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Dorigo-Zetsma, J.W.

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CHAPTER 7

Mycoplasma pneumoniae P1 type 1 and type 2 specific sequences within the P1 cytadhesin gene of individual strains

J.Wendelien Dorigo-Zetsma 1,2, Berry Wilbrink 2,
Jacob Dankert 1, Sebastian A.J. Zaat 1

1 Department of Medical Microbiology, University of Amsterdam, Academic Medical Center, Amsterdam. The Netherlands,
2 Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

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Chapter 7

ABSTRACT

*Mycoplasma pneumoniae* strains traditionally are divided into two types, based on sequence variation in the P1 gene. Recently, we have however identified eight P1 gene subtypes by P1 gene PCR restriction fragment length polymorphism (RFLP) analysis. In the present study the complete P1 gene nucleotide sequences of three type 1 and two type 2 *M. pneumoniae* strains were analysed. A variable P1 gene sequence in a type 1 strain was identified with partial similarity to a recently reported variable region in the P1 gene of a *M. pneumoniae* type 2 strain (Kenri et al. Infect Immun 67;1999,4557-4562). 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 can be present within the P1 gene of an individual strain. The new variable regions in the P1 genes of our strains were located in the RepMP2/3 region. Identical or nearly identical 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 4 of the 5 strains studied, single nucleotide polymorphisms different from the previously reported P1 type 1 and type 2 characteristic nucleotides were identified. The polymorphic sites are candidate targets for genotyping of *M. pneumoniae* by direct sequencing of amplicons from clinical specimens.
INTRODUCTION

*Mycoplasma pneumoniae* is a common cause of respiratory infections in humans. Colonisation of the respiratory epithelium by *M. pneumoniae* is mediated by a terminal structure of *M. pneumoniae* cells, the attachment organelle (1). Several surface proteins are involved in the formation of the attachment organelle and cytadherence of *M. pneumoniae* to the respiratory epithelium. The 170 kDa protein P1, densely clustered at the site of the attachment organelle, is a major adhesin protein (2). The protein is encoded by a gene of nearly 5000 bp, of which two-thirds is present as repetitive region in the *M. pneumoniae* genome (3).

Early studies showed the existence of two P1 gene types among *M. pneumoniae* clinical isolates (4,5). In these studies Southern blotting and PCR-RFLP analysis on P1 gene amplicons using 1 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 (6-9).

Since humans mount a strong immune response to the P1 protein during infection (10), the P1 gene is likely to display antigenic variation (2). Among a collection of 218 *M. pneumoniae* clinical isolates in Japan, a new variable sequence in the P1 gene was identified in 4 P1 type 2 strains (11). In addition, we were able to distinguish 8 P1 subtypes within the two P1 gene types using an extended panel of restriction enzymes in PCR-RFLP analysis (9). This indicates 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 M 129 (ATCC 29342), of the P1 type 2 reference strain FH (ATCC 15531), and of one P1 type 1 and two P1 type 2 clinical isolates have been reported (12). The P1 gene sequences of the type 1 isolate and of the two type 2 isolates were however identical to those of strains M129 and FH, respectively (12). In order to analyse P1 gene sequence variability, we performed sequence analysis of P1 genes of *M. pneumoniae* strains. We used two *M. pneumoniae* reference strains and three *M. pneumoniae* clinical isolates with variable P1 genes as detected by our PCR-RFLP (9).

MATERIALS AND METHODS

*M. pneumoniae* strains and DNA isolation

Two *M. pneumoniae* reference strains and 3 clinical isolates were selected from a collection of 23 *M. pneumoniae* strains, used in P1 gene PCR-RFLP typing experiments as described before (9). The 3 clinical isolates were selected for P1 gene sequence analysis based on their unique pattern as obtained by P1 gene PCR-RFLP. Reference strains were P1 1428
(ATCC 29085), a P1 type 1 strain (9) and MAC (ATCC 15492), a P1 type 2 strain (4). Clinical isolates 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. *M. pneumoniae* isolates were cultured in plastic flasks (Nunc™, Denmark) containing 60 ml of SP4 medium, at 37 °C. Cells were harvested upon colour change of the medium after 1 to 5 weeks and pelleted by centrifugation at 8,000 x g for 45 min. The supernatant was discarded, and DNA was extracted from the pelleted bacteria with the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany).

