Molecular Characterization of the P1-Like Adhesin Gene from Mycoplasma pirum

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A DNA fragment has been isolated from the genome of Mycoplasma pirum by use of a genetic probe derived from the conserved region within the genes for the major adhesins of Mycoplasma genitalium and Mycoplasma pneumoniae. A gene encoding an adhesin-like polypeptide was localized, and sequence analysis indicated a G+C content of only 28%, with A- and T-rich codons being preferentially used. A total of 91% of positions 3 were either A or T. The deduced polypeptide is 1,144 amino acids long (126 kDa) and shows 26% identity with the adhesins of M. genitalium and M. pneumoniae. Other features in common with these two membrane proteins include a similar hydropathic profile and a proline-rich C terminus. Antibodies were prepared by using as an immunogen a peptide derived from the C terminus of the M. pirum adhesin-like polypeptide and were found to recognize on immunoblots a 126-kDa polypeptide from an M. pirum cellular extract. The characterization of the adhesin-like gene is a first step toward a better understanding of the mechanisms allowing this human mycoplasma to attach to host cells.

Mycoplasma pirum is a mycoplasma whose natural host was until recently unknown and which was isolated only from cell cultures (7). However, M. pirum has since been isolated from a culture of peripheral blood mononuclear cells (PBMCs) obtained from the blood of a patient infected with human immunodeficiency virus (HIV) (9, 26). In addition, by a polymerase chain reaction-based assay (9), this mycoplasma has been directly detected, without prior cultivation, in PBMCs from HIV-seropositive individuals (8). This result indicates that M. pirum is a human parasite; this indication was also initially suggested by tracing back to the origin of cell culture contaminants (7). Furthermore, HIV-associated cytopathic effects were found in vitro to be enhanced in the presence of various mycoplasmas, including Mycoplasma fermentans (17, 19) and M. pirum (26). This result leads to the hypothesis that mycoplasmas can act as cofactors in natural HIV infections (25).

Phylogenetically, M. pirum is closely related to mycoplasmas belonging to the Mycoplasma pneumoniae group (21, 36). Like most members of this group, M. pirum is characterized by an organized terminal structure or tip, which is a specialized cellular structure for adherence (28). The cell components responsible for attachment are the adhesins, and these have been shown in at least two human mycoplasmas, M. pneumoniae and Mycoplasma genitalium, to be clustered at the tip of the cell. The major adhesin in M. pneumoniae, P1, and adhesin MgPa in M. genitalium have been characterized and found to have molecular masses of 170 and 153 kDa, respectively (for a review, see reference 28). The P1 and MgPa proteins are potent immunogens, and antibodies directed against these adhesins inhibit adherence of the corresponding mycoplasmas to host cells. Adhesin P1 or its derivatives are thought to be good candidates for vaccine development (10, 14). The genes for both proteins have been cloned and sequenced (5, 11–13, 33). In addition, cross-hybridization between these genes and the genomic DNA of another terminal-structure mycoplasma, Mycoplasma gallisepticum, was obtained (3), suggesting at least a partial homology between genes encoding adhesins from flask-shaped mycoplasmas.

An antibody prepared against a peptide derived from the M. genitalium adhesin reduced the infectivity of HIV type 1 strain LAI and HIV type 2 strain ROD in lymphoid cells (24). The peptide chosen was homologous to a region of the M. pneumoniae adhesin containing an epitope(s) mediating cytadherence (6). Although the exact mechanism for the reduction of HIV infectivity is unknown, it was found by other investigators that the chosen peptide was homologous to a region of HLA molecules (1, 29) and that an HLA-DR peptide from this region inhibited HIV-induced syncytia (18). It was of interest to determine whether a related mycoplasma, such as M. pirum, which is able to increase HIV-associated cytopathic effects in vitro, would have an adhesin protein showing a similar homology with HLA molecules.

The goal of this study was to identify the gene(s) of the P1-like component (if any) in M. pirum. This would lead to a better understanding of the mechanisms of cell colonization by this mycoplasma and eventually of its interaction with HIV.

MATERIALS AND METHODS

Mycoplasma strains and culture conditions. M. pirum BER was previously isolated from a culture of PBMCs from a patient infected with HIV type 1 (26). M. genitalium (strain G377), M. fermentans (strains PG181, K7, and incognitus), M. pneumoniae (strain Eaton1), and Mycoplasma penetrans (strain GTU-54) were kindly provided by J. G. Tully (National Institute of Allergy and Infectious Diseases, Frederick, Md.). Mycoplasmas were cultivated in SP-4 medium (35).

