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Mycoplasma pneumoniae P1 Type 1- and Type 2-Specific Sequences within the P1 Cytadhesin Gene of Individual Strains

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***Mycoplasma pneumoniae* strains traditionally are divided into two types, based on sequence variation in the P1 gene. Recently, however, we have identified 8 P1 subtypes by restriction fragment length polymorphism analysis. In the present study the P1 gene sequences of three P1 type 1 and two P1 type 2 *M. pneumoniae* strains were analyzed. A new P1 gene sequence in a type 1 strain with partial similarity to a recently reported variable region in the P1 gene of an *M. pneumoniae* type 2 strain (T. Kenri, R. Taniguchi, Y. Sasaki, N. Okazaki, M. Narita, K. Izumikawa, M. Umetsu, and T.Sasaki, Infect. Immun. 67:4557–4562, 1999) was identified. In addition, the P1 gene of the type 1 strain contained another region with nucleotide polymorphisms identical to a stretch in the P1 gene of one of our type 2 strains. These findings indicate that recombination between sequences specific for P1 type 1 and type 2 had occurred and that P1 type 1 and type 2 hybrid sequences can be present within the P1 gene of an individual strain. Identical or nearly identical variable P1 gene sequences were present in several repetitive regions outside the P1 gene locus in the genome of *M. pneumoniae* strain M129, implying recombination as a mechanism for generation of the P1 gene variation. Additionally, in the P1 gene sequences of four of the five strains studied, single-nucleotide polymorphisms different from the previously reported P1 type 1 and 2 characteristic sequences were identified. The polymorphic sites are candidate targets for genotyping of *M. pneumoniae* by direct sequencing of amplicons from clinical specimens.**

Mycoplasma pneumoniae is a common cause of respiratory infections in humans. Colonization of the respiratory epithelium by *M. pneumoniae* is mediated by the attachment organelle, a terminal tip structure of *M. pneumoniae* cells (17). Several bacterial surface proteins, including a 170-kDa protein, P1, are involved in the formation of the attachment organelle and cytadherence of *M. pneumoniae* to the respiratory epithelium. The 170-kDa protein P1 is a major adhesin protein which is densely clustered at the site of the attachment organelle (1). Protein P1 is encoded by a gene of nearly 5,000 bp, comprising copies of repetitive regions RepMP2/3 and RepMP4, which are present in the *M. pneumoniae* genome in 10 and 8 copies, respectively (8). Since humans mount a strong immune response to the P1 protein during infection (21; M. Pedersen, S. Birkelund, and G. Christiansen, Abstr. 13th Int. Congr. Int. Org. Mycoplasmol. 2000, p. 241), the P1 gene is likely to display antigenic variation (1).

Early studies showed the existence of only two P1 gene types among *M. pneumoniae* clinical isolates (22, 27). In those studies Southern blotting and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of P1 gene amplicons using one restriction enzyme were applied. Other approaches for genotyping of *M. pneumoniae* identified two genomic groups among *M. pneumoniae* clinical isolates, which corresponded to their P1 gene types (3, 6, 16, 30).

Among a collection of 218 *M. pneumoniae* clinical isolates in Japan, a new variable sequence in the P1 gene was identified in four P1 type 2 strains (15). We used an extended panel of restriction enzymes in PCR-RFLP analysis, and we recently reported that five subtypes could be discriminated among 13 P1 type 1 strains and that three subtypes could be discriminated among 8 P1 type 2 strains (6). These findings indicate that more variation in the P1 gene sequence exists than previously anticipated.

Until now, the complete sequences of the P1 genes of the P1 type 1 reference strain M129 (ATCC 29342), the P1 type 2 reference strain FH (ATCC 15531), and three clinical isolates (one P1 type 1 isolate and two P1 type 2 isolates) (26) have been reported. The P1 gene sequences of the clinical P1 type 1 isolate and of the two P1 type 2 isolates were identical to those of the respective reference strains M129 and FH (26). In order to analyze P1 gene sequence variability, we performed sequence analysis of the P1 genes of two *M. pneumoniae* reference strains and three *M. pneumoniae* clinical isolates with variable P1 genes as detected by our PCR-RFLP (6).

