (even when extended exposures were examined). All strains were also shown to hybridize in a dot blot assay using a M. hyorhinis-specific gene probe described elsewhere (31; data not shown).

Size variation and common amphiphilic region of surface proteins recognized by MAbs. The size, biochemical characteristics, and intraspecies distribution of antigenic mycoplasma components were monitored by using a panel of MAbs, the properties of which are listed in Table 1. These included reagents previously shown (5, 24, 39, 40) to recognize surface epitopes on the cognate proteins p120, p70, and p23 of a prototype strain (GDL-1), as well as two MAbs directed to epitopes on p51 and p38 which are not exposed at the surface (24, 39, 40). For simplicity, MAbs are referred to by the antigen recognized on strain GDL-1 (e.g., anti-p23/17 [Table 1]). Species and strain distribution of all antigens recognized by the MAbs was analyzed by immunologically staining filter blots of mycoplasma components separated by SDS-PAGE (Fig. 2) with a combination of the MAbs. Immunoblot analysis showed that all these MAbs recognized corresponding epitopes on distinct molecules of the prototype strain M. hyorhinis GDL-1 and that the epitopes recognized were species restricted, since all the MAbs failed to stain other mycoplasmas tested, including the three species described in Fig. 2A as well as previously described (38, 41) species M. flocculare, Mycoplasma bovis, and Mycoplasma pneumoniae (data not shown). The p17 component recognized by IgM MAb to p23/17 (5, 24, 39) is not apparent in Fig. 2A (lane 1) because of dilution of the MAb in the pool used for this study. The distribution of these antigens within the species was further determined by immunoblot analysis of GDL-1 and five additional M. hyorhinis isolates. Immunoblots using MAbs to p70, p51, and p38 (Fig. 2B) revealed common staining of identical components in all isolates tested, indicating invariance in the size of internal antigens p38 and p51 as well as the surface protein p70.

In contrast to the size-invariant antigens defined among isolates with these three MAbs, striking differences were observed in staining patterns obtained with two other MAbs directed to surface epitopes on p120 and p23/17 of the isolate GDL-1. Since these surface antigens have been shown by their partitioning into the hydrophobic phase during TX-114 phase fractionation to be integral membrane proteins of this prototype strain (5, 24), an immunoblot of TX-114 phase and
TABLE 1. Properties of MAbs to *M. hyorhinis*

<table>
<thead>
<tr>
<th>Clone designation</th>
<th>Isotype</th>
<th>Proteins recognized&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Epitope on surface&lt;sup&gt;b&lt;/sup&gt;</th>
<th>References</th>
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<tr>
<td>F146CIIIB</td>
<td>IgG1(κ)</td>
<td>p120&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>24, 39, 40</td>
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<tr>
<td>AB3C</td>
<td>IgG2(κ)</td>
<td>p70</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>F20C17F</td>
<td>IgM(κ)</td>
<td>p23/17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>5, 24, 39, 40</td>
</tr>
<tr>
<td>F192C17a</td>
<td>IgG2a(κ)</td>
<td>p23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>F81C39R</td>
<td>IgG1(κ)</td>
<td>p51</td>
<td>−</td>
<td>24, 39, 40</td>
</tr>
<tr>
<td>F24C4Fg</td>
<td>IgG1(κ)</td>
<td>p38</td>
<td>−</td>
<td>24, 39, 40</td>
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<sup>a</sup> Defined on prototype *M. hyorhinis* GDL-1 isolate; numbers indicate kilodaltons as measured by migration in SDS-PAGE (5).

<sup>b</sup> Defined on prototype *M. hyorhinis* isolate GDL-1, by indirect immunofluorescent staining of intact organisms by techniques described in references 37 and 40.

