Antigen Heterogeneity among Isolates of \textit{Mycoplasma bovis} Is Generated by High-Frequency Variation of Diverse Membrane Surface Proteins

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The protein and antigen profiles of 11 isolates of \textit{Mycoplasma bovis} were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of whole organisms. The isolates examined included the type strain PG45 and 10 other filter-cloned strains or purified isolates both from animals without clinical signs and from clinical cases of bovine mastitis, arthritis, or pneumonia. While the overall protein patterns visualized by silver staining were very similar, marked differences in the antigen banding profiles were detected by rabbit antiserum prepared against whole organisms from one of the strains analyzed. This antigenic heterogeneity was shown to be independent of the geographical origin, the type of clinical disease, and the site of isolation and was also observed among serial isolates from a single animal. Antigen profiles were further monitored throughout sequentially subcloned populations of the PG45 strain. This clonal analysis revealed a high-frequency variation in the expression levels of several prominent antigens. All of these variable antigens were defined by detergent-phase fractionation with Triton X-114 as amphiphilic integral membrane proteins. A subset of different-sized membrane proteins was identified by a monoclonal antibody raised against a PG45 subclone expressing a 63- and a 46-kDa variant antigen within that set. The selective susceptibility of these proteins to trypsin treatment of intact organisms and their ability to bind the monoclonal antibody in colony immunoblots demonstrated that they were exposed on the cell surface. In addition, their preferential recognition by serum antibodies from individual cattle with naturally induced \textit{M. bovis} mastitis or arthritis confirmed that they were major immunogens of this organism. These studies establish that the apparent antigenic heterogeneity among \textit{M. bovis} isolates reported here does not represent stable phenotypic strain differences generated from accumulated mutational events but reflects distinct expression patterns of diverse, highly variable membrane surface proteins.

\textit{Mycoplasma bovis} is a widespread and important prokaryotic pathogen of cattle that is well established as the major causative agent of bovine mycoplasma mastitis and has also been associated with bovine arthritis and pneumonia, as well as infections of the bovine genital tract (15, 16, 22). Because control of these diseases is hampered by the lack of effective antimicrobial agents and by the lack of suitable vaccines for active prophylactic immunization, \textit{M. bovis} infections are responsible for considerable economic losses in cattle and milk production (15, 16, 29). Recently, some efforts have been made to improve the specific detection of this agent in the early stages of disease (6, 13). However, in light of recent evidence (8, 19–21, 24, 25, 28, 31, 33, 34, 37) of antigenic variability in a number of pathogenic \textit{Mycoplasma} species (reviewed in references 35 and 38), these approaches based on monoclonal antibodies (MAbs) (6, 13) may also be fairly limited without a clear knowledge of the stability and intraspecies distribution of the antigens recognized. Although \textit{M. bovis} is not among the species that have been studied in detail, more recently, one of us (K. Sachse) reported slight differences between strains in the expression levels of individual antigens (26). These very preliminary data support the idea of the possible presence of variable surface antigens in this organism. Determining the identities and structural features of such components selectively and specifically recognized during infection could lead to an increased understanding of the pathogenesis of disease caused by this agent and may also be critical for constructing immunogenic reagents capable of detecting and preventing \textit{M. bovis} infections.

In the present study, a rabbit hyperimmune serum was initially used to demonstrate a striking antigenic variability among 11 strains and clinical isolates of \textit{M. bovis}, as well as within isogenic clonal lineages of the type strain PG45. To identify and monitor specific components involved in this variability and to later assess their possible role in host interactions, a MAb that identified a restricted set of prominent size-variant antigens selectively expressed in the cultured populations analyzed was generated. Using techniques developed previously (9, 23–25), we define these products as amphiphilic membrane surface proteins and establish in an accompanying paper (5) that they represent a family of antigenically and structurally related lipoproteins which spontaneously undergo high-frequency changes in size and expression. We further demonstrate that they are major immunogens.

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of *M. bovis* and consider that they may be good candidates for a potential vaccine design.

**MATERIALS AND METHODS**

*Mycoplasma* isolates and culture conditions. *M. bovis* isolates and their origin are listed in Table 1. They were obtained as lyophilized filter-cloned strains or as purified frozen broth cultures of clinical isolates from the following sources: *M. bovis* 422 and 454 were obtained from H. Pfützner, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Jena, Germany, and have been recently described (26); the type strain PG45 (2, 12) originated from the collection of the Institute for Microbiology and Infectious Diseases of Animals, School of Veterinary Medicine, Hannover, Germany, and was originally obtained from J. G. Tully. All other isolates originated in this institute. All isolates were propagated at 37°C in a modified standard mycoplasma broth medium (1) containing 19 g of heart infusion broth (Difco, Detroit, Mich.), 50 ml of liquid yeast extract (10% vol/vol); Oxoid, London, United Kingdom), 2 × 10⁶ U of penicillin G (Hoechst, Frankfurt, Germany), and 200 ml of heat-inactivated (56°C, 30 min) horse serum per liter. Stools of each isolate were prepared by freeing 1-ml portions of a 10-ml logarithmic-phase broth culture at −80°C. For subcloning, processing for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis as described below, frozen stocks were thawed, and 100-μl aliquots were expanded in 1-ml broth cultures.

