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Possible Origin of Sequence Divergence in the P1 Cytadhesin Gene of *Mycoplasma pneumoniae*

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Specific regions of the P1 adhesin structural gene of *Mycoplasma pneumoniae* hybridize to various parts of the mycoplasma genome, indicating their multiple-copy nature. In addition, restriction fragment length polymorphisms and sequence divergence have been observed in the P1 gene, permitting the classification of clinical isolates of *M. pneumoniae* into two groups, I and II. These data suggest that the observed P1 gene diversity may be explained by homologous recombination between similar but not identical multicopy P1-related sequences and the P1 structural gene. We used oligonucleotide probes specific to the diverged regions of the group I and group II P1 structural genes to clone and sequence multicopy P1-related DNA segments. We detected sequences in group I *M. pneumoniae* isolates that were homologous not only to the group I P1 structural gene but also to the diverged regions of the group II P1 structural gene. Likewise, sequences in group II clinical isolates that were homologous both to the group II P1 structural gene and the diverged regions of the group I P1 structural gene were detected.

Adherence of pathogenic microorganisms to specific tissue surfaces is a crucial step in the establishment of infection. *Mycoplasma pneumoniae*, a flask-shaped procaryote that causes primary atypical pneumonia in humans (3), requires a group of adhesins and accessory proteins to cytadhere (2, 13). A 170-kDa adhesin, designated P1, must cluster at the specialized tip-like organelle of *M. pneumoniae* for successful parasitism (2, 7, 8). Mutants of *M. pneumoniae* that lack P1 or are unable to cluster P1 at the tip cannot adhere to host cells and are avirulent (2, 13). During infection of humans and experimental animals, the P1 protein elicits a strong humoral immune response (14).

To further elucidate the structural and functional properties of P1, the P1 gene was cloned and DNA and protein sequences were determined (10, 29). Epitopes that reacted with cytadherence-blocking monoclonal antibodies were mapped to specific P1 regions (6, 12). Southern blot analysis of the entire *M. pneumoniae* chromosome with subclones of the P1 gene established that there is only one full-length copy of the P1 structural gene within single organisms; however, two-thirds of the P1 gene hybridized to multiple copies, indicating extensive homology between specific sequences of the P1 gene and other segments of the mycoplasma genome (25). This observation raised the possibility that P1 gene variation might result from homologous recombination. There are numerous examples in pathogenic microorganisms of recombination between homologous but slightly different gene copies leading to antigenic variation and evasion of host defense mechanisms (1, 20, 21, 23).

Southern blot analysis of *M. pneumoniae* clinical isolates with P1 gene subclones as hybridization probes detected restriction fragment length polymorphisms, permitting the classification of these mycoplasma isolates into two distinct categories, designated group I and group II (5, 27). Comparison of the P1 genes from these two groups revealed two stretches of major sequence variation (27). One area of divergence was located at nucleotides 640 through 1100 of the P1 gene, where the two groups shared only 72% homology. The other stretch was located at nucleotides 2818 through 3456, where 90% homology was observed (27). To trace the source of these diverged sequences and to explain the possible mechanisms of P1 gene diversity, oligonucleotide probes specific to the divergent regions of group I and group II P1 genes were synthesized. These probes were used in Southern blot hybridizations to detect the presence of related sequences in group I and group II mycoplasmas.

MATERIALS AND METHODS

Mycoplasma strains and growth conditions. Group I *M. pneumoniae* strains were M129-B16 (ATCC 29342) and a clinical isolate, PN 597, isolated from a patient from Washington state in the 1960s (5). Group II *M. pneumoniae* strains were FH (ATCC 15531), TW 7-5 (isolated in South Carolina in the 1970s), and UTMB (isolated from the synovial fluid of an arthritis patient in 1986) (5). Mycoplasmas were grown at 37°C for 3 days in 32-ounce (950-ml) glass prescription bottles containing 70 ml of SP4 medium (31). Glass-attached mycoplasmas were rinsed twice with 10 mM sodium phosphate (pH 7.2)–0.1 M NaCl, scraped into the same buffer, and collected by centrifugation at 9,500 × *g* for 20 min.

Oligonucleotide probes. Six pairs of 20-mer oligonucleotides corresponding to the regions of divergency observed in group I and group II P1 genes (27) were synthesized. These probes were purchased as purified preparations from O.C.S. Laboratories, Inc., Denton, Tex. Table 1 details the probes and the corresponding complementary nucleotide sequences in the P1 genes of group I and group II *M. pneumoniae* strains.

