NOTE**DNA sequencing reveals limited heterogeneity in the 16S rRNA gene from the *rrnB* operon among five *Mycoplasma hominis* isolates**

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8000 Aarhus C, Denmark**To investigate the intraspecies heterogeneity within the 16S rRNA gene of *Mycoplasma hominis*, five isolates with diverse antigenic profiles, variable/identical P120 hypervariable domains, and different 16S rRNA gene RFLP patterns were analysed. The 16S rRNA gene from the *rrnB* operon was amplified by PCR and the PCR products were sequenced. Three isolates had identical 16S rRNA sequences and two isolates had sequences that differed from the others by only one nucleotide.****Keywords:** *Mycoplasma hominis*, 16S rRNA, variation, RFLP analysis, intraspecies heterogeneity

Mycoplasmas are among the smallest self-replicating prokaryotes known. They differ from other prokaryotes in that they lack a cell wall, have low genomic DNA G+C content (Razin & Freundt, 1984), and have a small genome that varies in size from 580 kbp in *Mycoplasma genitalium* (Su & Baseman, 1990) to 1300 kbp in *Mycoplasma iowae* (Grau *et al.*, 1991). Furthermore, mycoplasmas have a special use for the codon UGA, which is used as a stop codon in other bacteria but encodes tryptophan in mycoplasmas. Because of their small coding capacity, mycoplasmas have a low copy-number of rRNA genes, with one or two copies of the operon (Amikam *et al.*, 1984). The order of genes in each operon is from 5' to 3': 16S, 23S and 5S.

Mycoplasma hominis is usually found as part of the normal flora of the human urogenital tract. It has been associated with neonatal infection (Quinn *et al.*, 1985) and is a risk factor for pre-term delivery (Hillier *et al.*, 1995). In spite of the association of *M. hominis* with the above-mentioned diseases, it has not been possible to reveal specific pathogenicity factors. This is partly due to the fact that *M. hominis* isolates show considerable heterogeneity, predominantly in their membrane proteins (Christiansen *et al.*, 1990; Olson *et al.*, 1991). Moreover, at present three variable surface

antigens have been described for *M. hominis*: Lmp (Jensen *et al.*, 1995; Ladefoged *et al.*, 1995, 1996), P120 (Nyvold *et al.*, 1997) and Vaa (Zhang & Wise, 1997). These proteins are probably involved in defence against the host immune system. In a study conducted by Nyvold *et al.* (1997), the surface antigen P120 was found to be composed of one hypervariable domain, two semivariable domains and four constant domains. The hypervariable domain was sequenced for several strains; four strains showed pairwise identity (strains P2/SC4 and 7488/DC63).

Considerable intraspecies heterogeneity has been described within the 16S rRNA genes (Christiansen & Andersen, 1988; Blanchard *et al.*, 1993). Christiansen & Andersen (1988) found pronounced heterogeneity among *M. hominis* strains when 26 strains were examined for heterogeneity by RFLP analysis using parts of rRNA genes as probes; pairwise analysis of strains P2/SC4 and 7488/DC63 also revealed differences. Blanchard *et al.* (1993) found significant differences in the 16S rRNA genes of two *M. hominis* strains, PG21^T and 183. Therefore, the aim of this study was to determine the intraspecies variability of the 16S rRNA genes of *M. hominis* by automated DNA sequencing and to relate this heterogeneity to the variability in the genes encoding membrane proteins (surface antigens). *M. hominis* strains P2, SC4, DC63, 7488, 183 and PG21^T were selected for the study.

Two *Escherichia coli* XL-1 Blue clones were obtained from previous investigations. The clone pBMhE6.6 (Özkökmen *et al.*, 1994) has an insert with DNA upstream of the 16S rRNA gene and part of the 16S

The EMBL accession numbers for the 16S rRNA gene sequences from the *rrnB* operon of *M. hominis* strains reported in this paper are as follows: strain PG21^T, AJ002265; strain DC63, AJ002266; strain SC4, AJ002267; strain P2, AJ002268; strain 7488, AJ002269; and strain 183, AJ002270.

