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Mycoplasma synoviae Has Two Distinct Phase-Variable Major Membrane Antigens, One of Which Is a Putative Hemagglutinin

A. H. NOORMOHAMMADI,[1] P. F. MARKHAM,[1] K. G. WHITHEAR,[1] I. D. WALKER,[1] V. A. GUREVICH,[1] D. H. LEY,[2] AND G. F. BROWNING[1]*

School of Veterinary Science, The University of Melbourne, Parkville, Victoria, Australia 3052,[1] *and College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606*[2]

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Mycoplasma synoviae **is a major pathogen of poultry, causing synovitis and respiratory infection. A cluster of 45- to 50-kDa membrane proteins is immunodominant in strain WVU-1853. Four distinct proteins were identified in this cluster by high-pressure liquid chromatography. Monoclonal antibodies and monospecific antisera against each established that they fell into two groups, MSPA and MSPB, each containing two members distinguishable by a difference in hydrophobicity. A 25- to 30-kDa membrane protein (MSPC) was shown to be antigenically related to the MSPB proteins. Considerable variation in the size and expression of MSPA and MSPB was observed among different strains of *M. synoviae*. Examination of expression in colonies of strain WVU-1853 established that both MSPA and MSPB (and MSPC) were phase variable. Immunostaining of MSPB (and MSPC) with monoclonal antibodies exhibited quantal variation, with three distinct levels observed between and within colonies. Hemadsorption by *M. synoviae* colonies was also found to be phase variable, with some colonies exhibiting sectorial expression of hemadsorption. Monospecific antisera against MSPA inhibited hemagglutination, but neither monoclonal antibodies nor monospecific antisera against MSPB could inhibit hemagglutination. However, loss of the capacity to hemadsorb by individual clones was associated with loss of expression of both MSPA and MSPB. These findings have elucidated the complexity of structure, function, and expression of the 45- to 50-kDa membrane protein cluster of *M. synoviae*, and they suggest that all members of the cluster may be involved in adhesion.**

Mycoplasmas are the smallest self-replicating organisms known. Due in part to their limited genome size and lack of cell wall, all mycoplasmas are parasites, unable to survive for long periods outside their natural host. The close relationship with their host is reflected most clearly in their membrane proteins, some of which are responsible for adherence to host cells and are major targets of the host immune response. Variable expression of distinct membrane proteins has been observed in several mycoplasma species and hypothesized to play a role in evasion of the host immune response (33).

Mycoplasma synoviae is a major pathogen of poultry worldwide, causing respiratory tract infection and arthritis in chickens and turkeys (17). A number of studies have used immune fowl sera to identify species-specific antigens of *M. synoviae* F10-2AS and WVU-1853, which may be useful in the diagnosis of infection (3–5, 13, 16, 19). Of the antigens reported, a cluster of bands of 41 to 53 kDa and a band of 22 kDa were shown to be antigenic and species specific. Recent studies in our laboratory, using monoclonal antibodies (MAbs), have identified antigens with similar molecular masses in the hydrophobic fraction of *M. synoviae* WVU-1853 cells solubilized in Triton X-114 (TX-114) (13). Given the established role of some surface proteins in both immunity to and pathogenesis of other mycoplasmas, these antigens may well be significant in events such as adhesion (11, 12, 15, 18).

Attachment of *M. synoviae* cells to erythrocytes (25, 26) and chicken embryo cells (1, 2) has been documented, and available evidence indicates a direct correlation between hemadsorption of erythrocytes to colonies of different clones of *M. synoviae* WVU-1853 and their hemagglutination activity (26). Furthermore, clonal variation in hemadsorption and hemagglutination of *M. synoviae* strains has been reported both in vitro and after passage in vivo (26), suggesting the occurrence of phase variation in the expression of proteins involved in these processes. The main aims of this study were to identify the major membrane proteins of *M. synoviae* recognized by the infected chicken, to investigate their role in hemadsorption and hemagglutination, and to examine them for phase-variable expression.

