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A Spontaneous Hemadsorption-Negative Mutant of *Mycoplasma pneumoniae* Exhibits a Truncated Adhesin-Related 30-Kilodalton Protein and Lacks the Cytadherence-Accessory Protein HMW1

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A spontaneous, hemadsorption-negative mutant of *Mycoplasma pneumoniae* lacks the cytoskeleton-forming HMW1 protein and exhibits a truncated adhesin-related 30-kDa protein. Genetic analyses revealed deletion of one nucleotide in the *hmw1* gene and loss of eight repeated sequences comprising 144 nucleotides in the gene for the adhesin-related 30-kDa protein.

Adherence of *Mycoplasma pneumoniae* to epithelial cells (cytadherence) is a highly complex multifactorial process and the prerequisite for colonization of the human respiratory tract and subsequent disease described as atypical pneumonia (2, 16, 32). Characterization of spontaneously cytadherence-negative mutants (9, 21, 22) or comparable mutants obtained after nitrosoguanidine treatment (12, 13, 43) resulted in the identification of proteins which are necessary for effective adherence to host cells. In addition to the adhesin P1 (170 kDa) and the adhesin-related 30-kDa protein (3, 4, 7, 10, 15, 16, 18, 40), which are not sufficient for *M. pneumoniae* cytadherence, three membrane proteins of 85, 72, and 37 kDa (designated B, A, and C, respectively, by Hansen et al. [12]) and five proteinaceous components (HMW1 to -5) of the cytoskeleton-like triton shell are involved in the attachment process (17, 24, 30, 35–38, 43). These proteins are generally designated accessory proteins.

We have considerable evidence that the two proteins B and C are identical with the cleavage fragments of the open reading frame 6 (ORF6) gene product (40- and 90-kDa proteins) of the P1 operon, which consists of three ORFs in the order ORF4, ORF5 (P1), ORF6 (11, 17, 19, 21, 22, 26, 27, 35, 43). Biochemical and immunological studies revealed the location of the ORF6 gene product at the tip-like organelle of *M. pneumoniae* in close proximity to the P1 protein (11, 26, 27).

In *M. pneumoniae* the loss of cytadherence is a very frequent event. About 0.7% of colonies derived from a cytadherence-positive clone have proved hemadsorption negative (22, 25). However, the molecular basis or the reasons for the high rate of mutants lacking proteins involved in cytadherence are still unclear. Until now, in only one case was the genetic basis for the loss of the proteins P1, A, B, and C in a single mutant determined (39).

In this article we describe the molecular basis of mutations in the *hmw1* gene and the adhesin-related 30-kDa-protein gene accompanied by loss of cytadherence.

Culture conditions and isolation of a hemadsorption-negative mutant of *M. pneumoniae*. *M. pneumoniae* M129-B18 (ATCC 29342) and the spontaneous mutant M6 were grown for 48 h at 37°C in 137-cm^2 Roux flasks containing 120 ml of modified Hayflick medium (14). The adherent *Mycoplasma* colonies were scraped off into phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate [pH 7.4]). After centrifugation at 8,000 × *g* for 10 min the cells were washed three times in PBS and the final pellets were frozen at −70°C.

For isolation of spontaneous mutants defective in adhering to erythrocytes, for the most part the procedures of Hansen et al. (12) and Krause et al. (22) were used. Briefly, a suspension of the virulent wild-type *M. pneumoniae* M129-B18 was plated onto agar plates containing Hayflick medium and 1.0% agar (Difco, Detroit, Mich.). This strain was able to multiply in hamster lungs after intranasal inoculation, and histological pneumonia was observed by H. Brunner (6). After 10 days of inoculation at 37°C the *M. pneumoniae* colonies on the agar plate were flooded with a suspension of human erythrocytes in PBS (blood group O; optical density at 600 nm, 1.8) and incubated at 37°C for 30 min. The plates were gently washed three times with PBS. The colonies were screened microscopically for bound erythrocytes. A single hemadsorption-positive colony was picked and resuspended in 1 ml of Hayflick medium (14). One-hundred-microliter aliquots of this suspension were plated on agar plates which were used for screening colonies lacking adhering erythrocytes. Hemadsorption-negative colonies were isolated as described above for the hemadsorption-positive colony. To ensure the homogeneity of the isolated hemadsorption-negative colonies, each hemadsorption-negative colony was colony cloned twice. One of those hemadsorption-negative mutants, designated M6, was defective in binding to the human colon carcinoma cell line WiDr and multiplied in culture medium about twice as fast as the wild type, as measured by radioactivity of [^{3}H]palmitic acid-labelled cells of cultures of the wild type and the mutant, which had been inoculated with equal amounts of [^{3}H]palmitic acid-labelled bacteria. Although this mutant M6 grew adherently to glass, it was not able to bind to plastic.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot). The procedure for cloning of certain regions of the protein P1, the adhesin-related 30-kDa protein, the 40- and 90-kDa proteins, and the cytoskeleton-like proteins HMW1 and HMW3 as well as the methods for expression and purification of the corresponding fusion proteins were described previously (26, 27, 31, 35).

