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Reacquisition of Specific Proteins Confers Virulence in *Mycoplasma pneumoniae*

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Hemadsorbing revertants were isolated from spontaneous hemadsorption-negative, avirulent mutants of *Mycoplasma pneumoniae*. The revertants simultaneously reacquired specific proteins absent in their homologous mutants, along with neuraminidase-sensitive adherence to the respiratory epithelium and virulence. Peptide mapping and immunological analysis indicated no precursor-product relationships among certain of these proteins.

Mycoplasma pneumoniae is a surface parasite of the human respiratory tract. *M. pneumoniae* infections are generally mild and self-limiting, occurring most frequently in children and young adults (11). Adherence of the invading mycoplasmas to the luminal surface of the respiratory epithelium is essential for successful colonization (23, 26). These organisms possess a differentiated tip structure, which is seen in close proximity to the host cell surface in infected tissue (3, 6, 8). Immunoferritin labeling, in conjunction with electron microscopy, has shown that protein P1, a trypsin-sensitive surface macromolecule previously implicated in cytadsorption (17), is concentrated about the tip organelle (2, 16). In vitro binding assays (18) and attachment inhibition studies with antibodies directed against P1 (10, 16; D. C. Krause and J. B. Baseman, Infect. Immun., in press) indicate that P1 plays a direct role in adherence and virulence.

While protein P1 is probably the most important mediator of mycoplasma cytadsorption, it is apparent that attachment is a complex process involving many elements. Hemadsorption has been used as a convenient measure of mycoplasma adherence to host cells. Characterization of nitrosoguanidine-derived mutants of *M. pneumoniae* which were unable to hemadsorb (HA^-) provided evidence that additional proteins were associated with mycoplasma adherence to host cells and virulence (1, 12, 15). While these findings were reinforced by the subsequent isolation and characterization of a homologous hemadsorbing (HA^+) revertant (14, 21), the potential existence of secondary mutations in these strains due to chemical mutagenesis prompted the screening of wild-type *M. pneumoniae* for spontaneously arising HA^- mutants. A total of 22 such mutants were isolated and grouped into four classes on the basis of their protein profiles (19). Mutants in class I lacked or were markedly deficient in four high-molecular-weight proteins, designated HMW1, HMW2, HMW3, and HMW4 or HMW1-4 (molecular weight range, 140,000 to 215,000). Other HA^- mutants (class III) (19) possessed normal levels of HMW1-4 but were missing three proteins previously described (13) which are detectable only on two-dimensional polyacrylamide gels (proteins A, B, and C; molecular weights, 72,000, 85,000, and 37,000, respectively). A single mutant (class IV), in addition to missing proteins A, B, and C, lacked protein P1 described above. The remaining mutants (class II) possessed protein profiles apparently identical to that of the wild-type strain. All of the HA^- mutants adhered poorly to hamster tracheal rings in vitro and were avirulent in hamsters (19). It was concluded from this study that although the presence of certain proteins does not necessarily ensure normal cytadsorption capability, the absence of these proteins causes a significant reduction in cytadsorption and virulence.

To reinforce the biological role of specific *M. pneumoniae* proteins, we have isolated and characterized HA^+ revertants from each major class of HA^- mutants (I, II, III, and IV). All of the revertant strains were comparable to the wild-type strain in their protein profiles, cytadsorption capabilities, and virulence in vivo. In addition, we have probed the biochemical relationship of HMW1-3 using peptide mapping and specific antisera directed against two of these mycoplasma proteins.

MATERIALS AND METHODS

Organisms and culture conditions. The *M. pneumoniae* strains used in this study were the virulent HA^+ parent strain M129-B25C and randomly selected representatives of each class of spontaneous HA^- mutants (classes I, I', II, III, and IV, as described elsewhere

[19]). *M. pneumoniae* were grown at 37°C in 32-ounce (ca. 960-ml) or 8-ounce (ca. 280-ml) glass prescription bottles containing 70 or 25 ml, respectively, of Hayflick medium. Exponentially growing mycoplasmas were harvested at 48 to 72 h post-inoculation, when the phenol red pH indicator in the growth medium became orange in color. After three washes with phosphate-buffered saline (PBS), pH 7.2, *M. pneumoniae* were collected by centrifugation at 9,500 × *g* for 15 min. Intrinsic radiolabeling of *M. pneumoniae* proteins was achieved by incubating log-phase mycoplasma cultures for 6 h at 37°C in Hanks balanced salt solution containing 10% dialyzed horse serum and 250 μCi of L-[^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.; specific activity, 1,050 Ci/mmol). After a 1.5-h incubation at 37°C in fresh Hayflick medium, radiolabeled *M. pneumoniae* were washed three times with PBS containing 1 mM unlabeled methionine and harvested. CFU were quantitated as described elsewhere (9).