Southern blot analysis of *M. pneumoniae* genome. DNA was purified from individual strains of *M. pneumoniae* by previously published procedures (26) and digested in batches overnight with excess amounts of appropriate restriction enzymes. Digested DNA (5 μg) was loaded into individual

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TABLE 1. Complementary oligonucleotide probes corresponding to diverged P1 sequences

M. pneumoniae group	Probe designation	Nucleotide nos.	Synthetic 20-mer oligonucleotide	Corresponding subclone region	No. of nucleotide differences
I	1A	651–670	TTC GCT GCT GTG GTA AGCCC	C	11
II	1B	651–670	TCC CTT GGT TAG GGA GCGCT		
I	2A	773–792	TGA AGA ACC AGT GGC CTCAG	C	12
II	2B	779–798	GGA ACT GGA ACT CGA TTCTT		
I	3A	954–973	CGG ACT GAC CCG ACT CCTCG	D	14
II	3B	975–994	CTT GCT GGT TGT TAT TGGAG		
I	4A	2720–2739	GGT GTG GTT TTC GTC ACTTT	H	6
II	4B	2735–2754	CTT GGT GTG GTC GTC ACTTG		
I	5A	3028–3047	TTG GAA TCG GAC CCA CTTCG	I	13
II	5B	3040–3059	TTA TCA TCT TGG CCG GACTT		
I	6A	3253–3272	AGG GTG GCT TTG GAA TCATT	I	15
II	6B	3268–3287	TTC GAC GTT GTG TTT GTGCC		

wells, and DNA fragments were separated on 0.7% agarose gels. Gels were stained with ethidium bromide and photographed, and the DNA was transferred to nitrocellulose filters by standard procedures (22). Oligonucleotide probes were labeled at the 5′ end with [γ-^{32}P]ATP by the T4-polynucleotide kinase reaction (15). Filters were prehybridized at 40°C overnight in a solution containing 6× SSC (1× SSC is 0.15 M sodium citrate–0.15 M sodium chloride [pH 7.0]), 5× Denhardt solution (0.1% [each] Ficoll [Pharmacia], polyvinylpyrrolidone, and bovine serum albumin), 50 mM sodium phosphate (pH 6.8), and 0.1 mg of herring sperm DNA per ml. Hybridizations were performed in prehybridization solution plus 10% dextran sulfate and ^{32}P-labeled oligonucleotide probes. After hybridization at 40°C for 24 h, filters were rinsed twice at 4°C (30 min each) with 6× SSC, washed twice in wash solution (3 M tetramethylammonium chloride, 50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 0.1% sodium dodecyl sulfate) at 40°C for 20 min, rinsed once with 6× SSC at 4°C, dried, and exposed to X-ray film (32). Under these experimental conditions, the 20-mer oligonucleotide probes hybridized only to the homologous P1 structural gene and homologous P1-related sequences.

Cloning of the P1-related sequences. *M. pneumoniae* genomic DNA (300 μg) was digested to completion with appropriate restriction enzymes, and DNA fragments were separated electrophoretically on 0.7% agarose gels. Fragments of the selected target sizes were transferred to NA45 ion-exchange filters (Schleicher and Schuell, Inc., Keene, N.H.), eluted with 20 mM Tris-HCl (pH 8.0) and 1 M NaCl at 65°C, and precipitated with ethanol.

Plasmid pUC9 or pUC19 was used as the cloning vector. Plasmids were digested with appropriate restriction enzymes and treated with intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to remove the 5′-end phosphate group. Purified mycoplasma DNA and vectors were mixed at a 1:1 molar ratio and ligated with T4 DNA ligase (15). The ligated DNA was used to transform competent *Escherichia coli* DH5α (BRL) according to the instructions of the manufacturer. Transformants were selected on LB agar plates containing 50 μg of ampicillin per ml (15). Colonies containing P1-related sequences were further selected by colony hybridization with ^{32}P-labeled oligonucleotide probes (15).

Analysis of clones containing P1-related sequences. Colonies that hybridized to selective probes were grown at 37°C overnight in 6 ml of LB broth containing 50 μg of ampicillin per ml, and cultures were harvested by centrifugation. Plasmids were released from bacteria by the alkaline lysis method (11) and precipitated with isopropanol (0.6 volume) at room temperature. Pellets were redissolved in 200 μl of TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA), treated with 1 mg of RNase A per ml at 37°C for 30 min, and extracted sequentially with phenol, phenol-chloroform (1:1), and chloroform. Finally, plasmid DNA was precipitated with isopropanol (0.6 volume) in the presence of 2.5 M ammonium acetate.

Plasmids were analyzed by digestion with restriction enzymes, and a partial restriction map was constructed for each clone. From the restriction maps, appropriate enzymes were selected to digest inserts into smaller pieces that were separated on agarose gels and transferred to nitrocellulose filters. Pools of oligonucleotide probes were used to hybridize to the filter to determine the precise location of related DNA sequences.