<sup>c</sup> Surface proteins of different sizes are recognized in other isolates, as described in the current report.

aqueous phase components from all six *M. hyorhinis* isolates was performed to determine the partitioning characteristics of antigens recognized by these MAbs in each isolate (Fig. 3A through C). The overall distribution of mycoplasma proteins into the TX-114 and aqueous phases was also monitored, as indicated in Fig. 3D, which shows the partitioning of [35S]methionine-labeled proteins from each isolate. Figure 3A shows that MAb to p23/17 identified the 23- and 17-kDa proteins on prototype isolate GDL-1, but discrete higher-molecular-mass components were stained on other isolates, including a 55-kDa variant on GDL-11, GDL-111, and BTS-7 and a 50-kDa form on isolate PG29. Proteins from the arthritogenic isolate SK76 did not express the epitope recognized by this MAb. In all cases, size-variant forms of this antigen were found exclusively in the TX-114 phase, characteristic of integral membrane proteins.

The staining pattern of immunoblots obtained with MAb to p120 also identified marked size differences in antigens from various isolates expressing this epitope (Fig. 3B). GDL-1 showed staining of a previously defined (40) component, p120, while isolate GDL-111 exhibited a prominent component stained by this MAb of 88 kDa. Isolate GDL-111 stained weakly with this MAb, revealing faint additional components of 100 and about 150 kDa. Again, all of these variants were found only in the TX-114 phase. The epitope detected by this MAb was not observed in immunoblots of TX-114 phase proteins from some isolates of *M. hyorhinis* (PG29, BTS-7, and SK76). The size-invariant surface protein p70 from each isolate was also shown to partition into the TX-114 phase (Fig. 3C). Indirect immunofluorescent staining of intact mycoplasmas by previously described methods (5, 37, 39, 40) showed that epitopes recognized by MAb to p70 were expressed on the surface of all six strains and that those recognized by MAbs to p120 or p23/17 were expressed at the surface of strains showing size-variant forms of the respective antigens (data not shown).

These results identified two distinct sets of *M. hyorhinis* surface antigens, each defined by a MAb, that exhibited marked differences in expression and apparent size polymorphism among isolates within the species *M. hyorhinis*. Immunoblots of isolates after 10 to 50 passages in broth culture revealed no differences in staining patterns, indicating that expression of a particular variant was an operationally stable property even after extended cultivation in vitro.

That the epitopes defining size-variant antigens were in fact associated with proteins in all isolates was confirmed by treatment of TX-114 phase proteins with trypsin prior to SDS-PAGE (24). This treatment completely abrogated binding of anti-p120 MAb in subsequent immunoblots of any strain. Similarly, trypsin treatment eliminated binding of the anti-p23/17 MAb to the p55, p50, or p23 size-variant proteins. However, as shown in Fig. 4, the epitope recognized by the latter MAb was shown in all cases to reside on an 18-kDa limit tryptic peptide generated from any of the size-variant forms of the antigens (p23, p55, or p50). These 18-kDa limit peptides were subsequently shown to partition into the TX-114 phase, as previously described (24) in analysis of the p23 form of the antigen (data not shown), thereby identifying an analogous amphiphilic region in each variant form of the antigen which is capable of interacting with the mycoplasma membrane.

Size-variant antigens showed a relatively delineated binding pattern in immunoblots. However, in large forms of the antigens, a regularly spaced ladder of epitope-bearing peptides descending in size was observed in immunoblots of some preparations. In addition, multiple discrete forms of antigens were in some cases apparent within a single isolate (e.g., the weak staining by MAb to p120 of an 88-kDa form of antigen in the GDL-1 isolate; Fig. 3B, lane 1). Although these characteristics were not typical of general proteolytic
To investigate respective variant isolates GDL-1 and GDL-11 were further shown to express their respective variant forms of p23 and p55 when grown in medium supplemented with horse serum. The possibility that some isolates might selectively elaborate soluble enzymes or other modifying factors that could determine large or small forms of antigens, mixed logarithmic-phase cultures of strains GDL-1 and GDL-11 were cocultivated for 48 h, harvested, applied to SDS-PAGE, and immunoblotted with MAb to p23/17. Both p23 and p55 forms of the antigen were observed, and no other components were stained (data not shown), suggesting that soluble substances secreted by either strain did not account for the size variation observed with this antigen. However, these results did not eliminate a possible role of proteolysis in differential processing of these membrane proteins within organisms.