MATERIALS AND METHODS

***M. pneumoniae* strains and DNA isolation.** Two *M. pneumoniae* reference strains and three strains from a collection of 23 *M. pneumoniae* patient isolates used in P1 PCR-RFLP typing experiments as described before (6) were selected. Reference strains were PI 1428 (ATCC 29085), a P1 type 1 strain (6), and MAC (ATCC 15492), a P1 type 2 strain (27). Patient strains were two P1 type 1 strains, Mp22 and Mp4817, isolated in Denmark in 1963 and 1993, respectively, and one P1 type 2 strain, Mp1842, isolated in Denmark in 1987. Selection of these strains for P1 gene sequence analysis was based on their unique P1 PCR-RFLP pattern. *M. pneumoniae* strains were cultured in plastic flasks (Nunc, Roskilde, Denmark) containing 60 ml of SP4 medium at 37°C. Cells were harvested upon color change of the medium, after 1 to 5 weeks, by centrifugation at 8,000 × *g* for 45 min. The supernatant was discarded, and DNA was extracted from the pelleted bacteria with a QIAmp Tissue Kit (Qiagen GmbH, Hilden, Germany).

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DNA sequencing. Fragments of approximately 2,280 and 2,580 bp, together comprising almost the entire P1 gene, were amplified with primer pairs ADH1-ADH2 and ADH3-ADH4, respectively, using AmpliTaq DNA polymerase (Roche Molecular Systems, Inc., Branchburg, N.J.) (22). ADH1-ADH2 and ADH3-ADH4 amplicons were purified from agarose gel with QiaEx (Qiagen) and used for sequencing by applying a primer-walking strategy using a BigDye terminator cycle sequencing kit and a 307 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.).

Primer pairs P1up (GCTTTAAAGTATGGTGGCGGGGG) and ADH1BR (AAGTCATACCGGCGTAACGC), ADH2BF (GTAGTAGTAGTAGTCACA ACG) and ADH3BR (TGTCCACTTGAAGCCTTATC), and ADH4AF (CCG CACAGGTATCAGTCAAG) and P1down (GGTGAGGGTGTTGTGGTCTT GG) were used to generate amplicons comprising the sequences upstream of the ADH1-ADH2 fragment, between the ADH1-ADH2 and ADH3-ADH4 fragments, and downstream of the ADH3-ADH4 fragment, respectively. Sequencing of these amplicons allowed completion of the P1 gene sequences.

Sequence analysis and nucleotide sequence accession numbers. Sequence analysis was performed with the ClustalW multiple-alignment tool (http://pbil.ibcp.fr/cgi-bin/alignclustalw.pl). P1 gene nucleotide sequences from *M. pneumoniae* strain M129 (GenBank accession no. M18639), strain 309 (GenBank accession no. AB024618), and strain TW 7-5 (26) were used for alignments. Protein translation was performed with "The Protein Machine," using the *Mycoplasma* codon table (http://www2.ebi.ac.uk/translate/).

Unless stated otherwise, nucleotide positions in this paper are designated according to the numbering in the P1 gene sequence of *M. pneumoniae* strain M129 (29), accession number M18639.

The nucleotide sequence data reported in this paper are listed in the GenBank nucleotide sequence database under accession numbers AF286371 (strain PI 1428), AF289999 (strain Mp22), AF290000 (strain Mp4817), AF290001 (strain MAC), and AF290002 (strain Mp1842).

RESULTS

Comparison of P1 gene sequences. The complete P1 gene nucleotide sequences of two *M. pneumoniae* reference strains, PI 1428 and MAC, and of strains Mp22, Mp4817, and Mp1842 were determined. The promoter and terminator regions of the P1 gene sequences of all five strains were identical to the corresponding regions in the P1 gene sequence of strain M129, the strain used to sequence the entire *M. pneumoniae* genome (29). The deduced translation products were full-length P1 proteins. The sequences of the P1 type 1 strains PI 1428, Mp22, and Mp4817 showed the highest similarity with the P1 gene sequence of the P1 type 1 M129 strain, while the sequences of the two P1 type 2 strains MAC and Mp1842 had the highest similarity with the P1 gene sequence of the P1 type 2 TW 7-5 strain. The P1 gene sequences of all five strains contained the previously reported specific sequences characteristic for their respective P1 types (26).