DNA sequence analysis. The dideoxy-chain termination method of Sanger et al. (19) was used to determine DNA sequences. Clones containing the P1-related sequences were grown at 37°C overnight in 500 ml of LB broth supplemented with 0.5% glucose plus 50 μg of ampicillin per ml. Cells were harvested by centrifugation, plasmids were extracted and further purified by CsCl gradient centrifugation (15), and inserts were released and fragmented with appropriate restriction enzymes. DNA fragments that hybridized to specific oligonucleotide probes were separated by electrophoresis and transferred to NA45 ion-exchange filter paper as described above. These fragments were cloned into M13 phages, and single-stranded phages were purified before DNA sequencing (29). Nucleic acid and protein sequence computer analyses were performed with the Microgenie program (Beckman Instruments Inc., Palo Alto, Calif.). In most cases, sequences from both complementary strands were determined.

Nucleotide sequence accession number. The nucleotide sequence of the P1 cytadhesin of *M. pneumoniae* has been assigned EMBL accession number M18639.

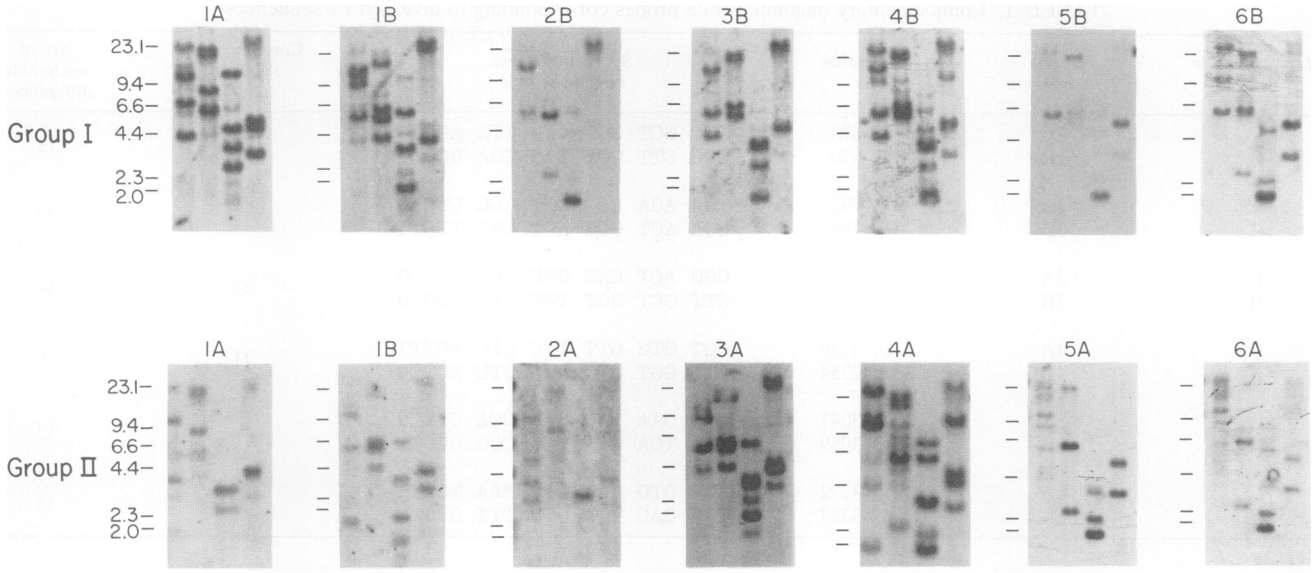

FIG. 1. Southern blot analysis of the *M. pneumoniae* genome with oligonucleotide probes specific to diverged regions of the P1 structural gene. Group I is represented by strain M129-B16, and group II is represented by strain TW 7-5. Other isolates from the same group exhibited similar patterns. Genomic DNA was isolated and digested with various restriction enzymes (from left to right in each panel: *Bam*HI, *Eco*RI, *Hin*dIII, *Sma*I). The DNA fragments were separated on 0.75% agarose gels by electrophoresis and then transferred to nitrocellulose filters and probed with various ^{32}P-labeled oligonucleotide probes. The sequences of these probes and their specificities are listed in Table 1. A probes are specific to the diverged regions of the group I P1 gene, and B probes are specific to the group II P1 gene. Numbers on the left side are DNA molecular size standards in kilobases.

