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Variant Colony Surface Antigenic Phenotypes within Mycoplasma Strain Populations: Implications for Species Identification and Strain Standardization

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Immunobinding assays with mycoplasma colonies on agar plates (immunofluorescence and immunoperoxidase techniques) or with imprints of colonies transferred to solid supports (colony immunoblotting) are widely used as standard diagnostic tests for serological species identification of mycoplasma isolates. However, in light of the high rate of variability of surface antigens in many mycoplasmas, diagnostic data obtained with these techniques require a more critical evaluation. In this report, we demonstrate with some examples that mycoplasma surface variability based on alterations in expression, in size, and in surface presentation of integral and peripheral membrane proteins may lead to misinterpretation of colony immunostaining reactions obtained by using specific monoclonal antibodies as well as conventional diagnostic hyperimmune sera. To more easily identify phenotypically mixed isolates or samples which contain more than one species, we have introduced some minor modifications of the colony immunoblot technique which provide sharp signals of positive as well as negative reactions and enable identification of cryptic epitopes. It is further demonstrated that because of the variability in colony surface antigenic phenotype, mycoplasma strains, including well-established reference and other prototype strains which are used under the same designation in many laboratories, can differ markedly in their antigen profiles and their potentially virulence-related surface properties, since they are usually purified by filter cloning and often propagated by subcultivation of randomly selected agar-grown subpopulations. We conclude from this study that because of this surface variability, the establishment of criteria for standardization of mycoplasma strains and diagnostic antisera is urgently required in order to obtain reproducible results in different laboratories.

Despite new molecular approaches (22), one of the most commonly used techniques for routine species identification of mycoplasmas isolated from clinical material is at present still the immunobinding assay performed with mycoplasma colonies on agar plates (3, 6, 9, 13, 14, 23) or more frequently with colony blots, i.e., imprints of colonies transferred to nitrocellulose (NC) (7, 18). Several modifications of this technique, including various immunofluorescent (3, 6, 9, 13, 23) and immunostaining (3, 7, 14, 18, 23) procedures with polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs) have been described. All of these assay systems are based on the detection of mycoplasma surface antigens and are believed to be highly specific and sensitive. The result indicating the presence of one, two, or even more than two species is regarded as showing a single or mixed mycoplasma infection, a diagnosis which can have profound clinical implications.

However, the previous reports cited above did not take into account that species identification may be complicated by a peculiar, perhaps ubiquitous property of mycoplasmas, namely, their capability to undergo high-frequency surface antigenic variation. Indeed, from studies in the last few years (1, 2, 10, 21, 22, 26–29, 32, 35–37, 39, 40, 41) it has become apparent that many mycoplasma species possess membrane surface protein antigens that vary in their expression (phase variation), in their structural features (size variation), and in the surface accessibility of their epitopes (epitope masking). Nevertheless, so far it has generally been anticipated—and this has also been documented in one earlier report on the swine pathogen *Mycoplasma hyorhinis* (28)—that unlike the heterogeneous reaction pattern of mycoplasma colonies obtained with MAbs to such variant surface antigens, the reaction obtained with PAbs (rabbit or mouse antisera against mycoplasma reference or prototype strains) is uniform both among strains and within subcloned populations of a given mycoplasma species. However, in a more recent study of the bovine pathogen *M. bovis* (26) we observed that in contrast to the generally accepted view, PAb reagents can indeed reveal clearly heterogeneous colony immunostaining patterns which from a diagnostic point of view may be quite ambiguous and therefore difficult to interpret.

The present study expands these very preliminary observations and further demonstrates that the variability in surface antigenic phenotype of mycoplasma colonies based on variation in expression, in size, and in presentation of surface antigens can in fact have a confounding effect on the interpretation of results obtained by using colony immunostaining techniques for diagnostic purposes. In addition, evidence that a positive result in a diagnostic colony immunoblot assay, i.e., the unambiguous identification of a suspect isolate, strongly depends on the origin or source of the immunological reagent used is presented. Moreover, to improve the sensitivity and reliability of the colony immunoblot technique as a diagnostic procedure, some modifications of the classical assay system (7, 18, 26, 28, 29) which (i) facilitate the identification of nonreacting colonies, (ii) uncover cryptic epitopes, and (iii) consequently allow for an easier and more objective interpretation of diagnostic data are described. Finally, a very real concern is discussed, namely, the fact that despite increasing published information

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on mycoplasma surface antigenic variability, many laboratories routinely purify well-established mycoplasma strains upon acquisition, without considering the possibility that subpopulations that are phenotypically different from the predominant population of the original stock culture may be selected.

MATERIALS AND METHODS

Mycoplasmas and culture conditions. The source and properties of *M. gallisepticum* R and PG31 (type strain) from our Jerusalem laboratory collection are described elsewhere (38). The Jerusalem PG31 strain was designated PG31b to distinguish it from the same strain (PG31a) in the collection of the Institute for Microbiology and Infectious Diseases of Animals, School of Veterinary Medicine, Hannover, Germany, whose antigen pattern was recently shown (39). *M. gallisepticum* A5969, originally described by Jungherr and Luginbuhl (17), was from the following two sources: strain A5969a was obtained from K.-H. Hinz, Clinic of Avian Diseases, School of Veterinary Medicine, Hannover, Germany, and strain A5969b was from our Jerusalem collection. *M. imitans* 4229 (type strain) and 30902, as well as *M. iowae* 695 (type strain), were obtained from J. M. Bradbury, University of Liverpool, Liverpool, United Kingdom. Sources for *M. bovis* PG45 (type strain) included the following: strain PG45a was from the collection of the Institute for Microbiology and Infectious Diseases of Animals, School of Veterinary Medicine, Hannover, Germany (its antigenic surface variability was recently described [24]); strain PG45b was obtained from H. Pfützner, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Jena, Germany (its protein and antigen profile was previously described by Sachse et al. [30]); strain PG45c was from the mycoplasma reference strain collection of the University of Aarhus, Aarhus, Denmark, and obtained from S. Levisohn, Kimron Veterinary Institute, Bet Dagan, Israel; and strain PG45d was from our Jerusalem laboratory collection. *M. fermentans* K7 was obtained from C. Bébéar, University of Bordeaux, Bordeaux, France. All mycoplasma strains were grown in broth culture; the avian strains were grown in a modified Edward medium (25), supplemented with 10% (vol/vol) horse serum, and all other strains were grown in a standard mycoplasma medium, supplemented with 20% (vol/vol) horse serum (26). Stocks of each strain were prepared from mid-logarithmic-phase cultures and stored at −80°C.

Subcloning. Subclones of mycoplasma strains were obtained and screened for variant colony surface antigenic phenotypes as described previously (26, 28, 29). Briefly, fresh broth-grown organisms from primary passages of stocks were diluted serially in broth medium and plated on solid medium containing 1% (wt/vol) agar. Plates were incubated at 37°C in a moist atmosphere with 5% (vol/vol) CO_2 for 5 to 6 days. Colonies which differed in their surface antigenic phenotypes were accurately located on agar plates by aligning them with corresponding colored or colorless (white) dots on NC membrane discs (see below). Well-separated colonies exhibiting the selected surface phenotype were isolated with Pasteur pipettes and propagated at 37°C in 1 ml of broth medium for 24 to 96 h. To confirm the selected colony phenotype, these cultures were subsequently plated and the resulting colonies were rescreened by immunostaining as described below. In some experiments, cultures representing clonal isolates, which have been selected for their distinct levels of immunostaining intensity (see below), were plated as mixtures to ensure that the various colony surface phenotypes seen on different agar plates were not artifacts related to minimal differences in growth conditions or to the colony blotting and immunostaining procedure.