MATERIALS AND METHODS

M. synoviae **strains and growth media.** All experiments were conducted with *M. synoviae* WVU-1853 unless otherwise noted. The origins of all *M. synoviae* strains and *M. gallisepticum* S6 have been described previously (22, 23). The organisms were grown in mycoplasma broth (MB) to the late logarithmic growth phase and harvested at approximately pH 6.8. To produce single isolated colonies on solid media for colony hemadsorption and colony immunoblotting, an appropriate dilution of *M. synoviae* cells was prepared in MB and inoculated onto mycoplasma agar (MA) plates. The plates were incubated at 37°C for approximately 6 days to obtain colonies with an average diameter of 0.3 mm (32).

TX-114 phase partitioning. TX-114 (Sigma) phase partitioning was performed as previously described by Bordier (9) with some modifications. Briefly, *M. synoviae* cells were harvested by centrifugation at 20,000 × *g* at 4°C, and the pellet was washed three times in phosphate-buffered saline (PBS). Cell proteins were solubilized in 0.5% (vol/vol) TX-114 in PBS, incubated for 1 h at 4°C, and centrifuged at 12,000 × *g* for 40 min. The supernatant was carefully loaded onto a sucrose cushion (0.6% [wt/vol] sucrose, 0.06% [vol/vol] TX-114), incubated for 10 min at 37°C, and centrifuged at 300 × *g* at 37°C for 5 min. The supernatant was removed, and the detergent phase was treated to eliminate TX-114 as described by Wessel and Flugge (31). Briefly, the detergent phase was mixed with a solution of 40% methanol–10% chloroform and centrifuged for 1 min at 9,000 × *g* and 4°C. After removal of the organic supernatant, the pellet was dried under vacuum and resuspended in either sodium dodecyl sulfate-polyacrylamide

* Corresponding author. Phone: 61 3 9344 7342. Fax: 61 3 9344 7374. E-mail: browning@muwayf.unimelb.edu.au.

gel electrophoresis (SDS-PAGE) sample buffer for SDS-PAGE or 8 M urea for high-pressure liquid chromatography (HPLC).

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting experiments were carried out as previously described (13).

HPLC. Samples (approximately 800 μl) containing less than 100 μg of total protein dissolved in 8 M urea were applied to a reversed-phase column (ProRPC 15-μm-bead-diameter C_8; Pharmacia) and eluted with a 0 to 90% (vol/vol) linear gradient of acetonitrile as specified by the manufacturer (Pharmacia). The gradient was buffered with 0.1% (vol/vol) trifluoroacetic acid. Chromatographic separation was monitored with a UV detector operated at a wavelength of 214 nm. The resultant fractions were dried with a vacuum centrifuge and used for further analysis.

Polyclonal antisera and MAbs. The origin of MAbs 50, 97, and 334 has been described previously (13). Polyclonal sera against *M. synoviae* and *M. gallisepticum* were raised in SPF White Leghorn chickens by air sac inoculation with cultures of *M. synoviae* WVU-1853 or 7NS or *M. gallisepticum* S6. Monospecific polyclonal antisera against each antigen of HPLC peaks were raised in rabbits as described by Harlow and Lane (14). Briefly, 10 to 15 μg of dried HPLC fractions was resuspended in SDS-PAGE sample buffer, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond C; Amersham). The nitrocellulose membrane was stained with 0.2% (wt/vol) Ponceau S (3-hydroxy-4-[2-sulfo-4-(sulfophenylazo)phenylazo]-2,7-naphthalene disulfonic acid) in 3% (wt/vol) trichloroacetic acid and 3% (wt/vol) sulfosalicylic acid to visualize protein bands. The bands of interest were excised from the membrane and sonicated in sterile PBS. The mixture was emulsified in an equal volume of complete Freund's adjuvant and injected intramuscularly into rabbits. To increase the antibody titer, the rabbits were boosted several times with the purified proteins emulsified in incomplete Freund's adjuvant.

HI test. Hemagglutination antigen was prepared by growing and harvesting *M. synoviae* as described above. The cell pellet was resuspended in PBS, mixed with an equal volume of glycerol, and stored at −70°C. The hemagglutination inhibition (HI) test was carried out with 4 hemagglutination units of antigen as previously described (7).

Colony hemadsorption. *M. synoviae* colonies on MA were overlaid with 15 ml of 0.5% (vol/vol) chicken erythrocytes in PBS and incubated for 30 min at 37°C. The erythrocyte suspension was removed, and the plates were gently washed with PBS and then examined at low magnification under a microscope. Hemadsorbing (HAd^+) and nonhemadsorbing (HAd^-) colonies were selected and grown in MB.

