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NOTES

Isolation of Mutants of *Mycoplasma pneumoniae* Defective in Hemadsorption

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Mutants of *Mycoplasma pneumoniae* incapable of hemadsorption were isolated by means of chemical mutagenesis. These hemadsorption-negative mutants did not attach to hamster tracheal rings in vitro at as high a frequency as that exhibited by the wild-type parent strain.

Mycoplasma pneumoniae is an important cause of human respiratory disease, as the etiological agent of primary atypical pneumonia. The mechanism(s) by which this organism mediates its pathologic effects are just beginning to be identified (4). It has been recognized for some time that *M. pneumoniae*, as well as some other *Mycoplasma* species, possess the ability to attach firmly to various types of epithelial cells (10). Studies employing tracheal organ culture have shown that attachment of viable *M. pneumoniae* to respiratory epithelium is required for the subsequent disruption of host cell metabolism and structural integrity (4). A proteinaceous component on the mycoplasma cell membrane has been implicated in this attachment process (5).

The majority of *M. pneumoniae* receptor sites on tracheal epithelial cells have been indirectly shown to contain sialic acid residues (9, 10). Similarly, sialic acid-containing receptor sites have been implicated in the binding of erythrocytes to colonies of virulent *M. pneumoniae*, in a process called hemadsorption (10). These data, coupled with the fact that trypsin-treated colonies of *M. pneumoniae* are unable to adsorb either erythrocytes or tracheal epithelial cells (10), suggested that *M. pneumoniae* might utilize the same mechanism(s) for attachment to both cell types. To further evaluate the relationship of the hemadsorption process to respiratory epithelium attachment by *M. pneumoniae*, we have isolated mutants of *M. pneumoniae* which fail to hemadsorb.

The virulent M129 strain of *M. pneumoniae* was grown in Hayflick medium at 37°C (6). Glass-attached organisms were exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a final concentration of 25 μg/ml in pH 6.7 phosphate-buffered saline for 30 min, which resulted in 95% mortality among the exposed cells. The mycoplasmas were then rinsed three times with pH 7.2 phosphate-buffered saline and scraped into 10 ml of Hayflick broth. A small portion (0.2 ml) of this suspension was incubated in fresh Hayflick broth for 96 h, at which time glass-adherent organisms were scraped into the spent growth medium. Serial dilutions of these cells were plated on PPLO agar plates (6) and incubated at 37°C for 8 days.

Hemadsorption-negative colonies were identified essentially by the method of Sobeslavsky et al. (10). The use of a Glarex optical projection screen (Carl Zeiss, 7082 Oberkochen, West Germany) coupled to a Zeiss WL microscope greatly facilitated the identification of hemadsorption-negative colonies, because it permitted rapid viewing of large numbers of the extremely minute mycoplasma colonies for their hemadsorption characteristics (Fig. 1). Tentative hemadsorption-negative mutants were purified by three successive applications of a single-colony isolation method involving suspension of the selected colony in broth, followed by filtration through a 0.45-μm filter, and subsequent serial dilution and plating of the filtrate.

Two hemadsorption-negative mutants were isolated from each of two independent mutagenesis experiments. These mutants were identified as *M. pneumoniae* by the use of specific antisera and by their characteristic beta-hemolysis on agar plates overlaid with sheep erythrocytes (2). Evaluation of the ability of these mutants to attach to hamster tracheal rings in vitro (9) showed that none of these mutants could adsorb to the respiratory epithelium at as great a fre-