Table 1. *M. hominis* isolates used in this study

<i>M. hominis</i> strain	Source	Year/place of isolation	Reference
PG21 ^T	Lower genitalia	1953/England	Nicol & Edward (1953)
DC63	Cavum oris	1963/England	Taylor-Robinson <i>et al.</i> (1963)
7488	Cervix	1988/Aarhus, Denmark	This laboratory
SC4	Male urethra	1969/England	Hollingdale & Lemcke (1969)
P2	Upper urinary tract	1978/Aarhus, Denmark	Thomsen (1978)
183	Vagina	1974/USA	Lin & Kass (1974)

rRNA gene. The clone pBMhE1.2 has an insert with part of the 16S rDNA gene and DNA downstream of the 16S rRNA gene. Plasmid DNA from the two *E. coli* clones was prepared by the alkaline lysis method as described by Sambrook *et al.* (1989), and the sequence was determined with primers 5' GCGTTCATCCTGAGCC 3' and 5' GTAATACGACTCACTATAGGGCGAATTGGG 3'. The sequences were obtained with the Thermo Sequenase Dye Terminator Cycle Sequencing kit (Amersham) using an ABI Prism 377 fluorescent sequencing instrument. *M. hominis* strains PG21^T, P2, SC4, 7488, DC63 and 183 (Table 1) were cultivated in BEa medium (Christiansen & Andersen, 1988) and genomic DNA was isolated as described by McClenaghan *et al.* (1984).

PCR was carried out both directly on proteinase-K-treated culture and on genomic DNA. The following primers were used for PCR: 5' CCGAGAGATAAA-TCTCTCAAAGCTGGATAC 3' and 5' GGAATA-TTCAAAGCTGAAGTTGGCG 3'. These primers were designed to amplify a fragment of approximately 2 kbp containing the entire 16S rRNA gene. PCR was performed in a total volume of 100 µl containing 5 U AmpliTaq DNA polymerase, 100 pmol each primer, 0.2 mM each of dATP, dTTP, dGTP and dCTP, 10 µl 10 × PCR buffer II, 10 µl 25 mM MgCl₂ solution, and 2.5 µl purified genomic DNA or 20 µl proteinase-K-treated culture (made up to 100 µl with water). The enzyme and buffers were purchased from Perkin Elmer. The reaction mixture was thermocycled in a GeneAmp PCR system 9600 thermal cycler from Perkin Elmer under the following cycling conditions: denaturation of template, 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The PCR products were purified with PCR Preps DNA Purification Resin and Wizard Minicolumns (Promega). The purification was carried out in accordance with the instructions of the manufacturer. The forward PCR primer is specific for the *rrnB* operon (Christiansen & Andersen, 1988) as *rrnA* and *rrnB* have different upstream regions. The reverse PCR primer is not specific as it is situated inside the operon and the two operons are very similar. Therefore the 16S rRNA genes amplified from genomic DNA are from *rrnB*.

The PCR products were sequenced with the primers described above in addition to the following primers:

5' GAAACTGGATACGAATACCATTAACGTC 3'; 5' GCCGGGTCGAGAGACTGAAC 3'; 5' GCG-TTCGTAGGCTGTTTGTAAAGTC 3'; 5' CGCAT-TAAATGATCCGCCTGAG 3'; 5' GCCTCTTACG-AGTGGGGCC 3'; 5' CTGCGATTACTAGCGAT-TCCGAC 3'; 5' CCATCTGTCACTCCGATAACC-TCC 3'; 5' GTAAGCTGCCTTCGCCTTTGG 3'; 5' CGAAGACCTTCATCCACG 3'; 5' GCGTTCATC-CTGAGCCAGG 3'; and 5' GCGTCCATCAAGC-TTTCGC 3'. These primers (DNA Technology) were designed to sequence the entire 16S rRNA gene as there were approximately 300 bp separating each primer. To eliminate errors introduced by PCR, different PCR amplification products were sequenced.

DNA sequences were assembled and analysed with software from the Genetic Computer Group sequence analysis software package. The program FASTA was used to search for similar sequences in the EMBL database. In addition, the GenBank database at The National Center for Biotechnology Information was searched via its text-searching system.