Colony immunoblotting. A round nitrocellulose membrane (Hybond-C; Amersham) was placed on MA containing *M. synoviae* colonies, marked for orientation, removed, and air dried on filter paper. The membrane was then blocked by incubation in 10% (wt/vol) bovine serum albumin in PBS for 30 min at room temperature and then treated for immunostaining as described previously (13).

RESULTS

Immunodominant cluster of 45 to 50 kDa. *M. synoviae* cells were solubilized with the detergent TX-114 and fractionated into aqueous (hydrophilic) and detergent (hydrophobic) phases. Aqueous- and detergent-phase proteins were separated by SDS-PAGE and immunoblotted. Immunostaining of detergent phase proteins with MAb 50, 97, or 334 showed that each MAb reacted with a broad band of 45 to 47 kDa and a diffuse band of 25 to 30 kDa (Fig. 1, lanes 1 to 3) while chicken anti-*M. synoviae* serum detected a broad band of 45 to 50 kDa (broader than that bound by the MAbs), a diffuse band of 25 to 30 kDa, and several other bands with higher molecular masses (lane 4). Chicken anti-*M. gallisepticum* and preinoculation sera reacted with a number of higher-molecular-mass bands but did not react with the bands of 45 to 50 or 25 to 30 kDa (lanes 5 and 6, respectively) when used to probe detergent-phase proteins. Immunoblotting of hydrophilic-phase proteins with either chicken anti-*M. synoviae* serum or MAb 50, 97, or 334 did not reveal any band.

Four distinct antigens in the cluster. The TX-114 hydrophobic fraction of *M. synoviae* cells was subjected to reversed-phase HPLC purification. The resultant fractions from 10 discrete chromatographic peaks (as detected by absorbance at 214 nm) were collected, further analyzed by SDS-PAGE, and tested for reaction with chicken anti-*M. synoviae* serum and/or MAb 50, 97, or 334 by immunoblotting. Three peaks contained antigenic proteins: peak 1, which eluted at an acetonitrile concentration of approximately 55% and contained one band of 50 kDa ($MSPA_1$); peak 2, which eluted at an acetonitrile concentration of approximately 56% and contained a doublet of 50 and 45 kDa ($MSPA_2$ and $MSPB_2$); and peak 3, which eluted after the delivery of the entire acetonitrile gradient during the reequilibration step and contained a band of 47 kDa ($MSPB_3$) and a diffuse band of 25 to 30 kDa ($MSPC_3$), as seen in Fig. 2A, lanes 2, 3, and 4, respectively. The chromatographic elution of these peaks was reproducible, and the resulting fractions 1, 2, and 3 contained approximately 3, 15, and 4 μg of protein (per 100 μg of protein fractionated), respectively.

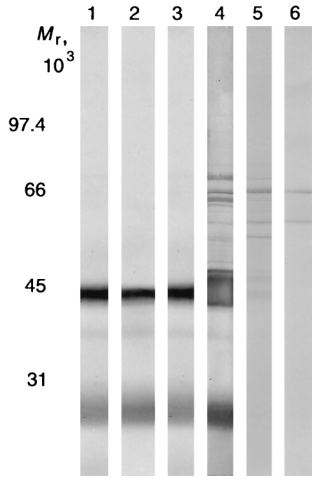

FIG. 1. Immunoblot of the hydrophobic TX-114 fraction of *M. synoviae* cells probed with MAbs 50, 97, and 334 (lanes 1, 2, and 3, respectively) and with chicken anti-*M. synoviae* (lane 4), anti-*M. gallisepticum* (lane 5), and preinoculation (lane 6) sera.

Two groups of antigens, MSPA and MSPB. Immunoblotting of HPLC fractions showed that $MSPA_1$, $MSPA_2$, $MSPB_2$, $MSPB_3$, and $MSPC_3$ reacted with chicken anti-*M. synoviae* serum and only $MSPB_2$, $MSPB_3$, and $MSPC_3$ reacted with a pool of MAbs consisting of MAbs 50, 97, and 334 (Fig. 2B) or with each MAb individually (results not shown).

