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Molecular Distinctions among Clinical Isolates of *Mycoplasma pneumoniae*

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Restriction enzyme fingerprinting of genomic DNA and Southern blots probed with subclones of the *Mycoplasma pneumoniae* cytadhesin P1 gene were used to characterize clinical isolates of *M. pneumoniae*. On the basis of the examination of 29 individual *M. pneumoniae* isolates, two distinct groups were established. Group 1, which displayed a 12-kilobase band following DNA digestion with *Hin*dIII, consisted of strain M129-B16 and three others obtained in the state of Washington during the 1960s. The remaining *M. pneumoniae* strains belonged to group 2, which lacked the 12-kilobase band and included samples from the 1940s, 1970s, and 1980s. This category also included the only *M. pneumoniae* strain isolated from the synovial fluid of an arthritic patient.

Mycoplasma pneumoniae, a cell wall-less, flask-shaped procaryote that infects the human lower respiratory tract, is the causative agent of primary atypical pneumonia (3). A 5-year surveillance study (1963 to 1968) in Seattle, Wash., indicated that 20% of all pneumonia cases in the general population were associated with *M. pneumoniae* (9). The incidence of *M. pneumoniae* infection in more restricted settings, such as among young adults in military installations, is even higher. Epidemiological data collected in a study with the U.S. Armed Forces suggested that between 20 and 45% of pneumonia cases were caused by *M. pneumoniae* (5). Current routine methods for the diagnosis of *M. pneumoniae* infection include direct isolation of microorganisms on complex media (22), demonstration of seroconversion during acute and convalescent phases of infection (14), and complement fixation (17) and metabolic inhibition assays (21). However, no method is consistently reliable, and none can differentiate among strains of *M. pneumoniae*.

Molecular analysis of virulent *M. pneumoniae* isolates revealed that a 170-kilodalton surface protein, designated P1, clusters densely at the tip-like organelle and mediates cytadherence (1, 8, 11). The P1 gene of wild-type *M. pneumoniae* M129-B16 has been cloned, and its entire nucleotide sequence has been determined (20). When the cloned P1 gene was used to probe other clinical isolates of *M. pneumoniae* by Southern blot hybridization, two distinct hybridization patterns were detected. In order to better understand the P1 gene variation observed among clinical isolates, we used P1 DNA probes to study *M. pneumoniae* isolates collected at different times and from different locations.

MATERIALS AND METHODS

Origin of *M. pneumoniae* strains. *M. pneumoniae* M129-B16 (ATCC 29342) was originally isolated from a patient in 1968 (15). Strain FH (ATCC 15531) (2, 7) and strain Mac (ATCC 15492) (7, 16) were obtained from the American Type Culture Collection, Rockville, Md. Other clinical isolates included the TW series and R32P, which were obtained from J. G. Tully of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., and originated from a *M. pneumoniae* vaccine trial conducted with military recruits in 1974 and 1975 (24). Strain UTMB was isolated from the synovial fluid of an arthritic patient by C. P. Davis at the University of Texas Medical Branch at Galveston (4). Strains PN 597, PN 6644, and PN 14366 were obtained from patients in the 1960s in Seattle, Wash. (9, 10, 23). Six *M. pneumoniae* strains were isolated in 1988 in France by C. Bebear and H. Renaudin and were provided by J. G. Tully.

Extraction of genomic DNA. *M. pneumoniae* isolates were grown at 37°C for 3 days in 32-oz (920-ml) glass prescription bottles containing 70 ml of SP4 medium (22). Glass-attached mycoplasmas were rinsed with phosphate-buffered saline twice (10 mM sodium phosphate [pH 7.2], 0.1 M NaCl) and harvested. Cell pellets were resuspended in phosphate-buffered saline and lysed by the addition of a 1/10 volume of 10% sodium dodecyl sulfate (19). Cell lysates were treated successively with RNase and proteinase K and then extracted with phenol, phenol-chloroform (1:1), and chloroform-isoamyl alcohol (24:1). Individual DNA preparations were precipitated with 2 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.4). Each DNA pellet was dissolved in Tris-EDTA buffer and quantitated by measuring UV A_{260} and A_{280}.

Restriction enzyme digestion and Southern blot analysis. DNA from each clinical isolate was digested overnight with sufficient amounts of restriction enzymes to ensure complete digestion. Digested DNA (5 μg) was loaded on 0.75% agarose gels, separated by electrophoresis, stained with ethidium bromide, and photographed. DNA was transferred to nitrocellulose filters as described by Southern (18) and probed with ^{32}P-labeled subclones of the P1 gene under stringent conditions (19).