Antibodies. The preparation and characteristics of MAb 1E5 have been described recently (26). It is an immunoglobulin (Ig) M isotype that recognizes a surface-exposed or cryptic epitope which is shared by at least seven integral or peripheral membrane proteins of four distinct *Mycoplasma* species: VspA, VspB, and VspC of *M. bovis* (1); PvpA and pMG41 of *M. gallisepticum* (27, 39); pMI41 of *M. imitans* (27); and pMIO41 of *M. iowae* (27). MAb 12D8 has been developed and described by Garcia et al. (11) and was kindly provided by S. H. Kleven, University of Georgia, Athens. It is an IgG1 MAb that binds to a highly immunogenic phase-variable *M. gallisepticum* membrane surface lipoprotein showing size polymorphism among strains (20, 27, 39). Five rabbit hyperimmune antisera (PAbs) which are used in diagnostics were included in this study. The preparation and properties of PAb anti-D490 directed against *M. bovis* D490 (26) and of PAb anti-PG31a directed against *M. gallisepticum* type strain PG31a (39) have been recently described; PAbs anti-PG18 against *M. fermentans* type strain PG18, anti-695 against *M. iowae* type strain 695, and anti-PPAV against *M. iowae* PPAV were obtained from J. G. Tully, National Institute of Allergy and Infectious Diseases, Frederick, Md. (the last two were provided by S. Levisohn). For colony and Western blot (immunoblot) immunostaining, antisera and hybridoma culture supernatants containing antibodies were diluted 1:50 (MAb 1E5), 1:250 (MAb 12D8), or 1:1,000 (PAbs) in phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.0 mM NaH_2PO_4, pH 7.2) containing 10% (vol/vol) fetal calf serum.

Colony immunoblotting. Variant colony surface antigenic phenotypes were detected by colony immunoblotting by using a procedure previously described (26, 28, 29), with slight modifications. Briefly, NC membrane discs (diameter, 82 mm; pore size, 0.45 μm; Bio-Rad, Richmond, Calif.) were placed on freshly grown mycoplasma colonies on the surfaces of agar plates. After 5 min, they were gently removed from the agar surface with blunt-ended forceps and placed with the colony side up in petri dishes containing blocking buffer (TS buffer [10 mM Tris, 150 mM NaCl, pH 7.4] supplemented with 3% [wt/vol] bovine serum albumin [Sigma, St. Louis, Mo.]). After being blocked for 1 h at room temperature, the NC discs were briefly washed (three times for 5 min each time) in TS buffer and then incubated with the primary antibody (MAb 1E5 or PAbs) overnight at 4°C. Unbound antibody was removed by three washes (5 min each) with TS buffer. The NC discs were then incubated for a minimum of 2 h at room temperature with peroxidase-conjugated secondary antibody (goat) against mouse IgM or rabbit Igs (Jackson Immuno Research Laboratories, West Grove, Pa.), each diluted 1:1,000 in PBS-fetal calf serum. After three washes (5 min each) in TS buffer, the colony blots were developed for 5 to 20 min with substrate solution containing either *o*-dianisidine or 4-chloro-1-naphthol as the chromogen. The reaction was stopped by washing blots in two changes of deionized water. In all incubation and washing steps, the colony blots were gently agitated on a rocking platform. Colonies that reacted with the MAb or PAb were stained either brown (*o*-dianisidine) or blue (4-chloro-1-naphthol).

Three modifications of the above-described colony immunoblot technique were used to allow MAb binding to cryptic epitopes: (i) in modification I, the washing period after blocking was extended to more than 12 h (at room temperature or at 4°C) with several additional changes of TS buffer; (ii) in modification II, the colony blots were washed three times in TS buffer containing 0.1% (vol/vol) Tween 20 (Bio-Rad) after blocking; (iii) in modification III, the lifted colonies were gently pressed onto the NC membrane with blunt-ended forceps during or immediately after the discs were peeled from the agar surface.

Examination of colony immunoblots. Differences in colony surface antigenic phenotype within mycoplasma strains and clonal populations were identified by using a stereo zoom dissecting microscope (Wild Heerbrugg, Heerbrugg, Switzerland) and oblique lighting. Immunostained colony imprints were examined on (i) completely dry, (ii) nearly dry, or (iii) freshly wetted (with deionized water) NC membrane discs.

Hemadsorption assay. Colonies of *M. gallisepticum* A5969a and A5969b were screened for their cytadherence properties by using a modification of the classical hemadsorption assay (12). To avoid artifacts due to removal of surface material through washing, colonies were first blotted on NC discs and then the remaining colonies on the plates were overlaid with a 0.2% (vol/vol) suspension of chicken or human erythrocytes in PBS and incubated for 30 min at 37°C. After two gentle washes with PBS, the colonies were examined microscopically (see above) for hemadsorption.

SDS-PAGE and Western immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for analysis of variant colony surface antigenic phenotypes was performed by the method of Laemmli (19) as reported previously (1, 26) by using whole organism samples and 9% polyacrylamide gels containing 3% (wt/vol) urea. All samples for SDS-PAGE were treated at 100°C for 5 min under reducing conditions. Separated proteins were electrophoretically transferred to NC membranes (0.45-μm pore size; Bio-Rad) and immunostained as described above for colony blots with 4-chloro-1-naphthol as the chromogenic substrate. Relative molecular masses were determined with prestained protein standards (low range; Bio-Rad) run simultaneously and transferred directly onto NC membranes.

RESULTS

Improved detection of phenotypically negative, nonreactive mycoplasma colonies. Figure 1 shows individual progeny colonies of a clonal population of *M. imitans* 30902 which has been selected for its negative phenotype by immunostaining with MAb 1E5 after demasking of the corresponding epitope (see below). The heterogeneous immunostaining pattern of this progeny population is similar to that we have recently described (27). Most of the colonies displayed the selected negative, i.e., "off," phenotype (Fig. 1, arrows) with respect to the expression state of the corresponding epitope-bearing peripheral membrane protein pMI41 (27); however, colonies displaying the phase-switched positive, i.e., "on," phenotype occurred at a high frequency. In addition, some colonies exhibiting a sectored pattern of immunostaining (Fig. 1, arrowheads) were seen. While negative and sectored colonies were barely identifiable on completely dry blots (Fig. 1A), they became clearly visible as white dots on a dark background during the final stage of drying (panel B). Alternatively, examination of dry colony blots immediately after wetting with deionized water similarly enabled detection of the negative colony phenotype at a remarkably high resolution (Fig. 1C; see also Fig. 2C and D and Fig. 3A); because of swelling processes, all