HPLC fractions 1, 2, and 3 were separated by SDS-PAGE and transferred to nitrocellulose membranes, and bands corresponding to $MSPA_1$, $MSPA_2$, $MSPB_2$, $MSPB_3$, and $MSPC_3$ were excised and used to immunize rabbits. Immunoblotting revealed that these antigens fell into two antigenically distinct groups, MAb-binding ($MSPB_2$, $MSPB_3$ and $MSPC_3$) and non-MAb-binding ($MSPA_1$ and $MSPA_2$) MSPs. Rabbit antisera against each MSP in either of these groups bound to all the other MSPs within its group. Figure 2C shows the immunostaining of HPLC peaks 1, 2, and 3 with rabbit anti-$MSPA_1$ and rabbit anti-$MSPB_3$ (lanes a and b), which were used in subsequent experiments. The reactivity of MSPs with different immunoreagents is summarized in Table 1.

Inhibition of hemagglutination by rabbit anti-MSPA. To further study the effect of MAbs and rabbit anti-MSP sera on the attachment of *M. synoviae* cells to chicken erythrocytes, dilutions of rabbit anti-$MSPA_1$, rabbit anti-$MSPB_3$, and preinoculation rabbit sera, MAbs 50, 97, and 334 were used in an HI assay. Rabbit anti-$MSPA_1$ inhibited hemagglutination up to a dilution of 1:32, while rabbit anti-$MSPB_3$ and preinoculation rabbit sera failed to inhibit hemagglutination beyond a 1:2 dilution. The significance of the rabbit anti-$MSPA_1$ serum HI titer was highlighted by the relatively low titer of this serum in immunoblots, compared with the rabbit anti-$MSPB_3$ serum. MAbs 50, 97, and 334 were unable to inhibit hemagglutination at the highest concentrations used.

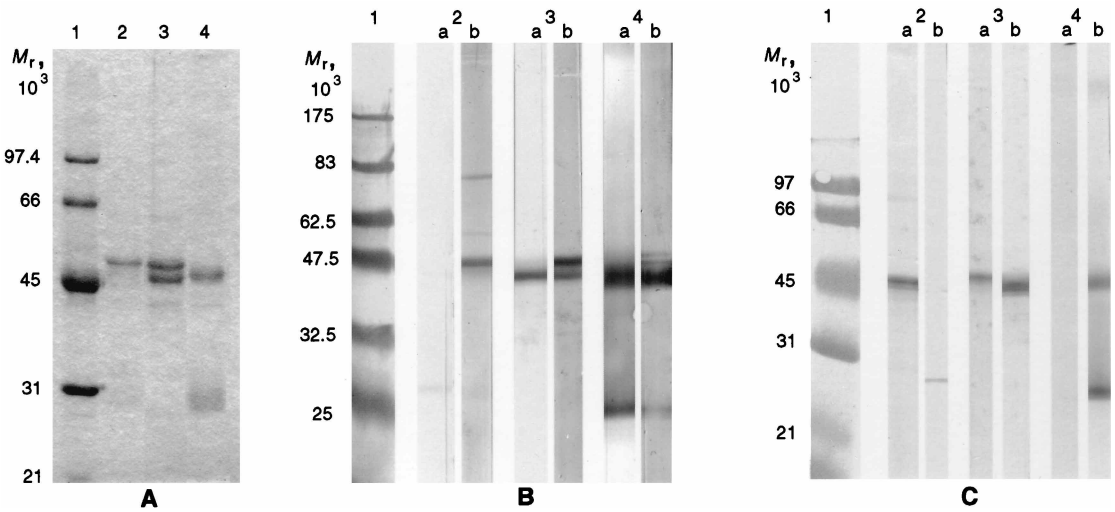

FIG. 2. (A) SDS-PAGE of HPLC peaks 1 (lane 2), 2 (lane 3), and 3 (lane 4). (B) Immunoblots of peaks 1 (panel 2), 2 (panel 3), and 3 (panel 4) separated by SDS-PAGE. The first lane (a) of each panel was probed with a pool of MAbs 50, 97, and 334, and the second (b) was probed with chicken anti-*M. synoviae* serum. (C) Immunoblots of peaks 1 (panel 2), 2 (panel 3), and 3 (panel 4) separated by SDS-PAGE and immunostained with rabbit anti-$MSPA_1$ (lanes a) or rabbit anti-$MSPB_3$ (lanes b). Lanes 1 show protein molecular mass markers.