**Subcloning.** Subclones of strain PG45 were obtained by using procedures described elsewhere (24, 25). Briefly, fresh broth-grown organisms from primary passages of the PG45 stock were vortexed and serially diluted 10-fold to 10⁻⁶. Samples (20 μl) of each dilution were spread evenly over the surface of 1% agar medium of the same composition as the broth medium described above. Two dilutions were inoculated onto separate halves of a plate petri dish (5 by 1 cm, with 8 to 9 ml of agar medium). The plates were incubated at 37°C in a moist atmosphere with 5% CO₂ for at least 6 days, and then well-isolated colonies were picked with Pasteur pipettes and each was inoculated into 1 ml of broth medium. These primary cloned isolates were propagated at 37°C for 3 days. These cultures typically contained approximately 1.45 × 10⁹ CFU/ml, representing an estimated 27 total generations from the single organism plated. Aliquots (100 μl) of these cultures were expanded in 1-ml broth cultures and stored as stock cultures at −80°C. The remainder was replated to obtain second-generation (and subsequent) subclones as described above and/or processed for SDS-PAGE and immunoblotting as described below.

**Antibodies.** Antigens for immunization of rabbits and mice were prepared as follows. Organisms from broth-grown, logarithmic-phase cultures were harvested by centrifugation at 12,000 × g for 30 min. The cell pellets were washed twice in phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.0 mM NaH₂PO₄, pH 7.2). Washed cell pellets were suspended in PBS and aliquots were stored at −80°C prior to use. The protein concentration of each antigen preparation was determined by using a modified biuret-based protein assay (Boehringer Ingelheim, Ingelheim, Germany), with bovine serum albumin as the standard.

Hyperimmun antiserum to whole broth-grown organisms from *M. bovis* D490 was generated in a female New Zealand White rabbit according to the protocol of Morton and Roberts (18) by injection with 5 mg of protein emulsified 1:1 (vol/vol) in incomplete Freund adjuvant (Difco) (0.5 ml into each of two subcutaneous and two intramuscular sites), followed by a second injection of the same dose and by the same route 4 weeks later. One week after the second injection, the animal was exsanguinated, and the serum was divided into equal portions and stored at −20°C.

MABSs to a clonal variant of *M. bovis* PG45, designated cl.7.8.2, were prepared as follows. Two 6-week-old BALB/c mice were inoculated intraperitoneally six times at 2-week intervals with 100 μg of protein (in 100 μl of PBS) in 100 μl of lipoprotein adjuvant N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine, which was obtained from W. Bessler, University of Freiburg, Freiburg, Germany. In both cases, hyperimmune serum was obtained by eye bleeding and measured for antibody activity by the dot blotting procedure described below. Two weeks after the last (sixth) inoculation, the mice were given a final immunizing dose of 200 μg of protein (without adjuvant). Three days later, splenocytes were obtained and fused with nonsecreting murine FO myeloma cells (10), which were provided by W.-D. Müller, University of Jena, Jena, Germany. Fusions were performed by using standard procedures described in detail elsewhere (11). After fusion, cells were distributed into 96-well microtiter plates in hybridoma growth medium (RPMI 1640 [Gibco BRL, Eggenstein, Germany], supplemented with 10% heat-inactivated fetal calf serum [Gibco BRL], 2 g of NaHCO₃ per liter, 2 mM L-

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**Table 1. M. bovis** isolates analyzed

