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Selection of *Mycoplasma hominis* PG21 Deletion Mutants by Cultivation in the Presence of Monoclonal Antibody 552

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Three mutants of *Mycoplasma hominis* PG21 were isolated and shown to contain alterations in the size of a repeat-containing gene encoding a surface-localized 135-kDa antigen designated Lmp1. The mutants were isolated by cultivating *M. hominis* for a 3-month period in the presence of Lmp1-specific monoclonal antibody (MAb) 552. The epitope for MAb 552 was localized at the repeated part of the protein. The gene encoding Lmp1 is part of a transcriptional complex that contains 9.5 direct repeats of 471 bp each. Pure cultures of mutant strains were obtained by subcloning, and three mutants were characterized. The mutants showed deletions of a various number of repeats. The deletions were accompanied by a decrease in size of the proteins. With increasing size of deletions, agglutination and growth inhibition by MAb 552 became less pronounced. Spontaneous aggregation of the mutant *M. hominis* cells in culture medium was, however, increased, indicating that the repeated elements may be of importance for repulsion of the cells.

A method used by several pathogenic microorganisms to avoid the host immune response is to vary surface antigens (27). Antigenic variation provides the microorganism with increased ability to colonize in different or changing host environments. Because they lack a cell wall making the plasma membrane the only barrier to the environment (30), mycoplasmas could be expected to have developed special strategies to obtain a versatile surface exposed to the environment. Strains of *Mycoplasma hominis* exhibit marked antigenic (1, 2, 19, 20, 22, 23) and genomic (6, 7, 24) variation, but so far no correlation between the pathogenicity and specific antigenic components of the *M. hominis* strains has been shown.

Christiansen et al. (8) and Ladefoged et al. (19) used monoclonal antibodies (MAbs) for detection of gene and antigenic variation in 26 *M. hominis* strains. MAbs raised against *M. hominis* PG21 were used to characterize the antigenic proteins. Whereas MAbs reacting with cytoplasmic antigens showed a uniform band pattern in immunoblotting, MAbs reacting with surface-exposed components showed a high degree of variability in their reactivities with the 26 *M. hominis* strains. MAb 30.3.1 and MAb 552, which showed variability in their reactivities with the 26 *M. hominis* strains, were used to identify gene probes in a recombinant *Escherichia coli* expression library. MAb 30.3.1 reacted in immunoblottings with 12 of the 26 strains. Southern blot analysis using the clone producing a fusion protein reacting with MAb 30.3.1 as the probe showed that this probe reacted only with DNA from the 12 strains that reacted in immunoblotting. This finding indicated a variation involving deletions (8). MAb 552 reacted with all strains in immunoblotting, in most strains with a protein of 93 kDa. In *M. hominis* PG21, a ladder pattern of proteins ranging in size from 93 to 135 kDa was observed. Results from Southern blot analysis with recombinant plasmids producing fusion proteins reacting with MAb 552 used as probes indicated a complex gene arrangement involving repetitive structures (16). The repeated elements were found at two genomic positions separated by more than 121 kbp.

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To reveal the structures and functions of the repetitious regions and to identify the gene(s) encoding the surface-localized polypeptide recognized by MAb 552, both gene areas have been cloned and sequenced. One region, containing the *lmp1* and *lmp2* genes, consisted of 9.5 nearly identical repeats of 0.5 kb flanked by unique nonrepeated sequences. In repeat 8 (R8), a base substitution gave rise to a stop codon which was directly followed by a methionine constituting the end of *lmp1* and the start of *lmp2* (16). The second gene area, *lmp3-lmp4*, contained 0.5-kb repeated structures similar to the repeated elements seen in the *lmp1-lmp2* region (unpublished results). A stop codon and a methionine separated by 51 bp, giving rise to two open reading frames, *lmp3* and *lmp4*, were found in R6. By hybridization, the repeated part of the *lmp1-lmp2* region was identified on a 10-kb *Bgl*II, an 11-kb *Eco*RI, and a 64-kb *Bam*HI fragment (16), while the *lmp3-lmp4* region was found on a 12-kb *Bgl*II, a 6-kb *Eco*RI, and a 29-kb *Bam*HI fragment (unpublished results). The MAb 552-defined epitope was encoded by the repeated sequences from both gene areas (16), and both gene areas thus have a potential capacity to encode polypeptides reacting with MAb 552.

The aim of this study was to determine whether it was possible, in an in vitro system, to select for changes in specific genes by adding a MAb reacting with the corresponding antigen. We report in this paper the identification of intragenic deletions resulting in antigenic variation. Since MAb 30.3.1 recognized a surface-exposed protein for which the encoding gene seemed to be associated with gene deletions, environmental influence could have been involved in causing the deletions. In light of the complex *lmp1-lmp2* gene structures including the repeated sequences, MAb 552 was also included in the study. *M. hominis* PG21 was continuously cultured in the presence of MAb 30.3.1 or MAb 552 or, as controls, without a MAb for 3 months. No alteration was seen in the cultures cultivated with MAb 30.3.1 or in the controls. Three antigenic variants of *M. hominis* PG21 cultivated in the presence of MAb 552 were isolated on the basis of altered DNA and protein profiles. Deletions were detected only in the region containing the *lmp1* and *lmp2* genes. Northern (RNA) blotting and immunoblotting of the mutant strains were performed, and comparisons were made with the reference strain *M. hominis* PG21.

MATERIALS AND METHODS

Strains and culture conditions. *M. hominis* PG21 (type strain) was obtained from the International Mycoplasma Reference Centre, Aarhus, Denmark. Cultures of *M. hominis* PG21 were grown in BEA medium (13). The *Mycoplasma* strain was filter cloned according to the recommendations from the Subcommittee of the Taxonomy of Mollicutes (15, 28) and subsequently freeze dried.

MAbs. MAb 552 and MAb 30.3.1 were obtained from hybridoma cell cultures cultivated in RPMI 1620 with 10% fetal calf serum to concentrations of 10 μg/ml for MAb 30.3.1 and 30 μg/ml for MAb 552.

Propagation of *M. hominis* with MAbs. *M. hominis* cultures were maintained in 1.7 ml of BEA medium to which was added 1/10 volume of hybridoma cell supernatants (1 μg of MAb 30.3.1 or 3 μg of MAb 552 per ml) or 1/10 volume of RPMI 1640 with 10% fetal calf serum alone. The cultures were examined once every day, and 50 μl of culture was passaged to 1.7 ml of fresh medium when growth was evident by a change in the color of the pH indicator. At appropriate time intervals, cultures were cultivated in 300 ml of BEA medium and used for protein and DNA preparation as well as for establishing of stock cultures, which were stored at −70°C.

Filter subcloning of *Mycoplasma* strains. Subclones were derived by filter cloning. Cell aggregates were disintegrated by gentle passing the cultures through a 0.45-μm-pore-size filter immediately prior to plating of the organisms on BE agar plates (28). Well-separated colonies were randomly picked in agar plugs, propagated at 37°C in 1.7 ml of BEA medium, and assigned a new strain designation. These cloning steps were carried out three times for each subclone. The cultures were stored at −70°C and used as stocks for subsequent experiments.

DNA cloning. *M. hominis* DNA was isolated as described by Christiansen et al. (9). DNA cloning was done by standard methods as described by Sambrook et al. (26), using pBluescript SK+ (Stratagene, La Jolla, Calif.) as the vector. Recombinant plasmids were transformed into *E. coli* XL1-Blue cells (Stratagene). Positive clones were selected by colony hybridization as described by Sambrook et al. (26), using clone pBMhAS-16, containing the nonrepetitious region of *lmp1*, as a probe (16).

DNA fragments used for nick translation or subcloning were purified by electrophoresis through 1% low-melting-temperature agarose (SeaPlaque GTG agarose; FMC Bioproducts, Rockland, Maine). Gel slices containing the appropriate fragments were excised, melted at 62°C in Tris-EDTA buffer, and then subjected to phenol-chloroform extraction and ethanol precipitation.

Plasmid DNA preparation, restriction enzyme cleavage, and Southern blotting. Plasmid preparation was done by alkaline lysis (26), with the following modifications. RNase treatment was followed by precipitation with 20% polyethylene glycol in 2.5 M NaCl at 0°C for 1 h followed by centrifugation at 20,000 × *g* for 10 min. Plasmid DNA was cleaved with restriction enzymes as recommended by the supplier (Boehringer, Mannheim, Germany). Digested DNA was separated on 0.7% agarose gels, transferred to Hybond-N membranes (Amersham International, Inc., Amersham, United Kingdom), and probed with nick-translated plasmid DNA (26). Probes were removed from the blots by washing the filters in 0.5× Denhardt solution–25 mM Tris-HCl (pH 7.5)–0.1% sodium dodecyl sulfate (SDS) for 2 h at 80°C.