RESULTS

Southern blot analysis of the *M. pneumoniae* genome. Southern blot analysis of mycoplasma genomic DNA with group-specific oligonucleotide probes is presented in Fig. 1. The A probes (1A through 6A, Table 1) were specific for the diverged sequence of the group I P1 structural gene, and the B probes (1B through 6B, Table 1) were specific for the group II P1 gene (27). Probe 1A, which corresponds to the C region of the P1 structural gene (25), hybridized to multiple copies of the homologous group I (M129 and PN597 strains) genomic DNA. The hybridization pattern is similar to our published data with the C-region subclone of the P1 gene (25). As expected, probe 1B hybridized to multiple copies of the homologous group II (FH, TW 7-5, and UTMB strains) genomic DNA. Additional comparisons showed that probes 1A and 1B also hybridized to multiple fragments of the heterologous group of mycoplasma clinical isolates (i.e., probe 1A hybridized with group II DNA and probe 1B hybridized with group I DNA; Fig. 1). Interestingly, comparison of the hybridization patterns displayed by each probe with group I and group II DNA revealed similarities. For example, in both group I and group II strains, probe 1A hybridized to the following fragments: a 10-kb *Bam*HI fragment, 20- and 9-kb *Eco*RI fragments, 3.5- and 2.5-kb *Hin*dIII fragments, and a 4.4-kb *Sma*I fragment. Probe 1B hybridized to a different set of fragments than did probe 1A, yet many common fragments were detected in both group I and group II genomes (Fig. 1). These data indicate that the overall genomic structure of group I and group II *M. pneumoniae* strains was conserved. Hybridization with other probes (Table 1) exhibited similar relationships (Fig. 1). Furthermore, when the hybridization patterns of the B probes (specific for the group II P1 structural gene) were examined with group I genomic DNA, several bands hybridized to more than one probe, indicating long stretches of homology to the diverged sequences of the group II P1 gene in group I isolates (Fig. 1). These bands include a 5.5-kb *Bam*HI fragment that hybridized to all six probes (1B through 6B); a 4-kb *Bam*HI fragment that hybridized to probes 1B, 3B, and 4B; a 6.0-kb *Eco*RI band that hybridized to probes 1B, 3B and 4B; a 5.2-kb *Eco*RI band that hybridized to probes 1B, 2B, 3B, and 4B; a 1.8-kb *Hin*dIII band that hybridized to all six probes; and a 5-kb *Sma*I band that hybridized to probes 3B, 4B, 5B, and 6B. Long stretches of homology were also observed when the A probes (specific to the group I P1 structural gene) were used to screen the group II genome (Fig. 1). For example, a 6-kb *Eco*RI band hybridized to probes 3A, 4A, 5A and 6A; a 3.2-kb *Hin*dIII band hybridized to all six probes (1A through 6A); a 2.1-kb *Hin*dIII band hybridized to probes 4A, 5A and 6A; a 1.8-kb *Hin*dIII band hybridized to probes 3A, 4A, 5A, and 6A; a 5-kb *Sma*I band hybridized to probes 3A, 4A, 5A, and 6A; and a 4-kb *Sma*I band hybridized to probes 2A, 3A, and 4A. Genomic DNA from strains within the same group displayed the same hybridization patterns, indicating that each group conserved its genomic organization.

Cloning and characterization of P1-related sequences. Specific P1-related group I DNA bands that hybridized to B probes (i.e., group II P1-specific sequences) and group II DNA bands that hybridized to A probes (i.e., group I P1-specific sequences) were cloned. *M. pneumoniae* M129-B16 was selected as the source of group I DNA, and strain TW 7-5 was selected as the group II representative. From M129-B16 group I DNA, libraries were constructed from the 4-kb *Bam*HI, 5.5-kb *Bam*HI, 6-kb *Eco*RI, 1.8-kb *Hin*dIII, and 5-kb *Sma*I regions and screened with a pool of the six B probes (Table 1). Positive clones were selected, and plasmids were extracted for further characterization. From TW 7-5 group II DNA, libraries of the 1.8-kb *Hin*dIII, 2.1-kb *Hin*dIII, 3.2-kb *Hin*dIII, 3-kb *Sma*I, 4-kb *Sma*I, and 5-kb

TABLE 2. Selected clones containing P1-related sequences

Group	Probes	Clone	Vector	Hybridization to probes		Description
				1, 2, and 3	4, 5, and 6	
I	B	B16B5-4	pUC19	+	+	5.5-kb *Bam*HI fragment
		B16E6-1	pUC19	+	−	6-kb *Eco*RI fragment
		B16B4-1	pUC18	+	−	4.0-kb *Bam*HI fragment
II	A	TWH2-5	pUC9	−	+	1.8-kb *Hin*dIII fragment
		TWH2-9	pUC9	−	+	2.1-kb *Hin*dIII fragment
		TWS-4	pUC19	+	+	4.0-kb *Sma*I fragment

*Sma*I regions were constructed and screened with a pool of the six A probes (Table 1). Several positive clones from each library were further characterized, and restriction maps were constructed.

Oligonucleotide probes 1B, 2B, and 3B, which hybridized to the diverged P1 structural gene sequences in the C and D regions of group II *M. pneumoniae* (Table 1) (25), were pooled and used to identify homologous regions in the selected group I clones for DNA sequence analysis. Oligonucleotide probes 4B, 5B, and 6B were also pooled and used to identify appropriate group I clones. Similar experiments were performed with pools of group I-specific probes to identify homologous regions in group II DNA. A list of selected clones is presented in Table 2. From each clone a partial restriction map was constructed, and regions that shared strong homology to the P1 structural genes were identified (Fig. 2).

