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Genomic and Phenotypic Analyses of *Mycoplasma pneumoniae* Strains

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Mycoplasma pneumoniae strains PI-1428, M129, B176, FH, and MAC were analyzed for homology by DNA-DNA hybridization, thermal elution midpoints of the DNA-DNA heteroduplexes, DNA cleavage patterns with restriction endonucleases, and protein gel electrophoretic profiles. These properties were compared with biological assays, such as antigenic reactivity with reference antisera in the growth inhibition and metabolic inhibition tests, as well as the ability to attach to human WiDr cell cultures. The avirulent, nonhemadsorbing strain B176 could be differentiated from the others by diminished attachment capacity to WiDr cells and by slightly reduced DNA homology. The other strains appeared to be identical when examined by these procedures. No significant differences were detected among any of the strains by the metabolic inhibition or growth inhibition tests. The DNA cleavage patterns of the five strains were also similar, although strains FH and MAC differed from the other three strains in a few bands. These results demonstrate that these five strains are similar and that the species *M. pneumoniae* is remarkably homogeneous.

Recent nucleic acid hybridization studies have revealed wide variations in DNA homology among different strains of *Acholeplasma laidlawii* and *A. axanthum* (E. B. Stephens et al., manuscript in preparation) and among strains of *Ureaplasma urealyticum* (7). Moreover, Razin, Harasawa, and Barile (submitted for publication) showed that restriction endonuclease cleavage patterns of DNA can differentiate the eight established serovars of *U. urealyticum* into two distinct clusters, whereas the endonuclease cleavage patterns of *Mycoplasma pneumoniae* strains appeared similar. Thus, these techniques were utilized to compare virulent and avirulent strains of *M. pneumoniae* to determine whether DNA analysis of these strains could be related to virulence. In this study, five *M. pneumoniae* strains with different pathogenicities were examined for DNA homology, DNA endonuclease cleavage patterns, protein gel electrophoresis profiles, antigenic properties, and adsorption to mammalian cells. Prior work has suggested that *M. pneumoniae* virulence is correlated with cytadsorption (9, 14, 18). The results presented indicate that the *M. pneumoniae* strains examined are remarkably homogeneous with regard to these genomic and phenotypic characteristics.

MATERIALS AND METHODS

Organisms used and cultivation techniques. The following *M. pneumoniae* strains were used. PI-1428, passage level (p.) 8 to 14, a clinical isolate; FH, high passage (p. > 300); and FH (Eaton), low passage (p. 3 to 10), were obtained from R. M. Chanock, National Institutes of Health, Bethesda, Md. Strains M129 (p. 15 to 20), a clinical isolate; B176, (p. 5 to 15 in our laboratory since spontaneous loss of hemadsorption at p. 169 of parent strain M129) (19); and MAC, high passage (p. > 300), were provided by A. M. Collier, University of North Carolina, Chapel Hill, N.C. Cultures were grown on glass surfaces in mycoplasma broth (12) as described previously (5). Glass-adherent organisms were washed with phosphate-buffered saline, pH 7.2, removed from the surface by scraping with a rubber policeman, and centrifuged at 27,000 × *g* for 30 min at 4°C. Organisms which did not attach to glass were collected by centrifugation at 16,000 × *g* and washed with phosphate-buffered saline by repeated centrifugation, as described above.

Nucleic acid hybridization studies. (i) DNA purification. The cell pellets obtained by centrifugation were used immediately for purification of DNA. The pellet from each strain was lysed in a solution containing 8 M urea, 1% sodium dodecylsulfate (SDS), 1 M $NaClO_4$, 1 mM EDTA, and 0.24 M NaH_2PO_4 (PB), pH 6.8 (lysing solution). The DNA was purified by the hydroxyapatite batch elution method (4) and further processed for hybridization studies as described previously (1, 2).