The P1 gene nucleotide sequence of P1 type 1 reference strain PI 1428 was completely identical to the P1 gene sequence of strain M129 (29). In strain Mp22 two synonymous point mutations were identified relative to the M129 P1 sequence: at nucleotide position (nt) 3451 (C→T) and at nt 3927 (G→A).

The P1 gene nucleotide sequences of P1 type 2 strain Mp1842 and of strain TW 7-5 were almost identical. In strain Mp1842 one nonsynonymous mutation was identified at nt 3904 (G→A), resulting in an amino acid change at position 1302 from V to I.

Novel sequence variation in the P1 gene of strain Mp4817. In the P1 gene of P1 type 1 strain Mp4817, six nonsynonymous nucleotide substitutions were present. Relative to the M129 sequence, the changed nucleotides were located at nt 688 and 689 (AC→CA), 748 (A→G), 3368 and 3369 (CG→GC), and 3370 (G→T). These changes resulted in amino acid changes at positions 230 (T→H), 250 (L→G), and 1123 and 1124 (TV→SL). At nt 1957 an insertion of three AGT triplets resulted in three additional serines, bordering a stretch of seven serines.

In addition, the P1 gene of strain Mp4817 contained a novel variable region of 586 bp between nt 3402 and 3991 (region A in Fig. 1). This new sequence was aligned with the P1 sequences of P1 type 1 strain M129 (29) and P1 type 2 strains TW 7-5 (26) and 309 (15) (Fig. 2). A 55-bp stretch within region A of strain Mp4817 and within the new sequence of the P1 gene of P1 type 2 strain 309 (15) was identical (region B in Fig. 1 and 2) and differed from the corresponding sequences of strains M129 (P1 type 1) and TW 7-5 (P1 type 2) at 22 positions (40%).

The major part of the novel variable region A in strain Mp4817 was localized within the RepMP2/3 repeat region of the P1 gene. Since recombination with other RepMP2/3 regions outside the P1 gene may have occurred, the entire genome of M129 (GenBank accession no. U00089) was searched. This revealed an identical sequence (100% identity) in the M129 genome from nt 13953 to 14539. Sequences homologous to region B, the 55-bp stretch of the novel sequence of strains Mp4817 and 309, were found at three locations in the M129 genome, at nt 76930 to 76985 (100% identity), nt 579414 to 579469 (one nucleotide mismatch), and nt 414624 to 414679 (one nucleotide mismatch).

P1 type 1 and 2 hybrid sequences in P1 type 1 strain Mp4817 and P1 type 2 strain MAC. In the P1 gene nucleotide sequences of the P1 type 1 strain Mp4817 and of the P1 type 2 strain MAC, an identical stretch of 261 bp (nt 2764 to 3025; region C in Fig. 1) was detected, by which Mp4817 differed from the prototype P1 type 1 strain M129 and strain MAC differed from the prototype P1 type 2 strain TW 7-5 (Fig. 1 and Table 1). At seven positions the P1 type 2 strain MAC had nucleotides characteristic for P1 type 1 strains (open boxes in Table 1). Conversely, the P1 type 1 strain Mp4817 had nucleotides characteristic for the P1 type 2 strains at six positions (gray boxes in Table 1). One of these nucleotides was localized at nt 2823 within region C, the 261-bp stretch identical in strains Mp4817 and MAC. The other five P1 type 2-specific nucleotides were localized in the sequence bordering region C, between nt 3093 and 3382 (Table 1). In addition, strains MAC and MP4817 had four identical nucleotide polymorphisms within region C, differing from both P1 type 1- and type 2-characteristic sequences (Fig. 1; boldface nucleotides in Table 1). Searching the M129 genome for sequences homologous to region C revealed one location (259 bp), from nt 14916 to 15175 (U00089), with two mismatches at positions other than the polymorphisms in MAC and Mp4817 and one location (237 bp), from nt 77698 to 77935 (U00089), with four mismatches. Two of these mismatches were at nucleotide positions characteristic for type 1 or 2.