From strain M129-B16 of group I *M. pneumoniae*, clones B16 B5-4, B16 E6-1, and B16 B4-1 (Table 2) were characterized. Other clones detected by the pool of 1B, 2B, and 3B probes were found to overlap with clones B5-4 and E6-1. Furthermore, although clone E6-1 comprised a 6-kb *Eco*RI fragment similar in size to the authentic P1 structural gene (29), restriction map analysis clearly distinguished between the two 6-kb *Eco*RI fragments. From strain TW 7-5 of group II *M. pneumoniae*, three clones were selected (Table 2).

Sequence analysis of the selected P1-related clones. For clone B16 B5-4, two regions of significant homology with the P1 structural gene were found (Fig. 2). One region of 1 kb shared 91% sequence homology with the group II P1 gene from nucleotides 234 to 1202 and 78% homology with the corresponding region of the group I P1 gene (Fig. 2). Within this region, the B16 B5-4 sequence was identical to the group II P1 gene at positions where the corresponding probes 1B and 3B were located (Fig. 3A). However, the B16 B5-4 sequence was not identical to either P1 structural gene at the probe 2B position (Fig. 3A). The other region of 1.7 kb shared 86% sequence homology with the group II P1 gene (from nucleotides 2355 to 4022) and 83% homology with the corresponding region in the group I P1 gene. At the positions where probes 5B and 6B were located, the B16 B5-4 sequence was identical to that of the group II P1 gene (Fig. 3B). However, at the position where probe 4B was located (Table 1), the sequence of clone B16 B5-4 was not identical to that of either the group I or the group II P1 gene (Fig. 3B). The B16 B5-4 clone also contained sequences similar to those of pSPT6 (Fig. 2), which was identified as a fragment containing two species of repetitive sequences in the *M. pneumoniae* genome (33).

Clone B16 E6-1, which hybridized to the pool of probes 1B, 2B, and 3B, was found to share 89% homology with the group II P1 gene from nucleotides 560 to 1202 and 79% homology with the corresponding region of the group I P1 gene (Fig. 2). The sequences of B16 E6-1 that correlated with probes 1B, 2B, and 3B were almost identical to those of the group II P1 structural gene. However, considerable sequence variation was observed in the regions located between these probes (Fig. 3A).

Clone B16 B4-1 was originally identified as a fragment that shared extensive homology with the P1 gene, and subsequent sequence analysis revealed two homologous stretches (Fig. 2). One region of about 1.1 kb exhibited 88% homology with the group II P1 gene from nucleotides 59 to 1202 and 93% homology with the corresponding region of the group I P1 gene. At nucleotide positions where probes 1B and 2B were located (Table 1), the sequence of clone B16 B4-1 was identical to that of the group I P1 structural gene. However, at sequence nucleotide positions where probe 3B was located, the sequences of B16 B4-1 were identical to those of the group II P1 structural gene (Fig. 3A). Another stretch of about 1 kb shared 83% homology with the group II P1 sequence from nucleotides 2996 to 4061 and 85% homology with corresponding regions of the group I P1 gene. These data were interesting because the observed sequence homologies (i.e., from nucleotides 2996 to 4061) corresponded to the H and I regions of the P1 gene (25), yet no hybridization was detected with the pool of 4B, 5B, and 6B probes (Table 2). The 20-mers represented by these probes comprised sequences distinct from those of the B16 B4-1 clone; thus, this clone is an unusual chimera of the parent P1 gene.

Clone TW H2-5 (Fig. 2) contained a 1.8-kb *Hin*dIII fragment that hybridized to the pool of 4A, 5A, and 6A probes (Table 2). Within this clone, a continuous stretch of 1.3 kb shared 95% homology with the group I P1 gene from nucleotides 2339 to 3584 and 91% homology with the corresponding region of the group II P1 gene. The sequence of this clone was identical to that of the group I P1 gene at positions where probes 4A, 5A, and 6A were located (Fig. 3B).

Clone TW H2-9 contained a 2.1-kb *Hin*dIII fragment that exhibited 88% sequence homology with the group I P1 gene from nucleotides 2340 to 3824 and 83% homology with corresponding regions of the group II P1 gene. This sequence was identical to that of the group I P1 gene at the positions of probes 5A and 6A, but in the region where probe 4A was located three mismatches were observed (Fig. 3B).

