Analysis of Polypeptides of Mutants of *Mycoplasma pneumoniae* that Lack the Ability to Haemadsorb

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Haemadsorption-negative mutants of *Mycoplasma pneumoniae* were isolated which varied in their capacity to adsorb erythrocytes of various animal species suggesting adherence to erythrocytes is mediated by different binding mechanisms. Trypsin treatment of the wild-type strain resulted in loss of haemadsorbing activity; several polypeptides, some of which regenerated with haemadsorbing activity following further incubation, were also trypsin sensitive. The haemadsorption-negative mutants could be divided into two groups according to their polypeptide pattern. In the first group (11 mutants) the PAGE pattern was identical to that of the wild-type strain. The second group comprised 7 mutants which differed from the wild-type by lack of one or more polypeptides with molecular weights of 190000, 90000 or 40000. During growth attachment to glass was weak or absent in the mutants. Surface hydrophobicity as measured by hydrophobic-interaction chromatography was nearly comparable in mutants and parent strain.

INTRODUCTION

Mycoplasma pneumoniae is a surface parasite adhering to and colonizing the epithelial linings of the human respiratory tract. Recent data provide evidence that *M. pneumoniae* adherence is mediated by polypeptides (Feldner *et al.*, 1979*a*; Gorski & Bredt, 1977; Hu *et al.*, 1977; Hu *et al.*, 1982; Krause *et al.*, 1982). Several approaches are possible to characterize the mycoplasma membrane components responsible for adherence: (i) isolation of binding sites from solubilized membranes (Banai *et al.*, 1980; Razin *et al.*, 1981); (ii) affinity chromatography using antibodies or receptor components as immobilized reaction partners; or (iii) examination of non-adhering mutants for potential differences from the parent strain (Hansen *et al.*, 1979; Hansen *et al.*, 1981). In the experiments described in this paper, haemadsorption-negative mutants of *M. pneumoniae* were tested for their adherence spectrum. Furthermore, we describe the reappearance of certain proteins after trypsin treatment and the reduction of polypeptide synthesis after glucose treatment of the mycoplasmas.

METHODS

Isolation of haemadsorption-negative mutants of M. pneumoniae. The FH strain of *M. pneumoniae* was grown in Hayflick medium as described previously (Feldner *et al.*, 1979*a*). According to Hansen *et al.* (1979), glass-attached mycoplasmas were exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Serva, Heidelberg, F.R.G.) at a final concentration of 25 µg ml⁻¹ in pH 6.7 phosphate-buffered saline for 30 min, which resulted in a 99% reduction in the number of c.f.u. After washing with fresh medium, serial dilutions of these cells were plated onto agar medium and incubated at 37 °C for 6 to 10 d. The agar plates were then overlaid with sheep erythrocytes (Manchee & Taylor-Robinson, 1968) for 30 min at 37 °C and examined microscopically for the presence of haemadsorption-negative colonies (Fig. 1). Tentative mutants were either picked from the agar with a tiny spatula or some material was removed with a platinum loop. The selected colonies were suspended in growth medium and finally purified

Abbreviations: GPE, Guinea pig erythrocytes; HE, human erythrocytes; RE, rabbit erythrocytes; SE, sheep erythrocytes.

Fig. 1. Photograph of colonies of the wild-type strain and of a haemadsorption-negative mutant strain. The bar marker represents 10 μ m.

by three successive cloning steps including filtration through a 0.4 μ m polycarbonate filter (Nuclepore Corp.) followed by plating of the diluted filtrate. The mutants were identified as *M. pneumoniae* by a growth inhibition test with specific antiserum and by their characteristic beta-haemolysis of sheep erythrocytes (Hansen *et al.*, 1979). The mutants and the wild-type strain were equally susceptible to erythromycin. Originally, more than 100 haemadsorption-negative mutants could be isolated in one mutagenesis experiment. During the cloning procedure, however, most of the mutants reverted to the haemadsorption-positive condition.

Assessment of mycoplasma adherence to erythrocytes. Adherence of [3 H]palmitic acid-labelled *M. pneumoniae* cells (Feldner *et al.*, 1979b) to erythrocyte suspensions was carried out as described previously (Banai *et al.*, 1978; Kahane & Bredt, 1983).