**Association of lipid with size-variant integral membrane surface protein antigens.** We have recently reported (5) that

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**FIG. 4.** Localization of surface epitope on tryptic fragments of size-variant antigens. TX-114 phase proteins from *M. hyorhinis* GDL-1 (lanes 1 and 2), GDL-11 (lanes 3 and 4), and PG29 (lanes 5 and 6) were treated with trypsin, applied to SDS-PAGE, and immunoblotted with MAb to p23/17. Numbers at left indicate positions of intact size-variant antigens p23/17, p55, and p50 in the respective strains. Arrows indicate 18-kDa limit tryptic fragments identified by the MAb.

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**FIG. 3.** Immunoblot of TX-114 phase-fractionated *M. hyorhinis* components with MAbs to surface antigens. Mycoplasmas were phase-fractionated with TX-114, and the detergent and aqueous phase components were applied to SDS-PAGE and immunoblotted as described in Materials and Methods. TX-114 (TX) and aqueous (AQ) phase components from six *M. hyorhinis* isolates were stained with MAbs to p23/17 (panel A), p120 (panel B), or p70 (panel C). Representative isolates are in the order shown in Fig. 2B. The molecular masses (in kilodaltons) of stained mycoplasma components are shown at left. Each lane represents phase-fractionated material from 15 \( \mu \)g of mycoplasma protein. Panel D represents an autoradiograph of TX-114 phase-fractionated proteins from \( ^{35}S \)methionine-labeled mycoplasmas prepared as described in Materials and Methods. The array of isolates and fractions is the same as in panels A through C. The molecular masses (in kilodaltons) calculated from markers are indicated at the left.
These coplasma isolates showed that isolate phase. Similarly, every BOYER Arrows indicate SDS-PAGE corresponding by SDS-PAGE phase-fractionated as SDS-PAGE phase proteins were strains major proteins specifically of 3H-labeled proteins were metabolically labeled with [3H]palmitate labeled with [3H]palmitate and TX-114 phase proteins were immunoprecipitated with MAbs and analyzed by SDS-PAGE and fluorography as described in Materials and Methods. (A) Fluorograph of [3H]palmitate-labeled TX-114 (TX) or aqueous (AQ) phase components from six M. hyorhinis isolates (lanes 1 to 6), corresponding to those described in the legend to Fig. 2B. (B to D) Fluorographs of [3H]labeled proteins immunoprecipitated from TX-114 phase proteins of the same six M. hyorhinis strains (lanes 1 to 6) by using MAbs to p23 (panel B), p120 (panel C), or p70 (panel D). Arrows indicate positions and molecular masses (in kilodaltons) of major proteins specifically immunoprecipitated.

lipid-associated proteins of the M. hyorhinis prototype isolate GDL-1 are numerous and partition exclusively into the hydrophobic phase during TX-114 phase fractionation and that surface antigens p120, p23, and p70 are among those proteins associated with lipids. To determine whether the size-variant surface antigens in other isolates of M. hyorhinis were similarly lipid associated, all isolates were labeled with [3H]palmitate, phase fractionated with TX-114, and analyzed by SDS-PAGE and fluorography. Figure 5A shows that in every strain, virtually all the [3H]-labeled proteins resolved on SDS-PAGE partitioned into the hydrophobic detergent phase. In contrast, analysis of [35S]methionine-labeled mycoplasma isolates showed that most proteins partitioned into the aqueous phase (Fig. 3D). (It has previously been noted [5] that some lipid-modified proteins, including p120 and p23 of isolate GDL-1, are not labeled with [35S]methionine.) These results ensured that [3H] labeling of proteins did not result from conversion of [3H]palmitate into general metabolite pools and that the [3H]-labeled proteins partitioning into the TX-114 phase were in fact a defined subset of proteins from each of these isolates. The selective partitioning of [3H]-labeled components showed strong correlation between fatty acid labeling and amphiphilic properties of the labeled proteins.