Clone TW S-4 contained three areas of sequence homology with the P1 gene. One region exhibited 93% homology with the group I P1 gene from nucleotides 2350 to 2468 (Fig. 2) and the corresponding region of the group II P1 gene. A second region shared 82% sequence homology with the group I P1 gene from nucleotides 2940 to 3285 and 83% homology with the corresponding region of the group II P1 gene. Within the region where probes 5B and 6B were located, substantial sequence divergency between group I

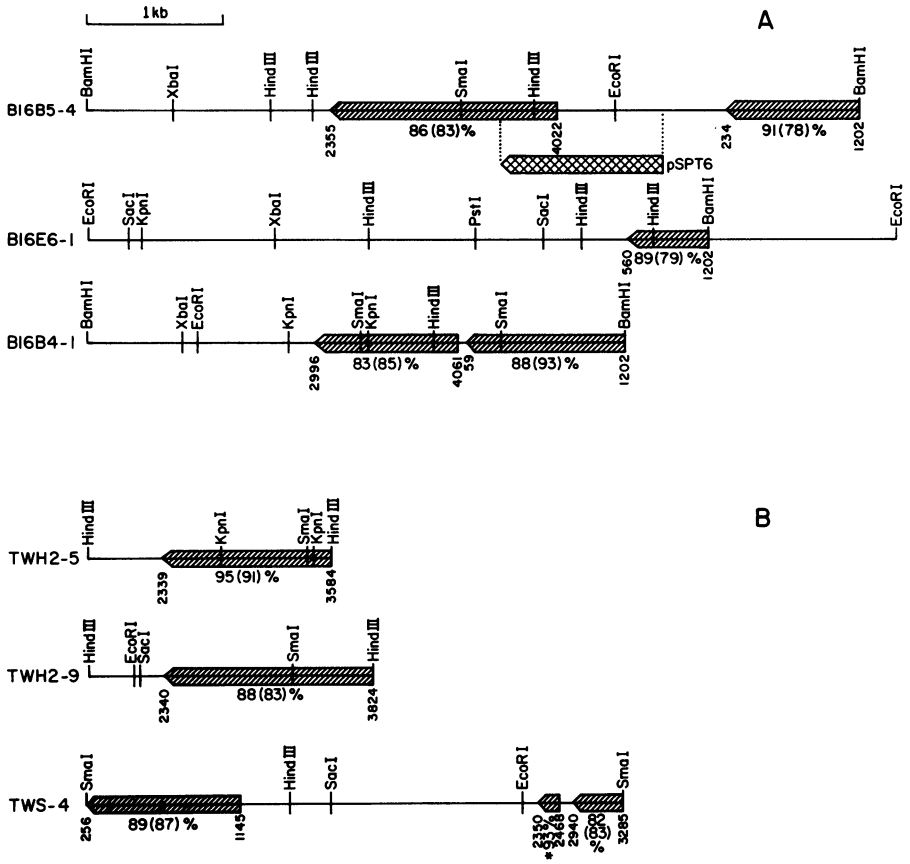

FIG. 2. Partial restriction maps and regions of sequence homology between selected clones and the P1 structural gene. Regions of significant homology to P1 are represented (▨) from 5′ to 3′. The portion of the sequence that matched to SPT6 (33) is also represented (▩). (A) Three clones selected from strain M129-B16 (group I). The numbers under each homologous region indicate the nucleotide number of the group II P1 structural gene with which the region is homologous and the percentage of homology. Numbers within parentheses represent the percentage of sequence homology when the clone is compared with the group I P1 structural gene. (B) Three clones selected from strain TW 7-5 (group II). The numbers under each homologous region indicate the nucleotide number with which the region is homologous and the percentage of homology when compared with the group I P1 gene. Numbers within parentheses represent the percentage of sequence homology when the clone is compared with the group II P1 structural gene; the asterisk (*) indicates the same percent homology (i.e., 93%) for both group I and group II P1 structural genes.

and group II P1 structural gene sequences was detected (Fig. 3B). A third region of about 0.9 kb demonstrated 89% homology with the group I P1 gene from nucleotides 256 to 1145 and 87% homology with the corresponding region of the group II P1 gene. Within this region where the two P1 sequences diverged, the TW S-4 sequence was identical to those of probes 1A and 2A but not to that of probe 3A (Fig. 3A).

DISCUSSION

Mycoplasmas are cell wall-less, self-replicating microorganisms with genome sizes ranging from 577 to 1,380 kb (17, 18, 24). Evolutionarily, mycoplasmas probably derived from low-G+C-containing gram-positive bacteria by chromosomal size reduction (34). It is generally believed that the mycoplasma genome contains only the minimal essential genetic information required for free-living microorganisms. Nonetheless, recent genetic studies have documented the existence of many short repetitive sequences in the genomes of several host-associated mycoplasmas (4, 9, 16, 25, 30, 33); the functions of these repetitive sequences are unclear. It is possible that mycoplasmas, like other pathogenic microorganisms, possess the ability to alter their surface molecules by homologous recombination among multiple copies of specific gene families (1, 20, 21, 23).

Previous studies by us (25) and others (4, 33) have demonstrated that specific repetitive elements are located in or adjacent to the operon of the cytadhesin P1 gene of *M. pneumoniae* (4, 25, 33). Within the P1 gene, about two-thirds of the coding region consists of repetitive sequences (25). A survey of clinical isolates revealed two types of P1 genes, resulting in the categorization of *M. pneumoniae* strains into two groups, I and II (5). In an attempt to understand the origin of the diverged P1 sequences, oligonucleotide probes specific to each diverged P1 region were synthesized and used to examine chromosomal fragments of selected group I and group II *M. pneumoniae* isolates. Data indicated that individual oligonucleotide probes hybridized to multiples copies within each mycoplasma group. In addition, specific probes hybridized to the genomes of both group I and group II strains.