Partial digestion of clones pBMhB616A, p74-5.7, p56-6.7, and p81-7.7 was performed by using *Hin*dIII and *Eco*RV in serial 10-fold dilutions with 2 to 0.016 U per μg of DNA. The samples were digested at 37°C for 30 min. Southern blots of partial digestions were hybridized with end-labelled oligonucleotides SK and KS (Stratagene) at 42°C (26). After hybridization, the filters were washed three times for 5 min each time at 42°C and exposed to X-ray films.

Sequence analysis. Sequencing of denatured double-stranded DNA was done by the method of Hattori and Sakaki (14), using the Sequenase sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and [α-^{32}P]dATP (Amersham). Primers with sequences 5′-GGTTAAAAGATGCTATTAC-3′ (3075), 5′-GGAGAACGAATGAATG-3′ (3076), 5′-CATTGTCACTTAATT GAGGC-3′ (3077), and 5′-CTTGTTTCGATAACTCG-3′ (3078) were used in addition to universal primers SK and KS. Sequence data were analyzed by the Genetics Computer Group sequence analysis software package version 7.2-Unix installed on Ultrix V.4.4 (11).

Pulsed-field gel electrophoresis (PFGE). Preparation and restriction endonuclease digestion of DNA in agarose blocks as well as contour-clamped homogeneous electric field electrophoresis were done as described by Ladefoged and Christiansen (17). One percent agarose gels were run for 20 h at 200 V with a pulse time ramped from 1 to 15 s. The bands were visualized by staining with ethidium bromide after gel electrophoresis.

SDS-PAGE and immunoblotting. Antigens were prepared from mycoplasmas harvested in log phase. The pellets were washed twice in phosphate-buffered saline and dissolved in SDS sample buffer (62.5 mM Tris HCl [pH 6.8], 10% [vol/vol] glycerol, 2.3% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 0.05% [wt/vol] bromphenol blue). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Andersen et al. (2). The gels were either stained with Coomassie blue or transferred to nitrocellulose and used for immunoblotting (2).

RNA analysis. Total RNA was isolated from early-log-phase cultures by the method of Chomczynski and Sacchi (5). RNA electrophoresis was carried out in formaldehyde buffer and Northern blotting was carried out in formamide buffer as described by Sambrook et al. (26).

Agglutination assay. Cultures were incubated overnight in the absence or presence of 3 μg of MAbs per ml added to BEA medium. When growth was evident from color change of the medium, 0.05 μg of bisbenzimide (Hoechst 33258; Sigma Chemical Co., St. Louis, Mo.) per ml was added, and the cultures were incubated for 1 h. Two microliters of each culture was mixed with a drop of glycerol. The samples were analyzed for number and size of aggregates compared with free mycoplasmas under a Leitz Orthoplan fluorescence microscope (Leitz GmbH, Wetzlar, Germany).

Growth inhibition. The reduction in the number of CFU caused by MAb 552 was determined essentially as described by Ladefoged et al. (16). *M. hominis* strains were incubated overnight at 37°C in BEA medium containing 3 μg of MAb 552 or MAb 30.3.1 per ml. To determine the number of CFU, three serial 10-fold dilutions in BEA medium were performed. Aliquots of 10 μl were plated in duplicates on BE agar plates (28) and incubated for 4 days at 37°C in 5% CO_2. The number of CFU was counted under a stereomicroscope (Leitz).

Nucleotide sequence accession numbers. The GenBank accessions numbers of the sequences determined in this study are U21961, U21962, and U21963 for Mh74, Mh56, and Mh81, respectively.

RESULTS

Propagation of *M. hominis* with MAbs. To establish if addition of a MAb to the growth medium could mediate the appearance of mutants in which major changes in DNA or protein occurred, *M. hominis* PG21 was cultivated in the presence of MAb 552 or MAb 30.3.1, both of which react with surface-localized membrane proteins. Four lines of *M. hominis* PG21 all originating from the same filter-cloned stock culture were established (Fig. 1).

Culture 1 (C1) was propagated without any antibody added and served as control, C2 and C3 were propagated with MAb 552 added to the medium, and C4 was propagated with MAb 30.3.1. At definite time points, 300-ml cultures were inoculated from the four culture lines as indicated in Fig. 1 and used for DNA and protein preparations. The following cultures were further subcloned: C1 at day 86, C2 at days 22 and 86, and C3 at day 44. The schedule used for subcloning was the same in each case; six colonies were randomly picked from agar plates, and each of them were further subcloned twice. The subcloned cultures were analyzed by immunoblotting and Southern blotting in parallel with the nonsubcloned cultures.

Immunoblotting of the uncloned *M. hominis* PG21 mutants. Immunoblotting with MAb 552 of the three C1 cultures showed a major band of 135 kDa and four additional weaker bands ranging in size from 93 to 130 kDa (Fig. 2A). The strains in which MAb 552 was added to the medium all showed different patterns. C2 showed one band of 135 kDa for both the day 22 and the day 86 preparation. C3 likewise showed a band of 135 kDa. The additional bands seen in C1 were not observed. C4 was subjected to immunoblotting with MAb 30.3.1. MAb 30.3.1 reacted with two bands of 49 and 38 kDa (results not shown). It was identical in reactivity to the original culture.

Southern blotting of the uncloned *M. hominis* PG21 mutants. DNA prepared from each of the eight uncloned cultures was digested with *Bgl*II, transferred to nitrocellulose, and hybridized with a probe containing the 471-bp repeat.

The three C1 cultures showed hybridization to two *Bgl*II bands of 12 and 10 kb in Southern blotting (Fig. 2B, lanes 1 to 3), identical to what was seen for *M. hominis* PG21 (16). In Southern blotting of C2 isolated at day 22, the probe hybridized to fragments of 12 and 10 kb and a weaker band of 6.5 kb (Fig. 2B, lane 4). For C2 isolated at day 86, hybridization to two fragments of 12 and 7.5 kb was seen (Fig. 2B, lane 5). Southern analysis of C3 isolated at day 44 revealed hybridization to two bands at 12 and 5.5 kb (Fig. 2B, lane 6). No hybridization to the 10-kb fragment was seen. Thus, subcultures cultivated with MAb 552 added to the medium all showed hybridization to additional bands. C4 which was cultivated with

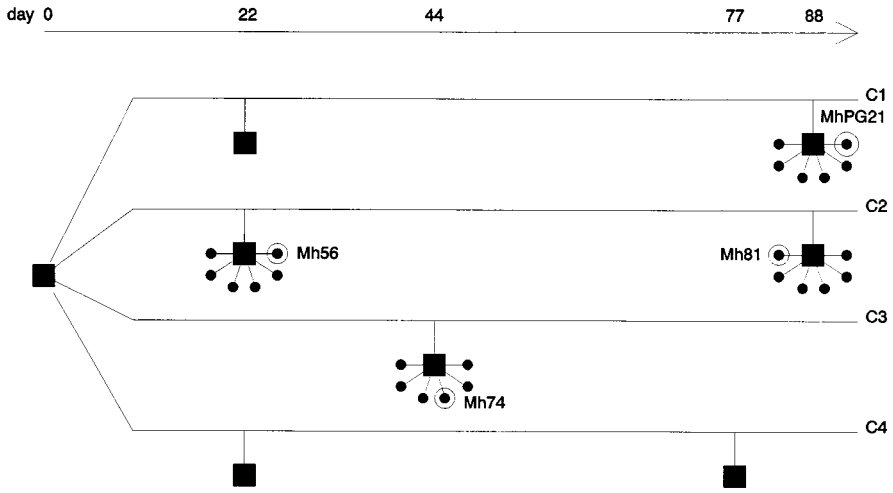

FIG. 1. Schematic diagram showing the cloning schedule, time of subcloning, and analysis by immunoblotting and Southern hybridization. C1, the original strain, *M. hominis* PG21, cultivated without MAb; C2 and C3, *M. hominis* PG21 cultivated with MAb 552; C4, *M. hominis* PG21 cultivated with MAb 30.3.1. Uncloned isolates (■) and filter-cloned isolates (●) were analyzed by immunoblotting and Southern hybridization. From each set of cloned isolates, one was selected for further analysis (○).

addition of MAb 30.3.1 was analyzed at days 22 and 77. Both preparations gave rise to two bands of 12 and 10 kb in Southern blotting (Fig. 2B, lanes 7 and 8).