The PG21^T 16S rDNA sequence is given in Fig. 1; the Pribnow box and the approximate positions of the mature rRNA molecule are shown. Table 2 shows that PG21^T, P2 and SC4 have identical sequences. The 16S rRNA sequence of strain DC63 differed from these three sequences by one nucleotide; position 1300 is thymine in DC63 and cytosine in the rest of the sequences. The sequence of strain 7488 also differed from the three identical sequences by one nucleotide; position 258 is thymine in 7488 and cytosine in the rest of the sequences including that of strain DC63. Furthermore, it is notable that, in strains PG21^T and SC4, the region upstream of the 16S rRNA gene differed from the rest of the sequences at one and four positions, respectively (position -107 in PG21^T and positions -233, -140, -123 and -86 in SC4). It is also notable that all of the strains have an *EcoRI* site at positions 763–768. Finding this site in the sequence of strain DC63 and the lack of variation was not expected according to Christiansen & Andersen (1988), even though lack of recognition at a specific restriction endonuclease site only reflects variation within a six nucleotide region. From the results of this previous study, the 16S rRNA sequence of strain DC63 was expected to lack the *EcoRI* site. This result was

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-244 ATTGAACAAT TTGGTAGAAA AGTTCCAAC GAATTTGATT ATGCAGATCT AGAAATAGTA GGTTAACAAT AAAATAATGA ATAGATATTA GAATCAGGAA
-144 AAAATCCTGA TTTTATTATT ATTTAAAAA TAAACAATT ATAATATATA CATATTTACT TTTCTGTTT AAAACAGCAA TTTTATATCG CTAAAAAATT
      -35          -10      +1
-44  TAATTTTTT TGAAAAAATA AAAAAAGTG TATATAATAT TTAAGGTTG TTTTGAAACA ACGAGATAGT TCTTTGAAAA CTGGATACGA ATACCATTAA

56  CGTCAATTTA TTTTATTATT TATAATCAAC CAATTCAAAT TTTT [TTATAA GAGTTTGATC CTGGCTCAGG ATGAACGCTG GCTGTGTGCC TAATACATGC
156 ATGTCGAGCG AGGTTAGCAA TAACCTAGCG GCGAATGGGT GAGTAACACG TGCTTAATCT ACCTTTTAGA TTGGAATACC CATTGGAAAC AATGGCTAAT
256 GCCGGATACG CATGGAACCG CATGGTCCG TTGTGAAAGG CGCTGTAAGG CGCCACTAAA AGATGAGGGT GCGGAACATT AGTTAGTTGG TGAGGTAATG
356 GCCCACCAAG ACTATGATGT TTAGCCGGGT CGAGAGACTG AACGGCCACA TTGGGACTGA GATACGGCCC AAACCTCTAC GGGAGGCAGC AGTAGGGAAT
456 ATTCCACAAT GAGCGAAAGC TTGATGGAGC GACACAGCGT GCACGATGAA GGTCTTCGGA TTGTAAAGTG CTGTTATAAG GGAAGAACAT TTGCAATAGG
556 AAATGATTGC AGACTGACGG TACCTTGTC AAGAGCGATG GCTAACTATG TGCCAGCAGC CGCGGTAATA CATAGGTCGC AAGCGTTATC CGGAATTATT
656 GGGCGTAAAG CGTTCGTAGG CTGTTTGTTA AGTCTGGAGT TAAATCCCGG GGCTCAACCC CGGCTCGCTT TGGATACTAG CAACTAGAG TTAGATAGAG
      EcoRI
756 GTAAGCGGAA TTCCATGTGA AGCGGTGAAA TCGTAGATA TATGGAAGAA CACCAAAGGC GAAGGCAGCT TACTGGGTCT ATACTGACGC TGAGGGACGA
856 AAGCGTGGG AGCAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAG CGATGATCAT TAGTCGGTGG AGAATCACTG ACGCAGCTAA CGCATTAAAT
956 GATCCGCCTG AGTAGTATGC TCGCAAGAGT GAAACTTAAA GGAATTGACG GGGACCCGCA CAAGCGGTGG AGCATGTGGT TTAATTTGAA GATACACGGA
1056 AAACCTTACC CACTCTTGAC ATCCTTCGCA AAGCTATAGA GATATAGTGG AGGTTATCGG AGTGACAGAT GGTGCATGGT TGTGTCGAGC TCGTGTCTGC
1156 AGATGTTTGG TCAAGTCCTG CAACGAGCGC AACCCCTATC TTTAGTTACT AACATTAAGT TGAGGACTCT AGAGATACTG CCTGGGTAAC TGGAGGAAG
1256 GTGGGGATGA CGTCAAATCA TCATGCCTCT TACGAGTGGG GCCACACACG TGCTACAATG GTCGGTACAA AGAGAAGCAA TATGGCGACA TGGAGCAAAT
1356 CTCAAAAAGC CGATCTCAGT TCGGATTGGA GTCTGCAATT CGACTCCATG AAGTCGGAAT CGCTAGTAAT CGCAGATCAG CTATGCTGCG GTGAATACGT
1456 TCTCGGTCT TGTACACACC GCCCGTCACA CCATGGGAGC TGGTAATACC CAAAGTCGGT TTGCTAACCT CGGAGGCGAC CGCCTAAGGT AGGACTGGTG
1556 ACTGGGTGTA AGTCGTAACA AGGTATCCCT ACGAGAACGT GGGGATGGAT CACCTCC ]