Marked size polymorphism was evident among the [3H]palmitate-labeled, TX-114 phase components of various M. hyorhinis isolates (Fig. 5A). Notably, some of these labeled components showed migration patterns corresponding to those obtained in immunoblot analysis by using MAbs to surface-associated variant proteins (e.g., Fig. 3A). To determine directly whether strain-variant forms of surface antigens were lipid labeled, immunoprecipitation of metabolically labeled TX-114 proteins from each M. hyorhinis isolate was performed with IgG MAb to size-variant antigens. Figure 5B shows that [3H]-labeled proteins identical to those recognized in immunoblots were immunoprecipitated by IgG MAb to p23. A 23-kDa protein was precipitated from the prototype isolate GDL-1, whereas M. hyorhinis isolates GDL-11, GDL-111, and PG29 exhibited 50- to 55-kDa [3H]-labeled proteins specifically recognized by this MAb. Labeled protein appeared not to be immunoprecipitated from isolate BTS-7, possibly because of the limited quantity of p55 antigen present, as suggested by immunoblots (Fig. 3A). In some experiments, a [3H]-labeled component of about 100 kDa was immunoprecipitated from isolate SK76 by MAb to p23 (not apparent in Fig. 5B because of the limited quantity represented).

The second set of size-variant antigens, defined by MAb to p120, was similarly analyzed by immunoprecipitation (Fig. 5C). Again, [3H]palmitate-labeled proteins precipitated from different isolates by MAb to p120 generally corresponded to the polymorphic components identified in immunoblot analysis (Fig. 3B), including components of 120 kDa on GDL-1, approximately 100 and 150 kDa on GDL-11, and 88 kDa on GDL-111. An additional component of yet another size was recognized on PG29. These results showed unequivocally that size-variant surface antigens defined by MAbs to p120 and MAb to p70 are expressed on distinct sets of lipid-associated TX-114 phase proteins. Immunoprecipitation with MAb to p70 (Fig. 5D) similarly identified a hydrophobic protein of approximately 70 kDa present on all M. hyorhinis isolates. This protein was also labeled with [3H]palmitate, although at much lower intensity. (Visualization of p70 in the SK76 strain is not apparent in Fig. 5B, again because of an underrepresentation of TX-114 phase proteins in this experiment.)

Confirmation of covalent lipid modification of integral membrane proteins of M. hyorhinis. The numerical prevalence and general size variation (Fig. 5A) of lipid- and labeled TX-114 phase proteins in M. hyorhinis (including the specific size-variant integral membrane surface antigens defined by MAbs) prompted additional analysis to determine the nature of the lipid association. To establish formally whether these proteins were covalently modified by lipids, we used previously described (38) methods to analyze methanolysis products derived from TX-114 phase proteins from mycoplasmas metabolically labeled with [3H]palmitate. Labeled TX-114 phase proteins from each M. hyorhinis isolate were precipitated with methanol and extensively delipidated with chloroform-methanol until no additional label could be extracted (38). Analysis of extracted proteins by SDS-PAGE and fluorography showed virtually identical patterns and intensities as those observed with proteins prior to delipidation (data not shown). Labeled products generated from acid
that lipid was covalently linked to these proteins. Figures 6A and B illustrate analyses of two *M. hyorhinis* isolates, GDL-1 and BTS-7, respectively. In parallel experiments, the other four isolates, GDL-11, GDL-111, PG29, and SK76, gave virtually identical chromatograms of labeled methanolysis products (data not shown).

Notably, only methyl palmitate and no other lipid form was recovered during methanolysis of TX-114 phase proteins from [*H*]palmitate-labeled *M. hyorhinis* (elution was monitored for 120 min). Although this finding was expected, it contrasted with earlier results from similar analysis of another mycoplasma, *M. hyopneumoniae*, where a major, additional [*H*]-labeled component was observed during analysis of methanolysis products from [*H*]palmitate-labeled TX-114 phase proteins (38). Since *M. hyopneumoniae* had been grown for that study in a different medium (containing 20% porcine serum), we analyzed methanolysis products from *M. hyorhinis* (GDL-1) propagated and labeled in exactly that same lot of medium (Fig. 6C). Again, only methyl palmitate was recovered under these conditions, indicating that the composition of the two media did not affect the nature of label recovered by methanolysis and that the apparent conversion of [*H*]palmitate to another lipid form associated with modified proteins of *M. hyopneumoniae* is a distinctive feature of that organism and does not occur in *M. hyorhinis*.

