Adherence of *Mycoplasma gallisepticum* Involves Variable Surface Membrane Proteins

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Received 10 October 1996/Returned for modification 10 December 1996/Accepted 4 March 1997

Adherence of *Mycoplasma gallisepticum* to erythrocytes was examined by colony immunoblotting, detergent phase fractionation, trypsin treatment, comparison of protein profiles, and comparison of erythrocyte-bound mycoplasma protein fractions of hemadsorption-positive and -negative mutants. The binding of *M. gallisepticum* to chicken or human erythrocytes was found to be mediated via surface-exposed membrane proteins undergoing high-frequency phase variation.

Mycoplasmas are a large group of diverse prokaryotic species comprising the class *Mollicutes*. Mycoplasmas lack a cell wall, carry a remarkably small genome, are phylogenetically related to gram-positive coccus bacteria, and are the smallest known self-replicating organisms (21, 22). Mycoplasmas are parasites in a wide range of hosts and typically cause persistent infections in humans and animals (27, 29). The infection process of the majority of human and animal pathogenic mycoplasmas involves colonization of the epithelial linings of the respiratory and urogenital tracts. An essential and initial step in the establishment of infection leading to colonization is the adhesion of the mycoplasmas to the host target cells (cytadherence). Many important pathogenic mycoplasma species have a flask-like shape with a tiny tip at one of the poles. This differentiated organelle, termed the tip organelle or terminal structure, functions as an attachment organelle (3, 5, 9, 23).

The critical role of cytadherence in virulence is reflected by the inability of noncytadhering mycoplasma strains to cause disease in experimentally infected animals (7, 12, 13, 16). Previous studies have shown that hemadsorption, the adherence of erythrocytes to mycoplasma cells, is a characteristic of several mycoplasma species (17, 24) and is an experimental indicator for the attachment capacity of the human pathogen *Mycoplasma pneumoniae* (3, 12, 13). The findings that (i) receptors for *M. pneumoniae* and for the avian pathogen *Mycoplasma gallisepticum* on both respiratory epithelial cells and erythrocytes contain sialic acid residues (2, 3, 18), (ii) trypsin pretreatment of mycoplasma cells eliminates attachment to both cell types (23), and (iii) *M. pneumoniae* mutants that lose their ability to adhere to and colonize the hamster respiratory epithelium also lose their ability to hemadsorb (7, 12, 13) suggest that at least these two species employ the same mechanism for attachment to the respiratory epithelium and to erythrocytes. Erythrocytes have been, therefore, the most frequently used model for studying mycoplasma adherence. The hemadsorption assay (17) enabled the identification of hemadsorption-negative (HA−) mutants of *M. pneumoniae* (7, 8, 12). The appearance of these mutants was spontaneous, and comparison of their protein profiles to those of mutants with HA+ phenotype revealed the absence of several proteins (11). Spontaneous reacquisition of the missing protein(s) resulted in reversion to the adherence-positive phenotype (12, 13). The appearance of the nonadhering mutants of *M. pneumoniae*, which occurred at a high frequency (12, 13), suggests that hemadsorption-mediating proteins in *M. pneumoniae* are subject to phenotypic switching.

A well-established test for determining whether a particular surface antigen undergoes high-frequency phenotypic switching is the colony immunoblot technique. Immunostaining with monoclonal or polyclonal antibodies allows the identification of colonies exhibiting variation in the expression of surface proteins (25, 26). One of the most conspicuous ways this heterogeneity takes shape in in vitro studies is by colony sectoring (25). A sector is defined as an immunologically distinct region in which a change in protein expression has occurred within a single colony. A typical feature of cells recovered from the sectoral region is the ability to generate colonies predominantly with the sectoral phenotype. Within that population, colonies exhibiting the nonsectoral phenotype can also be found, indicating high-frequency switching of the corresponding protein.