Southern blotting of the filter-cloned strains. The six subclones of C1 (Fig. 1) all showed identical hybridization patterns. One colony was selected for further analysis and named MhPG21 (Fig. 1, C1). Six colonies were analyzed from subclones of the C2 day 22 preparation. Five subclones hybridized to *Bgl*II fragments of 12 and 10 kb indistinguishable from MhPG21. One subclone showed hybridization to a 12-kb and a 6.5-kb *Bgl*II fragment. This subclone was selected for further analyses and was named Mh56 (Fig. 1, C2). The three bands of 12, 10, and 6.5 kb seen in the uncloned culture were thus represented in the subclones but with hybridization to only two fragments in each subclone. The six subclones from C2 day 86 were analyzed. All six subclones showed bands of 12 and 7.5 kb in Southern blotting. One of these colonies was named Mh81 and selected for further analysis. (Fig. 1, C2). Similarly, six colonies were analyzed from C3 day 44. All showed hybridization to two *Bgl*II bands of 12 and 5.5 kb. One of these was named Mh74 (Fig. 1, C3).

To compare the alterations in the *lmp1-lmp2* and *lmp3-lmp4* regions, DNA extracted from subclones MhPG21, Mh74, Mh56, and Mh81 was digested with *Eco*RI and *Bgl*II, gel electrophoresed, transferred, and hybridized with a probe containing the repeated 471-bp sequence (Fig. 3A). In all four subclones, the probe hybridized with two fragments in both digestions, indicating that the repeat still was present on both genomic localizations. In the *Bgl*II digests, a 12-kb fragment was present in all subclones. The 10-kb *Bgl*II fragment seen in MhPG21 disappeared, while the 5.5-, 6.5-, and 7.5-kb fragments were seen in strains Mh74, Mh56, and Mh81, respectively. The *Eco*RI hybridization correspondingly showed that the 6-kb fragment was unaltered, while the 11-kb fragment seen in MhPG21 disappeared and smaller fragments of 7, 8, and 9 kb were seen in Mh74, Mh56, and Mh81, respectively. These results thus indicated that deletions of 2, 3, and 4 kb had been generated in the *lmp1-lmp2* gene area.

Immunoblotting of the filter-cloned strains. SDS-PAGE was performed with freshly prepared antigens from MhPG21, Mh74, Mh56, and Mh81, and the gels were transferred to nitrocellulose for immunoblotting with MAb 552 (Fig. 3B). In MhPG21, MAb 552 reacted with polypeptides of 135 and 115 kDa and a double band of 93 kDa. In the mutant strains, the protein profiles were altered. In all mutants, a 135-kDa band was seen. In Mh74, and additional protein band of 105 kDa and a double band of 93 kDa were seen. In Mh56, MAb 552 reacted with an additional polypeptide of 130 kDa, while in Mh81, additional bands of 72 and 70 kDa were seen.

PFGE. To confirm the localization of the deletions and to exclude major chromosomal rearrangements, cultures of MhPG21, Mh74, Mh56, and Mh81 were cast in agarose blocks and analyzed by PFGE (Fig. 4). The genomic map of *M. hominis* PG21 was previously constructed by Ladefoged and Christiansen (18). In this work, the gene encoding the 135-kDa protein (Lmp1) was shown to be located on a 65-kb *Bam*HI fragment. Therefore, DNA from MhPG21, Mh74, Mh56, and Mh81 was digested with *Bam*HI and analyzed by PFGE. We compared the band patterns with those obtained by Ladefoged and Christiansen (18). Five bands of 269, 130, 94, 90, and 25 kb

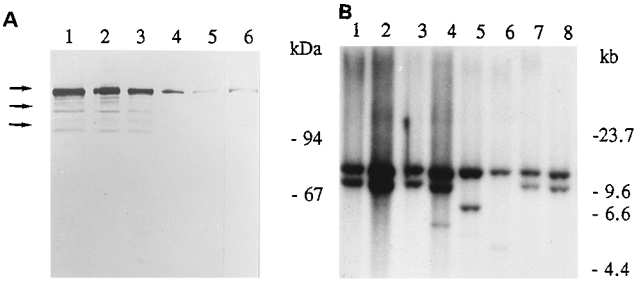

FIG. 2. Immunoblotting and Southern blotting of the uncloned *M. hominis* PG21 mutants. (A) Immunoblotting with MAb 552. Lanes: 1 to 3, C1 preparations from days 0, 22, and 86, respectively; 4, C2 day 22 preparation; 5, C2 day 86 preparation; 6, C3 day 44 preparation. Size standards are shown at the right. In all lanes, the same amount of protein was applied as determined by Coomassie blue staining of an SDS-gel electrophoresed in parallel. (B) Southern blotting of *Bgl*II-digested DNA hybridized with probe pBMhS38-1 containing the repeat. Lanes: 1 to 3, C1 preparations from days 0, 22, and 86, respectively; 4, C2 day 22 preparation; 5, C2 day 86 preparation; 6, C3, day 44 preparation; 7 and 8, C4, day 22 and 79 preparations. DNA standards are indicated at the right.

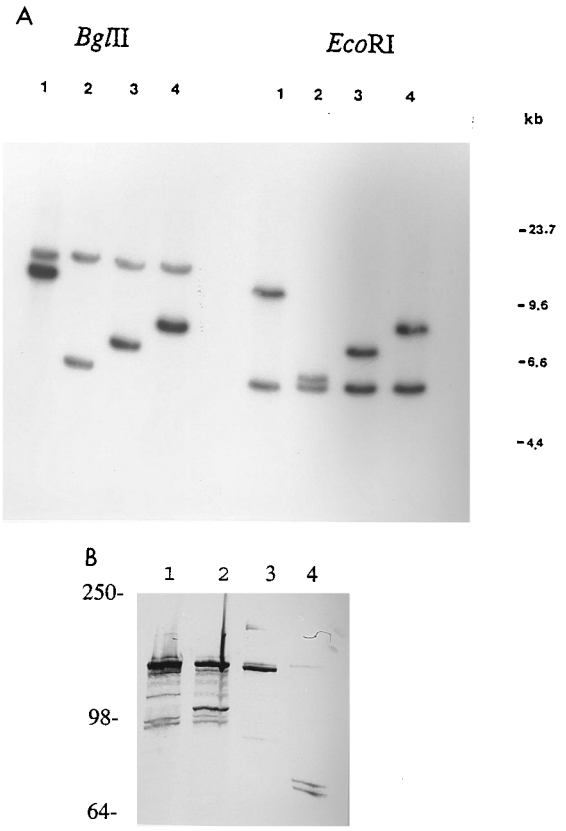

FIG. 3. Immunoblotting and Southern blotting of the filter-cloned strains. (A) Southern blot of *Bgl*II and *Eco*RI digestions of genomic DNA from the filter-subcloned strains were hybridized with probe pBMhS38-1 containing the 471-bp repeat. Lanes: 1, MhPG21; lanes 2, Mh74; 3, Mh56; 4, Mh81. Numbers at the right indicate DNA standards. (B) Immunoblotting with MAb 552 of MhPG21 (lane 1), Mh74 (lane 2), Mh56 (lane 3), and Mh81 (lane 4). The same amount of protein was applied to all lanes as determined by Coomassie blue-staining of SDS-gels. Numbers at the right indicate molecular masses in kilodaltons.

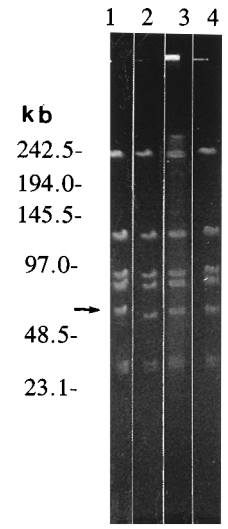

FIG. 4. Genomic DNA from the four strains was digested with *Bam*HI and analyzed by PFGE. Lanes: 1, MhPG21; 2, Mh74; 3, Mh56; 4, Mh81. Bacteriophage lambda DNA oligomers and *Saccharomyces cerevisiae* YNN295 chromosomes were used as molecular weight markers. Arrow indicates the fragment of 65 kb.

were observed in all strains, while the fragments corresponding to the 64-kb band of *M. hominis* PG21 were smaller in the mutants. Mh74, Mh56, and Mh81 showed bands of 60, 61, and 62 kb, respectively. The deletions found in the mutants were thus related to the region containing the *lmp1* and *lmp2* genes and were found to be of the sizes already predicted from the Southern blotting of *Bgl*II- and *Eco*RI-digested mutant DNA (Fig. 3A). Since the other bands were identical in all strains, we could exclude the possibility that major DNA rearrangements had occurred.