<table>
<thead>
<tr>
<th>Lane in Fig. 1 and 6</th>
<th>Isolate</th>
<th>Geographic origin</th>
<th>Bovine host</th>
<th>Site of isolation</th>
<th>Clinical condition</th>
<th>Yr of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PG45, type strain*</td>
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<td>Milk</td>
<td>Mastitis</td>
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<td>2</td>
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<td>Lung</td>
<td>Pneumonia</td>
<td>1975</td>
</tr>
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<td>3</td>
<td>D857⁵</td>
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<td>Milk</td>
<td>Mastitis</td>
<td>1990</td>
</tr>
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<td>4</td>
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<td>Milk</td>
<td>Mastitis</td>
<td>1990</td>
</tr>
<tr>
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<td>D1098</td>
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<td>Cow</td>
<td>Trachea</td>
<td>Mastitis</td>
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</tr>
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<td>6</td>
<td>D1499</td>
<td>Germany (West)</td>
<td>Calf</td>
<td>Joint Arthritis</td>
<td></td>
<td>1991</td>
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<tr>
<td>7</td>
<td>D1115</td>
<td>Germany (West)</td>
<td>Cow</td>
<td>Urine</td>
<td>Mastitis, arthritis</td>
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<td>D1511²</td>
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<td>Calf</td>
<td>Nose</td>
<td>Healthy*</td>
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<tr>
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<td>Germany (West)</td>
<td>Calf</td>
<td>Nose</td>
<td>Healthy*</td>
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<tr>
<td>10</td>
<td>422²</td>
<td>Cuba</td>
<td>Calf</td>
<td>Lung</td>
<td>Pneumonia</td>
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<tr>
<td>11</td>
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<td>Germany (East)</td>
<td>Cow</td>
<td>Milk</td>
<td>Mastitis</td>
<td>1981</td>
</tr>
</tbody>
</table>

* Isolates representing filter-cloned strains.
* Isolates from milk of different udder quarters of the same animal.
* Isolates from different animals of the same herd.
* Without clinical signs.

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glutamine, 50 mg of gentamicin [Serva, Heidelberg, Germany] per liter, and 0.05 mM 2-mercaptoethanol, which contained 0.1 mM hypoxanthine–0.0057 mM azaserine (Serva). Culture fluids from positive wells showing cell growth were tested for antibody production on a 96-well Minifold dot blot apparatus (model I SRC 96D; Schleicher & Schuell, Dassel, Germany). For the screening assay, 50 μl of a suspension of broth-grown whole organisms from M. bovis PG54c7.8.2 in PBS (containing 0.4 μg of protein) was deposited in each well of the Minifold apparatus, and the fluid was allowed to filter through a nitrocellulose membrane (0.45-μm pore size; Schleicher & Schuell) by gravity flow. After 30 min, the membrane was removed and blocked with PBS-T (PBS with 0.1% [vol/vol] Tween 20 [4; Serva]) containing 2% powdered milk. The membrane was washed three times with PBS-T and then returned to the Minifold. Hybridoima culture fluids, diluted 1:2 in PBS-T, were transferred to wells in the Minifold and again allowed to filter through by gravity flow. After 30 min, the membrane was again removed and washed three times with PBS-T. The membrane was then incubated for 1 h in alkaline phosphatase-conjugated sheep immunoglobulin G Fab fragments against mouse immunoglobulins (Boehringer Mannheim, Mannheim, Germany), diluted 1:500 in PBS-T. The membrane was again washed and subsequently developed with BCIP-NBT enzyme substrate solution (0.4 mM 5-bromo-4-chloro-3-indolyl phosphate [BCIP; Sigma, Deisenhofen, Germany] and 0.4 mM nitroblue tetrazolium [NBT; Sigma] in Tris saline [0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl2, pH 9.5]). Hybridomas producing reactive antibody were cloned by limiting dilution in 96-well microtiter plates, using hybridoima growth medium supplemented with 0.1 mM hypoxanthine–0.0057 mM azaserine, and were later adapted to serum-free growth medium (CG-Medium; Camon, Wiesbaden, Germany). The isotypes of MAbs were determined with the mouse monoclonal antibody isotyping kit (Amersham Buchler, Braunschweig, Germany), consisting of nitrocellulose membrane strips with immobilized heavy- and light-chain isotype-specific goat anti-mouse antibody and a peroxidase-based detection system. The protocol was performed as recommended by the manufacturer, and the reagents used were those supplied with the kit.

Other reagents included sera from cows with M. bovis mastitis (sample D1473) or M. bovis arthritis (sample D74) which were obtained from the serum collection of the Institute for Microbiology and Infectious Diseases of Animals, School of Veterinary Medicine, Hannover, Germany, and a recently described (6) immunoglobulin G2a(κ) MAb (5D8) to a 41-kDa antigen that was shown here to be an internal, hydrophilic protein (designated p41). The size of the p41 antigen recognized by this MAb was originally reported to be 37 kDa (6), but we have determined it here to be 41 kDa.

Sera and hybridoima culture supernatants containing MAbs were diluted in PBS-T or PBS for use in Western and colony immunoblotting, respectively, as described below.