Antisera against fusion proteins of certain regions of the protein P1, the adhesin-related 30-kDa protein, and the proteins of 40 and 90 kDa (ORF6 gene product) were produced in rabbits as described elsewhere (26, 27). Antisera against the cytoskeleton-like proteins HMW1 and HMW3 were prepared in rabbits as described by T. Proft and R. Herrmann (31).

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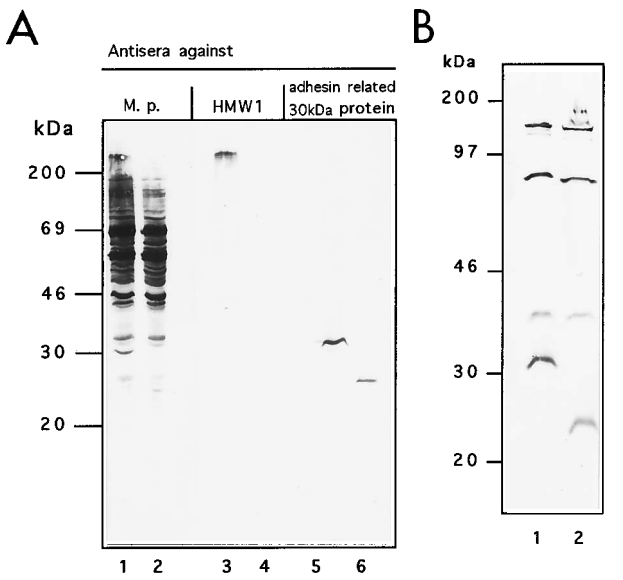

FIG. 1. (A) Comparative immunoblot analysis with total cell lysates of the wild-type *M. pneumoniae* M129 (lanes 1, 3, and 5) and the hemadsorption-negative mutant M6 (lanes 2, 4, and 6) by using the antisera against whole wild-type *M. pneumoniae* (M. p.) cells, the HMW1 protein, and the adhesin-related 30-kDa protein. (B) Immunoblot analysis with total cell lysates of the wild-type *M. pneumoniae* M129 (lane 1) and the mutant M6 (lane 2) by using antisera against the membrane proteins P1, the 90- and 40-kDa proteins (gene products of the P1 operon), and the adhesin-related 30-kDa protein.

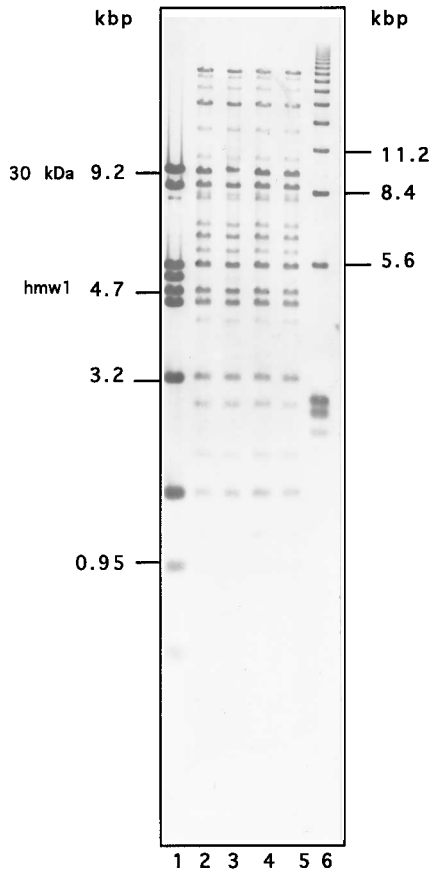

FIG. 2. Southern blot analysis of *Eco*RI-restricted genomic DNA of the wild-type *M. pneumoniae* M129 (lane 3) and of the mutant M6 (lanes 2, 4, and 5). Lane 1, *Eco*RI-digested pcosMP H8 DNA; lane 6, size marker. The DNA fragments were hybridized with the digoxigenin-11-dUTP-labelled pcosMP H8 DNA which contains the genes for the adhesin-related 30-kDa protein and the HMW1 protein.