Agglutination assay and viability assays. By the agglutination assay, we analyzed the effect of addition of MAb 552 to the cultivation media on aggregation of MhPG21 and the mutant subclones. Without antibody, the type strain MhPG21 did not aggregate (Fig. 5A). Most mycoplasmas were seen as single cells in addition to aggregates of a few cells. When MAb 552 was added, MhPG21 was observed in large aggregates of hundreds of mycoplasmas with nearly no free mycoplasmas (Fig. 5B). The mutants did exhibit some natural agglutination. Without addition of MAb 552, small aggregates with up to five mycoplasmas in addition to a few larger aggregates were typically observed in Mh74 (Fig. 5C). Agglutination was seen when MAb 552 was added (Fig. 5D), but the aggregates were smaller than for MhPG21. Mh56 exhibited a slight degree of natural aggregation. Aggregates of a few cells of mycoplasmas were predominant (Fig. 5E). When MAb 552 was added, large aggregates differing in size were observed (Fig. 5F). Mh81 aggregated most. Small aggregates of typically a few cells were predominant in addition to several larger aggregates. A few single cells were observed (Fig. 5G). When MAb 552 was added, aggregates similar in size to the Mh74 aggregates were observed (Fig. 5H). A control study was performed with MAb 30.3.1. This antibody did not agglutinate *M. hominis* cells. Compared with *M. hominis* PG21, all mutants thus showed a tendency to form spontaneous aggregates. Addition of MAb 552 increased the size of aggregates, but they never reached the size of the aggregates formed by addition of MAb 552 to *M. hominis* PG21. This result indicates that fewer epitopes on the mutants are available for reaction with MAb 552. We have previously shown that formation of aggregates of *M. hominis* PG21 with MAb 552 was accompanied with growth inhibition (16). To analyze whether the reduced size of aggregates formed when the mutants were incubated with MAb 552 would influence growth inhibition, we compared the decrease in viability measured as the reduction of CFU (Fig. 6). The surviving fractions in the assay were calculated as percentages on the basis of the control cultures with no antibody added. Addition of MAb 30.3.1 to the cultivation media did not influence the number of CFU. The reduction of CFU caused by the addition of MAb 552 was most pronounced for MhPG21 (Fig. 6), in which the number of CFU was reduced by 95%. Less reduction was seen for mutants Mh74, Mh56, and Mh81 (80, 72, and 82%, respectively). The decrease in CFU was correlated to the amount of added MAb 552.

Strategy for cloning of DNA fragments. To clone *Bgl*II fragments containing the *lmp1* and *lmp2* genes, DNA isolated from the Mh74, Mh56, and Mh81 was digested to completion with *Bgl*II and ligated to pBluescript SK+ opened with *Bam*HI. The recombinant bacteria were screened by colony hybridization using probe pBMhAS-16, containing the nonrepetitious part of *lmp1* (16). Positive colonies were examined by restriction enzyme cleavage and DNA sequencing of the 3′ and 5′ ends. The sequences were compared and shown to be identical to the known sequence of the corresponding *Bgl*II fragment,

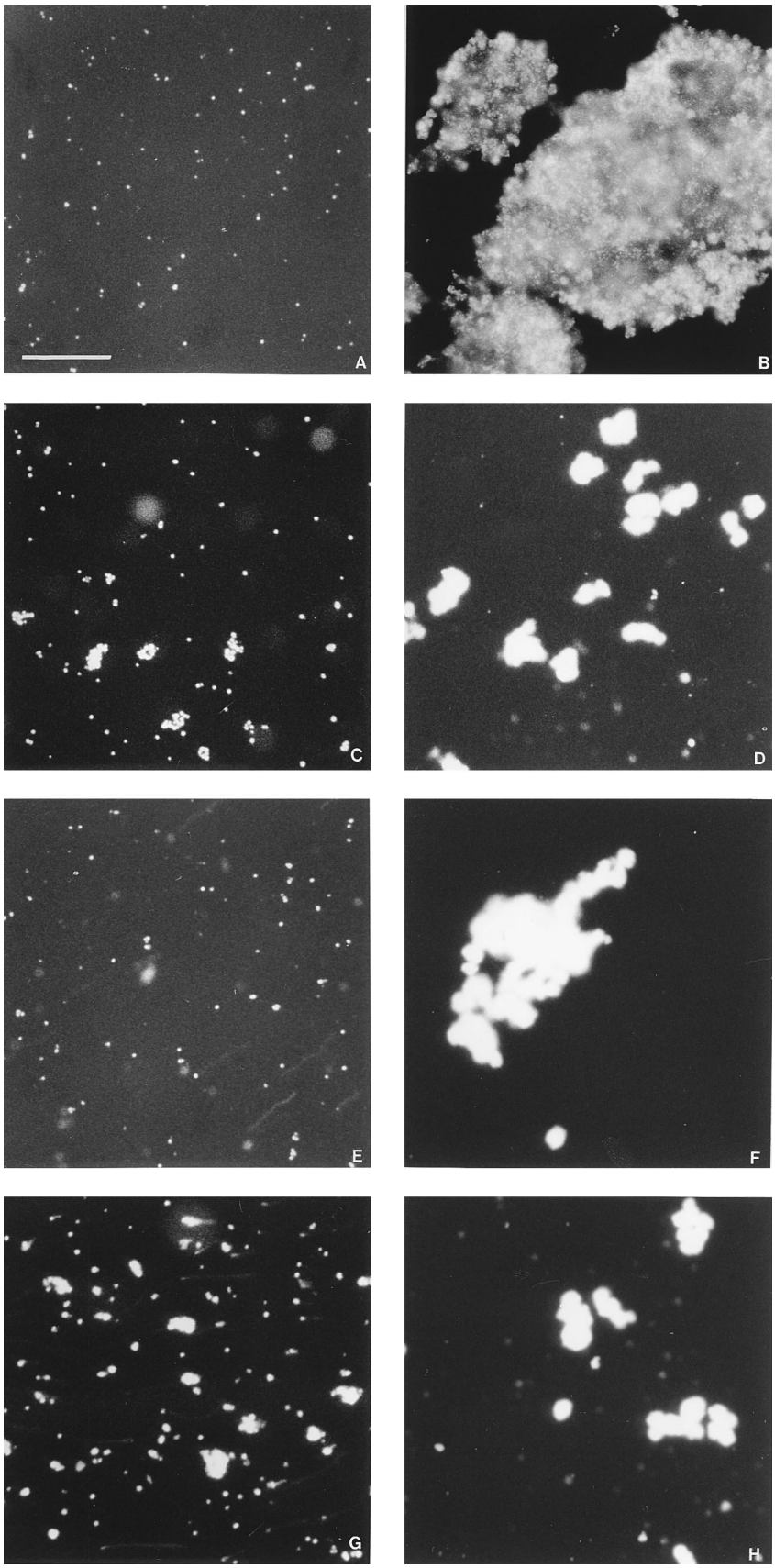

FIG. 5. Agglutination of MhPG21 (A and B), Mh74 (C and D), Mh56 (E and F), and Mh81 (G and H). Agglutination patterns were examined for *M. hominis* cultured without MAb 552 (A, C, E, and G) and with MAb 552 added to the medium (B, D, F, and H). The bar indicates 10 μm.

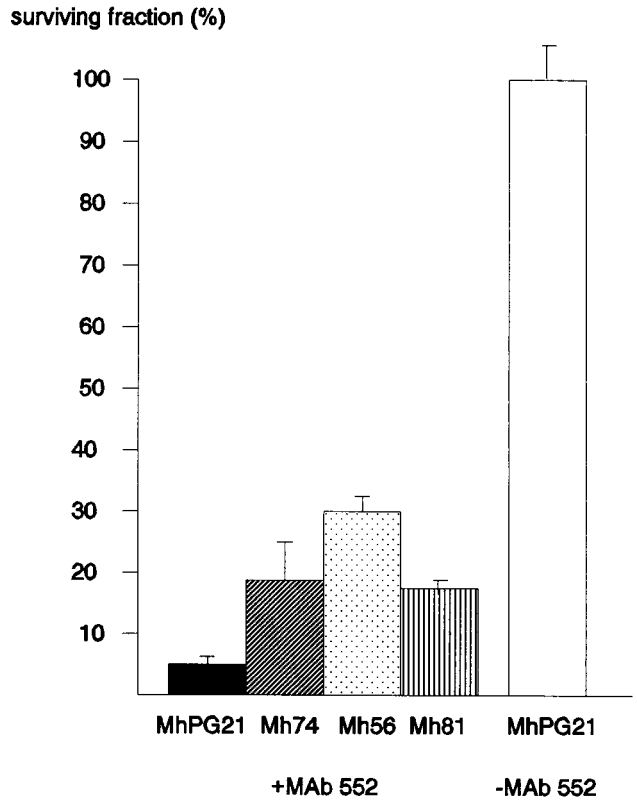

FIG. 6. Growth inhibition caused by the addition of 3 μg of MAb 552 per ml. The surviving fraction was calculated on basis of CFU from control cultures with no antibody added to the medium. Standard errors are indicated above the bars.

pBMhB616A of *M. hominis* PG21 (16). The clones containing *Bgl*II fragments from the mutants were named p74-5.7, p56-6.7, and p81-7.7 (Fig. 7).