Isolation of HA$^+$ revertants. Preliminary screening of each mutant class for HA$^+$ revertants proved unsuccessful (mycoplasma colonies grown on Hayflick agar plates [22] for 7 days were incubated with fresh chicken erythrocytes [19]). Therefore, we used a selective enrichment procedure described previously (14) for the isolation of revertant strains. Briefly, mutant mycoplasmas (approximately 5 × 10^9 CFU) were incubated in suspension with chicken blood for 45 min at 37°C with gentle mixing (chicken blood was <2 weeks old and diluted 1:1 in Alsever solution). Erythrocyte-associated mycoplasmas were purified by a series of three centrifugations at 600 × *g* for 20 min on a gradient of 0.5% (wt/vol) Methocel (Dow Chemical Co., Midland, Mich.)–20% (vol/vol) Hypaque (Winthrop Laboratories, New York, N.Y.). After expansion of the resultant mycoplasma population in Hayflick broth (72 to 96 h at 37°C), the enrichment procedure was repeated in its entirety with fresh chicken blood. The mycoplasma populations after the second enrichment were serially diluted and screened for hemadsorption; individual HA$^+$ colonies were identified and cloned by standard procedures (12).

One- and two-dimensional PAGE. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) was performed with a slab gel system consisting of a 3% stacking gel and a 5 or 7.5% separating gel. Whole-cell preparations containing approximately 100 μg of protein (24) were applied to each well. Gels were stained with Coomassie brilliant blue or processed for fluorography (4).

Revertant strains were examined by two-dimensional PAGE as detailed elsewhere (13). Proteins were separated in the first dimension by isoelectric focusing or non-equilibrium pH gradient electrophoresis, and in the second dimension by SDS-PAGE. Proteins were visualized with Coomassie brilliant blue or by fluorography.

Assay of attachment of HA$^+$ revertants to tracheal rings. Adherence of the revertant strains to the respiratory epithelium was examined with hamster tracheal rings in vitro (7, 19). Briefly, mycoplasmas radiolabeled intrinsically with [*methyl*-^{3}H]thymidine (Schwarz/Mann, Spring Valley, N.Y.; specific activity, 50 Ci/mmol) were incubated with tracheal rings which had been pretreated at 37°C for 60 min in PBS (pH 7.2), with or without neuraminidase (Sigma Chemical Co., St. Louis, Mo.; type VIII, 10 U/ml). After 3 h, rings were removed from the mycoplasma suspension, rinsed twice in PBS, solubilized overnight in 1% SDS, and counted for radioactivity in ACS scintillation cocktail (Amersham Corp.). Approximately 10^5 cpm, representing 2 × 10^7 CFU, were incubated with each tracheal ring. All assays were carried out in quadruplicate.

Virulence studies. Virulence of the HA$^+$ revertants was determined in Syrian golden hamsters on the basis of mycoplasma survival in the lungs and lung histopathology (9). Hamsters were inoculated intranasally with approximately 10^7 CFU of *M. pneumoniae* suspended in Hayflick medium or with Hayflick medium alone (sham-infected controls). At days 14 and 28 postinfection, the lungs were removed aseptically from anesthetized hamsters. The large lobe of the lungs was prepared for sectioning and staining, and the remaining lobes were homogenized, serially diluted, and plated on Hayflick agar plates. After 7 days at 37°C, the mycoplasma titers from the lung homogenates were determined (9) and expressed relative to lung weight. Lung sections were examined microscopically in a double-blind fashion for intrabronchial phagocytic exudate and peribronchial and perivascular lymphocytic infiltrate (9); the degree of histological pneumonia was scored from 0 to 9 (minimum to maximum pulmonary pathology, respectively).