Cloning and sequence analysis of selected DNA fragments revealed several interesting and unexpected features. For example, almost the entire group II P1 sequence in the

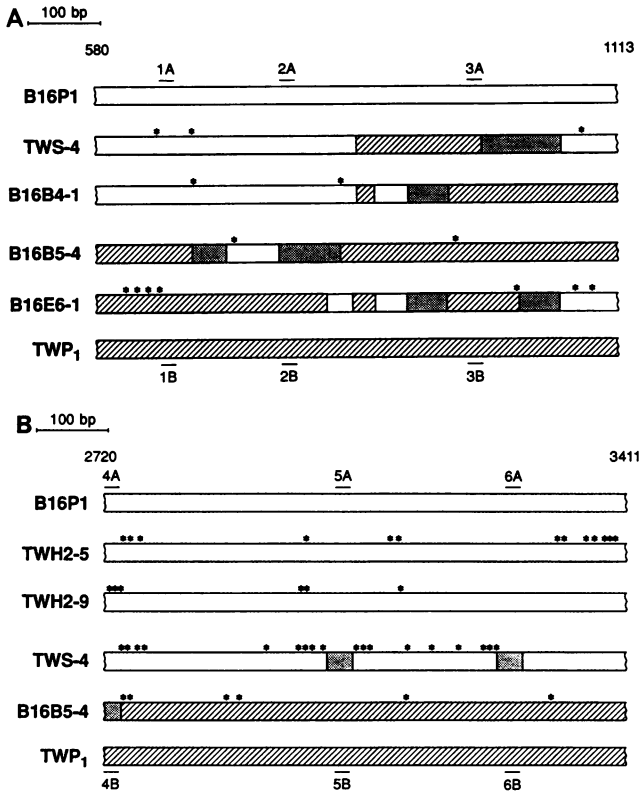

FIG. 3. Schematic summary of sequence relationships between oligonucleotide probes (1A to 3A and 1B to 3B [A]; 4A to 6A and 4B to 6B [B]; Table 1) and P1-related sequences from group I and group II *M. pneumoniae* clinical isolates. Symbols: ▭, regions of sequence homology with the group I P1 structural gene; ▨, regions of sequence homology with group II structural gene; ▩, regions of sequence dissimilarity in the group I and group II P1 structural genes; *, one or several base mismatches within the region.

diverged region from nucleotides 580 to 1113 could be generated by a double cross-over between group I clones B16 E6-1 and B16 B5-4 (Fig. 3A). Also of interest was the existence within clone B16 B5-4 of pSPT6 sequences that contained two species of *M. pneumoniae* repetitive sequences, RepMp1 and RepMp2 (33). The cloned 5.5-kb *Bam*HI fragment (Table 2) was probably identical to the 6-kb *Bam*HI fragment described by Wenzel and Herrmann (33). Although some DNA sequence discrepancies between our sequences and the published sequences were observed, Wenzel and Herrmann indicated that the published sequences were not 100% accurate (33). Although RepMp2 was part of the P1 gene, RepMp1 represented another species of about 300 bp that was repeated approximately 10 times within the *M. pneumoniae* genome. At the end of pSPT6, a stretch of 63-bp direct repeats was detected (Fig. 2).

Our results indicate that most of the diverged P1 sequences between group I and group II *M. pneumoniae* isolates are within the genomic DNA of both groups. Although probe 4B sequences were not detected in the three group I clones and probe 3A sequences were not detected in the two group II clones analyzed, both probes 3A and 4B hybridized to multiple bands in the *M. pneumoniae* genome. Therefore, it is possible that these diverged sequences occur within other chromosomal fragments that hybridized to the probes but were not sequenced.

Our previous studies indicated that *M. pneumoniae* undergoes a high frequency of spontaneous mutation to a noncytadhering phenotype (13, 24). One reason for the genome instability might be the existence of numerous species of repetitive sequences that share homology with the P1 structural gene (25, 33). Recombination among these sequences could result in many P1 gene variations. However, a survey of P1 genes by Southern blot analysis of 29 clinical isolates from different times and locations revealed only two types of P1 genes (5, 27, 28). A more extensive study of the mechanisms of homologous recombination in mycoplasmas should help to clarify these observations.