DNA extraction and DNA hybridization. Genomic DNA was purified by conventional procedures (22). DNA was digested with restriction enzymes according to the suppliers' recommendations (Boehringer Mannheim France SA, Meylan, France; and New England Biolabs, Beverly, Mass.). Following agarose gel electrophoresis, Southern blot hybridization was performed with a Hybond-N+ membrane (Amersham Inter-
buffer containing E. coli XL1-Blue competent cells was obtained following transformation of Escherichia coli XL1-Blue competent cells. DNA inserts from the recombinant plasmids were sequenced by dideoxy reactions. DNA fragments found to hybridize with probe 29NTS were amplified by PCR. DNA was ligated to plasmid vector pBlue-Script SK (Stratagene, La Jolla, Calif.). Recombinant plasmids were obtained following transformation of Escherichia coli XL1-Blue competent cells. DNA inserts from the recombinant plasmids were sequenced by dideoxy reactions. DNA fragments found to hybridize with probe 29NTS were amplified by PCR. DNA was ligated to plasmid vector pBlue-Script SK (Stratagene, La Jolla, Calif.). Recombinant plasmids were obtained following transformation of Escherichia coli XL1-Blue competent cells. DNA inserts from the recombinant plasmids were sequenced by dideoxy reactions (31) with modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio).

DNA cloning and sequencing. DNA fragments found to hybridize with probe 29NTS were purified by electrophoresis from an agarose gel (29) and ligated to plasmid vector pBlue-Script SK (Stratagene, La Jolla, Calif.). Recombinant plasmids were obtained following transformation of Escherichia coli XL1-Blue competent cells. DNA inserts from the recombinant plasmids were sequenced by dideoxy reactions (31) with modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio).

Analysis of DNA and polypeptide sequences. Sequence analysis was performed with the software programs provided in the GCG (Genetics Computer Group, Inc., Madison, Wis.) package. All previously published sequences were obtained from the GenBank (Los Alamos, N.Mex.) and European Molecular Biology Laboratory (Heidelberg, Germany) data bases.

Peptide synthesis and immunogen preparation. Two synthetic peptides modelling the N-terminal (MKKIKKFNYKYL LISLV) and C-terminal (AKKPNTPVFFPASQLTNDVSRA) regions of the P1-like polypeptide of M. piroli were synthesized by the Merrifield solid-phase method (23) and purified to homogeneity by high-pressure liquid chromatography. The last amino acid (A) of the C-terminal peptide was added for practical reasons during synthesis. Each peptide (10 mg) was coupled to 6 mg of keyhole limpet hemocyanin by the glutaraldehyde method as described by Pfaff et al. (27).

Immunization protocol. Two New Zealand rabbits were immunized with each peptide as follows: 200 µg of keyhole limpet hemocyanin-coupled peptide mixed with an equal volume of Freund's complete adjuvant was injected intradermally on day 0. This procedure was repeated subcutaneously with Freund's incomplete adjuvant on days 30, 60, and 90. Rabbits were bled 1 week following each injection.

Immunoreactivity of antipeptide antibodies. (i) Enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated for 2 h at 37°C with 500 ng of synthetic peptides in 50 µl of phosphate-buffered saline (PBS) (pH 7.4) per well. After saturation with 5% nonfat milk in PBS (400 µl) for 1 h at 37°C and washing with PBS (containing 0.5% milk and 0.01% Tween 20), 50 µl of serial dilutions of sera was added. After 2 h at 37°C, the wells were rinsed and 50 µl of peroxidase-labelled anti-rabbit swine antibodies (1/5,000 dilution; Dako A/S, Glostrup, Denmark) was added. After 1 h at 37°C and further rinsing, 100 µl of orthophenylendiamine was added and incubation was continued for 30 min at room temperature in the dark. The reaction was stopped by the addition of 50 µl of 4 N sulfuric acid, and the ratio of the optical density at 492 nm to that at 620 nm was determined.

(ii) Immunoblot analysis. A cell lysate of M. piroli in SDS was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis to a nitrocellulose membrane, saturation was obtained by incubation with 5% nonfat milk in PBS for 1 h at room temperature. The membrane was incubated for 2 h with antipeptide sera and washed in PBS. Peroxidase-labelled anti-rabbit swine antibodies (1/5,000 dilution) were added and incubated for 1 h. Finally, the antigen-antibody complexes formed were detected with dianisobenzamide as a staining reagent.

Nucleotide sequence accession number. The DNA sequence for the gene for the P1-like protein of M. piroli will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under accession number L19685.

