Distinctions in DNA and protein profiles among clinical isolates of *Mycoplasma pneumoniae*

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Clinical isolates of *Mycoplasma pneumoniae* previously shown to exhibit significant sequence divergency in a major 170 kDa adhesin, designated P1, were further characterized using restriction enzyme fingerprinting of genomic DNA and two-dimensional gel electrophoresis of total proteins. Numerous differences in DNA restriction patterns and protein profiles were found, possibly reflecting various degrees of virulence and antigenic potential.

Introduction

Mollicutes (mycoplasmas) are cell-wall-less prokaryotes representing the smallest self-replicating organisms. Their genomes are double-stranded circular DNA ranging in size from 400 to 1000 MDa (Morowitz & Wallace, 1973; Razin, 1985; Su & Baseman, 1990) and are distinguished by a low guanine plus cytosine (G + C) content, between 23 to 40% (Razin, 1985).

The genus *Mycoplasma* contains many species that are pathogenic for a wide variety of plants and animals (Freundt & Edward, 1979). In humans, *Mycoplasma pneumoniae* infects the lower respiratory tract and is the causative agent of primary atypical pneumonia (Chanock *et al.*, 1962; Eaton *et al.*, 1945; Grayston *et al.*, 1965). A 170 kDa protein (P1) has been identified as a major cytoadhesin and virulence factor (Baseman *et al.*, 1982; Feldner *et al.*, 1982; Hu *et al.*, 1982), and the gene has been cloned and sequenced (Inamine *et al.*, 1988; Su *et al.*, 1987). Using subclones of the cytoadhesin gene as probes in Southern blot analysis of clinical isolates, we classified *M. pneumoniae* into two groups (I and II) based upon different hybridization patterns (Dallo *et al.*, 1990; Su *et al.*, 1990b). Further analysis of their P1 genes revealed two stretches of significant sequence divergency (Su *et al.*, 1990a). One region was about 500 bp in which the two groups shared 72% DNA homology; the other was about 700 bp in which the two groups shared 90% homology. Both nucleotide stretches were located in multiple-copy regions of the P1 gene (Su *et al.*, 1988), raising the possibility that the observed sequence

divergency arose from recombination between homologous yet slightly different regions of the chromosome.

To elucidate further the distinctions between these two groups of *M. pneumoniae*, representative strains from each group were selected for further analysis using restriction enzyme fingerprinting and two-dimensional gel electrophoresis.

Methods

Origin of *M. pneumoniae* strains. *M. pneumoniae* M129-B16 (ATCC 29342) was originally isolated from a patient in 1968 (Lipman & Clyde, 1969). Strain FH (ATCC 15531) (Chanock *et al.*, 1962) was obtained from the American Type Culture Collection. Strain TW 7-5 was obtained from J. G. Tully (National Institute of Allergy and Infectious Diseases, Bethesda, Md., USA) and originated from a *M. pneumoniae* vaccine trial conducted with military recruits in 1974 and 1975 (Wenzel *et al.*, 1976). Strain UTMB was isolated from the synovial fluid of an arthritic patient by C. P. Davis (University of Texas Medical Branch at Galveston, USA) (Davis *et al.*, 1988). Strain PN 597 was obtained in the 1960s from a patient in Seattle (Wash., USA) (Foy *et al.*, 1970; Grayston *et al.*, 1965; Vu *et al.*, 1987).

DNA fingerprinting. DNA was purified from group I (M12-B16 and PN 597) and group II (FH, TW 7-5 and UTMB) *M. pneumoniae* strains according to published methods (Su *et al.*, 1987). Purified DNA was dissolved in TE buffer (10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA), quantified and stored at 4 °C. Restriction enzymes were purchased from BRL and NEB. Each DNA preparation (8 µg) was digested overnight with 20 to 30 units of specific restriction enzymes in a total volume of 50 µl at 37 °C, and the DNA was separated by agarose gel electrophoresis (Maniatis *et al.*, 1982). DNA from each group was digested with the same enzymes and loaded side-by-side during electrophoresis. Enzymes were selected that did not cut *M. pneumoniae* genomic DNA frequently and, therefore, were more likely to produce unique restriction patterns. Because of the low G + C content of the *M. pneumoniae* genome, enzymes were evaluated that specifically recognized 6 bp sequences in which at least four nucleotides were either

Abbreviation: NEPHGE, non-equilibrium pH gradient electrophoresis.

guanine or cytosine. After electrophoresis was completed, DNA gels were stained with ethidium bromide and photographed.