Variable colony hemadsorption. *M. synoviae* colonies grown on MA were examined for their ability to attach chicken erythrocytes in a hemadsorption assay. Approximately 5% of colonies of the original culture exhibited partial (sectorial) or uniform hemadsorption. Cloning of uniformly HAd^+ colonies and regrowth of the resultant cultures on MA increased the proportion of partially or uniformly HAd^+ colonies to 65%. A clear sectorial hemadsorption, or lack of it, was observed in some colonies. These sectors ranged from a small to large proportion of the colony. An example of the uniform presence or absence of hemadsorption is shown in Fig. 3A, with sectorial hemadsorption shown in Fig. 3B.

Lack of expression of both MSPA and MSPB in HAd^- colonies. Uniformly HAd^- and HAd^+ colonies were cloned, grown in MB, and subjected to TX-114 partitioning. Whole-cell and hydrophobic- and hydrophilic-phase proteins of these clones were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Comparison of the Coomassie brilliant blue profile of either whole-cell (Fig. 4A, lanes 2a and b) or hydrophilic-phase (lanes 4a and b) proteins revealed little difference between HAd^- (lanes a) and HAd^+ (lanes b) cells; however, examination of the hydrophobic-phase (lanes 3a and b) proteins revealed strongly staining bands of 45 to 50 kDa in the HAd^+ clones which were absent in HAd^- clones, and a strongly staining band of approximately 25 to 30 kDa in the HAd^- clones which was reduced in the HAd^+ clones. Immunostaining of the whole-cell proteins of HAd^- clones with a pool of MAbs 50, 97, and 334 (Fig. 4B, lane 1a), chicken anti-*M. synoviae* serum (lane 2a), or rabbit anti-$MSPA_1$ serum (lane 3a) indicated the absence of staining of $MSPA_1$ (and cross-reactive $MSPA_2$), $MSPB_2$, and $MSPB_3$ and the presence of a strongly staining band of approximately 25 to 30 kDa, similar in molecular mass to $MSPC_3$. In contrast, prominent antigens of 45 to 50 kDa were detected in the HAd^+ clones by chicken anti-*M. synoviae* serum (lane 2b), the pool of MAbs 50, 97, and 334 (lane 1b), and rabbit anti-$MSPA_1$ serum (lane 3b). The appearance of two different-sized bands staining with rabbit anti-$MSPA_1$ and rabbit anti-$MSPB_3$ is presumably due to size variants of MSPA and MSPB.

Phase-variable expression of MSPA and MSPB. Immunostaining of nitrocellulose lifts taken of *M. synoviae* colonies revealed that colonies (approximately 20%) exhibited sectorial expression when stained with rabbit anti-$MSPA_1$. In a similar study with MAbs 50, 97, and 334 (whether individually or pooled), three distinct levels of staining were seen in different colonies and also within individual colonies (Fig. 5). All colonies produced a uniform staining pattern when the colony lifts were probed with chicken anti-*M. synoviae* serum but did not stain when chicken anti-*M. gallisepticum* serum was used. This sectorial expression of MAb-binding and non-MAb-binding MSPs was also seen after cloning from individual colonies.

Variation of size and expression of MSPA and MSPB in different strains of *M. synoviae*. To investigate the presence of MSPs in other *M. synoviae* strains, rabbit anti-$MSPA_1$ and anti-$MSPB_3$ and MAbs 50, 97, and 334 were used to probe immunoblots of 14 different strains of *M. synoviae*. Rabbit anti-$MSPA_1$ reacted with bands of 80 kDa in strain K1723 (Fig. 6A, lane 2), 55 kDa in strain FMT (lane 5), and 50 kDa in strain WVU-1853 (lane 8). Rabbit anti-$MSPB_3$ bound one or more bands of different sizes in each strain tested (Fig. 6B). In similar studies, MAbs 50, 97, and 334 all reacted only with corresponding bands (MAb-binding MSPs) in strain WVU-1853 whereas MAb 50 reacted with an antigen of 40 kDa in strain K1858 as well (results not shown).