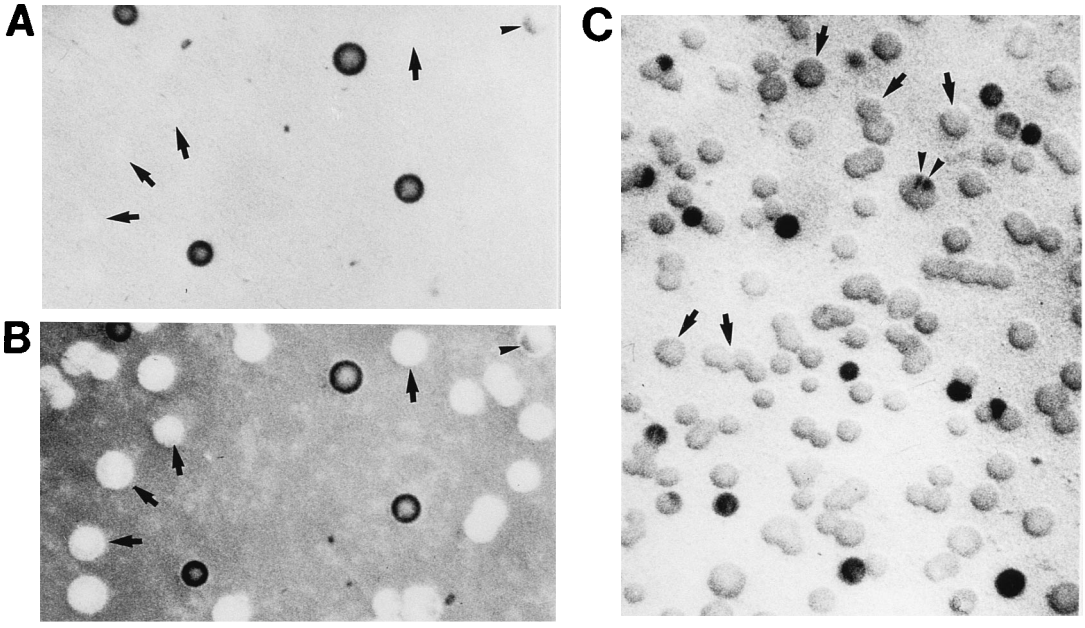

FIG. 1. Colony blots of a clonal population of *M. imitans* 30902 immunostained with MAb 1E5, demonstrating at different resolutions the variable expression of the peripheral membrane protein pMI41 among and within colonies. To obtain MAb binding to pMI41, blots were extensively washed with TS buffer after blocking, as described in Materials and Methods (modification I). (A and B) Phenotypically positive ("on") and negative ("off") colonies viewed on completely dry (A) and nearly dry (B) NC membranes. The same portion of a colony blot immunostained with MAb 1E5 is shown in both panels. While in panel A nonreactive "off" colonies are at the limit of visual detection, they are easily detectable in panel B as white dots on a dark background. Four such "off" variants are indicated by arrows, and one sectored colony is indicated by an arrowhead. (C) Phenotypically different (reactive and nonreactive) colonies visualized in three-dimensional form after rinsing of the dry blot with deionized water. Some nonreactive "off" colonies are indicated by arrows, and phenotypically positive backswitch sectors within one phenotypically negative colony are indicated by arrowheads.

colonies, i.e., both phenotypically positive and negative colonies, were hemispherical and thus were readily detected on the NC membrane surface.

Variation in immunoaccessibility of epitopes on surface-exposed mycoplasma proteins. Several possibilities may account for the differences in colony surface antigenic phenotype among and within mycoplasma strain populations, as detected in colony immunoblots. One possibility is variation in expression of surface-associated proteins. Figure 2B shows individual colonies of *M. gallisepticum* R immunoblotted with MAb 1E5, which recognizes the recently described (39) phase-variable integral membrane surface protein PvpA. Among the predominantly reactive, i.e., phenotypically positive, colonies, numerous nonreactive, phenotypically negative colonies (Fig. 2B, arrows) were present. In a previous study (39), we have shown by Western immunoblot analysis of selected individual colony variants of strain R that the positive and negative colony surface phenotypes correlated precisely with the "on" and "off" expression states of PvpA, respectively.

Another phenomenon that may explain the variability in colony surface antigenic phenotype is variation in immunoaccessibility of epitopes. When *M. gallisepticum* A5969b was plated and the resulting colonies were immunoblotted with MAb 1E5, more than 99.9% of the population exhibited the negative colony surface phenotype (Fig. 2C). However, comparative Western immunoblot analysis of equal amounts of stock cultures of strains A5969b (Fig. 2A, lane 2) and R (lane 1) revealed similar relative immunostaining intensities of PvpA, indicating no apparent differences in the relative amounts of PvpA associated with the broth-cultured organisms analyzed. This was confirmed with multiple preparations of both strains and is in contrast to our findings in a previous study (39), where strain A5969b showed only a very weak PvpA band, probably because of an underrepresentation of proteins in the reported experiment. The only striking difference between the two strains seen in Western immunoblots—and this is consistent with our previous data (39)—was the size of the expressed PvpA; while strain R exhibited a 55-kDa form of PvpA (Fig. 2A, lane 1), strain A5969b showed expression of a 46-kDa PvpA size variant (lane 2). In contrast to the situation with *M. gallisepticum* A5969b, which was shown to contain at least a minor subpopulation in which the PvpA-specific epitope recognized by MAb 1E5 was surface exposed (Fig. 2C), in *M. iowae* 695, which expresses the same epitope on a 41-kDa peripheral membrane protein (pMIO41; Fig. 2A, lane 3), no clonal variant which showed surface accessibility of that epitope was found (Fig. 2D). These results establish that epitopes of integral or peripheral mycoplasma membrane proteins are either exposed or masked at the surface and may also vary between these two states within strain populations.

Visualization of cryptic epitopes. To further demonstrate that in *M. gallisepticum* A5969b the PvpA-specific epitope was indeed present in the majority of the nonreactive colonies (Fig. 2C and 3A), colony blots were treated with TS buffer containing 0.1% Tween 20 as described in Materials and Methods. The result is shown in one example in Fig. 3B. Most of the colonies showing the negative surface phenotype (indicated by m [for masked] in Fig. 3) with the normal immunostaining procedure (MAb 1E5) turned positive (indicated by d [for demasked]) when they were restained with the MAb after detergent treatment, while only a small fraction of colonies remained unstained ("off" phenotype; data not shown). Likewise, expression of the corresponding pMI41 epitope on colony populations of *M. imitans* type strain 4229 was readily detected when colony blots were incubated in the detergent-buffer solution prior to immunostaining (Fig. 3C, right por-

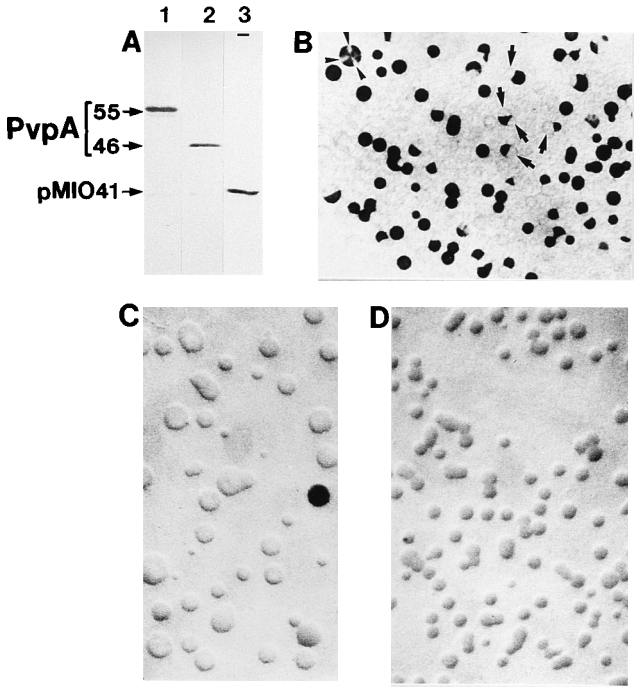

FIG. 2. Differences in the immunoaccessibility of epitopes on surface-associated proteins of *M. gallisepticum* and *M. iowae*. (A) Western immunoblot analysis of total proteins from broth-grown whole organisms of *M. gallisepticum* R (lane 1), *M. gallisepticum* A5969b (lane 2), and *M. iowae* 695 (lane 3) with MAb 1E5, demonstrating no apparent differences in the relative amounts of the specific antigens recognized. Each lane represents proteins from equal aliquots (400 μl) of mid-logarithmic-phase cultures. Arrows at left indicate two size variants (numbers indicate kilodaltons) of the membrane protein PvpA of *M. gallisepticum* and the PvpA-related pMIO41 of *M. iowae*. (B, C, and D) Immunoblots of colonies of *M. gallisepticum* R (B), *M. gallisepticum* A5969b (C), and *M. iowae* 695 (D) with MAb 1E5, showing marked differences in the surface accessibility of the MAb-defined epitope on PvpA55 (B), PvpA46 (C), and pMIO41 (D). (B) Colonies on a completely dry NC membrane disc; (C and D) colonies on NC membrane discs immediately after wetting with deionized water. Some nonreactive colonies in panel B are indicated by arrows, and nonreactive sectors within one phenotypically positive colony are indicated by arrowheads.