**Phase fractionation of mycoplasma proteins with TX-114.**

Detergent-phase fractionation of M. bovis proteins with Triton X-114 (TX-114; Boehringer Mannheim) was performed as described in detail elsewhere (9, 23, 25). Briefly, organisms from 1-ml logarithmic-phase cultures were harvested by centrifugation for 5 min at 12,000 × g, washed twice in PBS containing 0.5 mM phenylmethylsulfonyl fluoride (Fluka, Neu-Ulm, Germany), and solubilized at 4°C for 2 h in 400 μl of PBS-phenylmethylsulfonyl fluoride containing 1% (vol/vol) TX-114. After centrifugation at 4°C for 10 min at 12,000 × g to remove insoluble material, the supernatant was subjected to three cycles of phase fractionation. Proteins of the final TX-114 and aqueous phases were precipitated by adding methanol to a final concentration of 90% (vol/vol). After the preparations were maintained at −80°C for 16 h, the resulting precipitates were sedimented at 12,000 × g for 10 min at 4°C and subsequently processed for SDS-PAGE and immunoblot analysis as described below.

**SDS-PAGE and Western immunoblotting.** Samples were prepared for SDS-PAGE by heating PBS-phenylmethylsulfonyl fluoride-washed cell pellets or phase-fractionated proteins at 100°C for 3 min in sample buffer (2% SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 3% urea, 62.5 mM Tris, pH 6.8). They were then loaded at concentrations of 2, 5, 8, 10, 17, or 34 μg of protein (corresponding to [1, 3, 5, 6, 10, or 20] × 10^6 CFU) onto 0.75-mm SDS–9% polyacrylamide slab gels containing 3% urea. Electrophoresis was performed by the method of Laemmli (17) and carried out in a vertical slab gel electrophoresis unit (model SE 600; Hoefer, San Francisco, Calif.) at a constant current of 15 mA per gel in a pH 8.3 0.025 M Tris–0.192 M glycine buffer containing 0.1% SDS. After electrophoresis, mycoplasma proteins were either silver stained by the procedure of Blum et al. (7) or placed in a Transphor blotting apparatus (Hoefer) and transferred electrophoretically to nitrocellulose membrane filters (0.45-μm pore size; Schleicher & Schuell) for 1 h at 1 A, as described by Towbin et al. (30). Blots were incubated for 1 h at 60°C and then blocked overnight at 4°C with PBS containing 3% bovine serum albumin. After three washes in PBS-T, the blots were incubated overnight at 4°C with the primary antibody (rabbit antiserum to M. bovis D490 [diluted 1:1,000], bovine sera diluted 1:50, or hybridoima culture fluid supernatant containing MAbs [diluted 1:10]). The blots were again washed three times in PBS-T and then incubated for at least 2 h at room temperature in peroxidase-conjugated goat antisera (Nordic, Tilburg, The Netherlands) to rabbit, bovine, or mouse immunoglobulins, diluted in PBS-T 1:1,000, 1:500, and 1:400, respectively. After three washes in PBS, blots were developed with enzyme substrate solution (12 mg of 4-chloro-1-naphthol [Aldrich, Steinheim, Germany], 4 ml of methanol, 20 μl of 30% H2O2, 20 ml of PBS) for 5 to 20 min, and the color reaction was stopped by washing blots in water.

Relative molecular masses were determined with prestained protein standards (low range; Bio-Rad, Munich, Germany) run simultaneously.

**Colony immunoblotting.** Surface antigen heterogeneity within M. bovis populations was easily detected by colony immunoblotting, using a procedure previously described (24, 25), with minor modifications. Briefly, circles of nitrocellulose membrane filters (47-mm diameter, 0.45-μm pore size; Schleicher & Schuell) were placed on mycoplasma colonies on the surface of agar plates and left for 5 min. They were then gently removed from the agar surface and placed in petri dishes with transferred colonies facing up. Colony blots were immunostained (without blocking) as described above for Western immunoblots, except that PBS was used for antibody dilution and washing steps instead of PBS-T.

**Trypsin treatment of intact mycoplasmas.** Intact organisms from fresh broth cultures were treated with trypsin by a previously described method (24, 25). Briefly, mycoplasmas were harvested by centrifugation for 5 min at 12,000 × g from logarithmic-phase broth cultures, rinsed once with PBS at pH 7.2, and rinsed once with PBS at pH 8.0. Cells from approximately 170 μl of culture were incubated at 37°C for 1 h in 25 μl of PBS (pH 8.0) containing either no enzyme or various concentrations of 1-1-1-tosylamide-2-phenylethyl chloromethyl ketone-trypsin (Sigma) per ml. Samples were immediately processed for SDS-PAGE, and subsequent blots were pre-
Comparison of protein and antigen profiles of strains and clinical isolates of *M. bovis*. The silver-stained SDS-PAGE protein profiles of the 11 *M. bovis* strains and clinical isolates are shown in Fig. 1a. Although the overall protein banding patterns with major bands at 48, 45, 43, and 39 kDa were essentially identical, slight differences in the expression levels of some proteins could be detected. However, one strain (strain 422, lane 10), which was isolated in Cuba, was clearly distinguishable from the other isolates by the presence of three prominent protein bands at 50, 42, and 34 kDa.