TABLE 1. Summary of the reactivity of MSPs with various immunoreagents

MSP	Reactivity with:			
	Rabbit anti-$MSPA_1$	Rabbit anti-$MSPB_3$	Chicken anti-*M. synoviae*	MAbs 50, 97, and 334
$MSPA_1$	+	−	+	−
$MSPA_2$	+	−	+	−
$MSPB_2$	−	+	+	+
$MSPB_3$	−	+	+	+
$MSPC_3$	−	+	+	+

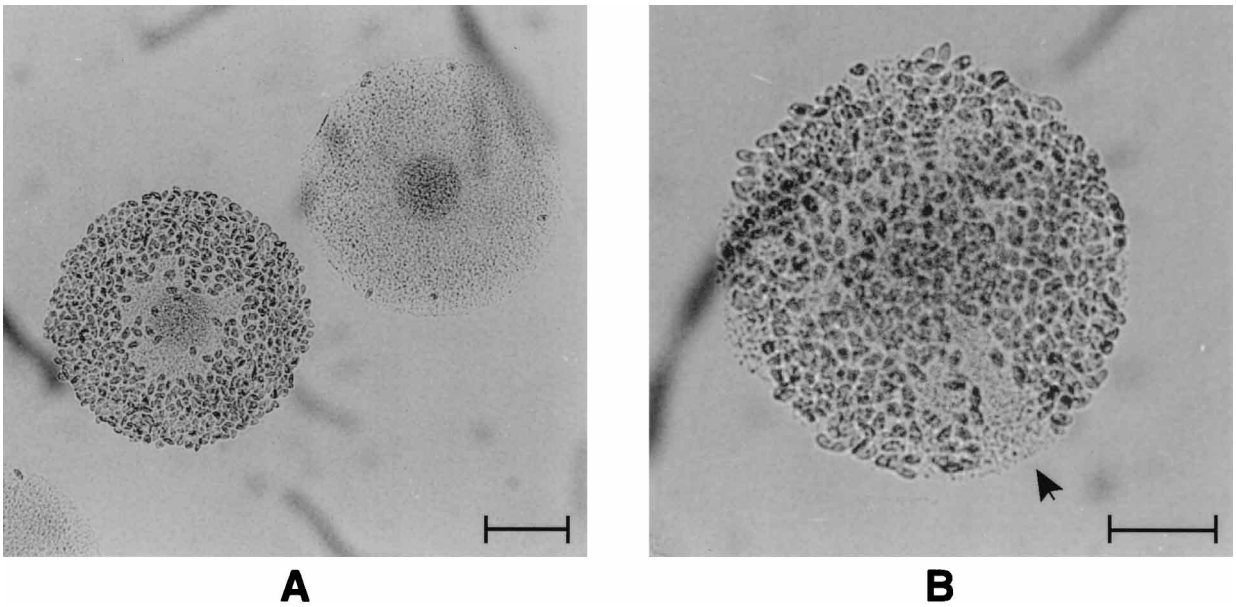

FIG. 3. (A) Uniformly HAd^+ (left) and HAd^- (right) colonies. (B) A nonhemadsorbing sector in a HAd^+ colony. The arrow in panel B indicates a sector which was not capable of hemadsorption. Bars, 100 μm.

DISCUSSION

This study has elucidated the complexity of the proteins in the immunodominant 45- to 50-kDa membrane protein cluster of *M. synoviae*. The four immunoreactive proteins in the cluster fell into two antigenically distinct groups (MSPA and MSPB), with the two members of each group clearly separable by reversed-phase chromatography. This suggests that there are two distinct proteins, with both proteins undergoing posttranslational modifications which alter their hydrophobicity, or that these proteins are encoded by two distinct multigene families and that two members of each family are expressed concurrently. While variation in posttranslational modification of mycoplasma membrane proteins has not been reported, a multigene family of a membrane protein has been found in *M. gallisepticum* (20, 21). In addition, a fifth membrane protein of 25 to 30 kDa, MSPC, was identified and found to be antigenically related to MSPB, suggesting that it could be a truncated form of MSPB.

Two observations indicated some association between MSPA and MSPB. The first was the correlation in size between the 80-kDa MSPA and 80-kDa MSPB in strain K1723, and the second was the loss of expression of both in clones of strain WVU-1853 which selected as HAd^-.