**DNA sequencing**

Fragments of approximately 2280 bp and 2580 bp, together comprising almost the entire P1 gene, were amplified with primer pairs ADH1-ADH2 and ADH3-ADH4 (5), respectively. 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 the BigDye terminator cycle sequencing kit and the DNA sequencer 307 (Perkin Elmer Applied Biosystems, Foster City, Calif.).

Primer pairs Plup (GCTTTAAAGTATGTTGGCGGGG) and ADH1BR (AAGTCATACCGCGTAACGC), ADH2BF (GTAGTAGTAGTAGTCACAACG) and ADH3BR (TGTCCACTTGAAGCCTTATC), and ADH4AF (CCGCACAGGTATCAGTC-AAG) and Pldown (GGTGAGGGTGTTGTGGTCTTGG) were used to generate amplicons comprising the sequence upstream of the ADH1-ADH2 fragment, between the ADH1-ADH2- and ADH3-ADH4 fragment, 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/align_clustalw.pl). P1 gene nucleotide sequences from strain M129 (GenBank accession no.: M18639), strain 309 (GenBank accession no.: AB024618), and from strain TW7-5 (12) 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 P1 gene sequence of *M. pneumoniae* strain M129 (13).

The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence database.
RESULTS

Comparison of P1 gene sequences

Complete P1 gene nucleotide sequences were obtained from 5 M. pneumoniae strains, PI 1428, MAC, Mp22, Mp4817 and Mp1842. The sequences had promoter and terminator regions identical to those of strain M129 (13) and the deduced translation products were full-length P1 proteins. The sequences of our P1 type 1 strains (PI 1428, Mp22, Mp4817) showed highest similarity with the M129 (P1 type 1) sequence, and the sequences of our P1 type 2 strains (MAC and Mp1842) with that of TW 7-5 (P1 type 2). In all cases the sequences contained the previously reported variable regions, characteristic for the 2 respective P1 types (12).

P1 type 1 sequences

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

P1 type 2 sequences

The P1 gene nucleotide sequence of P1 type 2 strain Mp1842 was almost identical to that of strain TW7-5. In strain Mp1842 one non-synonymous mutation was identified at position 3904 G→A, resulting in an amino acid change at position 1302 from V→I.

A novel variable region in the P1 gene of strain Mp4817, and P1 type 1 and 2 hybrid sequences in strains Mp4817 and MAC

In the P1 gene of type 1 strain Mp4817, four non-synonymous mutations were present at positions 688/9 AC→CA, 748 A→G, and 3368 C→G. These mutations resulted in amino acid changes at position 230 T→H, 250 L→G and 1123 T→S. At nucleotide position 1957 an insertion of 3 AGT triplets resulted in 3 additional serines, bordering a stretch of 7 serines.

In the P1 gene of Mp4817 a new variable sequence of 586 bp was found between nucleotide 3402 and 3991 (Fig. 1). This new sequence was aligned with the P1 sequences of strains M129 (13), TW 7-5 (12) and 309 (11)(Fig. 2). A 55 bp stretch within the new variable sequence of strain Mp4817 was identical to part of the new variable sequence in the P1 gene of P1 type 2 strain 309 (11)(Fig. 1 and 2). The major part of the new variable sequence in strain Mp4817 is localised in the RepMP2/3 repeat region of the P1 gene. Since recombination with other RepMP2/3 regions, outside the P1 gene, might be responsible for this variation, the entire genome of M129 (GenBank accession no. U00089) was searched.
ADH3-4 fragment of P1 gene (nt 2,468-4,768)

Type 1 strains

PI 1428

Mp22

Mp 4817

Type 2 strains

309

MAC

Mp 1842

* point mutation
\[\] identical nucleotide polymorphisms in strains Mp 4817 and MAC

\[
\begin{array}{c}
\text{type 1 configuration} \\
\text{type 2 configuration} \\
\text{identical sequence variation in Mp4817 and MAC} \\
\text{identical sequence variation in Mp4817 and 309} \\
\text{unique sequence variation in 309} \\
\text{unique sequence variation in Mp4817}
\end{array}
\]