Rabbits were immunized three times with 100 μl of a suspension of *M. pneumoniae* M129 (optical density at 600 nm, 1.5) in PBS in 4-week periods to obtain antisera against whole *Mycoplasma* cells. Cell pellets of *M. pneumoniae* M129 and the hemadsorption-negative mutant M6 were lysed in the SDS-PAGE buffer of Laemmli at up to 50 mg (wet weight)/700 μl, and the proteins (10 μl) were separated in SDS–12% polyacrylamide gels according to the method of Laemmli (23). Western blotting was carried out by the method of Towbin et al. (41). The procedure for immunoblot analysis was described earlier by Layh-Schmitt and Herrmann (26). Except for the antisera against whole bacteria and the HMW1 protein, which were diluted 1:10,000 or 1:5,000, respectively, all the other antisera (anti-P1, -40-, -90-, and -30-kDa protein) were used in dilutions of 1:2,000. The immunological studies revealed that in the mutant the HMW1 protein is not detected by the used antiserum and that this mutant expresses a truncated adhesin-related 30-kDa protein of 25 kDa (Fig. 1). All the other antisera tested (anti-P1, -40-, and -90-kDa protein [Fig. 1] and anti-HMW3 [data not shown]) did not show any differences in their reaction patterns with proteins of the mutant M6 compared with the wild type. Protein profiles of in vivo [^{35}S]methionine- and [^{35}S]cysteine-labelled wild-type and mutant M6 cells proved identical in SDS-PAGE (12 or 7.5% polyacrylamide) except for the missing HMW1 protein and the truncated 30-kDa protein in the mutant (data not shown).

Southern blot analysis. The cosmid pcosMP H8 was prepared by Wenzel and Herrmann as described previously (42). This cosmid, which carries the genes for the HMW1 protein and the 30-kDa adhesin-related protein (20), was labelled with digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) according to the supplier's instructions by using the nonradioactive labelling kit from Boehringer. Southern blot analyses showed that the *Eco*RI fragment of the wild type encoding HMW1 was of the same size (4.7 kbp) in the wild type, mutant, and cosmid clone (Fig. 2) (20, 42). However, the *Eco*RI fragment encoding the 30-kDa protein appeared to be approximately 200 bp smaller in the mutant (9.0 kbp) (Fig. 2, lanes 2, 4, and 5) than in the wild type or cosmid clone (9.2 kbp) (Fig. 2, lanes 1 and 3). This size difference was more obvious with *Hin*dIII digestion (data not shown), in which a 1.0-kbp fragment was detected in the wild type and cosmid digests, compared with a 0.85-kbp fragment in the mutant.

DNA sequence analyses. *Eco*RI fragments of the mutant M6 were ligated into the Bluescript vector pBC SK$^+$ (Stratagene, Heidelberg, Germany) according to the method of Sambrook et al. (33). *Escherichia coli* XL1-Blue was transformed with the recombinant pBC plasmid. Colonies which contained the vector with the *Eco*RI fragments carrying the *hmw1* or the 30-kDa-protein gene were detected by radiolabelled oligonucleotides (28) (nucleotide positions 591 to 615 of the 30-kDa-protein gene and 1611 to 1626 of the *hmw1* gene).

Plasmids were purified by the rapid alkaline lysis method (33), phenol-chloroform extracted, ethanol precipitated, and resuspended in TE (10 mM Tris-HCl [pH 8], 0.1 mM EDTA). DNA sequencing was performed by the chain termination method (34) using *Taq* polymerase and fluorescently tagged dideoxynucleotides in conjunction with an ABI 373A automated sequencer. Both strands were sequenced with specific oligonucleotides. Computer analyses were performed by using

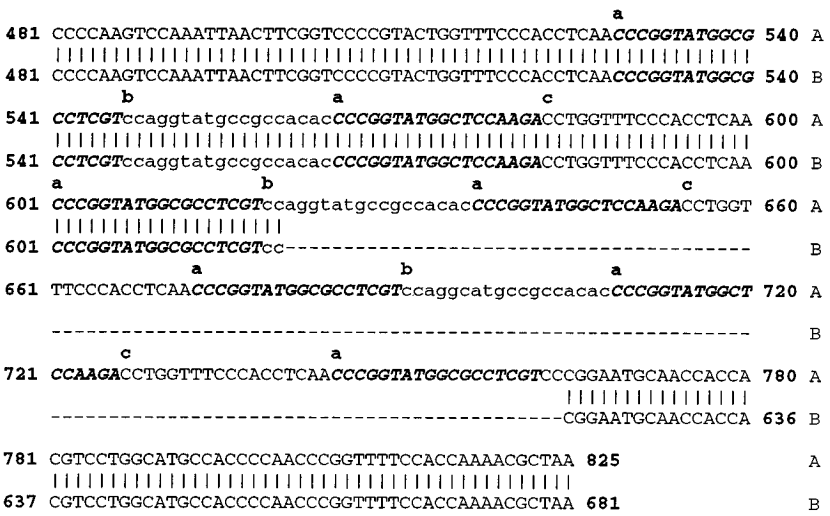

FIG. 3. Alignment of partial DNA sequences of the *M. pneumoniae* M129 wild-type gene (sequence A) for the 30-kDa adhesin-related protein and of the corresponding mutated gene (sequence B) of strain M6. a, b, and c, three types of repeats.

the HUSAR package (release 3) (Heidelberg Unix Sequence Analysis Resources) made available through the German Cancer Research Center.