TABLE 1. DNA-DNA hybridization of [^{3}H]DNA from strains MAC and M129 with unlabeled DNA from five strains of *M. pneumoniae*

[^{3}H]DNA probe	Unlabeled DNA	% Hybridization[a]	% Homology[b]	te_{50}[c]
MAC	M129	77.3	94.3	83.3
	MAC	82.0	100.0	83.2
	PI-1428	73.5	89.6	83.2
	FH (Eaton)	75.4	92.0	83.0
	FH	80.0	97.6	83.1
	B176	70.1	85.5	79.2
	Duck	2.0	2.4	ND[d]
M129	M129	81.8	100.0	ND
	MAC	80.9	98.9	ND
	PI-1428	78.8	96.3	ND
	FH (Eaton)	80.9	98.9	ND
	FH	75.8	92.7	ND
	B176	69.2	84.6	ND
	Duck	1.6	2.0	ND

[a] Average value from two separate experiments which did not differ by more than 1%.
[b] Hybridization values normalized to 100% for the homologous strain.
[c] Thermal elution midpoint temperature of the DNA-DNA duplexes.
[d] ND, Not done.

The purified DNA was pelleted in a Ti 60 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 40,000 rpm for 18 h at 20°C, suspended in 0.01 M Tris, pH 7.8, containing 0.1 M NaCl, and stored at 4°C.

(ii) [^{3}H]DNA probe synthesis. Purified native DNA was labeled in vitro with ^{3}H-labeled nucleoside triphosphates by the nick translation method (15, 21) and processed for hybridization as described earlier (23). The specific activity of the resultant single-stranded DNA probe was approximately 10^7 cpm per μg of DNA.

(iii) Hybridization procedures. The hybridization procedures of Aulakh and co-workers (1, 2) and Stephens et al. (23) were used. The hybridization mixture contained 1 mg of unlabeled sheared DNA per ml, 150,000 cpm of [^{3}H]DNA probe per ml, 0.2% SDS, 0.001 M EDTA, and 0.48 M PB. Reaction mixtures were denatured at 105°C for 5 min and incubated at 65°C overnight to a Côt value of >300 (2). Hybridized DNA was adsorbed to a hydroxyapatite column equilibrated at 60°C with 0.12 M PB containing 0.2% SDS and was then eluted with 0.48 M PB containing 0.2% SDS.

(iv) te_{50} determinations. The thermal elution midpoint temperature (te_{50}), an indicator of the amount of base mismatching in the DNA-DNA heteroduplex (17), was performed with hydroxyapatite columns prepared as described above. The hybridized DNA was adsorbed onto the column at 60°C and washed thoroughly with 0.12 M PB containing 0.2% SDS while the temperature was raised by 4°C increments to 100°C. All measurements of radioactivity were performed by adding 12 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) to 4 ml of eluate and counting in a Packard Tricarb liquid scintillation counter.

DNA cleavage by restriction endonucleases. The DNA was extracted and purified as detailed by Razin et al. (submitted for publication). For digestion by restriction endonucleases, the reaction mixture (total volume 20 μl) contained about 5 μg of mycoplasmal DNA, 50 to 60 U of *Eco*RI or *Xba*I (New England Biolabs, Beverly, Mass.), and 50 mM Tris-hydrochloride (pH 7.5)–10 mM $MgSO_4$–100 mM NaCl. Positive control reaction mixtures contained each specific endonuclease, the appropriate salt solution, and 1 μg of lambda phage DNA (Bethesda Research Laboratories, Gaithersburg, Md.). Digestions were carried out at 37°C for 60 min and were subjected to agarose gel electrophoresis as described previously (Razin et al., submitted for publication).

Polyacrylamide gel electrophoresis of *M. pneumoniae* cell proteins. Washed *M. pneumoniae* cells suspended in lysing solution were diluted 1:1 with a solution containing 2% SDS, 5% mercaptoethanol, and 20% glycerol and were boiled for 3 min. SDS-polyacrylamide gel electrophoresis of the boiled samples was carried out according to the method of Laemmli (16), using slab gels containing a 3% stacking gel and a 10% separating gel. Protein bands were stained with 0.05% Coomassie brilliant blue R-250 (BioRad Laboratories, Richmond, Calif.).

Serological procedures. New Zealand White rabbits, obtained from the Small Animal Section of the National Institutes of Health, were immunized by intravenous injection with washed cultures of strains PI-1428 or M129 at weekly intervals for 2 months. Mule anti-FH (Eaton) antiserum was obtained from the Research Resources Branch, National Institute for Allergy and Infectious Diseases, Bethesda, Md. The growth inhibition test was performed as described by Clyde (8), and the metabolic inhibition test was performed according to Taylor-Robinson et al. (24).