RESULTS

Because of the small genomic size (about 830 kilobases [kb]) of *M. pneumoniae*, digestion of mycoplasma DNA with restriction enzymes that recognize 6-base-pair sequences provided discrete and manageable DNA patterns (fingerprinting). When each DNA preparation was digested with restriction enzymes such as *Bam*HI, *Eco*RI, *Pst*I, or *Sma*I, all *M. pneumoniae* isolates exhibited the same restriction pattern. However, digestion with *Hin*dIII provided two

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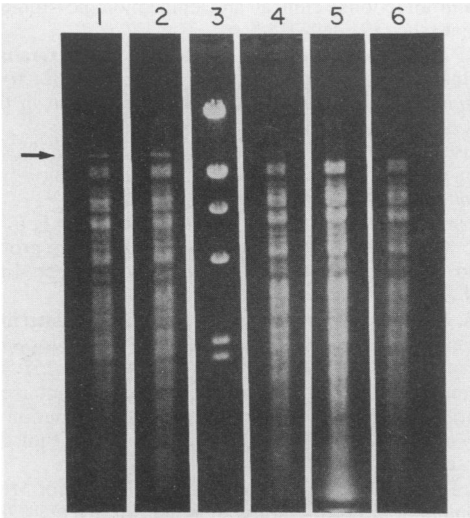

FIG. 1. Fingerprinting of *M. pneumoniae* genomic DNA. DNA (8 μg) was digested to completion with 40 U of *Hin*dIII restriction enzyme at 37°C overnight. The digested DNA was electrophoresed in 0.75% agarose gels to separate fragments by size. Gels were stained with ethidium bromide and photographed. Lanes: 1, B16 (group 1); 2, PN 597 (group 1); 3, *Hin*dIII-digested λ phage DNA used as a molecular weight standard; 4, FH (group 2); 5, TW 7-5 (group 2); 6, UTMB (group 2). The arrow indicates the 12-kb *Hin*dIII band present in group 1 but absent in group 2.

distinct patterns (Fig. 1). One pattern was representative of strains M129-B16 and PN 597 (group 1), in which a high-molecular-weight *Hin*dIII band of about 12 kb was observed. In contrast, three other isolates, TW 7-5, FH, and UTMB, were lacking this band (group 2).

The presence or absence of this 12-kb *Hin*dIII band correlated directly with the different P1 probe hybridization patterns of *Hin*dIII-digested *M. pneumoniae* DNA (Fig. 2). When subclone B, which represents a multiple-copy region from the P1 structural gene of wild-type strain M129-B16 (19), was used to probe *Hin*dIII-digested genomic DNA, it hybridized to 12-, 4.4-, and 3.1-kb bands in group 1 and to 5.2-, 3.1-, and 1.3-kb bands in group 2. Subclones F and G hybridized to a 4.4-kb band in group 1 and a 3.1-kb band in group 2. Subclones L and M hybridized to a 2.1-kb *Hin*dIII band in group 1 and a 4.1-kb band in group 2. No differences

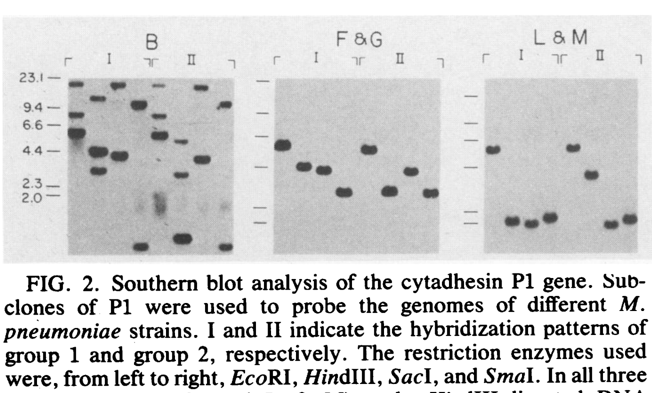

FIG. 2. Southern blot analysis of the cytadhesin P1 gene. Subclones of P1 were used to probe the genomes of different *M. pneumoniae* strains. I and II indicate the hybridization patterns of group 1 and group 2, respectively. The restriction enzymes used were, from left to right, *Eco*RI, *Hin*dIII, *Sac*I, and *Sma*I. In all three panels (B, F & G, and L & M), only *Hin*dIII-digested DNA discriminated between the hybridization patterns of group 1 and group 2 strains. The letter(s) on top of each panel indicates the P1 subclone(s) used (21). Numbers on the left indicate the sizes of molecular weight standards in kilobases.