This study links high-frequency phenotypic switching of variable surface membrane proteins and hemadsorption in *M. gallisepticum*, a respiratory pathogen of chickens and turkeys (32). We examined the possibility that the attachment of erythrocytes via a variable surface membrane protein(s) could be identified by the selective adherence of the erythrocytes to organisms within a single mycoplasma colony, thus generating a typical sector. *M. gallisepticum* AS969 was chosen for this purpose. This strain exhibits a predominant HA− phenotype which is consistent with its avirulent properties, e.g., its inability to colonize the natural host (15). However, within colonies of this organism, variants exhibiting the HA+ phenotype could be easily detected at a high frequency, 10−2 to 10−3 (Fig. 1A). During the screening of these colonies for their ability to hemadsorb, we discovered that in some colonies human or chicken erythrocytes bound selectively to a distinct portion of the mycoplasma colony, generating a distinguishable sector (Fig. 1). To our knowledge, this is the first report showing sectoral adherence of erythrocytes to mycoplasma colonies. This finding suggests that hemadsorption in *M. gallisepticum* involves the switching of a variable surface protein(s). The protein profiles associated with the two HA phenotypes were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting (14, 28) with serum raised in mice against total proteins of an *M. gallisepticum* AS969 clonal isolate exhibiting the HA+ pheno-
type (Fig. 2A). At least five proteins (p30, p48, p50, p80, and p82) were identified exclusively in the variant with the HA\(^{-}\) phenotype (Fig. 2A, lane 2), whereas two proteins (p69 and p72) were associated with the HA\(^{+}\) phenotype (Fig. 2A, lane 1). To confirm that the appearance of these proteins correlates with the hemadsorption phenotype and is not due to the random antigenic variation known to occur in several mycoplasma species (31), a lineage of clonal isolates of \textit{M. gallisepticum} A5969 representing hemadsorption phase transition during several generations, i.e., HA\(^{-}\) → HA\(^{+}\) → HA\(^{-}\), was generated (Fig. 2B). Total proteins of these isolates were analyzed by Western immunoblotting with the HA\(^{+}\)-clone-specific antiserum. The appearance of five proteins (p30, p48, p50, p80, and p82) correlated with the HA\(^{+}\) phenotype in the three consecutive clones tested (Fig. 2B, lanes 2 and 3). The HA\(^{-}\) phenotype was characterized by two proteins, p69 (also associated with the HA\(^{+}\) phenotype) and p72 (Fig. 2B, lanes 1 and 4). The reaction of p69 and p72 with the HA\(^{-}\)-clone-specific antiserum indicates the antigenic relatedness of these two HA\(^{-}\)-phenotype-related proteins to proteins expressed uniquely in the HA\(^{+}\) variant. The p69 polypeptide was also recognized by a polyclonal anti-pMGA serum (kindly provided by P. F. Markham, University of Melbourne, Melbourne, Australia) and apparently is pMGA, a surface agglutinin protein of \textit{M. gallisepticum} (6, 19). However, the p30, p48, p50, and p80 polypeptides were not detected by that serum (data not shown).

To further characterize proteins which were associated with the HA\(^{-}\) phenotype (Fig. 3), Triton X-114 (TX-114) phase fractionation was performed as described previously (4). This fractionation was performed as described previously (4). This
procedure differentiates amphiphilic integral proteins from hydrophilic cytoplasmic proteins. Polypeptides p30, p48, p50, and p80 were shown to be membrane proteins with amphiphilic properties. This was demonstrated by their exclusive partitioning into the TX-114 phase (Fig. 3A, lane 2). In addition, intact organisms from a mid-logarithmic-phase culture were treated with graded amounts of trypsin by a previously described method (4). This was done in order to determine whether these proteins are exposed on the cell surface. Western immunoblot analysis of trypsin-treated or untreated cells with preadsorbed HA\(^{-}\)-clone-specific antiserum (Fig. 2C) is shown in Fig. 3B. Increasing amounts of trypsin resulted in the complete disappearance of these proteins and the generation of epitope-bearing degradation products (Fig. 3B, lanes 2 to 5). The presence of other proteins that remained relatively unaltered during digestion (Fig. 3B, lanes 1 to 4) indicated the integrity of cells and demonstrated the selective susceptibility to trypsin and the surface localization of the p30, p48, p50, and p80 proteins.