tion), while it remained clearly undetected in untreated blots (Fig. 3C, left portion). Uncovering of the MAb 1E5-defined cryptic epitope of PvpA and pMI41 was also achieved by extended washing periods with multiple changes of buffer (Fig. 1) or by pressing the lifted colonies onto the NC membrane (Fig. 3D). However, all of these procedures failed to uncover the corresponding epitope of the peripheral membrane protein pMIO41 in *M. iowae* (Fig. 2D and data not shown). Thus, results obtained with the colony blot assay system depend largely on the conditions applied and appear to vary from one organism to another.

Variation in the level of colony immunostaining intensity within mycoplasma strain populations and correlation with size variation of the corresponding antibody-binding protein. To more precisely define the extent and nature of the surface epitope masking phenomenon occurring with *M. gallisepticum* A5969b (Fig. 2C, 3A, and 3B), the PvpA expression patterns of colony variants selected for their positive surface phenotype (i.e., with the MAb-defined epitope displayed in its surface-exposed unmasked form, as shown in one example in Fig. 2C) were monitored in Western blots. Surprisingly, all such positive variants screened to date expressed either a 55- or a 35-kDa size version of PvpA to similar levels (Fig. 4A, lanes 1 and 2); they did not, however, express the 46-kDa size form characteristic of the predominant, phenotypically negative colony population (Fig. 2A, lane 2, and data not shown). Replating of these variants and subsequent immunostaining of progeny colonies with MAb 1E5 revealed two reliably distinguishable degrees of colony immunostaining intensity, which were difficult to detect when the single positive variants were monitored within the colony population of the strain stock culture. Interestingly, while variants expressing the larger (55-kDa) version of PvpA showed an intermediate level of colony immunostaining (Fig. 4B), those expressing the smaller (35-kDa) version showed a strong immunostaining intensity (Fig. 4C), indicating differences in MAb reactivity, i.e., in the accessibility of the epitope recognized. The presence of strong versus intermediate MAb-binding phenotypes was confirmed by colony immunoblots of cloned populations plated as mixed cultures (Fig. 4D). These results demonstrate that size variation of surface-associated mycoplasma membrane proteins can profoundly affect the exposure of specific regions of these proteins and therefore their immunoaccessibility in their native state on the surface of intact mycoplasma cells grown on agar plates.

Heterogeneous or uniform colony immunostaining patterns of mycoplasma strains obtained with diagnostic hyperimmune antisera. It is still widely assumed in routine diagnostic laboratories that conventional polyclonal antibodies (i.e., rabbit or mouse antisera raised against whole organisms from broth-grown cultures of well-defined mycoplasma strains) are broadly reacting species-specific reagents which can reliably be used for mycoplasma species identification in colony immunobinding assays (3, 6, 9, 13, 14, 23). A recent study, however, has identified marked heterogeneity in immunostaining intensity among and within colonies of subcloned *M. bovis* populations by using such a conventional PAb (26). The present study confirms that careful interpretation of diagnostic data obtained with the colony blot assay system based on diagnostic hyperimmune antisera is in fact needed. Some of the difficulties encountered are illustrated in Fig. 5A to C, which show colony blots of stock populations of *M. bovis* PG45a (Fig. 5A), *M. fermentans* K (Fig. 5B), and *M. iowae* 695 (Fig. 5C), which were immunostained with rabbit antisera anti-*M. bovis* D490, anti-*M. fermentans* PG18, and anti-*M. iowae* 695, respectively. Each of the three diagnostic antisera revealed two colony subpopulations, namely, a major subpopulation that stained only weakly (Fig. 5, arrows) and a minor subpopulation that showed a strong level of reactivity. Some of the weakly as well as the strongly stained colonies in all three strain populations displayed a sectored pattern of immunostaining (Fig. 5, s; strongly stained sectors within weakly stained colonies are indicated by small arrows in Fig. 5A and C; weakly stained sectors within strongly stained colonies are not shown), indicating that the weakly stained colonies resulted from phase variation and do not represent contaminating populations of other cross-reacting mycoplasma species.

That the sectored appearance of colonies is indeed consistent with an "on-off" switching mechanism for the immunodominant antigens during colony growth and thus unambiguously identifies the lightly stained colonies as phase switchers and not as contaminants is further supported by the continued staining heterogeneity, as well as the continued presence of sectoring in cloned subpopulations. Examples of such clonal populations, derived from individual colonies of the original stock cultures of *M. bovis* PG45a (Fig. 5A), *M. fermentans* K (Fig. 5B), or *M. iowae* 695 (Fig. 5C) and screened with the corresponding antiserum, anti-*M. bovis* D490, anti-*M. fermentans* PG18, or anti-*M. iowae* 695, are shown in Fig. 6A, B, and C, respectively. Each of the three progeny populations clearly