Attachment studies. Attachment of *M. pneumoniae* (radiolabeled by the addition of [^{3}H]palmitic acid to the growth medium) to WiDr human cell culture monolayers on 5-mm cover slips was performed as described previously (6). In brief, confluent WiDr cell culture monolayers were incubated with 10 to 80 μg of radiolabeled *M. pneumoniae* suspensions in 100 μl of Hanks balanced salt solution (HBSS) for 1 hr at 36°C. Unattached organisms were removed by aspiration

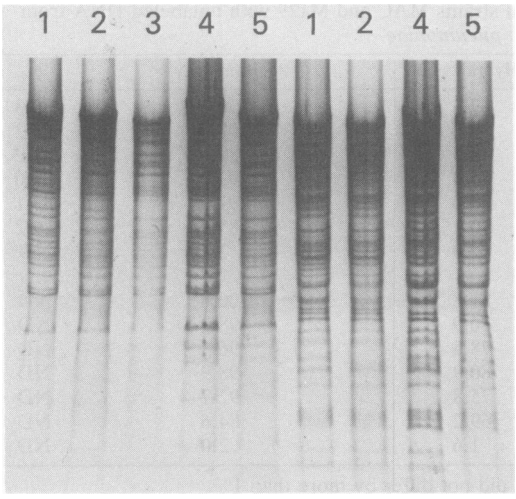

FIG. 1. Endonuclease cleavage patterns of the DNA of *M. pneumoniae* strains after agarose gel electrophoresis. The DNA of strains FH (lane 1), MAC (lane 2), M129 (lane 3), B176 (lane 4), and PI-1428 (lane 5) was digested by *Xba*I (first 5 lanes) or by *Eco*RI (last 4 lanes).

and the monolayers were washed twice with 0.5 ml of HBSS, transferred to minivials, and counted by liquid scintillation spectrometry. Protein content of *M. pneumoniae* cultures was quantitated by the Lowry procedure (20) with bovine serum albumin as the standard; the number of colony-forming units per microgram of protein was approximately 5×10^6.

RESULTS

Hybridization studies. The results of the hybridization studies with [^{3}H]DNA probes derived from *M. pneumoniae* strains M129 and MAC are presented in Table 1. The M129 and MAC probes hybridized 81.8 and 82.0% to their homologous DNAs, respectively. Both probes did not hybridize significantly to unrelated duck DNA (<2.5%), demonstrating the specificity of the probes used in the study. As shown in Table 1, the percent homology (i.e., hybridization values normalized to 100% for the homologous strain) was 90% or greater for all of the *M. pneumoniae* strains examined, except for strain B176. The unlabeled DNA from strain B176 was 85 to 86% homologous with [^{3}H]DNA from both M129 and MAC. These results indicate that strains M129, MAC, PI-1428, FH (Eaton) (low passage), and FH (high passage) are very similar to one another with respect to their DNA nucleotide sequences, whereas strain B176, a nonvirulent, noncytadsorbing strain derived from the virulent parent strain M129 (18), is slightly less similar. These results are supported by the te_{50} of the DNA-DNA duplexes, also shown in Table 1. The te_{50} value for the MAC homoduplex was 83.2°C, whereas the te_{50} values ranged from 83.0 to 83.3°C for the heteroduplexes of those strains highly related to MAC. The te_{50} value for the most distantly related heteroduplex, B176-MAC, was 79.2°C, indicating that strain B176 is slightly different from the other *M. pneumoniae* strains.

DNA cleavage patterns. The purified DNA extracted from *M. pneumoniae* strains FH, MAC, M129, B176, and PI-1428 was digested by the restriction endonucleases, *Eco*RI and *Xba*I, and the results are shown in Fig. 1. The cleavage patterns of the DNAs of the five strains appear very similar, with slight differences in only one or two bands.