Peptide mapping and immunological analysis of mycoplasma proteins HMW1, 2, and 3. The structural relatedness of proteins HMW1-3 was studied by (i) peptide mapping and (ii) radioimmunoprecipitation (RIP) analysis with antisera specific for certain of the proteins. Peptide mapping was performed according to Cleveland et al. (5). Total protein from four 32-ounce (ca. 960-ml) bottles of *M. pneumoniae* was separated by SDS-PAGE on discontinuous 3% stacking–4.5% separating gels. The gels were stained for 1 h with Coomassie brilliant blue (0.25% [wt/vol] in 50% [vol /vol] methanol–10% [vol/vol] glacial acetic acid) and were destained until the protein bands were discernible (approximately 1 to 1.5 h in 25% [vol/vol] methanol–7.5% [vol/vol] glacial acetic acid). The bands of interest were sliced from the gels, equilibrated for 30 min in 0.125 M Tris (pH 6.8) containing 0.1% SDS and 1 mM EDTA, and applied to individual wells of a second gel. The second gel consisted of a 3.0-cm 3% stacking gel and an 8.5-cm 12% separating gel. The gel slices containing the proteins were overlaid with 0.5 to 2.5 μg of α chymotrypsin (Sigma; type VII) and then electrophoresed until the samples were approximately 1.0 cm from the top of the separating gel. The current was turned off for 30 min to allow proteolysis to occur, and electrophoresis was resumed. Polypeptides were visualized with silver stain (25).

Antisera were raised in rabbits against proteins HMW1 and HMW3 purified by preparative SDS-polyacrylamide slab gel electrophoresis according to Tjian et al. (28). Rabbits were injected as described elsewhere (2) with approximately 100 μg of protein per injection. Reactivity of prebleed and immune sera was evaluated by RIP (2). Briefly, [^{35}S]methionine-labeled mycoplasmas were detergent solubilized in the presence of 1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × *g* for 45 min to remove insoluble debris, and the resulting supernatant was combined with the test serum. Immune complexes were precip-

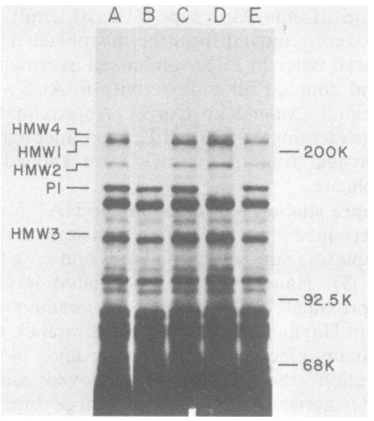

FIG. 1. Analysis of intrinsically labeled ([^{35}S]methionine) total protein from wild-type and HA$^+$ revertant strains of *M. pneumoniae* by SDS-PAGE and fluorography. Lane: (A) HA$^+$ M129-B25C, (B) class I HA$^-$ mutant, (C) HA$^+$ revertant of class I mutant, (D) class IV HA$^-$ mutant, (E) HA$^+$ revertant of class IV mutant. Proteins HMW1-4 and P1 are indicated to the left. Molecular weights were determined by coelectrophoresis of ^{14}C-labeled molecular weight standards. Their positions are indicated to the right. The gel system consisted of a 3% stacking–5% separating gel.

itated by protein A-bearing *Staphylococcus aureus*, eluted, and analyzed by SDS-PAGE and fluorography.

RESULTS

Isolation of HA$^+$ revertants. In all but one case, the selective enrichment technique resulted in mycoplasma populations which were 5 to 10% HA$^+$ (no HA$^+$ revertants were isolated from class I'). The high percentage of HA$^-$ mycoplasmas in the erythrocyte-associated populations can probably be attributed to either aggregation of HA$^-$ mycoplasmas to erythrocyte-adherent HA$^+$ mycoplasmas, or the pelleting of larger clumps of HA$^-$ mycoplasmas during centrifugation on Methocel-Hypaque, or both. The failure to obtain an HA$^+$ revertant from the class I' mutant may be a reflection of the stability of the molecular event(s) leading to the HA$^-$ phenotype in that class.

One- and two-dimensional PAGE. Examination of total protein from the wild-type strain B25C and specific HA$^+$ revertant strains by one-dimensional SDS-PAGE revealed that their protein profiles were virtually identical, both in fluorograms (Fig. 1) and with Coomassie blue staining (data not shown). Note in particular the return to normal levels of the high-molecular-weight proteins HMW1-4 in the revertant of the class I mutant, and of P1 in the revertant of the class IV mutant. Two-dimensional PAGE analysis demonstrated that the revertants of the class III and IV mutants had regained proteins A, B, and C (data not shown). The comparison of protein profiles of the wild-type, mutant, and revertant strains is summarized in Table 1.