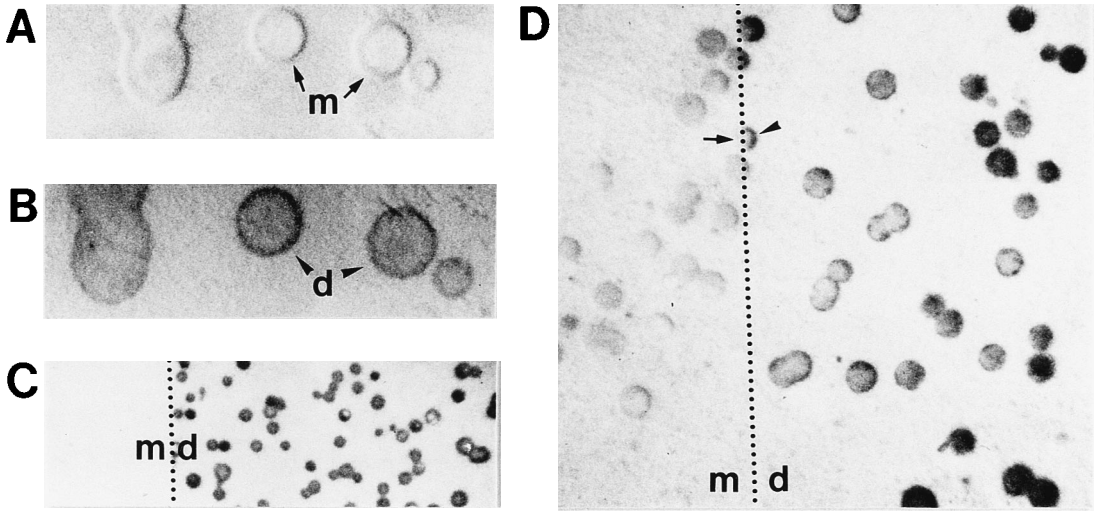

FIG. 3. Demasking of cryptic epitopes on surface-associated proteins of *M. gallisepticum* and *M. imitans*. (A and B) Colony blot of *M. gallisepticum* A5969b immunostained with MAb 1E5 specific to PvpA. The same portion of the colony immunoblot is shown in both panels. In all colonies of this area, the epitope was masked (m) (indicated by arrows) at the surface (A) but could be demasked (d) (indicated by arrowheads) by treatment with TS buffer containing 0.1% (vol/vol) Tween 20, as described in Materials and Methods (modification II) (B). (C and D) Colony blots of *M. imitans* 4229 immunostained with MAb 1E5, which recognizes pMI41. While in the untreated left portions of the blots the corresponding MAb-defined epitope was masked (m), it became accessible to MAb 1E5 (d) in the other (right) portions of the blots either by treating the lifted colonies with TS buffer containing 0.1% (vol/vol) Tween 20 prior to immunostaining (modification II) (C) or by slightly pressing them onto the NC membrane (modification III) (D). The two portions of the blots are indicated by a dotted line. (D) The line is dividing two colonies into halves. While the left halves of these colonies were not accessible to MAb 1E5, i.e., they show a negative surface phenotype (indicated in one colony by an arrow), the right halves show strong immunostaining (arrowhead). (A and D) Colonies are shown after wetting of the dry immunoblots with deionized water; (B and C) immunostained colonies are shown on completely dry NC membrane discs.

showed a mixed phenotype consisting of strongly stained, lightly stained, and sectored (Fig. 6, arrows) colonies.

Species identification with the colony immunobinding assay is further confounded by the fact that the reaction pattern is very much dependent on the antiserum used. For example, while the antiserum against *M. iowae* 695 revealed a marked phenotypic heterogeneity in the propagated stock population of the homologous strain 695 (Fig. 5C), the corresponding antiserum to strain PPAV revealed no detectable differences, i.e., all colonies of the strain 695 stock culture showed the same strong level of reactivity and thus could be easily identified as *M. iowae* (Fig. 5D).

These observations demonstrate that the interpretation of colony immunoblot results can be greatly complicated by the complexity of surface antigenic variation in some mycoplasma species and may require some expertise to distinguish between antigenic variants and truly mixed (or contaminating) organisms, even when conventional hyperimmune antisera are used.

Differences in surface properties and antigenic makeup of prototype mycoplasma strains classified as the same strains in different laboratories. Increasing numbers of reports during recent years have demonstrated that a high rate of variability of surface antigens is quite common among the mycoplasmas (1, 4, 10, 21, 22, 26–29, 32, 34–37, 39–41). In this report, we try to provide the message that because of this high-frequency phenotypic variability, any further round of purification by filter cloning or by subcultivation of randomly selected agar-grown subpopulations is ill-advised once a mycoplasma isolate has been given a strain designation. That quite different subpopulations of mycoplasma strains classified as the same have in fact been enriched over the years in different laboratories is demonstrated in Fig. 7.

In one example (Fig. 7A and D), the Hannover A5969 strain of *M. gallisepticum* (A5969a, Fig. 7A and D) is shown to differ in some surface attributes from the corresponding Jerusalem strain (A5969b, Fig. 7B and C). These differences are illustrated as follows. First, although both strain populations express the integral membrane surface protein PvpA to a similar degree and predominantly as the 46-kDa version (Fig. 2A, lane 2, and data not shown), its specific epitope recognized by MAb 1E5 was differentially exposed. While in the Hannover strain population this epitope was immunoaccessible on the organism's surface (Fig. 7A), in the Jerusalem strain population it was predominantly masked (see above) (Fig. 7B; also Fig. 2C). Second, another clear difference between the surface characteristics of the two A5969 strain versions was demonstrated with the hemadsorption assay with chicken (Fig. 7C and D) or human (data not shown) erythrocytes. While the colonies of the Jerusalem strain were consistently shown to be hemadsorption negative (Fig. 7C), those of the Hannover strain were hemadsorption positive (Fig. 7D).

For other examples (Fig. 7E to H), we have employed the Western immunoblot technique, which was recently shown by Washburn et al. (34) to be a valuable approach to assess the relationship among strains. Using this approach, we were able to demonstrate that two reference mycoplasma strains (*M. gallisepticum* type strain PG31 and *M. bovis* type strain PG45) obtained from different sources show marked differences in the distribution and structural features of specific membrane surface proteins (Fig. 7E and G) and, even more strikingly, in their overall antigen profiles (Fig. 7F and H). First, a clear difference between the Hannover (PG31a, Fig. 7E and F, lanes 1) and the Jerusalem (PG31b, Fig. 7E and F, lanes 2) versions of the type strain of *M. gallisepticum* in the size of the integral membrane surface protein PvpA (52 versus 46 kDa) was detected with MAb 1E5 (Fig. 7E). In another pattern, MAb 12D8 identified a 70-kDa form of a recently reported highly immunogenic membrane lipoprotein (20, 39) in the Jerusalem strain, while it revealed no detectable antigen in the Hannover strain (data not shown). The difference between the two samples was

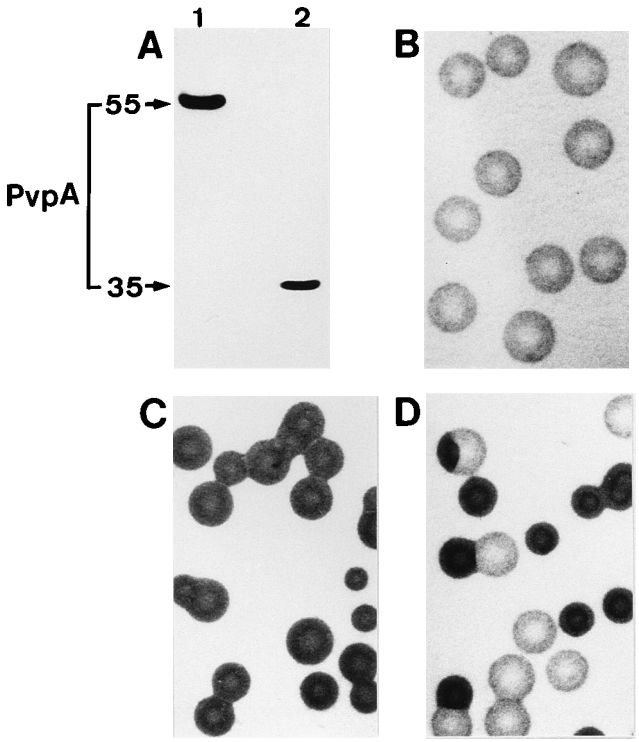

FIG. 4. Differential epitope accessibility on PvpA size variants in clonal populations of *M. gallisepticum* A5969a. (A) Western immunoblot analysis of broth-cultured whole organisms (400-μl mid-logarithmic-phase cultures) of two clonal isolates of strain A5969b which were selected in colony immunoblots with MAb 1E5 to PvpA (Fig. 2C) for their distinct levels of immunostaining intensity (lane 1, intermediate MAb-binding phenotype; lane 2, strong MAb-binding phenotype). Immunostaining of the Western blot with MAb 1E5 shows expression of PvpA by colonies of both surface phenotypes to similar degrees but in distinct size versions (indicated by arrows; numbers indicate kilodaltons). (B, C, and D) Colony immunoblots of individual progeny colonies of the two clonal isolates shown in panel A demonstrating correlation between the level of MAb 1E5 reactivity, i.e., the colony surface phenotype (intermediate [B] and strong [C]), and the size of the expressed PvpA (55 [B] and 35 [C] kDa). Colonies with intermediate and strong MAb-binding phenotypes expressing the large (55-kDa) and small (35-kDa) PvpA size variants, respectively, were easily detected in mixed cultures (D).