Protein electrophoretic patterns. To determine whether the slight DNA homology differences observed between strain B176 and the other strains could be detected in protein electrophoretic profiles, SDS-polyacrylamide gel electrophoresis analysis of the *M. pneumoniae* cell proteins was performed. No difference was seen in the protein patterns of the *M. pneumoniae* strains after one-dimensional SDS-polyacryl-

TABLE 2. Comparison of *M. pneumoniae* strains by the metabolic inhibition test

Antigen[a]	Titer of antiserum to:[b]		
	PI-1428	M129	FH (Eaton)
PI-1428	1,280 (>20,480)	1,280 (>20,480)	1,280 (>20,480)
M129	1,280 (>20,480)	1,280 (>20,480)	1,280 (>20,480)
FH (Eaton)	1,280 (>20,480)	1,280 (>20,480)	1,280 (>20,480)
FH	1,280 (>20,480)	1,280 (>20,480)	1,280 (>20,480)
MAC	1,280 (>20,480)	1,280 (>20,480)	2,560 (>20,480)
B176	1,280 (>20,480)	1,280 (>20,480)	2,560 (>20,480)

[a] Each culture contained 10^5 color changing units per 0.2 ml.
[b] Values in parentheses were obtained in the presence of complement.

TABLE 3. Comparison of *M. pneumoniae* strains by the growth inhibition test

Antigen[a]	Zone of growth inhibition (mm) for antisera prepared against:		
	PI-1428	M129	FH (Eaton)
PI-1428	2.8	2.9	3.0
M129	2.8	2.8	2.8
FH (Eaton)	2.6	2.6	2.5
FH	2.5	2.6	2.5
MAC	2.8	2.5	2.8
B176	2.8	2.8	4.3

[a] Each culture contained 10^4 color changing units per 0.2 ml. Analogous results were obtained with 10^3 or 10^5 color changing units per 0.2 ml.

amide gel electrophoresis (data not shown).

Serological analysis. Mule anti-FH (Eaton) antiserum and rabbit antisera against strains PI-1428 and M129 were used in the metabolic inhibition and growth inhibition tests to compare the antigenicity of the five *M. pneumoniae* strains. The results of the metabolic inhibition test are presented in Table 2. The titers obtained with each of the three antisera tested against the five *M. pneumoniae* strains were identical. Hence, the antigenic properties of these *M. pneumoniae* strains were very similar or identical. In the growth inhibition tests antisera against strains PI-1428, M129, and FH (Eaton) produced similar zones of growth inhibition against all of the strains tested (Table 3). Thus, the growth inhibition findings confirm the results obtained by the metabolic inhibition test and indicate that the antigenic properties of the *M. pneumoniae* strains detected by these two serological procedures are the same.

Attachment assays. Previous studies have shown that *M. pneumoniae* readily attaches to human WiDr carcinoma cell cultures (6). These cell culture monolayers were used to examine the attachment capacity of radiolabeled strains M129, MAC, PI-1428, FH, and B176. Cultures were grown from the same lot of medium and were radiolabeled by adding the same amount of [^{3}H]palmitic acid to the growth medium. The specific radioactivity (counts per minute per microgram of protein) and pH of the cultures at the time of harvest for all five strains were similar. Attachment of the radiolabeled strains to the WiDr cell monolayers is presented in Fig. 2. Virulent strains M129 and PI-1428, as well as laboratory strains MAC and FH, showed similar attachment curves. Apparent saturation of the monolayers was obtained at approximately 40 μg of mycoplasmal protein per assay. However, the attachment capacity of strain B176 was only about 20% of that of the other strains.

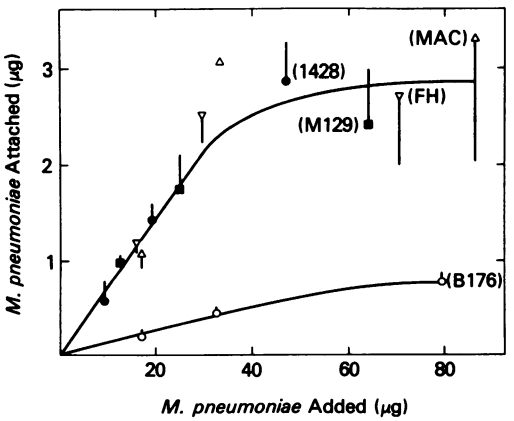

FIG. 2. Attachment of *M. pneumoniae* strains to WiDr cell culture monolayers. Symbols: ○, strain B176; ■, strain M129; ●, strain PI-1428; ▽, strain FH; △, strain MAC. Vertical bars indicate standard error of the mean.