Determination of the number of repeats. In *M. hominis* PG21, the *lmp1-lmp2* gene region was found to contain 9.5 repeats of 471 bp (16). In each repeat, a *Hin*dIII site was present, giving rise to eight *Hin*dIII repeats of 471 bp. In seven repeats an *Eco*RV site was present, giving rise to six *Eco*RV repeats of 471 bp. The number of 471-bp *Hin*dIII and *Eco*RV repeats in clones p74-5.7, p56-6.7, and p81-7.7 was determined and compared with the number in pBMhB616A. This was done by cutting out the inserts with *Sac*I and *Apa*I, thus leaving the part of the pBluescript polylinker that hybridizes to the oligonucleotide SK on the insert. The subsequent partial digestion with *Hin*dIII or *Eco*RV was followed by hybridization with the end-labelled oligonucleotide SK. The space between bands reflects the distance to the next restriction site; hence, the number of repeats could be determined by the number of spaces of 471 bp between bands.

The Southern blots of partially digested clones are shown in Fig. 8. In Fig. 8A, pBMhB616A was cleaved with decreasing amounts of *Hin*dIII and *Eco*RV. The number of bands varied depending on the endonuclease concentration. In lane 3 (0.5 U of *Hin*dIII per μg of DNA), the highest number of bands was seen. A band of 1.8 kb in addition to nine bands ranging from 4.6 to 8.6 kb spaced by 0.5 kb and a band of 9.7 kb were seen. For *Eco*RV digestions, lanes 12 and 13 showed bands at 4.6, 5.6, 6.1, 6.6, 7.1, 7.6, 8.1, 8.6, 9.1, and 9.7 kb, indicating a double *Eco*RV repeat (4.6 to 5.6 kb) followed by seven repeats. The repeats are followed by a 0.6-kb *Eco*RV fragment.

p74-5.7 (Fig. 8B) was shown not to contain any 471-bp *Hin*dIII fragment, since the SK primer hybridized to fragments of sizes 1.8, 4.6, and 5.7 kb. The pattern corresponded to the *Eco*RV digestions in which hybridizing fragments of 4.6, 5.1, and 5.7 kb indicated the presence of one 471-bp *Eco*RV fragment.

p56-6.7 was shown to contain two 471-bp *Hin*dIII fragments, since the SK primer hybridized to fragments of 1.8, 4.6, 5.1, 5.6, and 6.7 kb (Fig. 8C). The *Eco*RV hybridizations revealed bands of 4.6, 5.1, and 6.7 kb, indicating the presence of three repeats and no *Eco*RV site in R2, the same pattern as seen in *M. hominis* PG21.

In the hybridization of partial digested p81-7.7 (Fig. 8D), *Hin*dIII fragments of 1.8, 4.6, 5.1, 5.6, 6.1, 6.5, and 7.7 kb and *Eco*RV fragments of 4.6, 5.1, 5.6, 6.1, 6.5, 7.1, and 7.7 kb were observed; hence, p81-7.7 contained four *Hin*dIII and five *Eco*RV 471-bp repeats. *Eco*RV sites were present in all repeats.

The results were confirmed by reprobing the filters with the KS oligonucleotide, which hybridizes with the opposite end (results not shown). The region upstream the repeats was unaltered in all three clones.

On the basis of partial and complete digestions with *Hin*dIII, *Eco*RV, *Eco*RI, *Sau*3AI, *Hae*III, *Xba*I, and *Bgl*II, restriction maps for pBMhB616A and clones p74-5.7, p56-6.7, and p81-7.7 were constructed (Fig. 9).

Subcloning and sequence analysis. To compare the *lmp1-lmp2* homolog regions of Mh74, Mh56, and Mh81, the sequences of the cloned *Bgl*II fragments p74-5.7, p56-6.7, and p81-7.7 were determined and compared with the previously determined (16) sequence of pBMhB616A. The presence of nearly identical repeats required a special sequencing strategy (Fig. 7).

Subcloning of partial *Hin*dIII digests from p56-6.7 and p81-7.7 was performed. p56-6.7 and p81-7.7 were digested with *Hae*III and subsequently partially digested with *Hin*dIII to obtain clones containing various numbers of repeats. The appropriate fragments were purified from low-melting-temperature agarose gels and ligated to pBluescript SK+ opened with *Hin*dIII and *Hae*III. The sizes of inserts were determined by gel electrophoresis of the clones digested with *Sac*I and *Sma*I, which cut out the inserts from the vectors. The subclones of partial digests were named p56P-4.2, p56P-3.2, p56P-2.3 (Fig. 7B), p81P-6.1, p81P-5.2, p81P-4.1, p81P-3.1, and p81P-2.5 (Fig. 7C).

*Sau*3AI and *Hae*III fragments from p74-5.7 were subcloned and named p74S-4 and p74Hae-4, respectively. *Hin*dIII, *Eco*RI, and *Eco*RV subclones constructed by digesting p74-5.7 with the respective enzyme followed by religation were named p74Hoc, p74EIoc, and p74EVoc, respectively (Fig. 7A). Similar subclones constructed from p56-6.7 and p81-7.7 were named p56Hoc, p56EIoc, p56EVoc, p81Hoc, p81EIoc, and p81EVoc. From each of the three strains, a 1-kb *Hin*dIII fragment was subcloned, generating p74H1kb, p56H1kb, and p81H1kb. From the last *Hin*dIII restriction site in p74-5.7 and p56-6.7 to the next downstream *Hin*dIII site, 1.8-kb *Hin*dIII fragments were subcloned and named p74H-1.8 and p56H-1.8, respectively (Fig. 7).

According to the restriction maps (Fig. 9), the strains differed only in the number of repeats; thus, identical synthetic primers could be used for sequencing the nonrepeated regions. Since p74-5.7 contained 1.5 repeat, it was possible to sequence the *lmp1-lmp2* region by use of subclones p74S-4, p74Hae-4, p74Hoc, p74EIoc, p74EVoc, and p74H1kb in addition to synthetic primers (Fig. 7A). p56-6.7 contained 3.5 repeats. The sequencing was performed by use of synthetic primers, subclones p56H1kb, p56EVoc, p56EIoc, p56Hoc, and p56h-1.8,