RESULTS

Isolation of a gene encoding a P1-like adhesin in M. piroli. M. piroli genomic DNA was digested with restriction enzyme EcoRI, and DNA hybridization was performed following Southern transfer with probe 29NTS. This probe was chosen from the region showing the highest homology between the genes coding for the adhesins from M. pneumoniae and M. genitalium. Southern blot experiments with labelled oligonucleotide probe 29NTS were performed as described in Materials and Methods. The temperature during the washes was 55°C (lane 2) or 47°C (lane 3). DNA markers (DR1gest III; Pharmacia Biotech Europe, Brussels, Belgium) were run on the same gel (lane 1) and hybridized with themselves.
Each of these EcoRI fragments was subcloned into plasmid pBluescript SK−, and the nucleotide sequences of their extremities were determined. The open reading frames (ORF) were located with TGA as a tryptophan codon, as this codon usage has been shown elsewhere for Mycoplasma species (2), including M. pneumoniae (34). Homologies between these nucleotide and amino acid sequences were searched in the GenBank and Swissprot data bases by use of the FASTA program provided in the Genetics Computer Group package. Although no significant homology could be found at the nucleotide level, the deduced polypeptide sequence from one end of the 7.0-kbp EcoRI fragment was found to have the best scores of homology with the adhesins of M. pneumoniae and M. genitalium. Additional sequencing revealed that this fragment encoded sequences found at the NH₂ terminus of the P1-like protein in M. pneumoniae.

Since a HindIII site was found 87 bp upstream from the EcoRI site of the cloned DNA fragment (Fig. 2), an oligonucleotide (5'-CTTAAATATCGGAATGTA3') between these two sites was chosen and used as a probe in a Southern hybridization experiment. A single 10-kbp band from the genomic DNA of M. pneumoniae digested with HindIII hybridized to the probe (data not shown). This DNA fragment was cloned, and a restriction map was deduced (Fig. 2). Sequencing indicated that the region between the HindIII and EcoRI sites was identical to that sequenced from the 7.0-kbp EcoRI DNA fragment. Additional sequencing was performed to span the entire gene for the P1-like protein in M. pneumoniae (Fig. 2).

Sequence analysis. The ORF encoding the putative P1-like protein of M. pneumoniae was found to be 3,435 bp long (Fig. 3), with only a 28% G + C base content, compared with 53.5% for the P1 gene of M. pneumoniae and 40% for the MgPa gene of M. genitalium (5). The deduced P1-like polypeptide is 1,145 amino acids long (127 kDa) (Fig. 3); M. pneumoniae P1 is 1,627 amino acids long (176 kDa), and M. genitalium MgPa is 1,445 amino acids long (160 kDa).

The sequences of the three mycoplasmal adhesins were aligned by use of the BESTFIT program from the Genetics Computer Group package, and the percentages of identity and similarity (taking into account conservative changes) were higher for the amino acid residues of the adhesins from M. pneumoniae and M. genitalium than they were for the residues of these proteins and the P1-like molecule from M. pneumoniae (Table 1).

Although the N terminus of the mature P1-like protein of M. pneumoniae has not been determined, a comparison of its hydropathy plot with those of the adhesins from M. pneumoniae and M. genitalium suggests the presence of a signal sequence (Fig. 4). Indeed, these plots are almost superimposable, showing a positively charged amino terminus followed by a stretch of hydrophobic amino acids. If the P1-like protein of M. pneumoniae were processed in a manner similar to that of P1 (15) and MgPa (20), then the molecular mass of the mature protein would be approximately 121 kDa. The hydrophobic profiles of the three proteins are also similar at the C terminus and are characterized by a long hydrophobic stretch (from amino acids 1017 to 1060 for M. pneumoniae).

Since the P1 gene in M. pneumoniae and the MgPa gene in M. genitalium are part of a three-gene operon (12, 13), we examined sequences both upstream and downstream from the P1-like gene cloned from M. pneumoniae. No obvious large ORF could be found on either side; however, a Shine-Dalgarno-like sequence was found 7 nucleotides upstream from the initiation codon. Although oligonucleotide probe 29NTS used in this study is homologous to the C termini of the adhesins from M. pneumoniae and M. genitalium, it hybridized to M. pneumoniae EcoRI DNA fragment corresponding to the N-terminal end of the P1-like gene of this mycoplasma. A region located between nucleotides 387 and 415 was found to be highly homologous to this probe; 24 of 29 nucleotides were indeed identical. In addition, this region also corresponded to a small ORF (nucleotides 189 to 549 in Fig. 3), although it lacked an obvious initiation site. The deduced polypeptide is 120 amino acids long and shows striking homology with adhesins from M. pneumoniae (35% identity in a 92-residue overlap), M. genitalium (41% identity in an 82-residue overlap), and M. pneumoniae (36% identity in a 115-residue overlap).