Amino acid translation of the variable sequences and relation to antigenic domains. The deduced amino acid sequences of region A (Fig. 1) of strains Mp4817 and 309 were compared to those of M129 (P1 type 1) and TW 7-5 (P1 type 2) (Fig. 3). Differences between the Mp4817 and M129 sequences were found from position 1140 through 1330. The amino acid sequences of Mp4817 and 309 were identical from position 1243

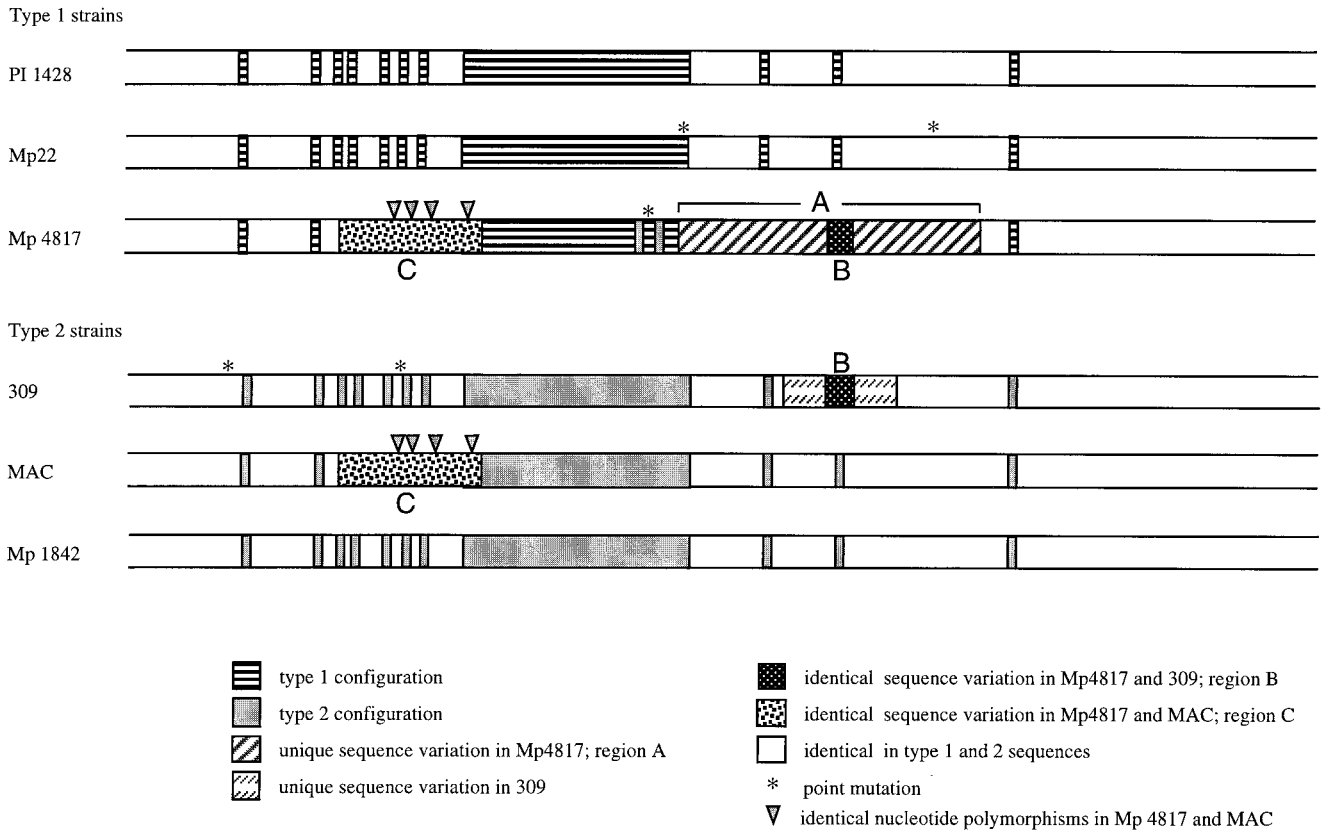

FIG. 1. Schematic representation of sequence divergence of ADH3-ADH4 amplicons (nt 2268 to 4768 relative to the start codon AUG of the P1 gene of strain M129 [29]) of the P1 genes of three P1 type 1 and three P1 type 2 *M. pneumoniae* strains. Sequence data for strain 309 are derived from reference 15. The figure is not drawn to scale.

through 1257. Differences between the translated amino acid sequences of region C, the 261-bp region of identity of strains Mp4817 and MAC, and the corresponding predicted amino acids for strains M129 and TW 7-5 are indicated in Table 1.