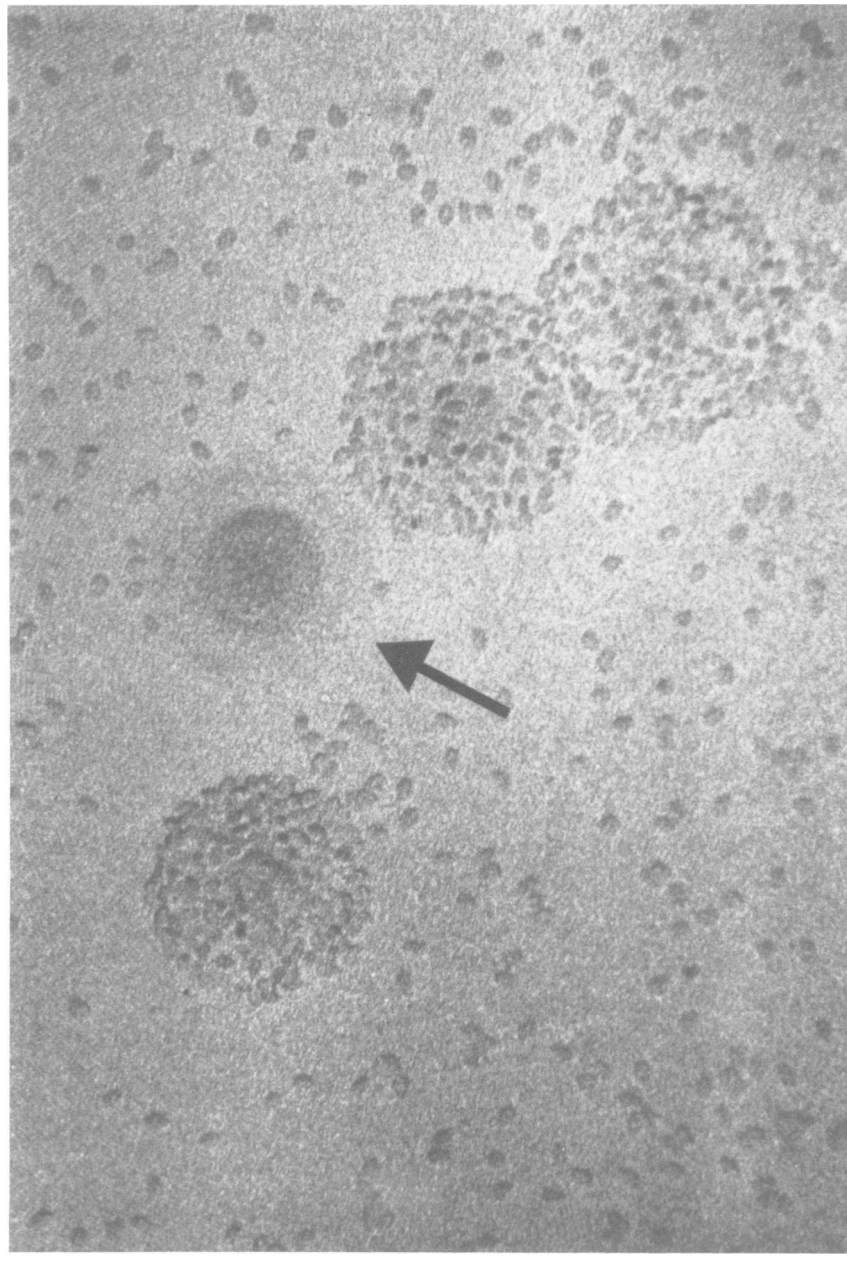

FIG. 1. *Photograph of image on Glarex viewing screen showing the hemadsorption characteristics of colonies of the wild-type parent strain and the hemadsorption-negative mutant strain HA2. Arrow indicates the hemadsorption-negative colony.*

quency as the wild-type parent strain (Table 1). Mutant strains HA2 and HB2 were found to attach to tracheal rings at a frequency about half as great as that of the wild-type strain. Mutant strains HA1 and HB1 exhibited attachment frequencies which were only slightly higher than the "background level" of adsorption obtained with wild-type *M. pneumoniae* incubated with tracheal rings at 4°C (9).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5) of wild-type and mutant cell proteins revealed that the protein profiles of mutant strains HA1 and HB1 were essentially identical to that of the wild-type strain (Fig. 2).

Mutant strains HA2 and HB2 differed from both the wild-type strain and the other mutants by their lack of a single protein with a molecular weight of 190,000. Correlation of these gel data with tracheal ring attachment data showed that the independently isolated mutants HA2 and HB2 both lacked the same high-molecular-weight protein and attached to tracheal rings at a higher frequency than did mutant strains HA1 and HB1. The exact relationship, if any, of this particular high-molecular-weight protein to the hemadsorption process is under investigation. The fact that the hemadsorption-negative mutant strains HA1 and HB1 apparently possess the same protein composition as the hemadsorption-positive wild-type strain indicates that components of the mycoplasma membrane surface other than proteins (3) may be involved in the hemadsorption process.

Strains of *M. pneumoniae* which were unable to hemadsorb have been previously isolated by indirect means (1, 7). All of these strains, however, possessed other significant differences from their parent strains apart from their hemadsorption characteristic. The use of the hemadsorption-negative mutants isolated in this study should contribute to the elucidation of the relationship between the hemadsorption process and respiratory epithelium attachment capability of *M. pneumoniae*. The data presented in this report indicate that a total lack of hemadsorption capability does not completely eliminate the ability of *M. pneumoniae* to attach to tracheal rings in vitro. Evaluation of the virulence of these mutants in both hamsters and tracheal organ culture systems is currently in progress.

TABLE 1. *Attachment of wild-type and mutant strains of M. pneumoniae to hamster tracheal rings*[a]

Strain	CFU added to ring[b]	cpm added to ring	cpm attached to ring	% Attachment
Wild-type M129	3.9×10^7	20,312	1,970	9.7
HA1[c]	8.5×10^7	23,780	546	2.3
HA2	3.8×10^7	23,682	1,442	6.1
HB1[c]	8.2×10^7	26,347	658	2.5
HB2	1.0×10^8	24,890	1,343	5.6
M129/4°C	3.9×10^7	20,312	302	1.4

[a] Tracheal ring attachment was monitored by the use of *M. pneumoniae* labeled with [*methyl*-^{3}H]thymidine (9).

[b] Colony-forming units (CFU) of wild-type and mutant strains were determined by plating serial dilutions of mycoplasmas on PPLO agar (2, 6). These data represent the mean from two separate experiments.

[c] HA series mutants and HB series mutants were isolated in independent mutagenesis experiments.

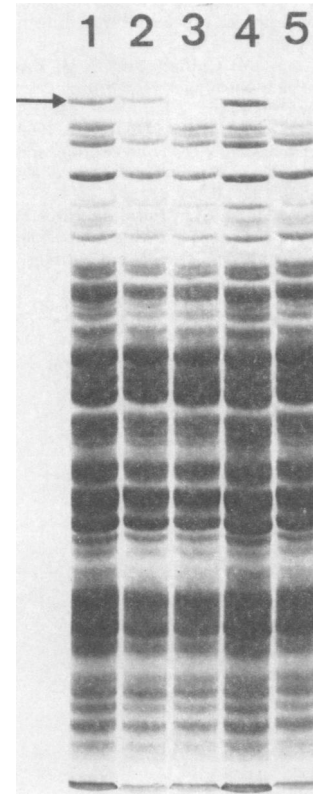

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of proteins from wild-type and mutant strains. The gel system was composed of a 4% polyacrylamide stacking gel and a 7.5% polyacrylamide separating gel. (1) M129 wild-type strain; (2) HA1; (3) HA2; (4) HB1; (5) HB2. Arrow indicates 190,000-molecular-weight protein band which is missing in strains HA2 and HB2. The molecular weight of this protein was determined by co-electrophoresis of known molecular weight standards.*

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