DISCUSSION

The *M. pneumoniae* strains used in this study were isolated in different laboratories and differed in the passage level in vitro, in their ability to adsorb to mammalian cells, and in their virulence for hamsters. Strains M129 and PI-1428 are clinical isolates (p. ≤ 20) and produce lung disease in hamsters. Strain B176, derived from strain M129, exhibits reduced attachment to cells and is avirulent for hamsters (3, 18). Strain MAC, an attenuated strain (p. > 300), and strain FH (p. > 300) produce reduced lung disease in hamsters (11, 18; M. F. Barile et al., manuscript in preparation), but virulence for hamsters can be regained when MAC and FH are passed successively in hamsters (11, 19). In some of the tests, strain FH at p. 3 to 10 (FH [Eaton]) was also studied. We have examined these *M. pneumoniae* strains for differences associated with virulence. The genome of *M. pneumoniae* is approximately 5×10^8 daltons, and extrachromosomal DNA was not detected (R. Harasawa and M. F. Barile, manuscript in preparation). Thus, the *M. pneumoniae* genome was readily analyzed by DNA-DNA hybridization and restriction endonuclease techniques.

Our results show *M. pneumoniae* to be a homogeneous species. The relative homology in the DNA-DNA hybridization studies ranged from 85 to 99% for all five strains, in spite of differences in passage level, adsorption to cells, and virulence. The relative homology is quite striking, with five strains showing 90 to 99% and one strain 85% homology. This genomic homogeneity observed with *M. pneumoniae* strains isolated from one host contrasts with the genomic variability observed for two *Acholeplasma* species which were isolated from diverse sources (i.e., human, animals, plants, and soil).

Nucleic acid hybridization analysis of 12 strains of *A. laidlawii* and six strains of *A. axanthum* showed wide variation within the same species, ranging from 54 to 100% homology (Stephens et al., manuscript in preparation).

The genomic similarity of the *M. pneumoniae* strains was also supported by the nearly identical DNA cleavage patterns obtained after treatment with restriction endonucleases. The restriction endonucleases *Eco*RI and *Xba*I cut the *M. pneumoniae* DNA at many sites, so that the identity or close similarity of the cleavage patterns indicates nucleotide sequence homology at multiple sites along the chromosome. Strains FH and MAC appeared to vary in a few bands from the other strains, although they were very closely related to one another. The genomic homogeneity of *M. pneumoniae* strains contrasts with the heterogeneity of *U. urealyticum* serovars observed in DNA cleavage patterns (Razin et al., submitted for publication) and in DNA-DNA hybridization studies (7). These investigators found that the DNA hybridization data and endonuclease cleavage patterns for the eight established serovars of *U. urealyticum* could be used to subdivide the *U. urealyticum* strains into two groups. Polyacrylamide gel electrophoresis of cell proteins also suggested that *U. urealyticum* strains fall into two groups (22).

The similarity among the *M. pneumoniae* strains, demonstrated by the hybridization studies and cleavage patterns of the genome, is also reflected in their phenotypic expression. The strains were indistinguishable in both the metabolic inhibition and growth inhibition tests. These results are consistent with an earlier report of serological identity of *M. pneumoniae* strains in double immunodiffusion, complement fixation, and metabolic inhibition tests (19). The one-dimensional protein electrophoretic profiles of these strains also showed marked phenotypic homogeneity. Similar results were reported by Hu et al. (14) for M129 and B176. However, Hansen et al. (10) have resolved differences in a few proteins between strains M129 and B176 by two-dimensional gel electrophoresis.

Our data indicate that the difference in virulence exhibited by the various *M. pneumoniae* strains may result from the modification or deletion of only a few genes. Thus, infectivity (3) remains the most critical procedure to assess virulence. However, the in vitro WiDr monolayer attachment assay can be used to quantitate the attachment capacity of virulent and nonvirulent strains. Other in vitro procedures for detecting pathogenic activities such as ciliostasis, protease, and hemagglutination (5) may also provide important information regarding the virulence of *M. pneumoniae* strains and help in their differentiation. Monoclonal antibodies directed to the mycoplasmal attachment or ciliostatic moieties may also be used to detect virulence factors among strains (13).

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