Repeated P1-like gene sequences in the M. pneumoniae genome. Southern hybridization experiments indicated P1-like gene sequences in the M. pneumoniae genome. A HindIII-XbaI DNA fragment (nucleotides 1469 to 3970 in Fig. 3) containing most of the coding region for the M. pneumoniae P1-like adhesin was used as a probe in a Southern hybridization experiment (Fig. 5). Six DNA fragments from the M. pneumoniae genome hybridized to the probe, indicating the presence of repeated P1-like gene sequences. In addition, under high-stringency conditions, no hybridization was obtained with DNA from other mycoplas-
FIG. 3. Nucleotide sequence and deduced amino acid sequences for the DNA fragment encoding the P1-like molecule in *M. pirum*. Underlined amino acids indicate amino acid sequences that were used to produce immune sera. The doubly underlined nucleotide sequence (387 to 415) is homologous to the oligonucleotide probe 29NTS.
mas, including *M. pneumoniae*, *M. genitalium*, *M. fermentans*, and *M. penetrans* (Fig. 5).

**Immunoreactivity of antipeptide antibodies to synthetic peptides and adhesin protein.** After immunization, only rabbits immunized with the C-terminal peptide produced specific antibodies against the corresponding peptide. The antibodies had a relatively high titer, since even at a 1/5,000 serum dilution, a positive reaction could be detected (Fig. 6). In contrast, no antipeptide antibodies were detected in the sera of rabbits inoculated with the N-terminal peptide. To demonstrate that the P1-like gene isolated from *M. pirum* really encodes an expressed protein, we analyzed the capacity of antipeptide antibodies to recognize specifically the corresponding protein in a Western blot (immunoblot) assay. The results depicted in Fig. 7 clearly demonstrate that antibodies produced against the C-terminal peptide recognized a protein with a relative molecular mass of 126 kDa, as determined by its electrophoretic mobility. This recognition seemed to be specific, as shown by the inability of preimmune sera to detect a positive reaction (Fig. 7, lane 3) and also by the capacity of soluble synthetic C-terminal peptide (lane 4) but not N-terminal peptide (lane 5) to totally inhibit this interaction. In addition, in perfect agreement with the ELISA results, no positive reaction was detected when rabbits immunized with the N-terminal peptide were tested (Fig. 7, lane 7). The lower bands detected in lanes 1, 2, and 5 seemed to be degradation products of the 126-kDa protein, since these bands were not detected by preimmune sera (Fig. 7, lane 3) and were totally inhibited in the presence of the synthetic C-terminal peptide (lane 4).

**DISCUSSION**

This study describes the cloning of the P1-like adhesin gene from *M. pirum* by use of an oligonucleotide probe corresponding to a conserved region between the adhesin genes from *M. pneumoniae* and *M. genitalium*. The *M. pirum* adhesin gene is characterized by a low G+C content, in accordance with the extremely low G+C content for the entire genome, 25.5%, as previously described by Del Giudice et al. (7). This base composition is also reflected in the codon usage, in particular that for tryptophan (Trp), since all 18 Trp residues are encoded by the triplet TGA. In contrast, in *M. pneumoniae* and in *M. genitalium*, 21 of 37 and 16 of 28 Trp residues are encoded by TGA, respectively (5). The percentage of codons with an A or a T residue in the third position in the P1-like gene of *M. pirum* was extremely high, 91%, compared with 65% for *M. genitalium* and 40% for *M. pneumoniae*. The percentages of similarity and identity among the adhesins of the three mycoplasmas (*M. pirum*, *M. genitalium*, and *M. pneumoniae*) are in accordance with their phylogenetic positions (21). The identity detected at the nucleotide level is lower than that measured among the deduced polypeptides and is not sufficient to obtain DNA cross-hybridization under high-stringency conditions with the *M. pirum* P1-like adhesin gene as a probe (Fig. 5).