In order to assess antigenic differences among the various isolates, Western immunoblot analysis of gels, similarly loaded with equal amounts of protein, was performed with a rabbit polyclonal antibody (PAb) raised against whole broth-grown organisms from strain D490. The result is shown in Fig. 1b. Marked differences can be seen between the isolates in the apparent expression levels of several prominent antigens with molecular masses between 25 and 80 kDa. No two strains gave identical immunoblot profiles. Only two antigens with molecular masses of 45 and 41 kDa were found in all isolates. In separate experiments, the strain-invariant 41-kDa antigen was shown to be an internal, hydrophilic protein which was recognized by MAb 5D8 (see below). The PAb immunoblot patterns for each isolate shown in Fig. 1b were highly reproducible and also did not change when organisms from different passages or different growth phases were analyzed (not shown).

Detection of antigen variability among clonal populations and within clonal lineages of *M. bovis* PG45 by PAb. Figure 2 shows the variety of antigen banding patterns found among second- to sixth-generation subclones of the type strain PG45 by using the PAb reagent against strain D490. The variations among subclones particularly included differences in the expression and the molecular masses of the uppermost, intensely stained bands, as well as in the overall banding patterns. Two separate antigen clusters could be distinguished: cluster I (lanes 1 to 6), which is characterized by a prominent band at 50 kDa and a set of bands with molecular sizes ranging from 33 to 36 kDa; and cluster II (lanes 7 to 14), which is characterized by a strongly reacting band between 62 and 77 kDa and a descending ladder of more weakly immunostained bands. In addition to these predominating immunoblot profiles, other subclones were found to express neither of the two antigen
were subjected to SDS-PAGE and the subsequent immunoblot was stained with rabbit antiserum to *M. bovis* D490 as described in the legends to Fig. 1b and 2. All samples represent approximately 10⁷ CFU. Sequential phenotypic transitions are indicated by arrows that represent a direct lineage of clonal variants obtained by serial subcloning of single colonies, as described in Materials and Methods. The clonal variant in lane 1 is identical to the variant shown in Fig. 2, lane 7. The relative molecular masses of five major variable antigens identified in this lineage are indicated in kilodaltons on the left and were calculated from prestained molecular mass markers run in the same gel. (b) Colonies derived from a subcloned population identical to the variant in panel a, lane 3, were transferred to nitrocellulose and immunostained with rabbit antiserum to strain D490, as described in Materials and Methods. A representative portion of that blot with colonies exhibiting various degrees of immunostaining intensity and sectoring is shown. Bar, 0.25 mm.

**FIG. 3.** Variation in expression and size of predominant antigens in a clonal lineage of *M. bovis* type strain PG45. (a) Whole organisms were subjected to SDS-PAGE and the subsequent immunoblot was stained with rabbit antiserum to *M. bovis* D490 as described in the legends to Fig. 1b and 2. All samples represent approximately 10⁷ CFU. Sequential phenotypic transitions are indicated by arrows that represent a direct lineage of clonal variants obtained by serial subcloning of single colonies, as described in Materials and Methods. The clonal variant in lane 1 is identical to the variant shown in Fig. 2, lane 7. The relative molecular masses of five major variable antigens identified in this lineage are indicated in kilodaltons on the left and were calculated from prestained molecular mass markers run in the same gel. (b) Colonies derived from a subcloned population identical to the variant in panel a, lane 3, were transferred to nitrocellulose and immunostained with rabbit antiserum to strain D490, as described in Materials and Methods. A representative portion of that blot with colonies exhibiting various degrees of immunostaining intensity and sectoring is shown. Bar, 0.25 mm.

Identification of variable antigens as integral membrane proteins. To investigate the possibility that the variable antigens detected in clonal populations of the PG45 strain might be membrane proteins, organisms from clonal isolates expressing variant antigens were subjected to TX-114 strain might be membrane proteins, organisms from clonal isolates expressing variant antigens were subjected to TX-114 phase fractionation. This procedure has been widely used to differentiate amphiphilic integral membrane proteins from hydrophilic internal cytoplasmic proteins of other mycoplasma species (3, 8, 9, 20, 23–25, 36, 37). Each prominent antigen of clusters I and II identified by the PAb reagent (Fig. 2 and 3a) was found to selectively partition into the hydrophobic TX-114 phase, thereby indicating the presence of hydrophobic domains capable of interacting with detergent micelles. An example of this immunoblot analysis is shown in Fig. 4a and b; a set of three putative size-variant antigens of cluster II (Fig. 4a) is shown to exclusively partition into the detergent phase (Fig. 4b). In contrast, antigen p41 (Fig. 4a) failed to demonstrate domains accessible to hydrophobic interactions with TX-114 (Fig. 4b) and segregated into the aqueous hydrophilic phase (not shown). On the basis of their highly selective partitioning properties, the variable antigens were therefore provisionally classified as integral membrane proteins. That they were in fact proteins in nature was shown by additional criteria (see below).