The sites of variation resulting in a divergent amino acid translation were compared to antigenic and cytadherence-mediating epitopes (5, 7, 13, 14) in order to assess whether nonsynonymous polymorphisms in the strains sequenced might affect these epitopes. According to the P1 membrane topology model proposed by Jacobs et al. (11), divergent amino acids at position 1302 in strain Mp1842 and at positions 1112 and 1113 in strain Mp4817 are localized within predicted membrane-spanning domains. In strain Mp4817 the divergent amino acids at positions 230 and 250, the three additional serines inserted adjacent to a stretch of seven serines at position 646 to 652, and the large new stretch of 190 amino acids (positions 1140 to 1330) at the C-terminal domain of the P1 protein are all predicted to be part of surface-exposed domains. They do not, however, localize within antigenic or adherence-mediating epitopes identified until now (5, 7, 13, 14). The divergent amino acids resulting from nonsynonymous polymorphisms in the identical stretch of strains Mp4817 and MAC encoded by region C (Table 1, boldface) are localized in predicted surface-exposed domains of the P1 protein. The divergent amino acid at position 1128 in strain Mp4817 is localized within a recognized antigenic epitope (14).

DISCUSSION

Only two types of *M. pneumoniae* P1 genes were assumed to exist, based on Southern blotting and the standard PCR-RFLP analysis of the P1 gene (2, 22, 27). However, recently a novel variable region in P1 type 2 strains was reported (15). Moreover, we have identified eight P1 subtypes among 23 *M. pneumoniae* isolates by applying an extended panel of restriction enzymes in the P1 PCR-RFLP (6). We now report the full-length sequence of the P1 cytadhesin genes of three P1 type 1 strains and two P1 type 2 strains. A novel sequence within the P1 cytadhesin gene was identified in P1 type 1 strain Mp4817. This sequence comprised 586 bp and was located at the 3′ end of the RepMP2/3 repeat region (region A in Fig. 1). A sequence completely identical to this variable region of the new P1 gene was found within the *M. pneumoniae* P1 type 1 M129 genome sequence. Comparison with a recently reported new variable sequence in the P1 gene of P1 type 2 strain 309 (15) revealed that both strains had a 55-bp region of 100% identity (region B in Fig. 1). This 55-bp region was present at three sites on the *M. pneumoniae* M129 genome; one site was located within one of the RepMP1 repeat sequences, and the two others were located in regions designated RepMP2/3-5 and RepMP2/3-6 by Kenri et al. (15). This finding suggests that sequences within repeat regions have been exchanged within the *M. pneumoniae* genome.