Figure 1
Schematic representation of sequence divergence of ADH3-ADH4 amplicon (nt 2,268-4,768) of the P1 genes of three P1 type 1 and three P1 type 2 M. pneumoniae strains. Nucleotide positions are indicated relative to the start codon AUG of M129 P1 gene (13). Sequence data of strain 309 are derived from (11). Figure is not drawn to scale.
Figure 2
Partial Pi nucleotide sequences of strains M129 (Pi type 1), TW7-5 (Pi type 2), and of the variable regions in clinical isolates Mp4817 (present study) and 309 (11). Nucleotide positions are indicated relative to the start codon AUG of the Pi gene of strain M129 Pi (13) and strain TW 7-5 (12). Identical nucleotides are indicated by dots. Differing nucleotides are indicated by characters. Nucleotide differences between Pi type 1 and 2 strain are marked with asterisks. The variable stretch which is identical in strains Mp4817 and 309 is underlined.
This revealed the presence of a 100% identical sequence in the M129 genome, from nt 13,953 to 14,539. The 55 bp stretch of the novel sequence of strain Mp4817 and strain 309, was also found at 3 other locations in the M129 genome, nt 76,930-76,985 (100% identity), nt 579,414-579,469 (1 nucleotide mismatch) and nt 414,624-414,679 (1 nucleotide mismatch).

In the P1 gene nucleotide sequence of P1 type 1 strain Mp4817 and that of P1 type 2 strain MAC, an identical stretch of 261 bp (nt 2764-3025) was detected, differentiating these strains from the prototype P1 type 1 strain and the P1 type 2 strain, respectively (Fig. 1 and Table 1). At 7 positions the type 2 MAC strain had the nucleotides characteristic for type 1 strains. Conversely, strain Mp4817, designated P1 type 1, had nucleotides characteristic for type 2 strains at 6 positions (Table 1). One of these was located within the 261 bp stretch identical in Mp4817/MAC, and 5 nucleotides bordered this stretch (Table 1). In addition, both strains had 4 identical nucleotide polymorphisms within the 261 bp stretch, differing from both P1 type 1 and 2 characteristic sequences (Fig. 1, Table 1). On searching the M129 genome for a similar 261 bp stretch, one location (259 bp) with 2 mismatches, at other positions than the polymorphisms in MAC and Mp4817, was found from nt 14,916-15,175 (U00089) and one location (237 bp), from nt 77,698-77,935 (U00089), with 4 mismatches. Two of these mismatches were on nucleotide positions specific for type 1 or 2.

The nucleotide polymorphisms in the P1 gene sequences of 4 strains related to the RepMP4 and RepMP2/3 regions in the P1 gene are indicated in Figure 3.

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**Figure 3**
Schematic representation of the P1 gene, with putative targets for genotyping by direct sequencing. Nucleotides shown are specific for the indicated strain. Extensive differences of 2 regions are shown in Table 1 and Fig. 2. Nucleotide positions are indicated relative to the start codon AUG of M129 P1 gene (13).
Amino acid translation of the variable sequences and relation to antigenic domains

The deduced amino acid sequences of the variable domain of Mp4817, and of strain 309 were compared to amino acid sequences of M129 (P1 type 1) and TW7-5 (P1 type 2)(Fig. 4).

Table 1. Polymorphisms in P1 gene from Mp4817 (P1 type 1) and MAC (P1 type 2) compared to reference strain M129 (P1 type 1) and strain TW 7-5 (P1 type 2)

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<th>amino acid position*</th>
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<td>MAC</td>
<td>A</td>
<td></td>
<td></td>
<td>ile</td>
<td></td>
</tr>
</tbody>
</table>

*numbers indicate the positions of nucleotides or corresponding amino acids in the P1 gene M129 (P1 type 1) (13) and in the P1 gene TW7-5 (P1 type 2) (12), counting from start codon AUG, as described in (16) and (17) for strain FH (P1 type 2); *

* indicates P1 type 1 or 2 specific nucleotide/amino acid, identical nucleotides, character: differing nucleotides
Differences between the predicted amino acid sequences of Mp4817 and M129 were found at position 1191 through 1330 (Fig. 4). The amino acid sequences of Mp4817 and 309 were identical from position 1243 through 1257. Differences between amino acid translation of the 261 bp region of identity of strains MAC and Mp4817 and the predicted amino acids for strains M129 and TW 7-5 are indicated in Table 1.

To uncover possible functional non-synonymous polymorphisms in the strains sequenced, the sites of variation resulting in a divergent amino acid translation were compared to antigenic epitopes, which have been reported to be recognised by cytadherence inhibiting anti P1 monoclonal antibodies (14-17).