While there was an association between the expression of both MSPA and MSPB and hemadsorption, only MSPA was found to be a hemagglutinin. Previous studies have shown that hemagglutination is not always correlated with hemadsorption in mycoplasmas (6), suggesting that hemadsorption is a multi-

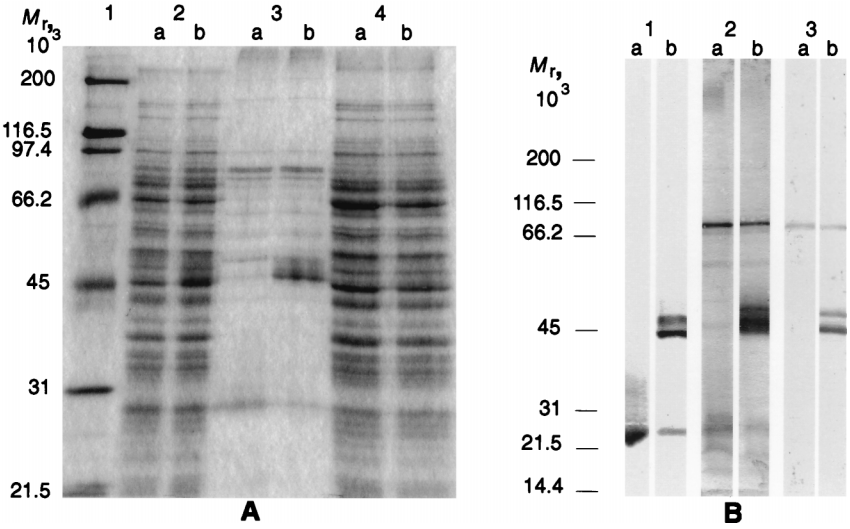

FIG. 4. (A) SDS-PAGE of whole-cell proteins (lane 2) and hydrophobic (lane 3) and hydrophilic (lane 4) proteins of cultures of uniformly HAd^- (lanes a) and HAd^+ (lanes b) *M. synoviae* colonies. Lane 1 shows protein molecular mass markers. (B) Immunoblots of the whole-cell proteins of the same cultures probed with a pool of MAbs 50, 97, and 334 (lane 1), chicken anti-*M. synoviae* (lane 2), and rabbit anti-$MSPA_1$ (lane 3).

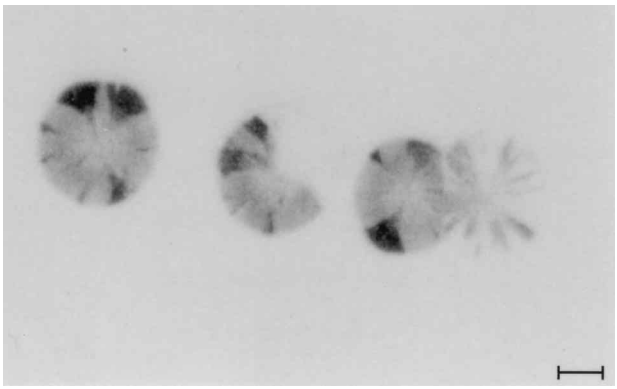

FIG. 5. Colony blot of *M. synoviae* immunostained with MAb 334. The colony on the right shows the intermediate level of staining with sectors failing to stain, and the colony on the left shows the intermediate level of staining with sectors staining at the maximal level. The colony second from the left shows all three levels of staining. Bar, 100 μm.

factorial process, with more than one protein involved in the process. A number of proteins have been shown to interact with the P1 hemagglutinin of *M. pneumoniae.*