In light of accumulating evidence that multiple forms of covalent lipid modification may occur in some members of the class *Mollicutes* (5, 7–9, 22), an initial attempt was made to identify possible variations in lipid linkages to TX-114 phase proteins of *M. hyorhinis* by using differential conditions of hydrolysis as previously described (38). SDS-PAGE gels containing TX-114 phase proteins from [*H*]palmitate-labeled *M. hyorhinis* isolates were treated with 1 M hydroxylamine under alkaline conditions, a method utilized to selectively release more labile *o*-ester- and thioester-linked fatty acids (26). Figure 7 shows that the majority of prominent, lipid-associated proteins retained label under these conditions, when compared with untreated control gels. This indicated that a significant proportion of palmitate was probably linked covalently to many proteins through amide bonds. A general reduction in labeling intensity of most proteins was observed in hydroxylamine-treated gels. This might be expected if proteins were acylated by the known procaryotic mechanism in which *o*-ester- and amide-linked fatty acids occur in a 2:1 molar ratio at a single glyceryl cysteine residue in bacterial lipoproteins (43). However, certain proteins showed complete loss of [*H*]label upon hydroxylamine treatment, notably a 120-kDa protein in the prototype isolate GDL-1 corresponding to the position of surface antigen p120 (Fig. 7A and B, lanes 1). Release of all [*H*]palmitate from this component after alkaline hydroxylamine treatment suggested linkage only through more labile thioester or *o*-ester bonds (26). Label was also completely released from a component of similar size from PG29 (Fig. 7A and B, lanes 4). These results suggest that although most fatty acylated proteins are probably modified in part by amide linkage, fatty acids may also be selectively linked to certain proteins only through more labile bonds. One such protein may be the p120 surface antigen of strain GDL-1.

**DISCUSSION**

This study extends our understanding of differential surface antigen expression in *M. hyorhinis* by demonstrating two sets of membrane lipoproteins which express specific
surface epitopes on selected isolates within this species and which show marked variation in the size of proteins expressing the epitopes on these isolates. Intraspecies antigenic differences involving surface lipoproteins have not been documented in other mycoplasmas but could reflect a general mechanism by which these wall-less organisms accommodate a variety of environmental changes, including those imposed during infection of an immunologically responsive host. Potential immunological ramifications of variation are further underscored by our earlier demonstration that one major variant antigen (p23, expressed on isolate GDL-1) mediated efficient, complement-mediated mycoplasmacidal activity in vitro (24). Lack of this epitope (or its cryptic presence) on other strains of M. hyorhinis (reference 24 and this report) could, in principle, influence the ability of an organism to survive in the face of an immune response. Indeed, the demonstration that multiple prominent surface antigenic structures vary widely among these isolates suggests a possibly powerful mechanism for generating antigenic diversity within this species.

The results reported here establish that size-variant surface antigens are among a remarkably large group of integral membrane proteins covalently modified with lipids; however, neither the biochemical nature of the epitope recognized nor the structural relationship between any two epitope-bearing size-variant proteins is fully determined. Epitope mapping suggested that structurally similar portions of the p23, p50, and p55 forms of proteins are recognized by the anti-p23/17 IgM MAb, since an amphiphilic, 18-kDa limit tryptic fragment bearing this epitope could be generated from each of these proteins isolated from its respective isolate. It is possible that the epitope recognized by MAb to p23/17 resides within a region of these antigens sharing common protein structure and that the difference in size derives from posttranslational modifications. Although reported lipid modifications (11, 16, 21) generally do not result in migration differences in SDS-PAGE of the magnitude observed here (e.g., 23 versus 55 kDa), the nature of the covalently bound, lipid-linked moiety has not been established; it could include a large (possibly carbohydrate) component capable of altering migration in this system. An alternative explanation of size variation is that specific epitopes recognized by the MAbs reside on a common posttranslational modification present on proteins of different size. This scheme would require highly distinctive modifications (to yield the specific epitopes recognized by different MAbs) as well as selective modification of precise proteins, since epitopes are indeed associated only with certain membrane lipoproteins among the many expressed on any individual strain.