The DNA sequence of the truncated adhesin-related 30-kDa-protein gene of the mutant M6 was aligned with the wild-type sequence reported by Dallo et al. (7) with the GAP program using the algorithm of Needleman and Wunsch (29). In the mutated gene a lack of eight repeat sequences of three different types was observed, with each consisting of 18 nucleotides and constituting a total sequence of 144 nucleotides from nucleotide position 620 to 764 (Fig. 3). This result is consistent with the finding that this protein encoded by the mutant M6 is truncated by about 5 kDa. From nucleotide 765 to 825 the mutated sequence continues in frame with the wild-type sequence. These repeat sequences code for a proline-rich, positively charged C-terminal region of the adhesin-related 30-kDa protein which is between 55 and 67% homologous with the P1 protein (7, 40). The loss of part of the proline-rich C terminus of the adhesin-related 30-kDa protein in the mutant might strongly affect the structure and probably the adhesin-related functions of the protein. Repeat sequences are generally known to be lost or reshuffled frequently (1), and our results indicate that this might be a common event also in *M. pneumoniae.*

The *hmw1* gene of the mutant was partially sequenced from nucleotide 449 downstream of the ATG start codon of the *hmw1* gene to nucleotide position 2172 upstream of the start codon. Alignment of the mutated DNA sequence to the corresponding wild-type sequence (accession number at the EMBL gene bank, U11381), which was determined by Dirksen et al. (8), revealed a deletion of one thymidine at nucleotide position 1709 in the mutant resulting in a frameshift and a premature termination codon after nucleotide position 1765 (Fig. 4). This kind of mutation might be due to the lack of proofreading functions of DNA polymerases in mycoplasmas as described by Boxer and Korn (5). With antisera directed against a fusion protein containing amino acids 136 to 258 of the HMW1 protein or against whole *M. pneumoniae* cells a truncated HMW1 protein has not been identified in the mutant, suggesting that this protein is not expressed or degraded very fast.

The HMW1 protein might contribute to effective cytadherence since mutants lacking all five cytoskeleton proteins (HMW1 to -5) are cytadherence negative and avirulent (22). Those mutants exhibit a random distribution of the P1 adhesin in the membrane in contrast to the wild type, which clusters the P1 protein on the tip structure of *M. pneumoniae* (22). Thus, the described mutant, lacking only the HMW1 cytoskeleton-

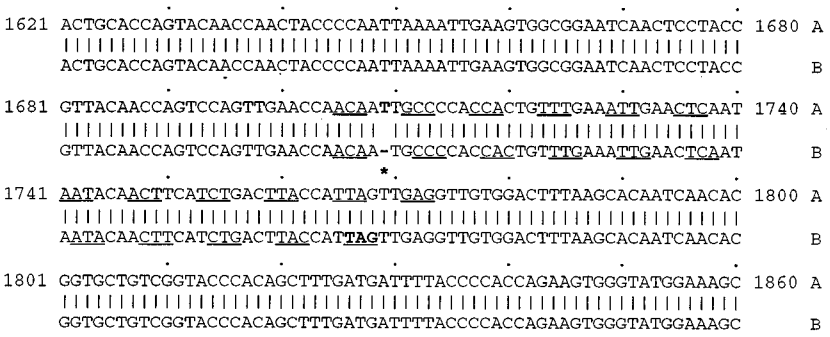

FIG. 4. Partial DNA sequence of the gene for the HMW1 protein of the wild-type *M. pneumoniae* M129 (sequence A) compared with the corresponding DNA sequence of the hemadsorption-negative mutant M6 (sequence B). Deletion (*) of one nucleotide (T) results in a frameshift and premature termination of the *hmw1* sequence.

forming protein, is an appropriate tool for studying the influence of an individual cytoskeleton protein on the distribution of membrane proteins which are involved in cytadherence, such as the P1 protein or the ORF6 gene product.

The reason for two independent mutations in one clone is unclear. Since we isolated several different double mutants (data not shown), we assume that the screening method, which selects for hemadsorption-negative colonies, favors mutants exhibiting defects in more than one adhesion-relevant protein.

Nucleotide sequence accession numbers. The DNA sequences have been submitted to the EMBL gene bank. The accession numbers are Z46228 for the 30-kDa M6 and Z46229 for the HMW1 M6.

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