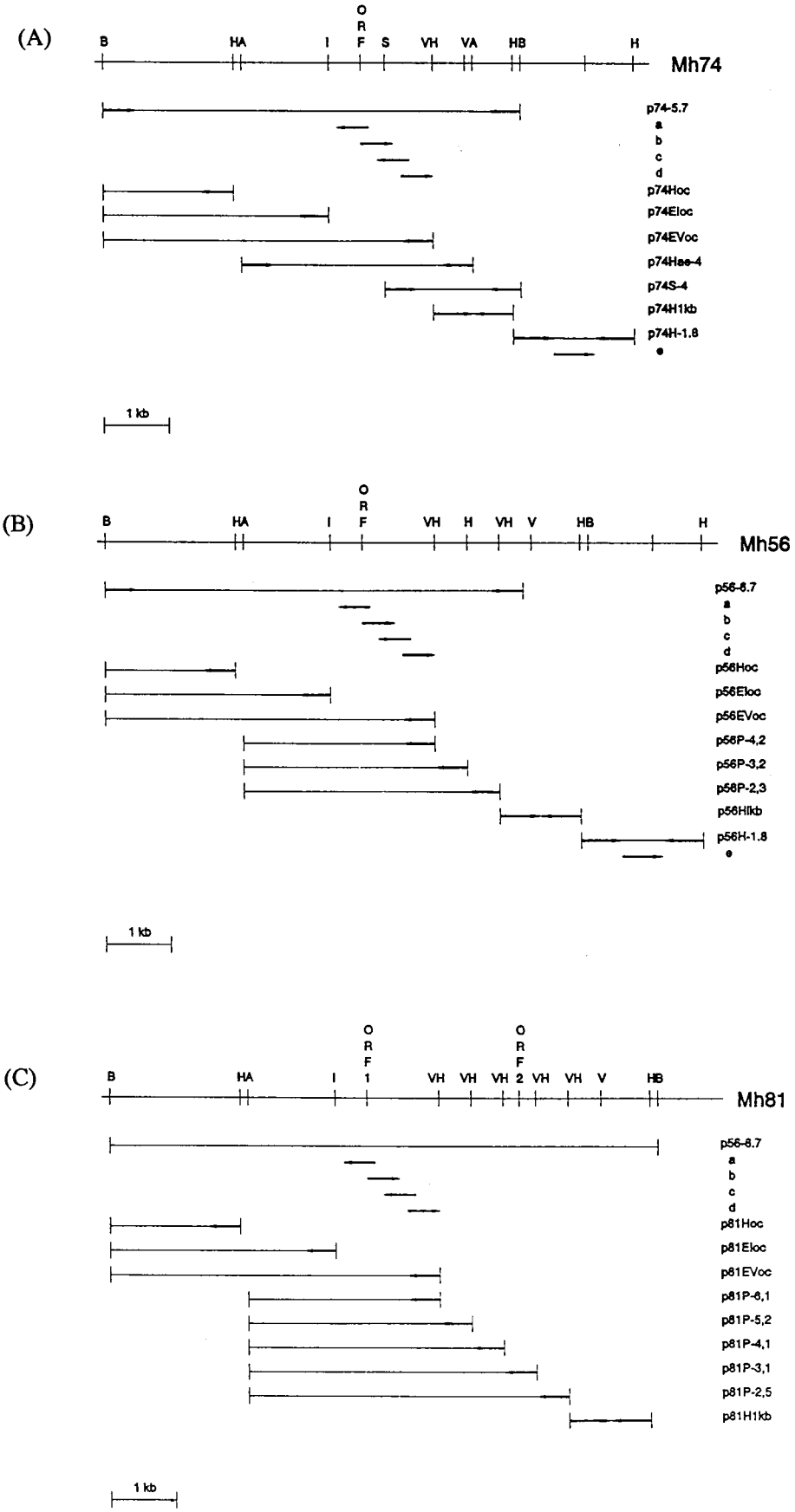

FIG. 7. Cloning and sequencing strategies for Mh74 (A), Mh56 (B), and Mh81 (C). Restriction cleavage sites: A, *Hae*III; B, *Bgl*II; H, *Hin*dIII; I, *Eco*RI; S, *Sau*3AI; V, *Eco*RV. Only restriction sites used for cloning are shown. ORF indicates the start of an open reading. Small letters at the right indicate synthetic primers: a, 3078; b, 3076; c, 3077; d, 3075; and e, 3524. Subclone designations are shown at the right. Arrows indicate the sequenced regions.

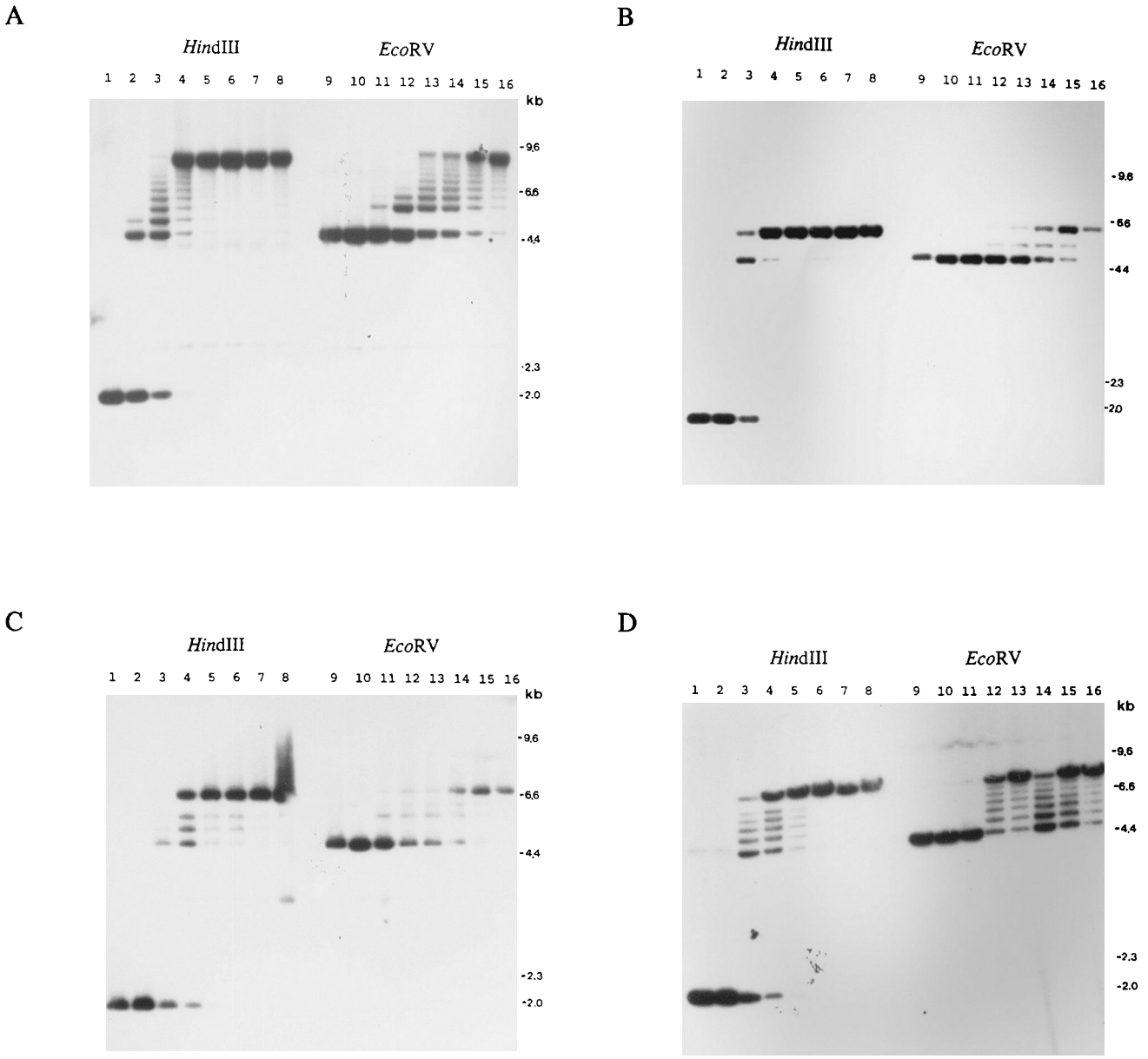

FIG. 8. Southern blots of partially digested pBMhB616A (A), p74-5.7 (B), p56-6.7 (C), and p81-7.7 (D). The inserts were cut out and digested with decreasing amount of *Hin*dIII (lanes 1 to 8) and *Eco*RV (lanes 9 to 16). The partially digested DNA was hybridized with an end-labelled SK oligonucleotide probe, complementary to the 5′ end of the insert.

and the subclones of partial digests (Fig. 7B). p81-7.7 contained 5.5 repeats. The sequencing was performed by use of synthetic primers, subclones p81H1kb, p81EVoc, p81EIoc, and p81Hoc, and the five subclones of partial digests, p81P-6.1, p81P-5.2, p81P-4.1, p81P-3.1, and p81P-2.4 (Fig. 7C).

By sequencing, six of the nine repeats in pBMhB616A were found to be identical. This sequence was determined on the basis of the consensus repeat (Fig. 10). Four variant repeats were found. In variant A, a A→G base substitution had occurred at nucleotide 451, resulting in the generation of a *Sau*3AI site. Variant B contained both this A→G base substitution and a C→A base substitution at nucleotide 11, resulting in the generation of a stop codon. Variant C was characterized by the base substitution A→G at nucleotide 451 (*Sau*3AI site) and a C→T base substitution at nucleotide 249, resulting in loss of the *Eco*RV site. Variant D, which was present only in MhPG21, had a C→G base substitution at nucleotide 32, resulting in a serine-to-tryptophan substitution. Variant E had, compared with the consensus repeat, a G→A base substitution at nucleotide 439. This variant was seen only in the mutants. The base substitutions in the different repeat variants were used to localize the deletions. Since the variant E repeat was present only in the mutants, the sequence alterations could not be explained exclusively by deletions. The assumption that a single-base substitution had occurred in the last complete repeat either in the colony picked for sequencing of pBMh B616A or in each of the three mutants made suggestions for deletion models possible. In the following discussion, we assume that variant E corresponds to R9 of MhPG21.