Hydrophobic interaction chromatography. Retention of [3 H]palmitic acid-labelled wild-type or mutant strains was measured as described elsewhere in detail (Jann *et al.*, 1981). Briefly, a sample (100 μ l) of a mycoplasma suspension filtered through a 0.6 μ m polycarbonate filter was placed on a Pasteur pipette packed with 1 ml of phenyl-Sepharose. The column was equilibrated with 10 mM-phosphate buffer (pH 6.8) and washed with the same buffer containing 1 M-ammonium sulphate. The radioactivity of the eluate was measured by scintillation spectrometry as described before (Feldner *et al.*, 1979a) and compared to that of the original mycoplasma suspension, i.e. 100 μ l in 5 ml of the phosphate buffer containing 1 M-ammonium sulphate. The radioactivity value of the original suspension was set at 100%.

Trypsin treatment of M. pneumoniae. Glass-grown *M. pneumoniae* wild-type cells were scraped into 1 ml of growth medium which was supplemented with 250 μ Ci of L-[35 S]methionine (ml medium) $^{-1}$ [specific activity, 1215 Ci mmol $^{-1}$ (44.95 TBq mmol $^{-1}$); Amersham Buchler, F.R.G.]. The organisms were incubated for 4 h at 37 $^{\circ}$ C. After two washings in Tris buffer (Feldner *et al.*, 1979a) the cells were resuspended in 1 ml of the same buffer. Trypsin (Feldner *et al.*, 1979b) was added to the mycoplasmas to a final concentration of 10 μ g (mg cell protein) $^{-1}$. After incubation (30 min at 37 $^{\circ}$ C) and subsequent inhibition of trypsin (Feldner *et al.*, 1979b), the suspension was washed once in Tris buffer and resuspended in 1 ml of growth medium containing 250 μ Ci [35 S]methionine. The organisms were incubated for 5 h at 37 $^{\circ}$ C, washed twice in Tris buffer and finally processed

Table 1. *Adherence characteristics of haemadsorption-negative mutants of M. pneumoniae*

Haemadsorption tests were performed with sheep erythrocytes (SE), rabbit erythrocytes (RE), guinea pig erythrocytes (GPE) and human erythrocytes (HE).

Mutant	Adherence to mutant colonies*				Adherence of mutants in suspension test			Glass adherence*	Mol. wt of proteins missing†
	SE	RE	GPE	HE	SE	RE	GPE		
MpFH (wild-type)	+++	+++	+++	+++	100‡	100‡	100‡	+++	—
2	—	—	—	—	1.0	NT	4.6	+	—
8	+++	—	+++	+	85	11.3	28	++	90 000
14	—	(+)	—	—	0	NT	NT	—	90 000 190 000
17	—	—	+++	—	0.5	0.6	1.2	—	—
22	—	—	—	—	NT	NT	NT	—	40 000
39	—	—	+++	—	0.9	NT	0.8	+	—
42	—	—	—	—	8.5	NT	NT	+	—
45	—	+++	—	—	12.0	0.1	0.2	(+)	—
60	—	—	—	—	NT	NT	NT	++	—
98	—	—	—	—	8.1	NT	NT	—	90 000
115	—	+++	—	—	0	0.2	0.1	+	90 000
120	—	—	+++	—	1.8	2.7	2.8	—	—
122	—	—	—	—	7.5	0.8	0.8	(+)	—
166	—	—	(+)	—	10.0	NT	NT	(+)	190 000
171	—	—	—	—	NT	NT	NT	—	—
180	+	+++	+++	+++	72.0	NT	NT	(+)	—
188	—	++	(+)	—	0.8	NT	NT	—	90 000
191	—	+++	+++	—	0	0.3	0.9	—	—

NT, Not tested.

* Adherence was scored visually on a — to +++ basis.

† The molecular weight of these proteins was determined by co-electrophoresis of known molecular weight standards.

‡ Adherence of the wild-type parent strain to SE, RE and GPE was set at 100% being equivalent to 2449, 2690 and 2352 c.p.m., respectively.

for SDS-gel electrophoresis according to Laemmli (1970). The gels were then impregnated with En³hance (New England Nuclear, Boston, Mass., U.S.A.) and, after drying, were exposed at —70 °C using Kodak X-Omat R X-ray film.