TABLE 1. Categorization of *M. pneumoniae* clinical isolates on the basis of cytadhesin P1 gene polymorphism

Strain	Yr(s) of isolation	Origin
Group 1		
M129-B16	1968	North Carolina
PN series[a]	1964, 1969, 1974	Washington
Group 2		
Mac	1944	California
FH	1959	California
TW series[b]	1974	South Carolina
R32P	1974	South Carolina
UTMB	1986	Texas
French series[c]	1988	France

[a] Includes strains 597, 6644, and 14366.
[b] Includes strains 1-6, 2-4, 7-4, 7-5, 7-6, 8-6, 10-5, 10-6, 11-4, 14-4, 23-5, 25-6, 48-5, and 49-5.
[c] Includes six strains isolated in France.

were detected between group 1 and group 2 strains when DNA was digested with *Eco*RI, *Sac*I, or *Sma*I. The observed restriction length polymorphism is apparently caused by the divergence of the P1 genes among different strains of *M. pneumoniae*, on the basis of detailed analysis of these two classes of P1 genes (unpublished observation).

Because of the prominent role of P1 in the cytadherence (1, 8, 11) and virulence (12, 13) of *M. pneumoniae* and in the host immune response to *M. pneumoniae* infection (14, 23), DNA fingerprinting and Southern blot analysis with P1 subclones were used to characterize *M. pneumoniae* strains collected at different times and from different locations. These results are summarized in Table 1. Of the 29 isolates examined, strain M129-B16 and three other clinical isolates obtained from the state of Washington during the 1960s (PN 597, PN 6644, and PN 14366) were classified as being in group 1, whereas the remaining 25 isolates belonged to group 2.

DISCUSSION

Although it is well established that *M. pneumoniae* is a human pathogen of the respiratory tract (3, 5), other anatomical sites for colonization and subsequent disease have been implicated (4, 21). Investigations concerning variations in *M. pneumoniae* strains that might be associated with different epidemics or virulence potentials have not been possible, because current diagnostic tests are unable to differentiate among *M. pneumoniae* isolates. The DNA fingerprinting method and Southern blot analysis using subclones of the P1 cytadesin gene as probes allowed us to classify clinical isolates into two groups. The basis of our classification is the divergence of the major cytadhesin gene among these mycoplasmas. Since P1 is a key virulence factor that also stimulates a strong host immune response (14, 23), the observed differences could be significant in understanding *M. pneumoniae* disease pathogenesis.

Our results clearly indicate that group 2 *M. pneumoniae* strains are more frequently isolated from clinical specimens than group 1 strains, since only 4 of 29 mycoplasma isolates examined belonged to group 1. Group 2 *M. pneumoniae* isolates include Mac and FH strains, which were originally isolated in California in the 1940s and 1950s (6, 7, 16), all TW isolates from the South Carolina study of military recruits in the early 1970s (24), the French isolates derived from clinical specimens in 1988, and the single isolate from an infectious site other than the respiratory tract (strain UTMB from synovial fluid; 4). It is interesting that *M. pneumoniae* isolates from a given study belong to the same group, as seen with all TW (group 2), PN (group 1), and French (group 2) strains (Table 1). Further research should attempt to correlate the P1 structural gene differences of clinical isolates with tissue tropism, host immune responsiveness, and severity of disease.