Thus far, the data suggested that p30, p48, p50, and p80 are integral surface-exposed membrane proteins expressed only in variants with the HA\(^{-}\) phenotype and that they thus may be responsible for adherence to erythrocytes. At this point, we examined the binding of the detergent-solubilized _M. gallisepticum_ (HA\(^{-}\)) preparation to glutaraldehyde-fixed human erythrocytes, as previously described (10, 11), with a modification of the solubilization buffer as described by Krause and Baseman (11). Briefly, mycoplasmas from a mid-logarithmic-phase culture (equivalent to 1 mg of cell proteins) were harvested by centrifugation, rinsed twice with phosphate-buffered saline, and suspended in 750 \(\mu\)l of solubilization buffer. After incubation for 30 min at 37°C, the cell extract was centrifuged at 30,000 \(\times\) g for 20 min at 4°C to remove insoluble material. The supernatant was incubated with 5% fixed erythrocytes for 24 h at 4°C. Erythrocytes were pelleted and washed extensively three times with solubilization buffer to remove nonspecific bound membrane components. Bound proteins were eluted by boiling the preparation for 3 min in a solution containing 62.5 mM Tris (pH 6.8), 2% (vol/vol) \(\beta\)-mercaptoethanol, 2% SDS, 10% (vol/vol) glycerol, and 0.003% bromophenol blue. The eluted fraction was concentrated by lyophilization and analyzed by SDS-PAGE and Western immunoblotting with the HA\(^{-}\)-clone-specific antiserum preadsorbed with total proteins of the variant with the HA\(^{-}\) phenotype, a process which eliminates protein bands common to variants with the two phenotypes (Fig. 2C, lane 2). Two proteins, p50 and p80, were identified in the eluted fraction from the fixed erythrocytes (Fig. 4A, lane 2). These proteins corresponded to the p50 and p80 products, which were shown to be surface-exposed membrane proteins exclusively expressed in the variant with the HA\(^{-}\) phenotype (Fig. 4A, lane 1; also Fig. 2). No proteins were detected in the fraction eluted from the fixed erythrocytes alone or after incubation with HA\(^{-}\) cells prepared in the same way (data not shown). Antiserum raised against the HA\(^{-}\) eluted fraction containing the p50 and p80 proteins was used to immunostain SDS-PAGE-separated total proteins or colonies of the HA\(^{-}\) phenotype (Fig. 4B, lane 1, and 4C) or of the HA\(^{+}\) phenotype (Fig. 4B, lane 2, and 4D). Interestingly, in addition to p50 and p80 products, the p50/p80 antiserum recognized two additional proteins: an invariant 65-kDa protein, present in variants with either HA phenotype, and the p48 protein, shown to be expressed only in the HA\(^{-}\) isolate (Fig. 4B).

Immunostaining of _M. gallisepticum_ HA\(^{-}\) or HA\(^{+}\) colonies (Fig. 4C and D, respectively) also revealed within the expected predominant population cells with antigenically positive or negative phenotypes. Some of these phenotypes were accompanied by a sector (Fig. 4D). Taken together, our results suggest that the cytadherence of _M. gallisepticum_, as monitored by the ability of the mycoplasma colonies to hemadsorb, involves several surface membrane proteins undergoing high-frequency phenotypic switching. Cytadherence phase variation is not unique to mycoplasmas and has been described for a variety of bacterial pathogens of mucosal surfaces, among them, _Escherichia coli_, _Neisseria gonorrhoeae_, and _Bordetella pertussis_ (1, 20, 30).

However, it is not yet known whether phenotypic switching of mycoplasma cytadherence as monitored in vitro also occurs in vivo during natural infections. Variation of attachment proteins may facilitate adherence and colonization of the mycoplasmas in the appropriate niche, or such variation enables exit from the host as a first step in initiating a new infection. The identification during hemadsorption phase transition of several proteins unique to the HA\(^{-}\) phenotype (p30, p48, p50, and p80) or the HA\(^{+}\) phenotype (p72) or of proteins expressed at different levels in variants with the two phenotypes (p69) underscores the multifactorial nature of the cytadherence process. The approach described here will be useful in the cloning of the corresponding cytadherence variable genes of _M. gallisepticum_.

FIG. 4. (A) Selective binding of detergent-solubilized proteins of _M. gallisepticum_ A5969 to glutaraldehyde-fixed human erythrocytes. Lane 1, detergent-soluble fraction containing the p48, p50, and p80 proteins, as indicated; lane 2, erythrocyte-bound fraction containing the p50 and p80 proteins, as indicated. (B) Western blot analysis of total proteins from HA\(^{+}\) (lane 1) or HA\(^{-}\) (lane 2) isolates with anti-mouse p50/p80 serum. Proteins p48, p50, p65, and p80 are indicated. (C and D) Phase variation in the expression of p48, p50, and p80 in _M. gallisepticum_ A5969 HA\(^{+}\) (C) or HA\(^{-}\) (D) populations. Imprints of colonies were transferred to nitrocellulose filters and immunostained with the anti-mouse p50/p80 serum. Colonies with the positive (open arrows) and negative (solid arrows) phenotypes are indicated. A positive sector within a negative colony is indicated by an arrowhead.
plasma gallisepticum, leading to a better understanding of the molecular mechanism of its adherence and pathogenicity.

This work was supported by a grant from the Israel Science Foundation (D.Y.). A.A. was a recipient of a postdoctoral fellowship from the Golda Meir Foundation. R.R. was supported by a fellowship (Ro739/5-1 and 5-2) from the Deutsche Forschungsgemeinschaft.

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Editor: S. H. E. Kaufmann