Identification by MAbs of a set of size-variant integral membrane proteins. A set of variable integral membrane proteins of *M. bovis* was defined by the experiments described above, using a rabbit antisera to recognizing either specific or shared epitopes on these proteins. To further assess the possible antigenic relatedness among these proteins and to later define their possible surface association, MAbs were generated from mice immunized with whole cells from a clonal isolate that predominantly expressed a 63-kDa variant membrane protein of cluster II (Fig. 4a and b, lanes 3) and also showed in colony blots of direct progeny populations a marked heterogeneity in staining intensity (Fig. 3b). To identify those MAbs reacting with surface-associated antigens, the MAbs were initially screened for the ability to bind whole organisms immobilized on nitrocellulose membrane filters. From a library of approximately 150 stable hybridoma cell lines, seven MAbs were found to react in that dot blot assay. One of these MAbs [MAb 1E5; isotype immunoglobulin M(κ)] was subsequently shown in Western immunoblots to recognize a shared epitope on the different-sized integral membrane protein antigens of cluster II (Fig. 4c), including the immunizing 63-kDa variant of this protein set (Fig. 4c, lane 3). In addition, this MAb was shown to react with a 46-kDa component (Fig. 4c, lane 3) that failed to react with the PAb reagent (Fig. 4a and b, lanes 3). These data suggest that two antigenically related but distinct components may exist within the set of variable integral membrane proteins identified by the MAb and further support the idea that at least one of these products occurs in discrete size-variant forms.
**Surface location of size-variant membrane proteins.** In order to determine if the variable membrane proteins bound by MAb 1E5 were in fact located on the external surface of M. bovis, freshly harvested intact cells from clonal variants of M. bovis PG45 that expressed individual epitope-bearing, different-sized membrane proteins were treated with graded amounts of trypsin or enzyme buffer alone. Sets of trypsin-treated and untreated cells were concurrently subjected to SDS-PAGE and Western immunoblotting with MAb 1E5. One example of this Western immunoblot analysis is shown in Fig. 4d. Incubation of intact organisms in buffer without trypsin yielded the native protein size variant (lane 1). The addition of various amounts of trypsin to the incubation buffer resulted in the generation of prominent epitope-bearing degradation products with molecular masses of 43 (lanes 2 and 3) or 41 (not shown) kDa and the complete disappearance of the intact proteins (lane 3). In contrast, immunostaining of corresponding blots with MAb 5D8 to antigen p41 revealed that this protein remained unaltered during digestion (Fig. 4e), thereby demonstrating the integrity of cells and the selective susceptibility of surface proteins to trypsin treatment and further confirming that p41 was an internal mycoplasma constituent. In a control experiment, the hydrophilic p41 was shown to be sensitive to similar levels of trypsin in aqueous-phase prepara-

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**FIG. 4.** Identification of a set of size-variant surface-exposed integral membrane protein antigens in clonal populations of M. bovis type strain PG45. (a to c) Clonal variants of M. bovis type strain PG45 were subjected to TX-114 phase fractionation, and proteins from whole organisms (a and c) or TX-114 phases (b) were separated by SDS-PAGE and immunoblotted with either rabbit antiserum to strain D490 (a and b) or MAb 1E5 (c), as described in Materials and Methods. The clonal populations represented in lanes 1 to 3 correspond to the variants in Fig. 2, lane 12, and Fig. 3, lanes 1 and 3, respectively. Samples contained proteins from approximately 10^6 (a, lane 3), 3 \times 10^6 (a, lanes 1 and 2; and c), or 6 \times 10^6 (b) CFU. Positions and molecular masses (in kilodaltons) of related size-variant amphiphilic membrane proteins identified by both reagents are indicated in each panel. A 46-kDa variant antigen which is exclusively recognized by the MAb is indicated in panel c. The internal p41 antigen which selectively partitioned into the aqueous phase (not shown) and is detected by the antiserum is indicated in panel a; in lane 3, the p41 band is weak because of the limited quantity represented. (d) Intact broth-grown cells (approximately 5 \times 10^6 CFU per sample) from the clonal variant expressing a 77-kDa size-variant membrane protein antigen (a to c, lanes 1) were incubated with different concentrations of trypsin and subjected to SDS-PAGE, as described in Materials and Methods. The subsequent immunoblot was stained with MAb 1E5. Lane 1 contains organisms incubated in enzyme buffer; lanes 2 and 3 contain organisms treated with 5 or 200 μg of trypsin per ml, respectively. Positions of the intact size-variant antigen and its 43-kDa digestion product identified by the MAb are indicated. (e) Immunostaining of a corresponding blot (as in panel d) with MAb 5D8 revealed that the internal p41 antigen was unaffected by external trypsin treatment. In a separate experiment, the TX-114 phase-fractionated p41 was shown to be sensitive to similar amounts of trypsin (not shown). The position of p41 is indicated. (f and g) Imprints of colonies representing the progeny of the clonal variant shown in lanes 3 of panels a to c were transferred to nitrocellulose and immunostained with MAb 5D8 (f) or 1E5 (g) as described in the legend to Fig. 3. Representative portions of these colony blots are shown. Bar, 0.25 mm.
bovis-induced mastitis antigens. Membrane proteins recognized by expression, (ii) mastitis and arthritis (b) or with serum from a noninfected healthy animal (c). Each lane represents proteins from approximately $3 \times 10^6$ CFU. The size of each variant antigen is indicated in kilodaltons.