even more evident when a recently described antiserum (PAb anti-PG31a; see reference 39) was used for immunostaining: several antigens that did not have any prominent similarly sized antigenic counterpart in the Hannover strain were detected in the Jerusalem strain (Fig. 7F). Second, although the overall expression pattern of the major *M. bovis* membrane surface lipoproteins VspA and VspB (as revealed with MAb 1E5) did not show much variation among the four PG45 type strains analyzed (Fig. 7G), there were some differences in the apparent level of VspB and in the size of VspA. For example, the stock culture of the Jerusalem PG45 strain (PG45d, Fig. 7G, lane 4) showed a less prominent VspB band and a slightly larger version of VspA (67 versus 65 kDa) than did the Hannover (PG45a, lane 1), the Jena (PG45b, lane 2), and the Aarhus (PG45c, lane 3) PG45 strains. When the blot shown in Fig. 7G was restained with the PAb anti-D490 (Fig. 7H), it was evident that the Hannover (lane 1), the Jena (lane 2), and the Aarhus (lane 3) strains are no longer quite the same in their antigenic composition while the Jerusalem strain (lane 4) and the Jena strain (lane 2) showed more closely related antigen patterns.

Taken together, these results establish that strains of various mycoplasma species, including well-defined prototype and reference strains, which were originally classified as the same strains but had been maintained in different institutions for many years may show significant differences in their antigenic makeup and in their structural and functional surface properties.

DISCUSSION

Since the first description of the immunofluorescence technique as a serological procedure to identify mycoplasma species on agar plates (9), the colony immunobinding assay in its various modifications has gained wide acceptance and is still considered the most suitable method for routine species identification (3, 6, 7, 9, 13, 14, 18, 23). One particular advantage over other existing serological and molecular identification methods underscores its unique diagnostic capability, namely, that more than one mycoplasma species within a mixed culture can be readily detected, even on primary isolation plates. However, none of these previous reports have discussed the possibility that the antigenic surface variability which has now emerged in many mycoplasma species (1, 2, 4, 10, 21, 22, 26–29, 32, 34–37, 39, 40, 41) could cause serious problems with the various colony immunobinding techniques. Only very recently, Bradbury (5) pointed out that the immunofluorescence technique as a diagnostic tool may have some limitations due to this phenomenon. Indeed, in a study of *M. bovis* done at about the same time we observed that a rabbit anti-*M. bovis* anti-

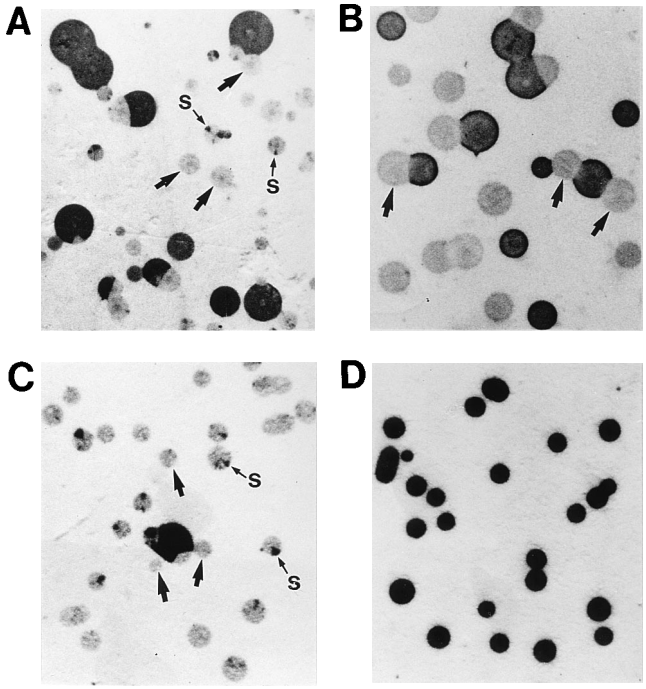

FIG. 5. Colony blot immunostaining patterns of mycoplasma strain populations obtained by using conventional diagnostic rabbit hyperimmune antisera. Colony blots of *M. bovis* PG45a (A), *M. fermentans* K (B), and *M. iowae* 695 (C and D) were immunostained with PAb anti-D490 (A), anti-PG18 (B), anti-695 (C), or anti-PPAV (D). A representative portion of each colony immunoblot is shown. While only a few colonies in panels A, B, and C are strongly stained, the majority show a very low degree of color intensity (indicated by arrows). A sectored pattern of staining within some colonies in panels A and C is indicated (s, small arrows). In contrast to the heterogeneous immunostaining pattern obtained with PAb anti-695 (C), the colonies of *M. iowae* 695 show a uniform strong immunostaining with PAb anti-PPAV (D).

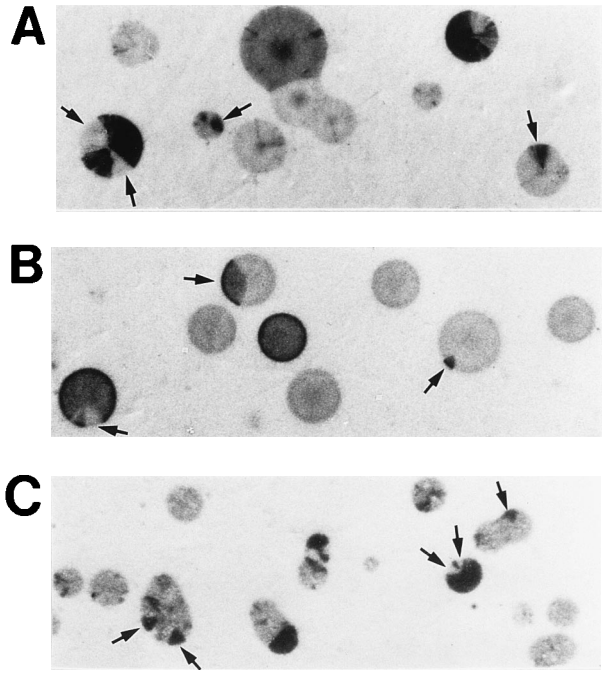

FIG. 6. Heterogeneous colony blot immunostaining pattern of clonally derived mycoplasma populations obtained by using conventional diagnostic antisera. Colony blots of progeny populations derived from single colony isolates of *M. bovis* PG45a (A), *M. fermentans* K (B), and *M. iowae* 695 (C) were immunostained with PAb anti-D490 (A), anti-PG18 (B), or anti-695 (C). Colonies expressing strong and weak PAb-binding phenotypes are present. Also present are sectored colonies which express both phenotypes within the same colony (arrows).

serum that has been used for diagnostic purposes revealed quite heterogeneous reaction patterns on colony blots of *M. bovis* clonal populations (Fig. 3b in reference 26), a picture which in the first place was highly reminiscent of a mixed culture containing different but antigenically related mycoplasma species. As a result of these observations, the present study was undertaken to answer the following questions. First, does this phenomenon affect species identification when the colony blot assay system is used as a diagnostic procedure? Second, how can this system be improved to make the identification of species and mixed cultures more reliable? And finally, what are the implications of mycoplasma phenotypic variability for the maintenance of specific properties that have been described to be characteristic of a particular strain?