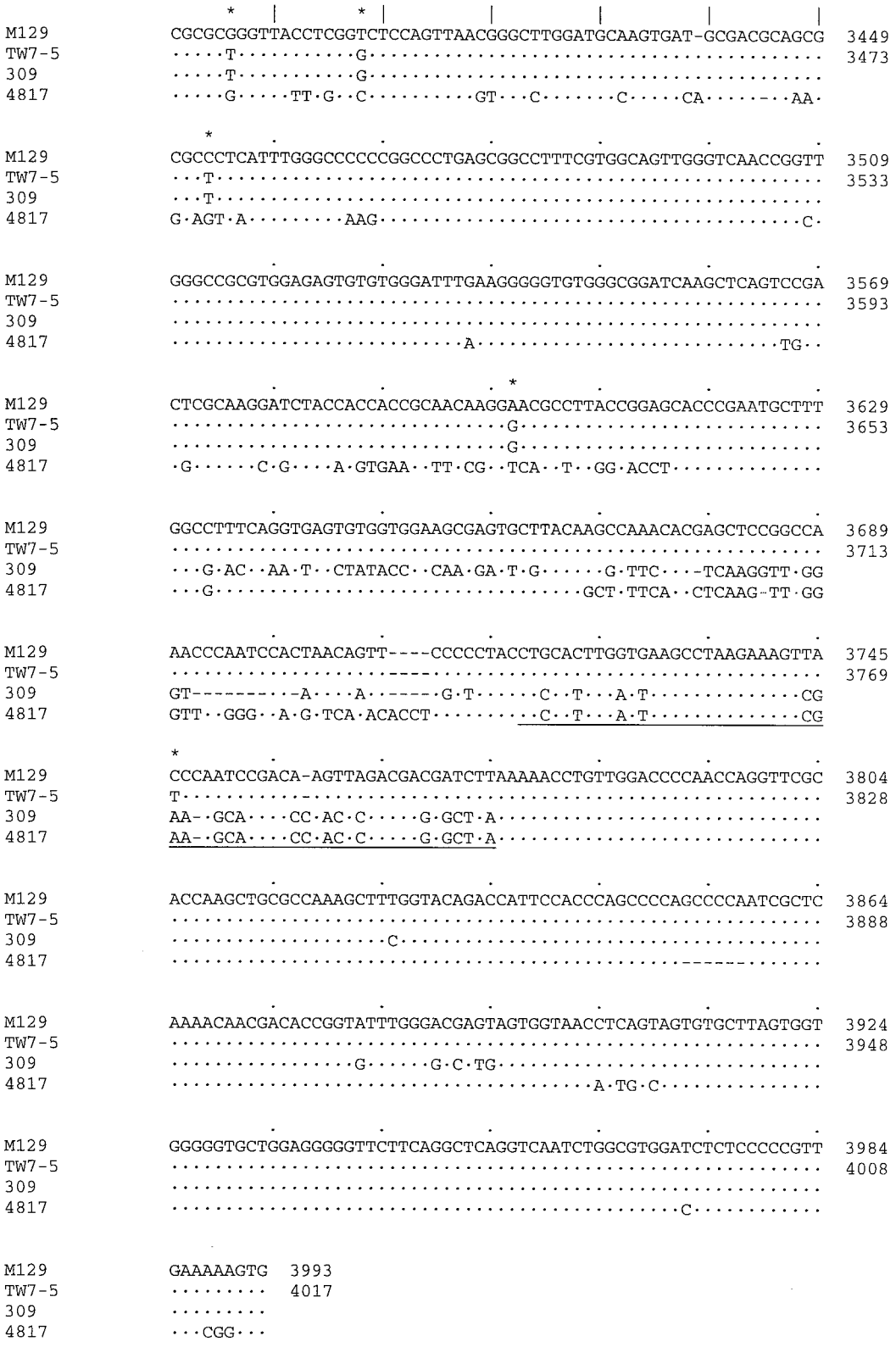

FIG. 2. Partial P1 nucleotide sequences of strains M129 (P1 type 1) and TW 7-5 (P1 type 2) and of the variable regions in strains Mp4817 (present study) and 309 (15), corresponding to region A in Fig 1. Nucleotide positions are indicated relative to the start codon AUG of the P1 genes of strain M129 (29) and strain TW 7-5 (26). Identical nucleotides are indicated by dots, and gaps are indicated by dashes. Differing nucleotides are shown. Nucleotide differences between reference P1 type 1 and 2 strains are marked with asterisks. The variable stretch which is identical in strains Mp4817 and 309 (region B in Fig. 1) is underlined.

TABLE 1. Polymorphisms in the P1 gene sequences of strains Mp4817 (P1 type 1) and MAC (P1 type 2) compared to the P1 gene sequences of strains M129 (P1 type 1) and TW 7-5 (P1 type 2)[a]