Assay of attachment of HA$^+$ revertants to tracheal rings. All HA$^+$ revertants attached to tracheal rings at levels comparable to that observed with the wild-type strain (Table 2), in contrast to the low level of adherence observed with the HA$^-$ mutants (19). Furthermore, attachment of wild-type and revertant strains exhibited equivalent sensitivities to neuraminidase pretreatment of the tracheal rings.

Examination of virulence of HA$^+$ revertants in hamsters. The hemadsorbing revertants were

TABLE 1. Summary of the protein profiles of the spontaneous HA$^-$ mutant classes and the homologous class-specific HA$^+$ revertants

Strain	Phenotype	Protein profile[a]							
		HMW1	HMW2	HMW3	HMW4	A	B	C	P1
M129-B25C	Wild type	+	+	+	+	+	+	+	+
Class I	HA$^-$ mutant	±	−	−	−	+	+	+	+
	HA$^+$ revertant	+	+	+	+	+	+	+	+
Class I'[b]	HA$^-$ mutant	±	−	−	−	+	+	+	+
Class II	HA$^-$ mutant	+	+	+	+	+	+	+	+
	HA$^+$ revertant	+	+	+	+	+	+	+	+
Class III	HA$^-$ mutant	+	+	+	+	−	−	−	+
	HA$^+$ revertant	+	+	+	+	+	+	+	+
Class IV	HA$^-$ mutant	+	+	+	+	−	−	−	−
	HA$^+$ revertant	+	+	+	+	+	+	+	+

[a] +, Protein present; ±, protein markedly deficient or a minor comigrating polypeptide present; −, protein absent.

[b] No HA$^+$ revertant isolated.

TABLE 2. Adherence of wild-type and HA$^+$ revertant *M. pneumoniae* to control and neuraminidase-treated hamster tracheal rings in vitro[a]

Strain	Adherence	
	% of control[b]	% Inhibition by neuraminidase[c]
M129-B25C[d] at 37°C	100	64
M129-B25C at 4°C[e]	22	
Class I-R[f]	102	55
Class II-R	116	66
Class III-R	94	62
Class IV-R	114	56

[a] Values represent the means from two separate experiments. All assays were performed in quadruplicate. The data were evaluated using the nonparametric Kurskall-Wallis analysis of variance (27). Differences observed among wild-type and revertant strains (at 37°C) were statistically insignificant ($P > 0.05$).

[b] % of control = [(revertant counts per minute bound/revertant counts per minute added)/(wild type counts per minute bound/wild type counts per minute added)] × 100%.

[c] % Inhibition = {1 − [(counts per minute bound to neuraminidase-treated rings/counts per minute added)/(counts per minute bound to control rings/counts per minute added)]} × 100%.

[d] Wild-type strain.

[e] Tracheal rings incubated with B25C at 4°C served as a negative control.

[f] Designation for HA$^+$ revertants from each class of HA$^-$ mutants.

TABLE 3. Titration of viable *M. pneumoniae* in hamster lungs after intranasal inoculation with wild-type or HA$^+$ revertant strains[a]

Strain	Inoculum[b]	Titer[c] at the following times postinfection	
		14 days	28 days
M129-B25C	6.98	6.46	6.06
Class I-R[d]	6.94	6.92	6.32
Class II-R	6.99	6.32	7.14
Class III-R	6.11	6.21	6.47
Class IV-R	6.84	6.31	6.38
Sham-infected control	0	0	ND[e]

[a] Data were evaluated using a two-factor analysis of variance. Differences between the means of wild-type and revertant titers were not statistically significant ($P > 0.05$).

[b] Log_{10} CFU per hamster.

[c] Log_{10} CFU per g of lung tissue; values represent the mean obtained from five hamsters per time point.

[d] Designation for HA$^+$ revertants from each class of HA$^-$ mutants.

[e] ND, Not done.

evaluated for their survival and growth in experimentally infected hamsters (Table 3). Mycoplasma titers in lung tissue of hamsters infected with wild-type or revertant strains were similar over the course of this study (Table 3). Slight differences observed were statistically insignificant ($P > 0.05$). These results are in contrast to data obtained with the HA$^-$ mutants, which did not survive in vivo (or survived at barely detectable levels in the case of the class II mutant) (19). Lung histopathology in wild-type-infected and HA$^+$ revertant-infected hamsters was similar. By day 14 postinfection significant histological pneumonia had developed in these animals. In contrast, lung lesion scores in HA$^-$ mutant-infected and sham-infected hamsters were approximately fourfold lower (data not shown) (19).