Selective recognition of the MAb-defined size-variant surface antigens of *M. bovis*-infected cows. Total protein from clonal variants of *M. bovis* PG45 (corresponding exactly to those in Fig. 4) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose as described in the legends to Fig. 4a and c. Identical filter blots were separately probed with bovine sera from clinical cases of *M. bovis* mastitis (a) or arthritis (b) or with serum from a noninfected healthy animal (c). Each lane represents proteins from approximately $3 \times 10^6$ CFU. The size of each variant antigen is indicated in kilodaltons.

**Selective binding of size-variant membrane surface proteins by serum antibodies from *M. bovis*-infected cattle.** To determine whether the variable membrane surface proteins recognized by MAb 1E5 were immunogenic in the natural bovine host, immunoblots of whole organisms from clonal variants expressing the same set of size-variant antigens as in Fig. 4 were stained with sera from two individual cows with *M. bovis*-induced mastitis or arthritis. As can be seen in Fig. 5a and b, the two serum samples showed strong and specific staining of the size-variant surface proteins, whereas serum from a noninfected healthy animal showed no reaction (Fig. 5c). Western immunoblot analysis of several other serum specimens from *M. bovis*-infected cattle confirmed these data. All animals developed strong preferential antibody reactions to these proteins (not shown). These results demonstrated that the variable membrane surface proteins defined by MAb 1E5 are major immunogens of *M. bovis* recognized during infection and disease.

**Distribution of size-variant membrane surface proteins among strains and clinical isolates.** The MAb developed here provided a tool to more specifically address the antigen heterogeneity among strains and clinical isolates initially observed with the PAb reagent noted above (Fig. 1b). Using this MAb in Western immunoblots, we were able to identify striking differences in the distribution and size of specific antigenically related surface proteins among all 11 isolates analyzed (Fig. 6). While in nine isolates one or two different size-variant forms of these antigens were identified, no corresponding antigen was detected in either strain D490 (Fig. 6, lane 2) or strain 422 (Fig. 6, lane 10). These differences were found to be consistent after repeated broth passages. Only a few of these size-variant and strain-variant proteins recognized by MAb 1E5 were also stained by the rabbit hyperimmune serum (Fig. 1b and 6, lanes 1, 4, 5, 7, 8, and 11), indicating that the PAb recognized additional epitopes on these proteins distinct from that detected by the MAb. Collectively, these findings strongly suggest that the set of MAb-defined membrane proteins identified here includes multiple size forms of related but distinct surface antigens which are either absent or present at different levels in particular isolates.

**DISCUSSION**

The overall evidence obtained in this study supports the recently developed concept that many mycoplasma species represent highly dynamic populations (24, 38). The data presented here clearly demonstrate that the bovine pathogen *M. bovis* is among those mycoplasma species which have the heritable capacity for rapidly generating extreme phenotypic diversity. This completely contradicts the definition proposed by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (14), according to which a mycoplasma species represents a cluster of isolates which exhibit a high degree of phenotypic relatedness. The first indirect evidence of phenotypic variation among *M. bovis* strains stems from the fact that this organism has been isolated from various tissues of both clinically healthy cattle and cattle with clinically diverse diseases (16, 22), indicating possible strain differences in virulence and in host tissue tropism. More substantial evidence on intraspecies strain variation emerged from a more recent study involving the
comparison of protein profiles of *M. bovis* strains and their antigen patterns obtained with rabbit and calf hyperimmune sera (26). In this earlier study, it was shown that amounts of individual proteins and antigens may differ among *M. bovis* strains. The present study describes these initially reported antigenic differences more precisely and extends our understanding of the structural basis of the observed heterogeneity.