We have now confirmed that mycoplasma surface antigenic variability can indeed profoundly affect the interpretation of results obtained with the colony blot assay in species identification of clinical isolates or in quality control of existing laboratory strains. The assay performed with well-established reference or prototype strains and corresponding species-specific diagnostic antisera clearly indicated the limitations of this technique, since of many standard antisera tested only a few revealed a uniformly heavy immunostaining of all colonies (e.g., Fig. 5D) and thus allowed an unambiguous species identification. Other antisera used for routine diagnosis revealed rather unsatisfactory results, namely, a very heterogeneous reaction pattern (Fig. 5A to C and data not shown) which in the first place could be misinterpreted as the reaction pattern of at least two mycoplasma species that show a sort of cross-reaction. The sole discriminating criterion that indicated the presence of only one mycoplasma species in these cases was a clearly visible sectored pattern of immunostaining within at least some of the colonies screened (e.g., Fig. 5A and C and data not shown), which demonstrated that phase variation of immunodominant surface antigens occurred in the respective strain or isolate. Nevertheless, the unambiguous distinction between the heterogeneous colony immunostaining of a truly mixed culture and that of a pure culture can sometimes be very difficult and requires some expertise of the investigator. In light of recent evidence that phase-variable surface antigens of different mycoplasma species may share the same epitope (27, 39), it is indeed quite possible that variable antigens contribute to the overall serologic cross-reaction among different species. Although this cross-reaction would not explain the high-intensity staining of sectors as illustrated in Fig. 5A and C, the most persuasive indicator that an antigenically mixed culture of a mycoplasma strain or isolate can indeed be considered pure is perhaps the finding that subcloned isogenic populations (Fig. 6) show similar heterogeneous immunostaining patterns, as well as the presence of sectoring, a result that cannot be overlooked, not even by an unexperienced investigator.

Another diagnostically important result of this study is the observation that antisera raised against different strains of the same mycoplasma species do not necessarily give similar colony immunostaining reactions with one and the same isolate or strain. In contrast, the reaction pattern was shown to depend very much on the antiserum applied (e.g., Fig. 5C and D). This finding indicates that antigenic differences among mycoplasma strains may account for antibody diversity among diagnostic antisera. We therefore suggest that in unclarified cases (such as that shown in Fig. 5C), at least two different antisera or a cocktail of all species-specific antisera available should be used in the colony immunoblot assay system. The application of such cocktails of antisera raised against different strains of the same species (e.g., an anti-*M. iowae* cocktail consisting of equal volumes of anti-*M. iowae* 695 and anti-*M. iowae* PPAV, each at a working dilution of 1:1,000) indeed proved in several examples (data not shown) to be a useful approach to obtaining unambiguous results (i.e., a uniform strong immunostaining, as in Fig. 5D). Previous recognition of the problem associated with mycoplasma antigenic variability in diagnostic serologic tests has recently prompted the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes to give a similar recommendation, namely, to utilize typing antisera against to several strains of a given species (16).

As recently shown by Theiss et al. (32) and Behrens et al. (1), two phenomena may contribute to the presence of variant colony surface antigenic phenotypes within stock cultures of mycoplasma strains or within subcloned populations, namely, (i) changes in expression states of surface antigens and (ii) the differential accessibility of their epitopes to antibody. The present study confirms that the antibody-binding phenotype of a mycoplasma colony, as revealed in the colony blot assay system, indeed not only reflects actual levels of expression of surface antigens but also can reflect complete or partial masking of surface epitopes. *M. gallisepticum* A5969b, for example, displayed three levels of reactivity to the PvpA-specific MAb 1E5, as determined by visual assessment of the degree of staining of each colony (Fig. 2C and 4B to D). Interestingly, a comparison of PvpA membrane proteins derived from colonies expressing strong and intermediate reactivities with MAb 1E5 revealed a clear difference in the size of PvpA on Western immunoblots (Fig. 4A). This result indicated that differential MAb binding of PvpA and thus its differential epitope presentation may occur as a result of variation in the structural attributes of this protein, a finding which is the first reported example to show that changes in the length of a mycoplasma