Among the models of surface antigen variation known in procaryotic organisms, perhaps the most intriguing analog to strain-specific appearance and size variation documented in M. hyorhinis is represented in the gram-positive eubacteria. Size variants of the M protein surface antigens are widely distributed among streptococci (12, 17). These proteins bear strain-restricted epitopes, define type-specific antigens, and are important in immunologic clearance of organisms (12, 17, 35). Recent analysis of amino acid sequence at the DNA level (15, 19, 20) indicates that M proteins have homologous, repetitive internal sequences consistent with a coiled-coil conformation over much of the molecule and that size variants may arise among strains by using different numbers of repetitive sequences within these proteins. Additionally, multiple sets of nonhomologous repeated units are utilized within a single M protein, and repetitive units may be nonhomologous between M proteins of two different strains (15, 19, 20). Whether or not an analogous system exists in mycoplasmas remains to be determined. However, we have recently demonstrated (M. J. Boyer and K. S. Wise, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, G12, p. 130) a highly repetitive, graded series of trypsin and submaxillary protease peptides bearing the epitope recognized by the MAb to p23. That these fragments show a highly regular spacing of about 3.5 kDa is consistent with an internally repetitive protein structure in some surface lipoproteins of M. hyorhinis (M. J. Boyer, R. K. Watson-McKown, and K. S. Wise, manuscript in preparation). These observations, along with accumulating genomic analysis suggesting a phylogenetic relationship of mycoplasmas and gram-positive eubacteria (42), provide support for a testable model of size-variant antigen structure mimicking that of streptococcal M proteins. However, other mechanisms for generating size variation cannot be ruled out; these include alternative procaryotic forms of phase variation (27) and assembly of small peptides into aggregates of various sizes, as suggested in a recently described size-variant antigen system of M. pulmonis (34). Interestingly, striking genomic polymorphism has been documented within the species M. hyorhinis (10), including a recently described repetitive element defining restriction-fragment-length polymorphism among the same isolates described in the current report (29). More complete biochemical characterization of size-variant lipoproteins and analysis of corresponding genomic sequences will be required to fully understand the structure and genetic mechanisms underlying this phenomenon.

An important feature of size-variant surface antigens established in this report is their association with lipids. Whether this occurs by mechanisms demonstrated in other procaryotes (43), through yet undefined linkages suggested in other members of the class Mollicutes (8, 22), or by systems analogous to those used by eucaryotes (11, 23, 26)
remains to be determined. The apparently selective acylation of some *M. hyorhinis* proteins through bonds labile to alkaline hydroxylamine raises the possibility that multiple forms of fatty acylation occur in a single organism. Covalent lipid modification of surface antigens is a common occurrence in *M. hyorhinis* and is undoubtedly a critical feature for organisms communicating with their environment through the external face of a single plasma membrane. The striking difference in the numerical prevalence of lipoproteins in *M. hyorhinis* versus that in *M. hominis* (38) emphasizes the possible diversity of acylation systems operating in different species of mycoplasmas. Rapid evolutionary emergence of systems to accommodate membrane protein anchorage or function may have had great selective advantage for these divergent organisms. Collectively, mycoplasmas may provide a rich source of alternative prokaryotic acylation systems that can be systematically defined and analyzed. Moreover, lipid-modified membrane proteins may play an important role in dictating intraspecies differences in surface antigenic structure and host interactions for a variety of mycoplasma species.

ACKNOWLEDGMENTS

We thank Robyn Watson-McKown for technical contributions to these studies.

This work was supported in part by Public Health Service grant AR28147 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases. K.S.W. was the recipient of Public Health Service Career Development Award AR00848 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

LITERATURE CITED