Strain	P1 type	Nucleotide position	Nucleotide sequence	Amino acid position	Amino acid sequence
			* *		* *
M129	1	2764-2775	GAC CAG CAG GGA	922-925	asp gln gln gly
TW 7-5	2	2776-2787	·G· ··· ··· ·A·	926-929	gly · · glu
Mp4817	1		·A· ··· ··· ·G·		asp · · gly
MAC	2		·A· ··· ··· ·G·		asp · · gly
			*		*
M129	1	2821-2823	··T	941	asn
TW 7-5	2	2833-2835	··G	945	lys
Mp4817	1		··G		lys
MAC	2		··G		lys
			* *		* *
M129	1	2866-2877	GCG ATC GAT CAA	956-959	ala ile asp gln
W 7-5	2	2878-2889	··· ··G ··A ···	960-963	· met glu ·
Mp4817	1		··· ·CC ·GT ···		· **thr gly** ·
MAC	2		··· ·CC ·GT ···		· **thr gly** ·
M129	1	2887-2889	ACC	963	thr
TW 7-5	2	2899-2901	···	967	·
Mp4817	1		··T		·
MAC	2		··T		·
			* * *		
M129	1	2983-3000	CTC TTC CTC CGC GGC TTG	995-1000	leu phe leu arg gly leu
TW 7-5	2	2995-3012	··G ··· ··G ··· ··· C··	999-1004	· · · · · ·
Mp4817	1		··C ··· ··T ··· ··· T··		· · · · · ·
MAC	2		··C ··· ··T ··· ··· T··		· · · · · ·
			*		
M129	1	3022-3024	GTG	1008	val
TW 7-5	2	3034-3036	··T	1012	·
Mp4817	1		··G		·
MAC	2		··G		·
			*		*
M129	1	3091-3093	··T	1039	his
TW 7-5	2	3106-3108	··G		gln
Mp4817	1		··G		gln
MAC	2		··G		gln
			** * *		* *
M129	1	3334-3339	ACG GTG	1112-1113	thr val
TW 7-5	2	3358-3363	·GC T·C	1120-1121	ser phe
Mp4817	1		·GC G·G		ser leu
MAC	2		·GC T·C		ser phe
			*		*
M129	1	3382-3384	GTC	1128	val
TW 7-5	2	3406-3408	A··	1136	ile
Mp4817	1		A··		ile
MAC	2		A··		ile

[a] Numbers given for nucleotide and amino acid positions indicate the positions of nucleotides or corresponding amino acids in the P1 genes of strains M129 (P1 type 1) (29) and TW 7-5 (P1 type 2) (26), counting from start codon AUG. Dots indicate identical nucleotides, asterisks indicate nucleotide and amino acid differences between P1 genes and proteins of P1 type 1 and 2 strains M129 and TW 7-5, open squares indicate P1 type 1-specific nucleotides and amino acids in P1 type 2 strain MAC, and gray squares indicate P1 type 2-specific nucleotides and amino acids in P1 type 1 strain Mp4817. Nucleotides and amino acids different from those of P1 type 1 strain M129 and P1 type 2 strain TW 7-5 but identical in strains Mp4817 and MAC are in boldface. Amino acids divergent in only one strain due to nucleotide variation are underlined.

In strain Mp4817 and reference strain MAC, a region of full identity was present in the central part of the RepMP2/3 region of the P1 gene (region C in Fig. 1). This region of full identity appeared to be a hybrid sequence, combining P1 type 1- and type 2-specific sequences. At two locations outside the P1 gene locus within the M129 genome, regions nearly identical to region C were found, within RepMP2/3 copies. In contrast to the general contention, our data indicate that recombination

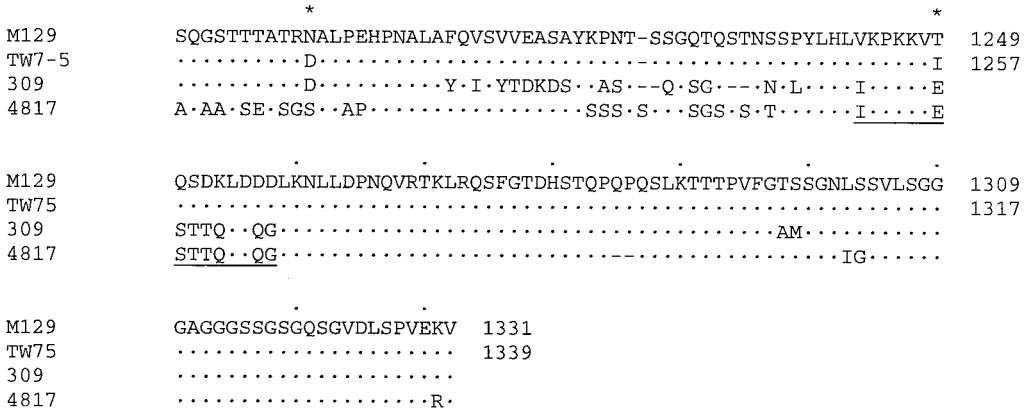

FIG. 3. Deduced partial amino acid sequences of P1 proteins of strains M129, TW 7-5, Mp4817, and 309. The regions shown correspond to the nucleotide sequences from nt 3571 onwards in Fig 2. Amino acid differences between reference P1 type 1 and 2 strains are marked with asterisks. The variable stretch which is identical in Mp4817 and 309 (corresponding to region B in Fig. 1) is underlined. Amino acid positions of the P1 proteins of M129 (29) and TW 7-5 (26) are indicated according to the published sequences.