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**TABLE 1. Amino acid homologies among the adhesins of *M. pneumoniae* (P1), *M. genitalium* (MgPa), and *M. pirum* (P1-like)**

<table>
<thead>
<tr>
<th>Adhesin</th>
<th>% Homology for:</th>
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<tbody>
<tr>
<td></td>
<td>P1</td>
</tr>
<tr>
<td>P1</td>
<td>100</td>
</tr>
<tr>
<td>MgPa</td>
<td>67*</td>
</tr>
<tr>
<td>P1-like</td>
<td>47*</td>
</tr>
</tbody>
</table>

* Percent identity

* Percent similarity

FIG. 3—Continued.
In contrast to the absence of cysteine residues in the *M. pneumoniae* and *M. genitalium* adhesins, 3 cysteine residues were found in the P1-like gene sequence of *M. pirum*. These would potentially allow the formation of intracysteine bonds and lead to a more stable and structured protein. Like PI and MgPa (5, 12, 13), the P1-like protein in *M. pirum* has a proline-rich C-terminal region. In the protein, 9 of 55 proline residues are clustered within the last 29 amino acids of the C terminus. This high content of proline is likely to reduce the flexibility of this region, in accordance with the immunogenicity of the C-terminal peptide for the corresponding antibodies reacting with the native protein (Fig. 7). The homology found

FIG. 4. Hydrophyt plots, determined by the method of Kyte and Doolittle (16), for the C termini and N termini of the adhesins of *M. pneumoniae*, *M. genitalium*, and *M. pirum*. Positive values on the ordinate indicate hydrophobic regions of the proteins, and negative values indicate hydrophilic regions. The numbers on the abscissa indicate the positions of the amino acid residues. The arrowheads indicate the location of the cleavage site for the signal sequence.
FIG. 5. Detection of repeated P1-like gene sequences in the \textit{M. pirum} genome. Genomic DNAs (10 µg) from \textit{M. pirum} BER (lane 2), \textit{M. pneumoniae} Eaton (lane 3), \textit{M. genitalium} G377 (lane 4), \textit{M. fermentans} PG18T (lane 5), \textit{M. fermentans} K7 (lane 6), \textit{M. fermentans} incognitus (lane 7), and \textit{M. penetrans} GTU-54 (lane 8) were digested with restriction enzyme \textit{EcoRI} and subsequently analyzed by electrophoresis on a 0.8% agarose gel. DNA markers (lane 1) are the same as those in Fig. 1. The probe for the Southern blot experiment was the HindIII-XbaI DNA fragment derived from the P1-like adhesin gene of \textit{M. pirum}.

FIG. 6. Immunoreactivity of antipeptide antibodies. Hyperimmune sera were produced in rabbits against C-terminal and N-terminal peptides as described in Materials and Methods and were tested in an ELISA. The tested sera were preimmune serum (● and △), immune sera obtained with the C-terminal peptide in two different animals (□ and △), and immune sera obtained with the N-terminal peptide in two different animals (○ and ○; these two curves are superimpossible). O.D., optical density.

FIG. 7. Immunoreactivity of antipeptide antibodies to \textit{M. pirum} polypeptides. Immunoblot membranes with whole-cell lysates of \textit{M. pirum} were analyzed with the following sera: serum to the C-terminal peptide (lane 1, 1/10 dilution; lane 2, 1/100 dilution), preimmune serum (lane 3), serum to the C-terminal peptide (1/10 dilution) in the presence of 100 µg of C-terminal peptide per ml (lane 4), serum to the C-terminal peptide (1/10 dilution) in the presence of 100 µg of N-terminal peptide per ml (lane 5), immune serum to the N-terminal peptide (lane 7, 1/10 dilution), and corresponding preimmune serum (lane 6, 1/10 dilution).

homologous to the P1-like gene. One of them, homologous to probe 29NTS, was located 34 nucleotides upstream from the initiation site for the P1-like gene. For adhesin P1 of \textit{M. pneumoniae}, it has been shown that although the gene encod-
ing this protein exists in multiple copies, there is only one full-length functional copy of the gene (32). Multiple copies of the adhesin gene have also been found in M. genitalium (4). We are currently investigating the possibility of the presence of a promoter for the P1-like adhesin gene of M. genitalium to determine whether the cloned gene is functional or not. No significant homology was found between the M. piriun P1-like adhesin and the M. genitalium peptide that was used to produce antibodies reducing HIV infectivity (24). This result indicates that the increased HIV-associated cytopathy in the presence of M. piriun cannot be attributed to homology in the region corresponding to this M. genitalium peptide. In addition, no significant homology was found between the M. piriun P1-like adhesin and HLA molecules.

The above-described results indicate that we have identified the gene for the P1-like adhesin in M. piriun. This will allow the design of immunological tools to study the putative location of this protein at the tip of the cell and its role in cytadherence.

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