MhPG21 contained 9.5 repeats. R1 was of variant type A

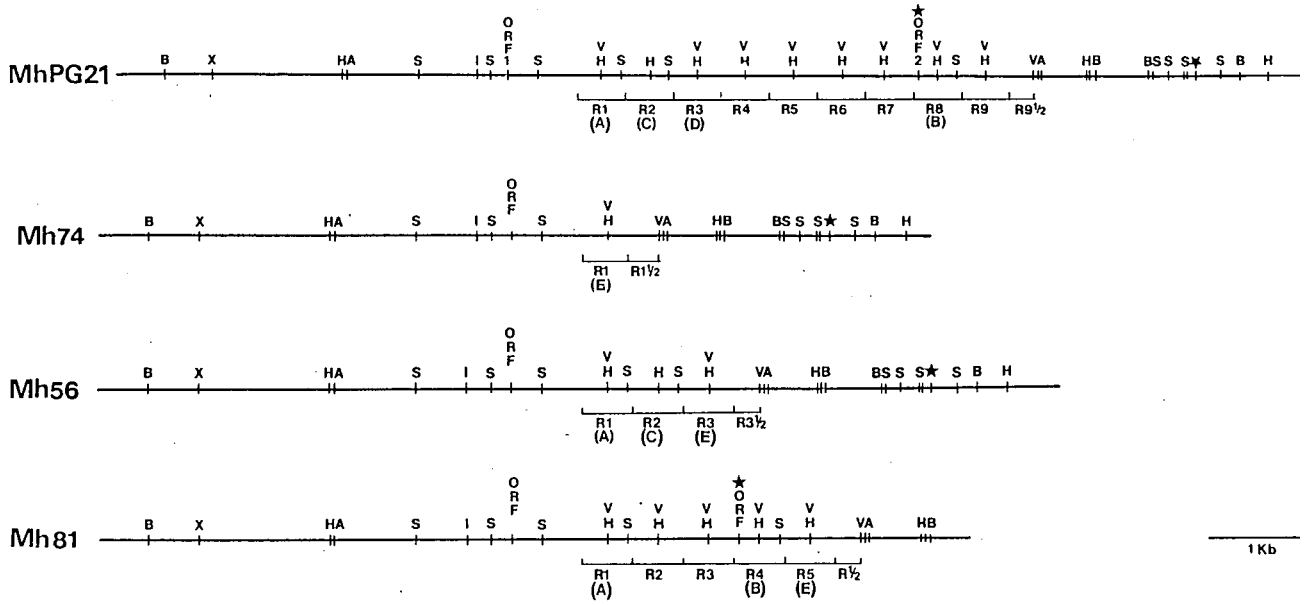

FIG. 9. Restriction maps of the *lmp1-lmp2* gene areas of MhPG21, Mh74, Mh56, and Mh81. The maps are based on complete and partial restriction enzyme cleavages by *Hae*III (A), *Bgl*II (B), *Hin*dIII (H), *Eco*RI (I), *Sau*3AI (S), *Eco*RV (V), and *Xba*I (X). ORF1 and ORF2 indicate the starts of *lmp1* and *lmp2*, respectively. ORF indicates the corresponding open reading frames in the mutants. Stars indicates stop codons. Letters in parentheses refer to the four variant repeats deviating from the consensus repeat.

(*Sau*3AI site). R2 was of type C (*Sau*3AI site and lack of *Eco*RV site), R3 to R7 and R9 were shown to be of the consensus type, and R8 was of type B (*Sau*3AI site and stop codon). The last half repeat was identical to the 5′ end of the consensus repeat.

p74-5.7 contained 1.5 repeat. The complete repeat was of type E. The half repeat was of the consensus type. On the basis of this arrangement, it seems likely that R2 to R9 were deleted compared with MhPG21.

p56-6.7 contained 3.5 repeats. R1 was of variant type A, R2 was of type C, and R3 was of type E. The half repeat was identical to the consensus sequence. These findings suggested a deletion of R3 to R8 compared with the MhPG21 gene.

p81-7.7 contained 5.5 repeats. R1 was of variant type A, while R2 and R3 were of the consensus type. R4 was of variant type B, while R5 was of variant type E. The half repeat was identical to the half consensus repeat, as seen for the other strains. Compared with the MhPG21 gene, a deletion of R2 to R5 could account for this arrangement.

The sequences were analyzed by the Genetics Computer Group program, and open reading frames were determined. Since in mycoplasmas UGA is translated to tryptophan, the universal stop codon UGA was translated as tryptophan (32). In all subclones, the open reading frames started in the unaltered nonrepetitious region. pBMhB616A and p81-7.7 contained a repeat with the UAA stop codon directly followed by a methionine, creating new open reading frames. p74-5.7 and p56-6.7 had no repeat containing a stop codon; hence, the open reading frames were continued to the next stop codon downstream of the repeated region.

Putative promoters, Shine-Dalgarno sequences, and termination structures were identical to those in the pBMhB616A sequence. No Shine-Dalgarno sequence was found upstream *lmp2*; hence, Lmp2 seemed not to be expressed. Identical putative very hydrophobic leader sequences were found at amino acids 1 to 26 in all strains. From the deduced amino acid sequence, the sizes and isoelectric points of the putative proteins were calculated and estimated to be 145 kDa and 10.02, respectively, for Lmp1 of *M. hominis* PG21, 116 kDa and 10.03 for Lmp1 in Mh74, 151 kDa and 10.03 for Lmp1 in Mh56, and 77 kDa and 9.93 for Lmp1 of Mh81. If Lmp2 were expressed, it would have a size of 110 kDa and a pI of 10.08. These results are in agreement with the results obtained by immunoblotting (Fig. 3B), which revealed an additional band of 105 kDa in Mh74, a strong band of 130 kDa in Mh56, and a double band of 70 to 72 kDa in Mh81.

RNA analysis. Northern blotting analysis was carried out with three different probes: pBMhAS-16 (containing the nonrepetitious part of *lmp1*), pBMhS-38 (containing the repeat) (16), and 0.9lmp3 (specific for the *lmp3-lmp4* region). The hybridization patterns were compared with the results obtained for *M. hominis* PG21 (16). In MhPG21, probes pBMhAS-16 and pBMhS-38 both hybridized to fragments of 7.2 kb. For Mh74, Mh56, and Mh81, pBMhAS-16 and pBMhS-38 hybridized to fragments of 3.0, 3.8, and 5.0 kb, respectively (Fig. 11). 0.9lmp3 did not hybridize to RNA (data not shown). For all four strains, transcripts with sizes predicted from the DNA sequence thus were found only from the *lmp1-lmp2* region. In all mutants, a 135-kDa protein varying in intensity was seen in immunoblottings with MAb 552. From sequencing data of the *lmp3-lmp4* gene area, an open reading frame encoding a putative polypeptide of 142 kDa has been determined (unpublished data). Since the open reading frame contained several repeats, it could not be excluded that the 135-kDa band originated from translation of the region even though no mRNA could be detected by Northern blotting.

DISCUSSION

In *M. hominis* PG21, two genomic regions have the capacity to encode polypeptides that react with MAb 552, since the MAb 552-defined epitope is localized within the regions en-

```
                A(VarB)                 G(VarD)
  1  AATACTGCTTCAATGCAATCAGCTAAATCTTCATTAGATGCAAAAGTTGCTGAAATTACT   60

 61  AAAAAATTAGAAACATTCAATAAAGATAAAGAAGCTAAATTCAATGAATTAAAACAAACA  120

121  AGAAATCAAATTCAAGAATTTATAAATACAAATAAAAACAATCCAAATTATTCAGAATTA  180

181  ATTTCACAATTAACTTCAAAAAGAGATTCTAAAAATTCAGTAACTGATTCTTCAAACAAA  240

           T(VarC)
241  TCAGATATCGAATCAGCAAACACCGAATTAAAACAAGCTTTAGCAAAAGCAAATGCTGAC  300

301  AAAGTTCAAGCTGATAATTTAGCAAAATCAATTAAAGAACAATTAAACAACTCAGTTTCT  360

361  AATGCAAATACATTATCAGCAAAATTAACTGACAAAGATAATACAATTCAACAAGCAAAA  420

                       A(VarE)     G(VarA, VarB, VarC)
421  ACTGAATTAGAAAAAGAAGTTCAAAAAGCAAATCAAGCAATTAAATCTAAT  471
```

FIG. 10. Nucleotide sequence of the 471-bp consensus repeat. The base substitutions corresponding to the five repeat variants A to E (VarA to VarE) are indicated above the sequence.

coded by the 471-bp repeat. Both regions have been cloned and sequenced (16). In each region, a unique region is followed by multiple repeats of very high homology, and a base substitution within a repeat gives rise to a stop codon. This stop codon is followed by a methionine, after which a second open reading frame continues in frame with the first reading frame. Despite the potential capacity for producing proteins, only one open reading frame has been shown to be translated in *M. hominis* PG21, and only one of the genomic regions, the *lmp1-lmp2* region, was shown to be transcribed. Whereas the 471-bp repeats showed greater than 99% homology, the regions upstream and downstream of the repeats were found to be unique. The repeats may thus serve as hot spots for gene rearrangements.