Peptide mapping and immunological analysis of proteins HMW1, 2, and 3. Results from a representative peptide mapping experiment involving HMW1-3 are shown in Fig. 2; insufficient quantities of HMW4 prevented its inclusion. Only limited proteolysis was observed with untreated controls (Fig. 2a), which might be attributed to the staining and destaining steps after protein separation in the first polyacrylamide gel (5). Because of the close proximity of HMW1-3 to other mycoplasma proteins, slight contamination could have occurred when the protein bands were sliced from the original acrylamide gel. Great care was taken in removing HMW1-3 from the gel; comparison with a mutant class I protein profile provided verification of the location of the appropriate proteins. With a low concentration (0.5 μg per well) of chymotrypsin, both similarities and differences appeared in the resultant peptide patterns (Fig. 2b, lanes A to C). With increasing concentrations of chymotrypsin, however, fewer similarities were observed (Fig. 2b to d). Note particularly the differences in the extent of proteolysis of HMW1-3 at the highest concentration of protease (Fig. 2d). Similar findings were obtained when papain (Sigma; type III, 0.25 μg per well) was substituted for chymotrypsin (data not shown).

RIP analysis of antisera raised against mycoplasma proteins HMW1 and HMW3 is shown in Fig. 3. Anti-HMW1 antiserum precipitated both HMW1 and HMW4, but did not react with HMW2 (molecular weight intermediate to HMW1 and HMW3, see Fig. 1) or HMW3 (Fig. 3a, lane C). That the single intense band seen in lane C of Fig. 3a consisted of the two high-molecular-weight proteins HMW1 and HMW4 was determined by examining a very brief exposure of the fluorogram. Anti-HMW3 antibodies reacted strongly with protein HMW3, but failed to precipitate any of the other higher-molecular-weight proteins (Fig. 3b, lane C). Attempts to raise specific anti-HMW2 serum were unsuccessful.

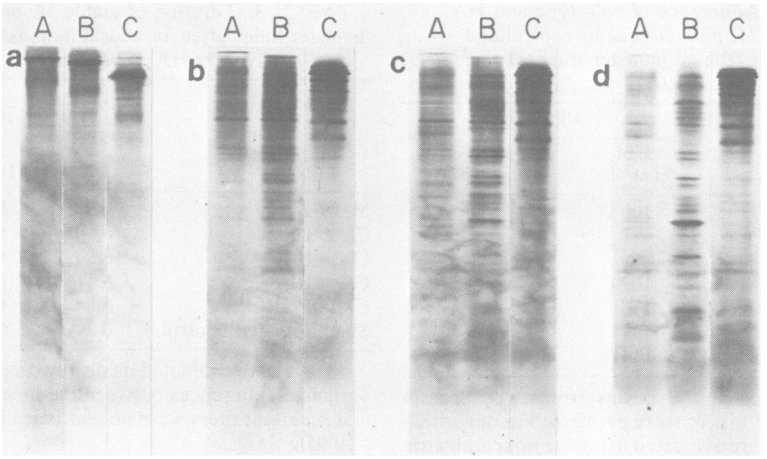

FIG. 2. Peptide mapping analysis of *M. pneumoniae* proteins HMW1-3. (a) Control profiles without protease; (b to d) profiles after treatment with 0.5, 1.25, and 2.5 μg of chymotrypsin, respectively. Lane: A, HMW1; B, HMW2; C, HMW3. The gel system consisted of a 3% stacking–12% separating gel. Protein bands were visualized by the silver stain (20).

DISCUSSION

The return of the capacity of HA^- *M. pneumoniae* to hemadsorb was accompanied by restoration of (i) a normal complement of mycoplasma proteins, (ii) the ability to adhere to neuraminidase-sensitive sites on the respiratory epithelium, and (iii) the ability to survive in vivo and produce pneumonia in experimentally infected hamsters. The biochemical changes in the class II mutants responsible for the loss of hemadsorption and virulence remain undefined. It is apparent, however, that the alterations were not irreversible, as a virulent HA^+ revertant was successfully isolated from this mutant class.

These data confirm the association of specific mycoplasma proteins with cytadsorption and virulence. With the exception of protein P1, which mediates adherence of *M. pneumoniae* to host cells (2, 16, 18; D. C. Krause and J. B. Baseman, Infect. Immun., in press), the precise roles in cytadsorption of the specific proteins described herein remain undefined. Other data suggest that some (or all) of these proteins may interact with P1 to maintain the proper distribution, disposition, or both, of P1 (2).