Reverse field electrophoresis of DNA. Because digestion with restriction enzymes as above generated high molecular mass DNA bands that were not well-resolved by conventional electrophoresis, reverse field gel electrophoresis (Carle *et al.*, 1986) was employed. *M. pneumoniae* strains were encapsulated in agarose beads according to the method of Jackson & Cook (1985). Cells were lysed *in situ* by addition of 1% SDS and 0.25 M-EDTA, and proteins were digested with proteinase K (Boehringer) at 50 °C overnight. The agarose beads were washed three times with 10 vols of wash buffer (10 mM-Tris/HCl, pH 8.0, 10 mM-EDTA) containing 2 mM-PMSF and stored at 4 °C. Before restriction enzyme digestion, the DNA-containing agarose beads were equilibrated with the appropriate reaction buffer. The amount of agarose beads used was determined empirically to produce sharp bands (about 20 to 50 µl of agarose beads) under the selected conditions. DNA was digested, loaded into wells and sealed with 0.5% agarose solution before electrophoresis. Reverse field electrophoresis was performed on 0.8% agarose gels in 0.5 × TBE buffer (1 × TBE is 90 mM-Tris, 45 mM-boric acid, and 0.5 mM-EDTA) at room temperature with buffer recirculation using a M.J. programmable power inverter PPI-200 (M.J. Research Inc., Cambridge, Mass., USA). The voltage was set at 7 V cm⁻¹ and electrophoresis was continued for 18 to 20 h. Preset programme 3 was used to resolve DNA bands in the 10 to 50 kb range; programme 4 was used to resolve bands in the 30 to 200 kb range.

Preparation of cell samples for two-dimensional gel electrophoresis. Glass-adherent cells of *M. pneumoniae* isolates from a single culture bottle (about 950 ml) were washed six times with cold phosphate-buffered saline (PBS; 0.145 M-NaCl, 0.017 M-Na₂HPO₄, 0.003 M-NaH₂PO₄, pH 7.2), scraped into 5 ml PBS and centrifuged at 9500 g for 20 min. Cell pellets were resuspended uniformly in 190 µl 0.05 M-Tris/HCl (pH 6.8) and 15 µl 2-mercaptoethanol according to Ames & Nikaido (1976). Individual samples were vortexed for 10 min followed by the addition of 53 µl 10% (w/v) SDS and 15 µl 2-mercaptoethanol. Tubes were heated at 100 °C for 4 min, cooled to room temperature and 95 µl of the resulting preparation was added to 55 mg urea in a new tube. After dissolution of the urea at 37 °C, 100 µl of the resulting preparation was added to 205 µl of lysis buffer (Ames & Nikaido, 1975). A 20 µl vol. of this solution, representing approximately 75 µg protein, was used for two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis. Non-equilibrium pH gradient electrophoresis (NEPHGE) was performed in 100 µl capillary tubes by the procedure of O'Farrell *et al.* (1977), using pH 3 to 10 ampholines (Bio-Rad) and an electrophoresis period of 2 h at 500 V·h. Tube gels were equilibrated for 15 min in SDS sample equilibration buffer (12.5 ml 0.5 M-Tris/HCl, pH 6.8, 23 ml 10% SDS, 5 ml 2-mercaptoethanol, 8 ml glycerol, 2.5 ml 0.05% bromophenol blue, 49 ml water to make 100 ml total volume). SDS gel electrophoresis was performed using 7.5% polyacrylamide separating gels and 4% polyacrylamide stacking gels, prior to staining with silver (Merril *et al.*, 1979; O'Farrell *et al.*, 1977). Immunoblots were performed according to the method of Towbin *et al.* (1979) using hyperimmune rabbit sera raised against *M. pneumoniae* strain M129-B16.