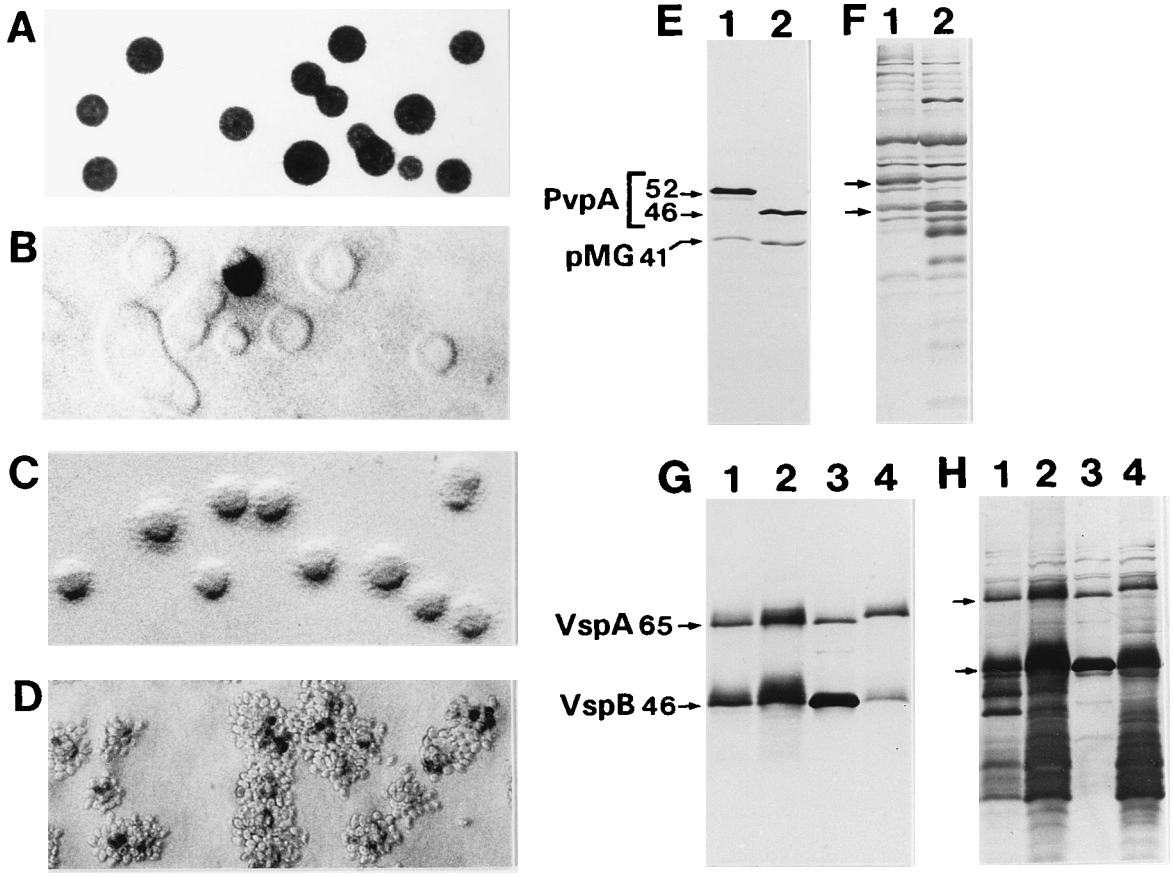

FIG. 7. Differences in surface properties and antigenic profiles among well-established mycoplasma strains used under the same designation in different laboratories. (A and B) Colony immunoblots of the Hannover strain of *M. gallisepticum* A5969 (A5969a) (A) (dry blot) and the corresponding Jerusalem strain (A5969b) (B) (wet blot) with MAb 1E5 to PvpA, showing striking differences in the surface presentation of the MAb-defined epitope. (C and D) Differences in hemadsorption properties of strain A5969a (D) and A5969b (C) demonstrated with chicken erythrocytes. (E and F) Comparative Western blot analysis of total proteins from broth-grown whole organisms of the Hannover (lanes 1) and the Jerusalem (lanes 2) versions of type strain PG31 of *M. gallisepticum* (PG31a and PG31b, respectively) with MAb 1E5 (E) or with PAb anti-PG31a (F), demonstrating that the antigen profiles of the two samples representing the same strain are not identical. Each lane represents proteins from equal aliquots (300 μl) of mid-logarithmic-phase cultures. Arrows at the left indicate the positions of the MAb-defined PvpA (numbers indicate kilodaltons) (E and F) and pMG41 (E). (G and H) Comparative Western blot analysis of total proteins from broth-grown whole organisms (20 μl of culture per sample) of the Hannover (lanes 1), Jena (lanes 2), Aarhus (lanes 3), and Jerusalem (lanes 4) versions of type strain PG45 of *M. bovis* (PG45a, PG45b, PG45c, and PG45d, respectively). The blot was immunostained with MAb 1E5 (G) and restained with PAb anti-D490 (H). The positions of a 65-kDa size variant of VspA and a 46-kDa size variant of VspB identified by the MAb are indicated by arrows.

surface antigen may correlate with differences in antibody binding.

Three examples of how to uncover masked mycoplasma surface epitopes in colony blots were presented in this report (Fig. 1 and 3): (i) by pressing lifted mycoplasma colonies onto the membrane, (ii) by extensive washing of colony blots, and (iii) by pretreatment of colony blots with Tween prior to immunostaining. All three procedures enabled detection of cryptic epitopes in two of three mycoplasma species tested (*M. gallisepticum* and *M. imitans*), the application of Tween proving to be the most reliable one, resulting in consistent and reproducible immunostaining reactions. This might be one of the reasons that other investigators did not report any ambiguous results (7, 18), since they have used Tween as a blocking reagent without being aware of its demasking capability. Therefore, it is important to emphasize the value of this reagent as a means of increasing the sensitivity of the colony blot assay as a diagnostic tool. Since the inaccessibility of certain surface antigens to antibody is due to steric hindrance by other surface molecules, there are two possible mechanisms by which unmasking of cryptic epitopes could take place: (i) by detachment of loosely bound interfering surface structures from the membrane (extensive washing) or (ii) by considerable rearrangements in the membrane caused by detergent (Tween) or shearing forces (pressure) that may lead to increased immunoaccessibility of otherwise masked membrane antigens.

One other drawback of the colony blot assay that has not been mentioned in previous communications is that nonreactive, unstained colonies are often hard to detect or even invisible because of the lack of a contrasting background (Fig. 1A). In particular, when the colony density is high, a few unstained colonies in a mass of immunoreactive ones become increasingly difficult to identify. This means that (i) a second or third mycoplasma species in a sample might be overlooked and (ii) a culture believed to be pure is in fact mixed. As we have demonstrated in this and a previous study (26), intense and sharp signals of unstained colonies can be achieved as follows. First, omission of the blocking step reveals a background color, which allows easy detection of nonreactive colonies as white dots (Fig. 4f in reference 26 and data not shown). Second, a similar picture (white colonies on a dark background) is obtained when immunostained colony blots are examined during

the process of drying (Fig. 1B). And third, each lifted colony (reactive or nonreactive) can easily be identified when the surfaces of immunostained and dried blots come in contact with water (Fig. 1C). Moreover, for species identification of such unstained colonies within a mixed culture (data not shown) we recommend, instead of preparing replicate blots as described by others (7, 18), restaining of the same colony blot with the antibody(ies) of choice by using a second chromogenic substrate which gives a color reaction that is distinct from the previous one (e.g., blue versus brown). This multiple-color technique, which also proved useful to identify specific surface antigenic phenotypes within a colony population (2, 20), offers a considerable advantage over the usually applied replicate screening technique in terms of sensitivity, resolution, and cost.

The results reported here further established that perhaps the most important consequence of mycoplasma surface antigenic variation in practical terms is the need to standardize mycoplasma strains. In other words, as soon as an isolate has been characterized and given a definite strain designation, it should always be maintained as a broth culture and preferentially propagated in medium of the same composition but never subcultivated via agar plates. However, despite emerging information indicating that highly variable surface antigens seem to be quite common among mycoplasmas, many laboratories continue to purify newly acquired mycoplasma strains or frequently used laboratory strains by multiple rounds of filter cloning (i) to remove minor subpopulations that may have arisen via rare spontaneous mutations after the strain was originally described and (ii) to avoid any possible cross-contamination with other commonly used laboratory strains, without being aware that any subpopulation selected has an unpredictable phenotype which might be quite different from the predominant phenotype of the original strain population. The filter-cloning procedure was described many years ago as an essential step in the characterization and taxonomic description of mycoplasma isolates (15, 33) or in the preparation of antisera (31), at a time when virtually no published data regarding the existence of surface antigenic variation as an important feature of mycoplasmas were available. In light of this new information, it is, however, somewhat surprising that the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes in its recently published revision of the minimum standards for description of new mycoplasma species (16) did not point out that any further purification of a defined mycoplasma (type) strain should be omitted, since any single colony picked from an agar plate of a strain stock culture may represent a "new" strain, i.e., an organism that can differ from the original (type) strain in several important features. In this report we have in fact demonstrated with some representative examples, including *M. gallisepticum* and *M. bovis* (Fig. 7), that strains which are considered to be the same but have been maintained over many years in different laboratories may no longer be identical and may show considerable differences in (i) their overall antigenic profiles, (ii) the expression pattern and structural attributes of specific antigens, and (iii) their adherence properties and thus their possible pathogenic potential. This inadvertent selection for completely new strain properties can also give an accurate explanation for the fact that data of different laboratories obtained with the "same" strain have been controversial. For example, while Garcia et al. have reported MAb 12D8 specific to *M. gallisepticum* to recognize a 79-kDa protein in their laboratory R strain (11), we have recently shown that it identifies a protein of 67 kDa in our Jerusalem R strain (20). All of these controversial results illustrate a serious concern, considering that structural alterations and in particular the "loss" of a diagnostically and functionally important antigen as a result of further processing of stock cultures through a series of clonings may have important consequences. First, differences in antigen structure and expression may profoundly affect the outcome of serodiagnostic assays which utilize whole-cell antigen preparations as test antigens and may lead to false-negative results. Second, diagnostic antisera prepared with different subpopulations of the same mycoplasma strain may differ in their antibody repertoire and may therefore cause different test results. And third, results related to the virulence properties of a particular strain may vary from experiment to experiment and from laboratory to laboratory and may lead to incorrect conclusions.

Taken together, the results presented here, along with those recently reported by Washburn et al. (34) and Czifra et al. (8), underscore the increasing need (i) to critically reevaluate published data obtained with the "same" strain or the "same" antiserum in diagnostic or experimental studies, (ii) to determine the degree of variation in phenotype among those strains which are frequently used in different laboratories for diagnostic or research purposes, and, most importantly, (iii) to establish standards for preventing diversification of mycoplasma strain characteristics in different laboratories.

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