It should be noted that the correlation between cytadsorption and virulence is not absolute. A HA^+ revertant of a nitrosoguanidine-derived HA^- mutant is capable of normal levels of neuraminidase-sensitive adherence to the respiratory epithelium. However, this strain survives in vivo only at intermediate levels and produces no significant pneumonia (14, 21). The factor(s) responsible for reduced virulence in

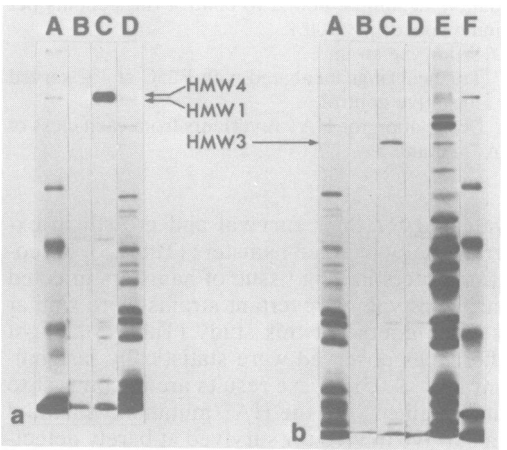

FIG. 3. RIP analysis of the relatedness of *M. pneumoniae* proteins HMW1-3 using anti-HMW1 (a) and anti-HMW3 (b) antisera. In part (a) lane A contains the ^{14}C-labeled molecular weight standards: myosin (200,000), phosphorylase B (92,500), bovine serum albumin (68,000), ovalbumin (43,000), and α chymotrypsinogen (25,700); lanes B and C are RIP profiles obtained with prebleed (lane B) and immune (lane C) anti-HMW1 serum; lane D is the profile of total radiolabeled *M. pneumoniae* protein. In part (b) lane A contains total radiolabeled *M. pneumoniae* protein; lanes B and C are the RIP profiles obtained with prebleed (lane B) and immune (lane C) anti-HMW3 antiserum; lanes D and E are the RIP profiles obtained with prebleed serum (lane D) or with antiserum from a rabbit immunized with *M. pneumoniae* (lane E); lane F contains the ^{14}C-labeled molecular weight standards as in part (a), lane A. The gel system consisted of a 3% stacking–7.5% separating gel.

vivo has not been identified. This observation in no way undermines the importance of adherence in virulence. Rather, it underscores the complexity of the host-parasite interaction and the need for spontaneously arising mutants and their homologous revertants for studies on pathogenicity.

After confirming the association of several specific mycoplasma proteins with cytadsorption and virulence, we attempted to probe the biochemical relationship of certain of the proteins described. The concurrent loss and return of groups of proteins in mutant and revertant mycoplasmas were intriguing, particularly in the case of the high-molecular-weight proteins (HMW1-4). Due to the small genome size, and, hence, limited biosynthetic potential of *M. pneumoniae*, it seemed unlikely that these four proteins were in fact distinct polypeptides, as this would require the commitment of a relatively large amount of genetic information for cytadsorption. Therefore, we felt there might exist a precursor-product relationship among at least some of these proteins. The peptide mapping data indicate, however, that proteins HMW1-3 are in fact distinct polypeptides, with no apparent precursor-product relationship. The observed similarities in the peptide patterns of HMW1-3 at low concentrations of protease may have been coincidental or may reflect minor parallels in primary structure associated with similar functions. The observations that antibodies to HMW1 fail to precipitate HMW2 or HMW3 and that antibodies to HMW3 do not react with HMW1 or HMW2 are consistent with the peptide mapping data. It is not clear why anti-HMW1 antibodies precipitated protein HMW4. It is possible that these two proteins were not separated sufficiently during purification of HMW1 for immunization, and as a result, antibodies were also raised against HMW4. Alternatively, these two large polypeptides might be structurally related. Clarification of this matter, as well as evaluation of the relationship between proteins A, B, and C, will require further investigation.

In conclusion, these results verify the importance of a number of mycoplasma proteins in cytadsorption and virulence. Additionally, the data reinforce the complexity of mycoplasma attachment and virulence and demonstrate the usefulness of spontaneously arising HA^- mutants and their homologous HA^+ revertants in the molecular characterization of mycoplasma disease pathogenesis.

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