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REFERENCES

1. **Barbour, A. G.** 1990. Antigenic variation of a relapsing fever *Borrelia* species. Annu. Rev. Microbiol. **44**:155–171.
2. **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.
3. **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.
4. **Colman, S. D., P. C. Hu, and K. F. Bott.** 1990. Prevalence of novel repreat sequences in and around the P1 operon in the genome of *Mycoplasma pneumoniae*. Gene **87**:91–96.
5. **Dallo, S. F., J. R. Horton, C. J. Su, and J. B. Baseman.** 1990. Restriction fragment length polymorphism in the cytadhesin P1 gene of human clinical isolates of *Mycoplasma pneumoniae*. Infect. Immun. **58**:2017–2020.
6. **Dallo, S. F., C. J. Su, J. R. Horton, and J. B. Baseman.** 1988. Identification of P1 gene domain containing epitope(s) mediating *Mycoplasma pneumoniae* cytadherence. J. Exp. Med. **167**:718–723.
7. **Feldner, J., U. Gobel, and W. Bredt.** 1982. *Mycoplasma pneumoniae* adhesin localized to tip structure by monoclonal antibody. Nature (London) **298**:765–767.
8. **Hu, P. C., R. M. Cole, Y. S. Huang, T. A. Graham, D. E. Gardner, A. M. Collier, and W. A. Clyde.** 1982. *Mycoplasma pneumoniae* infection: role of a surface protein in the attachment organelle. Science **216**:313–315.
9. **Hu, W. S., R. Y.-H. Wang, R. S. Liou, J. W.-K. Shih, and S.-C. Lo.** 1990. Identification of an insertion-sequence-like genetic element in the newly recognized human pathogen *Mycoplasma incognitus*. Gene **93**:67–72.
10. **Inamine, J. M., T. P. Denny, S. Loechel, U. Shaper, C. H. Huang, K. F. Bott, and P. C. Hu.** 1988. Nucleotide sequence of the P1 attachment-protein gene of *Mycoplasma pneumoniae*. Gene **64**:217–229.
11. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid vector cloning. Nucleic Acids Res. **9**:2989–2998.
12. **Jacobs, E., A. Pilatschek, B. Gerstenecker, K. Oberle, and W. Bredt.** 1990. Immunodominant epitopes of the adhesin of *Mycoplasma pneumoniae*. J. Clin. Microbiol. **28**:1194–1197.
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. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. **Nur, I., D. J. LeBlanc, and J. G. Tully.** 1987. Short interspersed and repetitive DNA sequences in spiroplasma species. Plasmid **17**:110–116.
17. **Pyle, L. E., L. N. Corcoran, B. G. Cocks, A. D. Bergemann, J. C. Whitley, and L. R. Finch.** 1988. Pulse-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes. Nucleic Acid Res. **16**:6015–6025.
18. **Razin, S.** 1985. Molecular biology and genetics of mycoplasmas (mollicutes). Microbiol. Rev. **49**:419–455.
19. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitor. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
20. **Segal, E., P. Hagblom, S. H. Seifert, and M. So.** 1986. Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. Proc. Natl. Acad. Sci. USA **83**:2177–2188.
21. **Smith, N. H., P. Beltran, and R. K. Selander.** 1990. Recombination of *Salmonella* phase I flagellin genes generates new serovars. J. Bacteriol. **172**:2209–2216.
22. **Southern, E. M.** 1975. Detection specific sequence among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–517.
23. **Stern, A., M. Brown, P. Nickel, and T. F. Meyer.** 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. Cell **47**:61–71.
24. **Su, C. J., and J. B. Baseman.** 1990. Genome size of *Mycoplasma genitalium*. J. Bacteriol. **172**:4705–4707.
25. **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.
26. **Su, C. J., A. Chavoya, and J. B. Baseman.** 1989. Spontaneous mutation results in loss of the cytadhesin (P1) of *Mycoplasma pneumoniae*. Infect. Immun. **57**:3237–3239.
27. **Su, C. J., A. Chavoya, S. F. Dallo, and J. B. Baseman.** 1990. Sequence divergency of the cytadhesin gene of *Mycoplasma pneumoniae*. Infect. Immun. **58**:2669–2674.
28. **Su, C. J., S. F. Dallo, and J. B. Baseman.** 1990. Molecular distinctions among clinical isolates of *Mycoplasma pneumoniae*. J. Clin. Microbiol. **28**:1538–1540.
29. **Su, C. J., V. V. Tryon, and J. B. Baseman.** 1987. Cloning and sequence analysis of cytadhesin gene (P1) from *Mycoplasma pneumoniae*. Infect. Immun. **55**:3023–3029.
30. **Taylor, M. A., R. V. Ferrell, K. S. Wise, and M. A. McIntosh.** 1988. Reiterated DNA sequences defining genomic diversity within the species *Mycoplasma hyorhinis*. Mol. Microbiol. **2**:665–672.
31. **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.
32. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base composition-independent hybridization in tetramethylammonium chloride. A method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82**:1585–1588.
33. **Wenzel, R., and R. Herrmann.** 1988. Repetitive DNA sequences in *Mycoplasma pneumoniae*. Nucleic Acid Res. **16**:8337–8350.
34. **Woese, C. R., J. Maniloff, and L. B. Zablen.** 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. USA **77**:494–498.