The complexity of the 45- to 50-kDa membrane protein cluster was further emphasized by the observation that both MSPA and MSPB were capable of high-frequency phase variation. Such phase variation has been observed in a range of mycoplasmas including *M. gallisepticum* (8, 24), *M. hyorhinis* (27), *M. pulmonis* (29), *M. bovis* (35) and *M. pneumoniae* (18). However in each of these species, the variably expressed proteins have been distinct and the mechanisms used for control of variation, where investigated, differ. Furthermore, the complete genome sequence of *M. genitalium* does not appear to contain homologs of any of the three best-characterized variably expressed membrane protein families, the V-1 antigen of *M. pulmonis*, the Vlp family of *M. hyorhinis*, and the pMGA family of *M. gallisepticum*. It appears likely that a range of membrane proteins, each fulfilling distinct functions, may be capable of phase variation and that mycoplasmas may utilize several distinct mechanisms to control this variable expression. While the role of this phase variation in vivo has not been established, it has been hypothesized that it may play a role in virulence and in immune evasion (30, 34). Most of the phase-variable membrane proteins previously characterized in mycoplasmas have had only two possible expression states: "on" or "off". However, a recent report has observed differential levels of expression in the "on" state of *M. hyorhinis vlp* genes (10). Similarly, Rosengarten and Yogev (28) have reported that differential levels of immunostaining of *M. gallisepticum* colonies with MAbs against PvpA were associated with significant variation in the size of PvpA. In both these instances, the lower level of staining intensity has been correlated with a significant increase in the size of the gene product. In this study, we also observed three distinct levels of staining intensity with MAbs against MSPB and MSPC, suggesting distinct levels of expression or the phenomenon of epitope masking. However, unlike the instances observed previously in other mycoplasmas (10, 28), this does not appear to be associated with a significant increase in the size of MSPB or MSPC. The alternative explanation, that it is due to epitope masking, as reported by Rosengarten et al. (28), is unlikely because the method used for colony immunostaining in our studies approximated the detergent treatment used in their study.

High-frequency phase variation of MSPA and/or MSPB may be reflected in variation in the capacity of individual colonies to hemadsorb and also in the novel observation that sectorial variation in hemadsorption could be seen within individual colonies. With such variation in hemadsorption, it will be ineresting to establish whether hemadsorption is as closely correlated with adhesion in *M. synoviae* as has been found in *M. pneumoniae*. The role of MSPA and MSPB in hemadsorption and adhesion will have to be examined, because both are phase variable, and neither was expressed in selected HAd^- clones in the limited studies described here. If the role of phase variation is to reduce exposure of major adhesion molecules to the host immune response, coordinate regulation might be expected if both MSPA and MSPB share a functional role. The alternative explanation is that the function of phase variation is to provide a range of alternative adhesion capabilities to maximize the number of potential target tissues.

Further examination of the genes that encode the different members of the *M. synoviae* 45- to 50-kDa membrane protein cluster, and the control of their expression, will shed further light on the function of the phase variation in mycoplasmas and is likely to reveal novel regulatory mechanisms.

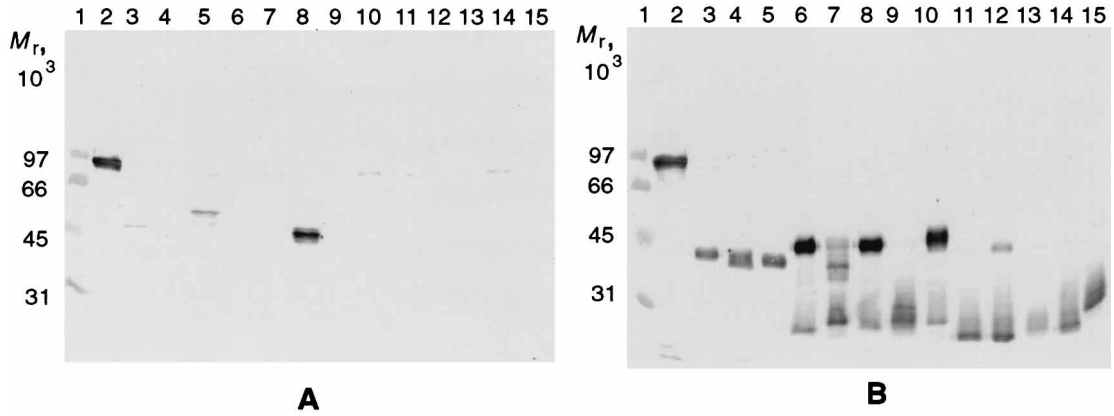

FIG. 6. Immunoblots of whole-cell proteins of 14 different strains of *M. synoviae* probed with rabbit anti-$MSPA_1$ (A) or rabbit anti-$MSPB_3$ (B). Lanes: 1, protein molecular mass markers; 2, K1723; 3, K1858; 4, K870; 5, FMT; 6, YA; 7, 7NS; 8, WVU 1853; 9, MS-H; 10, K1968; 11, T2; 12, 4GP H3; 13, 85099/1F; 14, 85099/303; 15, 88604/FP3.

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