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LITERATURE CITED

1. **Baseman, J. B., R. M. Cole, D. C. Krause, and D. K. Leith.** 1982. Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. J. Bacteriol. **151**:1514–1522.
2. **Chanock, R. M., L. Hayflick, and M. F. Barile.** 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. USA **48**:41–49.
3. **Clyde, W. A., Jr.** 1979. *Mycoplasma pneumoniae* infections of man, p. 275–306. *In* J. G. Tully and R. T. Whitcomb (ed.), The mycoplasmas II: human and animal mycoplasmas. Academic Press, Inc., New York.
4. **Davis, C. P., S. Cochran, J. Lisse, G. Buck, A. R. DiNuzzo, T. Weber, and J. A. Reinarz.** 1988. Isolation of *Mycoplasma pneumoniae* from synovial fluid samples in a patient with pneumonia and polyarthritis. Arch. Intern. Med. **148**:969–970.
5. **Denny, F. W., W. A. Clyde, Jr., and W. P. Glezen.** 1971. *Mycoplasma pneumoniae* disease: clinical spectrum, pathophysiology, epidemiology and control. J. Infect. Dis. **123**:74–92.
6. **Eaton, M. D., G. Meiklejohn, and W. Van Herick.** 1944. Studies on the etiology of primary atypical pneumonia. A filterable agent transmissible to cotton rats, hamsters, and chick embryos. J. Exp. Med. **79**:649–668.
7. **Eaton, M. D., G. Meiklejohn, W. Van Herick, and M. Corey.** 1945. Studies on the etiology of primary atypical pneumonia. II. Properties of the virus isolated and propagated in chick embryos. J. Exp. Med. **82**:317–328.
8. **Feldner, J., U. Gobel, and W. Bredt.** 1982. *Mycoplasma pneumoniae* adhesin localized to tip structure by monoclonal antibody. Nature (London) **298**:765–767.
9. **Foy, H. M., G. E. Kenny, R. McMahan, A. M. Mansy, and J. T. Grayston.** 1970. *Mycoplasma pneumoniae* pneumonia in an urban area: five years of surveillance. J. Am. Med. Assoc. **214**:1666–1672.
10. **Grayston, J. T., E. R. Alexander, G. E. Kenny, E. R. Clarke, J. C. Fremont, and W. A. MacColl.** 1965. *Mycoplasma pneumoniae* infections: clinical and epidemiologic studies. J. Am. Med. Assoc. **191**:369–374.
11. **Hu, P. C., R. M. Cole, Y. S. Huang, T. A. Graham, D. E. Gardner, A. M. Collier, and W. A. Clyde, Jr.** 1982. *Mycoplasma pneumoniae* infection: role of a surface protein in the attachment organelle. Science **216**:313–315.
12. **Krause, D. C., D. K. Leith, and J. B. Baseman.** 1983. Reacquisition of specific proteins confers virulence in *Mycoplasma pneumoniae*. Infect. Immun. **39**:830–836.
13. **Krause, D. C., D. K. Leith, R. M. Wilson, and J. B. Baseman.** 1982. Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. Infect. Immun. **35**:809–817.
14. **Leith, D. K., L. B. Trevino, J. G. Tully, L. B. Senterfit, and J.B. Baseman.** 1983. Host discrimination of *Mycoplasma pneumoniae* proteinaceous immunogens. J. Exp. Med. **157**:502–514.
15. **Lipman, R. P., and W. A. Clyde, Jr.** 1969. The interrelationship of virulence, cytadsorption and peroxide formation in *Mycoplasma pneumoniae* (34061). Proc. Soc. Exp. Biol. Med. **131**:1163–1167.
16. **Low, I. E., and M. D. Eaton.** 1965. Replication of *Mycoplasma pneumoniae* in broth culture. J. Bacteriol. **89**:725–728.
17. **Schmidt, N. J., E. H. Lennette, J. Dennis, and P. S. Gee.** 1966. On the nature of complement-fixing antibodies to *Mycoplasma pneumoniae*. J. Immunol. **97**:95–99.
18. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
19. **Su, C. J., A. Chavoya, and J. B. Baseman.** 1988. Regions of *Mycoplasma pneumoniae* cytadhesin P1 structural gene exist as multiple copies. Infect. Immun. **56**:3157–3161.
20. **Su, C. J., V. V. Tyron, and J. B. Baseman.** 1987. Cloning and sequence analysis of cytadhesin P1 gene from *Mycoplasma pneumoniae*. Infect. Immun. **55**:3023–3029.
21. **Taylor-Robinson, D., J. M. Gumpel, A. Hill, and A. J. Swannell.** 1978. Isolation of *Mycoplasma pneumoniae* from the synovial fluid of a hypogammaglobulinaemic patient in a survey of patients with inflammatory polyarthritis. Ann. Rheum. Dis. **37**:180–182.
22. **Tully, J. G., D. L. Rose, R. F. Whitcomb, and R. P. Wenzel.** 1979. Enhanced isolation of *Mycoplasma pneumoniae* from throat washings with a newly modified culture medium. J. Infect. Dis. **139**:478–482.
23. **Vu, A. C., H. Foy, F. D. Cartwright, and G. E. Kenny.** 1987. The principal protein antigens of isolates of *Mycoplasma pneumoniae* as measured by levels of immunoglobulin G in human serum are stable in strains collected over a 10-year period. Infect. Immun. **55**:1830–1836.
24. **Wenzel, R. P., R. B. Craven, J. A. Davis, J. O. Hendley, B. H. Hamory, and J. M. Gwaltney, Jr.** 1976. Field trial of an inactivated *Mycoplasma pneumoniae* vaccine. I. Vaccine efficacy. J. Infect. Dis. **134**:571–576.