In this study, we have analyzed three antigenic variants of the type strain *M. hominis* PG21. Restriction fragment length polymorphism accompanied by antigenic variation was introduced by cultivating *M. hominis* PG21 in the presence of MAb 552. All three mutants were characterized by deletions located in the repeated sequence *lmp1-lmp2*. No other major alterations could be detected in the genome. The sequence upstream the repeats was preserved and thus identical in the four strains. Northern blotting showed that the *lmp1-lmp2* sequence was transcribed in all strains. Thus, no transcriptional shift between spatially separated genes seemed to be involved, as proposed for the *Neisseria gonorrhoeae* pilin gene system (21) and *Borrelia hermsii* Vmp system (3), in which silent genes distributed over the genomes can be transferred to expression sites located elsewhere in the genomes.

Aggregation studies revealed spontaneous aggregation of the mutant strains, in contrast to MhPG21, which did not spontaneously form aggregates. When MAb 552 was added, all of the strains showed large aggregates. MhPG21, which contained the highest number of repeats in Lmp1 and thus of epitopes, produced the largest aggregates.

The isoelectric points of Lmp1 of MhPG21 and the mutants were nearly identical. The net charge at the cellular surface at a given pH could, however, because of the different numbers of repeats, be different and hence be responsible for the differences in solubility and aggregation patterns observed for MhPG21 and the mutants. Repulsion between highly charged surfaces may prevent aggregation. This possibility is consistent with the fact that MhPG21, harboring seven repeats in Lmp1 at the surface, did not aggregate. Mh74, containing 1.5 polypeptide repeats in Lmp1, and Mh56, containing 3.5 repeats in Lmp1, exhibited some natural aggregation. The natural aggregation was most pronounced for Mh81, which contained three repeats on the Lmp1 surface. When MAb 552 was added, MhPG21 exhibited very large aggregates, while the mutants aggregated in smaller but still considerably clumps. These findings are consistent with the number of polypeptide repeats containing the epitope for MAb 552.

Agglutination may be an important factor in colonization. Nonagglutinating microorganisms like MhPG21 might easily be distributed, whereas natural aggregation might provide the microorganisms with better chances to avoid an antibody response since some of the epitopes may be hidden within aggregates of mycoplasmas. The difference in agglutination patterns seen between MhPG21 and the mutants is confirmed by the growth inhibition assays in which each CFU due to agglutination contains multiple individual organisms. PG21, which exhibited the largest aggregates, gave rise to the lowest number of CFU. It was previously suggested that agglutination of mycoplasmas may result in decreased growth and metabolic activity (12). We have previously demonstrated for *M. hominis* PG21 inhibition of growth and metabolic activity in addition to reduction of cellular DNA caused by MAb 552 (16) and show in this study a correlation of size of agglutinates and growth inhibition.

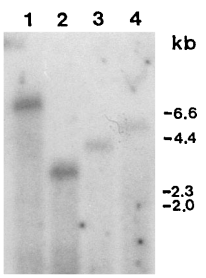

FIG. 11. Northern blot of mRNA extracted from log-phase cultures of MhPG21 (lane 1), Mh74 (lane 2), Mh56 (lane 3), and Mh81 (lane 4). Hybridization was performed with the *lmp1*-specific probe, pBMhAS-16. A molecular weight standard was provided by *Hin*dIII-digested bacteriophage lambda DNA and hybridization with the rRNA probe pmyc147 (6).

Spontaneous antigenic variation has been described for several bacteria, including *M. hominis*. From a patient with chronic arthritis in an artificial knee, Olson et al. (22, 23) consecutively isolated *M. hominis* from the synovial fluid of the knee and prepared three MAbs against the primary isolate, *M. hominis* 1620. These MAbs were reacted with 14 serial isolates obtained over a period of 6 years from the same patient. Immunoblotting with all three MAbs revealed marked differences in the protein patterns among the 14 strains. However, the restriction and hybridization patterns seemed to be nearly identical when probed with fragments of the conserved rRNA and elongation factor Tu genes from *E. coli*. This latter observation indicated antigenic variation rather than reinfection with different strains, since among other strains, hybridization studies with these probes frequently resulted in different restriction patterns (6, 17).

In vitro selection of antigenic variants with antibodies has been described for several viruses, including coxsackievirus B (29) and hepatitis B virus (4), and for parasites (31). For bacteria, only a few reports describe this approach. Recently, in vitro selection of *Borrelia burgdorferi* antigenic variants has been studied. By use of a growth inhibition assay, it was observed that a few persisting, still motile bacteria were present in addition to the major proportion of aggregates of nonmotile spirochetes in the cultures when MAbs were added to the medium (25). Sadziene et al. (25) consistingly passaged cultures in medium containing either polyclonal antibodies or MAbs raised against OspA or OspB. The proportion of motile cells increased dramatically from $<1\%$ in the first cultures to $>90\%$ in cultures passaged only two times, indicating that the motile spirochetes were antigenic variants not bound by the antibodies. After a couple of passages, the growth inhibition was fully reversed. These resistant cultures were subjected to SDS-PAGE and accordingly divided into four groups of mutants. All four classes of mutants arose from cultivation in antibody-containing medium, whereas controls with no or irrelevant antibodies passaged in parallel showed no alterations after 6 to 20 serial passages. In classes I and III, the described phenotypes were associated with documented genetic alterations. Whether this was the case for classes II and IV remains to be determined.

Cinco (10) has described the in vitro selection of antigenic variants of *B. burgdorferi* by cultivation in the presence of increasing amounts of homologous immune serum. The original strain, BITS, was cultured in the presence of different amounts of immune serum. The culture with the highest concentration of immune serum in which a few bacteria were motile and agglutinated was passaged to medium containing the same amount of immune serum. Subsequently cultures were passaged to media containing twice the amount of immune serum. It was shown that the *B. burgdorferi* became less and less sensitive to the inhibitory effect of the immune serum during a few passages. Further analysis by immunoblotting revealed the lack of an 8- to 11-kDa band (the lipooligosaccharide) in the last passaged culture. However, no correlation was shown to genomic alterations.

In our study, the starting culture originated from thrice filter-cloned single colonies of *M. hominis* PG21. In the starting culture and in both the culture that was propagated without a MAb and the culture that was propagated with MAb 30.3.1, no genomic alterations were observed. From the cultures propagated with MAb 552, mutants showing deletions in the *lmp1-lmp2* genomic region were detected after 22 days of propagation. It is therefore considered most likely that the mutations were generated during the cultivation with MAb 552. Because of a lower number of epitope-bearing repeats on the surface of the Lmp1, such cells would have less tendency to be agglutinated by MAb 552 and thus have a preference in growth rate. The marked higher survival fraction for the mutants compared with MhPG21 detected in the viability assay and the presence of the mixed culture after only 22 days of propagation with MAb 552 compared with the almost pure mutant cultures seen after 44 and 86 days strongly indicate the preference in growth rate of the mutant cells when cultivated in the presence of MAb 552.

The introduction of restriction fragment length polymorphism accompanied by antigenic variation was observed only in cultures in which *M. hominis* PG21 was cultivated in the presence of MAb 552. This MAb is directed against Lmp1 encoded by the very complexly structured *lmp1* gene containing repeats. The nonrepetitious gene encoding the polypeptide harboring the epitope recognized by MAb 30.3.1 was not associated with deletions, indicating the role of repeats as tools for deletion recombination. The role of the MAb as a selection agent may contribute to the understanding of antigenic variation in terms of microbe-to-host adaptability.

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