Results

Several enzymes produced DNA restriction patterns that discriminated between all group I and group II *M. pneumoniae* strains using conventional agarose gel electrophoresis (Fig. 1). However, because many high

molecular mass DNA fragments generated by these enzymes could not be resolved by conventional gel electrophoresis, reverse field gel electrophoresis (Carle *et al.*, 1986) was employed. Fig. 2 shows the reverse field electrophoresis patterns. When digested with *Apa*I both group I and group II genomes were resolved into at least 11 bands, some of which appeared to be doublets or triplets. The position of at least two bands differed between group I and group II strains. In group I the top two bands were at 250 kb and 110 kb, whereas in group II these bands were at 190 kb and 130 kb. *Nar*I digestion also revealed differences between group I and group II genomes. Bands at 130 kb and 90 kb in group I appeared as 150 kb and 100 kb in group II; other bands appeared identical. When digested with *Xho*I, besides the 12.5 kb band detected by conventional electrophoresis, group I displayed an extra band at 50 kb (possibly a doublet). In contrast, group II possessed a band at 110 kb not present in group I. Other restriction enzymes (*Sac*I and *Pvu*I) revealed additional differences between group I and group II DNA (Fig. 2). However, *Rsr*II digested DNA of both groups into at least 11 bands with apparently identical sizes (Fig. 2). Three of these bands appeared to be doublets (bands II, V and VII from top to bottom, Fig.

Table 1. DNA restriction fragment differences between group I and group II *M. pneumoniae* strains

Enzyme	Restriction fragments (kb)	
	Group I	Group II
<i>Apa</i> I	250	—
	—	190
	—	130
	110	—
	2.5	—
<i>Nar</i> I	—	150
	130	—
	—	100
<i>Pvu</i> I	90	—
	—	—
	25	—
	—	12
<i>Sac</i> I	—	7
	—	30
	22	—
	20	—
	—	12
<i>Sac</i> II	—	5
	8.5	—
	—	5.0
	—	4.0
<i>Sal</i> I	3.2	—
	—	18
	—	3.3
<i>Xho</i> I	—	110
	50	—
	12.5	—

Fig. 1. Restriction fragment differences between group I and group II *M. pneumoniae* chromosomes as indicated by conventional agarose gel electrophoresis. (a) DNA separated on 0.7% agarose gels; (b) DNA separated on 0.5% agarose gels. Numbers on the left are molecular size standards in kb. Arrows point to the observed differences in restriction fragments. Under each restriction enzyme name, group I DNA (I) is represented by strain M129-B16, and group II DNA (II) is represented by strain UTMB (Su *et al.*, 1990b).

Fig. 2. Restriction fragment differences between group I and group II *M. pneumoniae* chromosomes as indicated by reverse field gel electrophoresis. For *RsrII* and *ApaI*, 1 and 5 represent group I DNA of strains M129-B16 and PN597, respectively; lanes 2, 3 and 4 are group II DNA of strains UTMB, TW7-5 and FH, respectively (Su *et al.*, 1990b). For the other enzymes, group I DNA (I) is represented by strain M129-B16 and group II DNA (II) by strain UTMB. For *RsrII* II, V and VII indicate three doublet DNA bands. For the other enzymes, arrows indicate fragments of different sizes between group I and group II strains. Numbers are the estimated molecular size in kb.

Fig. 3. NEPHGE-SDS electrophoretic analysis of proteins (silver-stained) from (a) *M. pneumoniae* M129-B16 and (b) clinical isolate TW7-5. Protein spots that show differences between (a) and (b) are numbered. Arrows on the left represent possible differences in separation of proteins upon migration in first-dimension electrophoresis; arrows on the right represent possible quantitative differences in specific proteins. Orientation of NEPHGE is basic (left) to acidic (right). Positions of molecular mass standards (kDa) are indicated. All strains within each group displayed identical electrophoretic patterns.