Incorporation of [³⁵S]methionine into mycoplasmas. Freshly harvested *M. pneumoniae* cells were washed three times in Tris buffer, suspended in a small volume of the same buffer and divided into two 1 ml portions. 250 µCi [³⁵S]methionine (ml mycoplasmas)^{−1} was added. Finally, attachment-stimulating amounts of glucose (0.5 mg ml^{−1}) were added to one vial and attachment-repressing (Feldner *et al.*, 1981*b*) amounts of glucose (5 mg ml^{−1}) to the other. After incubation (3 h at 37 °C), the cells were washed three times in Tris buffer and processed for the SDS-gel electrophoresis. The gels were then impregnated and exposed as described above.

RESULTS

Characteristics of haemadsorption-negative mutant strains

Mutants grew distinctly slower than the wild-type strain. The ability of these mutants to grow and to adhere to glass surfaces (Table 1) was either considerably reduced (10 strains) or completely abolished (8 strains). By the use of SDS-gel electrophoresis the mutants could be grouped into two different classes. The protein profiles of 11 strains were essentially identical to that of the wild-type (e.g. 120 in Fig. 2), whereas 7 strains differed from it by their lack of either a single protein or of several proteins with molecular weights of 190 000, 90 000 or 40 000 (Table 1 and Fig. 2). The mutants varied in their capacity to adhere to erythrocytes from various species (Table 1). Nine mutants showed no adherence of erythrocytes from sheep (SE), rabbits (RE), guinea-pigs (GPE) or humans (HE). Mutants 45 and 115 adhered only to RE, whereas strain 191 adhered only to RE or GPE. Originally, strain 8 was SE-negative but lost this property after

Fig. 2. SDS-PAGE patterns of wild-type strain FH (WT) and mutant strains (22, 120, 115, 166).

several passages in liquid medium, probably due to a back mutation event which apparently did not affect its RE-negative adherence characteristics.

The visual estimation of adherence of the mutant strains on agar plates was compared with a quantitative adherence assay in which mycoplasmas and erythrocytes interacted in suspension. By this method, rather low radioactivity values were obtained with mutants (17, 39, 45, 115, 120, 191) for which positive adherence was found by the agar colony haemadsorption technique (Table 1).

The *M. pneumoniae* wild-type parent strain bound avidly to hydrophobic gels. Of the

Fig. 3. Electrophoretic analysis of *M. pneumoniae* wild-type proteins with and without exposure to trypsin. Tracks A, B and WT show bands detected by Coomassie blue staining; tracks WT1, A1 and B1 are autoradiographs of the same samples as in tracks WT, A and B. The samples in A and A1 were treated with trypsin; the samples in B and B1 were trypsin-treated and then reincubated in fresh culture medium for 5 h; WT denotes wild-type. The following polypeptides are of particular interest, with molecular weights of: *a*, 73 000–190 000; *b*, 70 000; *c*, 60 000; *d*, 55 000; *e*, 45 000; *f*, 190 000; *g*, 90 000.

radioactivity placed on a phenyl- or octyl-Sepharose column only 1 to 2% could be eluted by 1 M-ammonium sulphate. If hydrophobic interactions play a major role in adherence, the mutant strains may be expected to possess less hydrophobic properties than the wild-type strain. However, the mutant strains were retained to nearly the same extent as the parent strain (87–95% of wild-type).

Trypsin treatment of M. pneumoniae wild-type strain

Trypsin treatment of *M. pneumoniae* cells is known to abolish their capacity to adhere to erythrocytes (Feldner *et al.*, 1979*a*; Gorski & Bredt, 1977) and tissue cells (Hu *et al.*, 1977; Sobeslavsky *et al.*, 1968), suggesting that membrane surface proteins might function as mediators of adherence. Incubation of trypsinized mycoplasmas for about 3 to 5 h in growth medium restored their adherence capabilities. Comparison of polypeptide patterns of native, enzyme-treated and reincubated organisms (Fig. 3) showed that the high molecular weight polypeptides were most affected by trypsin treatment and that most of these were regenerated during the reincubation period. Of particular interest was the marked reappearance of polypeptides with molecular weights of 190 000 and 90 000 (Fig. 3*f* and *g*) because these were the components that were missing in several of the mutant strains.