By using a rabbit hyperimmune serum which was prepared with whole cells of the field strain D490, we were able to detect dramatic differences in the antigen expression patterns between the type strain PG45 and 10 other strains and field isolates which were much more striking (Fig. 1b) than those recently reported (26). That these various isolates were indeed characteristic of the species *M. bovis* was confirmed by the extensive degree of similarity among their overall protein profiles (Fig. 1a). Though further analysis is required, the results suggest that there is as much antigen heterogeneity among isolates from one geographical region as there is among isolates from different areas (Fig. 1b). Furthermore, the antigenic differences could not be correlated with either the site of isolation or the clinical condition (Fig. 1b). In addition, the antigen banding pattern obtained with the PAb reagent was shown to vary not only among *M. bovis* strains and field isolates but also among clones and subclones (Fig. 2) and within clonal lineages (Fig. 3) of individual strains. Since the rate of this variation of \(2 \times 10^{-3}\) per cell per generation is too high to be explained by random mutational events, it is very unlikely that the antigen heterogeneity among strains represents stable phenotypic strain differences due to antigenic shifts over the years; rather, it reflects distinct expression patterns of highly variable antigens, as has been reported for other mycoplasma species (24, 25, 33-35, 37, 38). Moreover, since antigenic differences were also observed among serial isolates from the same animal (Fig. 1b), we can conclude that antigenic variation of *M. bovis* may also occur in vivo, perhaps in response to the immune system, a hypothesis which was similarly proposed for *Mycoplasma hominis* (19) and *Mycoplasma pulmonis* (27) but which needs further investigation. TX-114 phase partitioning of *M. bovis* proteins utilized to identify these antigens with hydrophobic domains demonstrated that all variable antigens of *M. bovis* detected by the PAb have a sufficient degree of hydrophobicity to be provisionally defined as integral membrane proteins. In this regard, the strain-variant antigens of *M. bovis* resemble several recently described variable antigens of other mycoplasma species (3, 8, 20, 24, 25, 37).

The MAb developed in this study, 1E5, recognizes one subset of antigens within the set of variable membrane protein antigens identified by the PAb. The use of this MAb in trypsin digestion experiments and in colony immunoblots has demonstrated that these antigens (i) to be surface-exposed components, (ii) to be clearly associated with proteins, and (iii) to bear a surface epitope which undergoes high-frequency variation in expression. Although similar techniques have demonstrated the surface localization and variability of other mycoplasma membrane proteins (24, 25, 32, 37), the colony immunoblot technique as a tool for easily distinguishing between surface-exposed and non-surface-exposed antigens has not been formally established. The analysis described in this report, however, represents the first comparison of such external and internal antigens using this technique and demonstrates its usefulness as a convenient tool to differentiate between them (Fig. 4f and g). Moreover, the results reported here demonstrate for the first time that PABs raised against whole broth-grown organisms from individual strains can also serve as useful probes for the general detection of mycoplasma surface epitope switching in colony immunoblots (Fig. 3b), while in previous reports this phenomenon was detected only by specific MAbs (24, 25), not by PABs (24).

The PAB and MAB reagents generated here were used to further characterize the surface antigenic variation of *M. bovis*. In the present study, PAB and MAB immunoprofiles of serial subclones of the reference strain PG45 indicated that there are at least two antigenically related but distinct products which are involved in this surface variability, one of which was shown to occur in discrete size-variant forms (Fig. 4c). In an accompanying paper (5), we demonstrate that several features regarding the variation and variability of these proteins are quite similar to the variable lipoproteins (Vlps) of *Mycoplasma hyorhinis* (24, 25, 39). The results presented here also demonstrate the immunogenic nature of these molecules during natural infection and disease caused by *M. bovis*. Moreover, since they appear to be the dominant targets of the bovine immune response to this organism and are readily detected by cognate antibodies (Fig. 5), they may have diagnostic as well as immunoprophylactic value. Our data are therefore also encouraging toward (i) developing tests based on these proteins for improving the diagnosis of *M. bovis* infections and (ii) testing their potential use as vaccinogens to prevent or to manage disease caused by this agent.

Taken together, our results have shown that the antigen heterogeneity among strains and isolates of *M. bovis* reflects distinct expression patterns of diverse, highly variable membrane surface proteins. Detailed structural and functional analyses of these antigens with emphasis on their possible role as ligands of adhesion to host cells and on their interaction with the bovine immune system during experimental infection will be necessary to gain insight into how *M. bovis* causes disease and may lead to the development of a vaccine to control it. The results presented here and in an accompanying paper (5) provide the necessary basis for further studies in this direction.

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REFERENCES