2). Based upon these restriction patterns the total kb value of the mycoplasma genomes was determined to be approximately 850 kb which is in good agreement with published data on *M. pneumoniae* genome size (Wenzel & Herrmann, 1988; Krause & Mawn, 1989). Table 1 summarizes the observed differences between the group I and group II *M. pneumoniae* genomes. Overall, the two groups of *M. pneumoniae* showed much similarity in their DNA restriction patterns as would be expected, but minor differences existed that might have clinical and biological significance (Dallo *et al.*, 1990; Su *et al.*, 1990a, b).

Since the observed differences in DNA restriction patterns may result in changes at the protein level, two-

dimensional SDS-PAGE was performed to compare group I and group II *M. pneumoniae* strains. The silver-stained gels revealed many differences, mostly reflected in apparent changes in the isoelectric point of specific proteins (Fig. 3). Spots corresponding to proteins numbered 14 and 17 were not readily identifiable in group I *M. pneumoniae* under these experimental conditions while protein number 18 was missing in group II clinical isolates. Other protein differences are indicated by numbers and arrows.

In order to determine whether the proteins associated with altered migration properties were antigenic, immunoblots of two-dimensional gels of each *M. pneumoniae* strain were prepared using hyperimmune rabbit sera

to *M. pneumoniae* M129-B16. In most cases the proteins identified in group I mycoplasmas along with the corresponding proteins in group II mycoplasmas were antigenic (data not shown).

Discussion

Previously, we classified clinical isolates of *M. pneumoniae* into two groups, I and II, based upon the diversity of their major cytoadhesin P1 gene (Dallo *et al.*, 1990; Su *et al.*, 1990a,b). Further analysis of these mycoplasma strains by restriction enzyme fingerprinting of the chromosomal DNA and two-dimensional gel electrophoresis of proteins has further established molecular distinctions among these two groups. With DNA fingerprinting, in addition to the 12 kb *Hind*III band reported previously (Su *et al.*, 1990b), enzymes such as *Apa*I, *Nar*I, *Pvu*I, *Sac*I, *Sac*II, *Sal*I and *Xho*I further discriminated between group I and group II *M. pneumoniae* isolates. Within each group, we have not yet observed variations in the restriction enzyme fingerprinting profile. On the other hand, restriction fragments generated by other restriction enzymes, such as *Eco*RI or *Sma*I (Dallo *et al.*, 1990), and *Rsr*II (Fig. 2) reveal no differences indicating that *M. pneumoniae* as a species has preserved overall genetic homology. Chandler *et al.* (1982) studied the genomic and phenotypic differences of five strains of *M. pneumoniae* (PI-1428, M129, B176, FH and Mac). They found that DNA cleavage patterns produced by *Xba*I and *Eco*RI were similar with the exception of strains FH and Mac (group II) that differed slightly from the other three strains (M129-B16 belongs to group I). They concluded that because of its restricted ecological niche, *M. pneumoniae* as a species was remarkably stable (Su *et al.*, 1988).

The differences in DNA fingerprinting patterns observed between the two groups could reflect distinct restriction site arrangements and unique DNA sequences in each genome. We have detected two stretches of major differences in nucleotide sequences (72% and 90% homology) in the P1 cytoadhesin genes in these two groups (Su *et al.*, 1990a). In addition, some alterations in DNA fingerprinting patterns could be caused by distinct restriction modification patterns of the genomes involving methylation of cytosine or adenine at specific restriction sites (Brooks & Roberts, 1982; Razin & Razin, 1980), since all the restriction endonucleases that discriminate between group I and group II isolates are also sensitive to this type of restriction modification (Nelson & McClelland, 1987). Razin & Razin (1980) found that several species of mycoplasmas contained modified deoxynucleotides.

Analysis of *M. pneumoniae* proteins by two-dimensional SDS-PAGE demonstrated that the overall protein profiles of all mycoplasma strains within each group were identical. However, protein changes were readily detected between the two groups based upon isoelectric points, quantities of protein and the absence of specific protein species. These results further confirmed that the observed differences in DNA restriction patterns correlate with differences at the gene level. Since many of the proteins that demonstrate charge variations remain antigenic, infection by different *M. pneumoniae* clinical groups may result in modified host immune responses (Su *et al.*, 1990a). Whether any of these observed differences contribute to the overall survival and virulence potential of these mycoplasmas is unknown.

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