Incorporation of [³⁵S]methionine into glucose-energized mycoplasmas

Cells labelled with [³⁵S]methionine in the presence of 5.0 mg (ml glucose)⁻¹ showed a reduced incorporation of the label into several polypeptides when compared with cells labelled at 0.5 mg (ml glucose)⁻¹ (152 000 c.p.m. versus 188 000 c.p.m. per 100 µg mycoplasma protein). A polypeptide in the 100 000 range was consistently affected (not shown).

DISCUSSION

The data so far available suggest that proteins are involved in adherence of erythrocytes to *M. pneumoniae* colonies (Feldner *et al.*, 1979*a*; Gorski & Bredt, 1977) and tissue cells (Collier & Clyde, 1971; Hu *et al.*, 1977; Hu *et al.*, 1982; Krause *et al.*, 1982) and probably also in attachment of *M. pneumoniae* to glass surfaces (Feldner *et al.*, 1981*a*). Approaches used to identify such binding proteins on the mycoplasma membrane include the electrophoretic examination of polypeptide patterns of haemadsorption-negative mutants, or the analysis of pattern changes after protease treatment of the wild-type parent strain with subsequent reincubation in growth medium. However, the polypeptide patterns of phenotypically identical mutants varied and did not always reveal defects. The latter finding could indicate that either the binding site(s) was altered by the mutational event(s) without grossly affecting the electrophoretic mobility of a particular polypeptide, or that mycoplasma surface components other than proteins were involved in the adherence process (Hansen *et al.*, 1979; Krause *et al.*, 1982). A few of the mutants, however, showed changes in proteins with an approximate molecular weight of 90 000 and 190 000. Proteins with similar defects were recently described in mutants of a different *M. pneumoniae* strain (Hansen *et al.*, 1979; Hu *et al.*, 1982) and also in spontaneously non-haemadsorbing variants of *M. pulmonis* (Taylor-Robinson *et al.*, 1981). This and the fact that both proteins were regenerated by trypsin-treated mycoplasmas suggest that these substances may play a major role in the adherence process.

In a previous paper we demonstrated that the process of *M. pneumoniae* glass attachment (Feldner *et al.*, 1979*b*) is subject to a repression mechanism elicited by higher doses of glucose (Feldner *et al.*, 1981*b*). A similar finding was reported on adhesion of *Salmonella typhimurium* (Saier *et al.*, 1978), platelets (Salzman *et al.*, 1972) and granulocytes (Bryant & Sutcliffe, 1974). In the case of *S. typhimurium* high amounts of glucose were found to suppress formation of fimbrial protein. In a comparable experiment with *M. pneumoniae* wild-type cells, attachment-inhibiting amounts of glucose caused reduced incorporation of methionine into some polypeptides of which a 100 000 component was most affected. Whether this substance is related to the 90 000 polypeptide missing in some mutants is so far unknown. In contrast to this glucose-mediated suppression, inhibitors of protein synthesis like erythromycin and tetracycline were not effective in doses at, or moderately above, their minimal inhibitory concentration (data not shown).

The data presented in this report indicate that some mutants that do not adhere to sheep erythrocytes were capable of adhering to erythrocytes of other species (RE or GPE). This finding confirms previous studies which suggested different binding mechanisms for SE and RE (Feldner *et al.*, 1979*a*) and supports recent experiments showing that a total lack of haemadsorption capability does not completely eliminate the ability of *M. pneumoniae* to adhere

to tracheal rings (Hansen *et al.*, 1979). In our experiments, several mutant strains lost their haemadsorbing capacity for RE or GPE after multiple passages in liquid medium. This seems to be a common feature for *Mycoplasma* strains because similar observations were recently made with a multipassaged haemadsorbing *M. pulmonis* strain (Taylor-Robinson *et al.*, 1981).

The findings that most of the haemadsorption-negative mutants did not exhibit growth rates similar to that of the wild-type strain (data not shown) and that nearly all mutants failed to grow on glass are not in accordance with the results reported for two other haemadsorption-negative mutants (Hansen *et al.*, 1981). This discrepancy may be due to (i) the different wild-type strains (FH strain versus M129 strain) having different susceptibilities to chemical mutagenesis, (ii) undetected secondary mutations in the isolated strains, or (iii) the different number of mutant strains investigated (18 versus 2). That haemadsorption and glass attachment are mediated via related or identical substances is suggested by results obtained by use of monoclonal antibody directed specifically against haemadsorption sites on *M. pneumoniae* (Feldner *et al.*, 1982).

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