between sequences specific for P1 types 1 and 2 may occur. The finding that these sequences are also present at other locations in the genome, associated with repetitive sequences, strongly supports a possible role for intragenomic recombination between repetitive sequences in the P1 gene and those present elsewhere in the genome (15, 24, 28, 31). Similar intragenomic repeat recombinations may occur within other *M. pneumoniae* genes encoding immunogenic surface proteins, such as the 30- and 90-kDa proteins. It may be hypothesized that recombination events are a general mechanism causing variation in immunogenic surface proteins of *M. pneumoniae*, contributing to evasion of the host immune response. Such mechanisms have also been described for the closely related *Mycoplasma genitalium* (4), *Mycoplasma hominis* (32), and other bacterial species such as *Neisseria* spp. (20) and group A streptococci (9).

The five completely sequenced P1 genes encode full-length P1 proteins. Mutations at the start of the P1 gene, such as the frameshift mutation due to insertion of an adenine causing premature termination of translation in a cytadherence-negative *M. pneumoniae* strain (25), were not found. The first 59 amino acid residues of the P1 translation product are assumed to be a leader sequence that is processed to yield the mature P1 protein (10, 12). This putative leader sequence was present in all five new P1 sequences and in all cases was identical to that of strains M129 (P1 type 1) and TW 7-5 (P1 type 2), implying that the five P1 genes all encode P1 proteins which can be processed to functional adhesins.

The P1 protein topology has been partly resolved by identification of cytadherence-mediating and antigenic epitopes (5, 7, 13, 14) and by predicting membrane-associated helices (7, 11). A model has been proposed where three regions, an N-terminal domain (N-reg) (13), a middle domain (D1) (13), and a C-terminal domain (D2) (5) cooperate to form the cytadherence-mediating binding sites (7). The N terminus of the mature P1 protein is considered to be located outside the cell (12). According to this model, one of the divergent amino acids in the P1 protein of our strains was localized in a part of the P1 protein recognized as an antigenic epitope (14), whereas several others were localized in predicted surface-exposed loops. It therefore may well be that these amino acid changes cause antigenic variation of the P1 proteins.

Recently, the C-terminal domain of the P1 protein of *M. pneumoniae* strain FH was found to be highly antigenic (M. Pedersen, S. Birkelund, and G. Christiansen, Abstr. 13th Int. Congr. Int. Org. Mycoplasmol., p. 241, 2000). We identified sequence variation in this region. This may imply that the amino acid changes in the C-terminal domain of the P1 protein in strain Mp4817 contribute to evasion of the host immune response. Because of this variation, the use of peptides derived from the corresponding region of the P1 protein for development of a diagnostic test (Pedersen et al., Abstr. 13th Int. Congr. Int. Org. Mycoplasmol.) should also be cautioned.

M. pneumoniae is notoriously lacking in genomic variation, as only two genomic types have been distinguished by randomly amplified polymorphic DNA analysis (30), by RFLP analysis of long PCR amplicons of interrepeat regions (6), and by sequencing of the 16S-23S rRNA gene spacer region (6). Only one additional genomic variant has recently been described (3). Therefore, typing of *M. pneumoniae* based on analysis of usual targets such as housekeeping genes (19) presumably will not allow intraspecies differentiation. Genes encoding surface-exposed proteins, such as the P1 cytadhesin gene, are expected to be more variable and therefore to be more suitable as typing targets. We showed that in two of the five strains studied, P1 type 1 and 2 hybrid sequences were present. In addition, in four of the five strains several nucleotide polymorphisms were identified in the P1 gene sequence. In order to enable more refined strain differentiation, putative genotyping targets in the P1 gene may be derived from our sequence data. Direct sequencing of the generated fragments will increase insight into the variability of these sites in the P1 gene and can be used to study molecular epidemiology of *M. pneumoniae*. In addition to the variable sequences within the P1 gene, targets for typing through direct sequencing may be found in other genes encoding surface-exposed proteins, such as the 30-kDa protein (18). Ideally, a multilocus direct sequence typing system (19, 23) could be developed for *M. pneu-*

moniae, using variable sequences of outer surface protein genes as targets.

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