The divergent amino acid in strain Mp1842 was located at position 1302, which is within a cytadherence mediating epitope in domain D2 (16). In strain Mp4817, divergent amino acids were situated at positions 230, 250 and 1123. The amino acid at position 230 borders amino acid 231-238, which sequence has been recognised as cytadherence mediating epitope. The amino acid at position 1123 is situated within an immunodominant epitope (16,17). The amino acid stretch of 7 serines (aa 646-652), where insertion of 3 AGT triplets resulted in 3 additional serines, has as yet not been recognised as an epitope. The large variable stretch of 149 amino acids (1191-1330) in strain Mp4817 was situated at the C-terminal end of the P1 protein and includes an epitope in domain D2 (1303-1310)(16).

Amino acid changes in both strains MAC and Mp4817 in the central part of the P1 protein were situated in domain D1 (16).

### Figure 4
Deduced partial amino acid sequences of P1 proteins of strains M129, TW7-5, Mp4817 and 309. The regions shown correspond with the nucleotide sequences in Fig 2. Amino acid differences between P1 type 1 and 2 strain are marked with asterisks. The variable stretch which is homologous in Mp4817 and 309 is underlined. Amino acid positions of the P1 protein of M129 (13) and TW7-5 (12) are indicated according to the published sequences.
DISCUSSION

In the present study, a novel sequence within the P1 cytadhesin gene was identified in P1 type 1 strain Mp4817. The variable sequence comprised almost 600 bp, and was located at the 3' end of the RepMP2/3 repeat region, with a small overlap of the previously reported region differentiating the two P1 types (Fig. 1). A sequence completely homologous to the variable region of this new P1 gene was found in the \textit{M. pneumoniae} M129 genome. Comparison with a recently reported new variable sequence in the P1 gene of P1 type 2 strain 309 (11), revealed a 55 bp region of complete identity in these strains. This small region was present at 3 sites on the \textit{M. pneumoniae} M129 genome, one of which was located in a RepMP1 copy, and the two others in regions designated RepMP2/3-5 and RepMP2/3-6 as described by Kenri et al. (11).

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. This region of full identity appeared to be a hybrid sequence between type 1 and type 2 specific sequences. At two locations outside the P1 gene locus in the M129 genome, nearly identical regions were found in RepMP2/3 copies. Our data show that, in contrast to the general contention, recombination between sequences specific for P1 type 1 and 2 can occur.

The 5 completely sequenced P1 genes encode full length P1 proteins. No mutations were found at the start of the P1 gene, in contrast to the reported frameshift mutation due to insertion of an adenine causing premature termination of translation in a cytadherence negative \textit{M. pneumoniae} strain (18). In the P1 gene sequences of the 5 strains, non-synonymous nucleotide polymorphisms were translated into amino acids, of which the majority was located in domains described to contain cytadherence mediating epitopes (16,17). Our data strongly support a possible role for intragenomic recombination between sequences within the RepMP4 and RepMP2/3 repetitive sequences in the P1 gene, and those present elsewhere in the genome, as has been suggested before (11,19-21). The putative recombinations have produced a high number of amino acid changes, possibly causing antigenic variation of the P1 proteins. Similar recombinations may occur within other \textit{M. pneumoniae} genes encoding immunogenic surface proteins, such as the 30 kDa and 90 kDa proteins. It is likely that recombination events are a general mechanism for \textit{M. pneumoniae} to circumvent host immune response, as has been described for \textit{Mycoplasma genitalium} (22), \textit{Mycoplasma hominis} (23), \textit{Neisseria} (24) and group A streptococci (25).

In 2 of the 5 strains studied, P1 type 1 and 2 hybrid sequences were present. In addition, in 4 of the 5 strains several nucleotide polymorphisms were identified in the P1 gene sequence. In order to enable more refined strain differentiation, we identified putative targets in the P1 gene for genotyping by direct sequencing (Fig. 3). Primers for amplification of the targets should be selected outside repetitive regions, or be specific for the repeat regions within the
P1 gene, to ensure amplification of P1 gene fragments. 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*. Although usually genotyping targets are chosen within housekeeping genes (26), in the case of *M. pneumoniae* these genes presumably are too conserved to enable intraspecies differentiation. Therefore, *M. pneumoniae* genes encoding surface exposed proteins may be more suitable for genotyping. Additional targets for typing through direct sequencing may be present in other genes encoding surface exposed proteins, such as the 30-kDa protein, and carrying copies of repeat sequences. Ideally, a multilocus direct sequence typing system (26,27) could be developed, using variable sequences of outer surface protein genes as targets.
REFERENCES