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Fig. 1. DNA sequence of the 16S rRNA gene of the *rrnB* operon from PG21^T. The Pribnow box and *EcoRI* site are shown. The approximate beginning and end of the mature rRNA molecule are marked with bold brackets.

Table 2. Nucleotide differences in the 16S rRNA sequence of *M. hominis* strains

Strain	Nucleotide at position:						
	-233	-140	-123	-107	-86	258	1300
PG21 ^T	T	T	T	A	C	C	C
SC4	C	A	C	G	T	C	C
P2	T	T	T	G	T	C	C
7488	T	T	T	G	T	T	C
DC63	T	T	T	G	T	C	T

examined as follows. A freeze-dried culture of the original DC63 strain was propagated to ensure that no mutations had accumulated. The cells were subjected to proteinase K treatment and PCR was performed directly on the proteinase K-treated cells. The PCR product was cleaved with *EcoRI* and the expected fragments were obtained (results not shown). Christiansen & Andersen (1988) conducted their re-

striction analysis by Southern blotting on DC63 genomic DNA, which probably was modified at the *EcoRI* site, thus preventing the restriction endonuclease from cleaving the sequence into the expected fragments.

In a study conducted by Weisburg *et al.* (1989), the 16S rRNA sequence from *M. hominis* PG21^T was obtained,

although it is not a full-length sequence and it contains a long stretch of undetermined nucleotides. In a later study conducted by Blanchard *et al.* (1993), the 16S rRNA sequence from *M. hominis* 183 was obtained (this sequence was not full-length either) and compared with the PG21^T sequence obtained by Weisburg *et al.* (1989). On the basis of this comparison, Blanchard *et al.* (1993) concluded that significant heterogeneity is found in *M. hominis* 16S rRNA genes, verifying the results obtained by Christiansen & Andersen (1988). These sequence data have now been updated as the part of the strain 183 16S rDNA sequence which aligns with the long stretch of N nucleotides in the Weisburg PG21^T sequence has also been determined (positions 602–1109). The new sequence stretch from strain 183 is identical to that of the other strains.

The *M. hominis* strains selected for this study were chosen to represent isolates that had been previously shown to have differences within their 16S rRNA genes (Christiansen & Andersen, 1988); isolates showing pairwise identity within the hypervariable region of the P120 gene were also included. No correlation between heterogeneities in the 16S rRNA gene and differences within the hypervariable domain of the P120 gene was seen, indicating that different genetic mechanisms are involved in the development of genetic heterogeneity in *M. hominis*.

In conclusion, these results show that the *M. hominis* 16S rRNA gene is not as variable as previously thought. Strains PG21^T, P2 and SC4 had identical 16S rRNA sequences and strains DC63 and 7488 differed from the other sequences by one nucleotide. These results are in agreement with the results obtained by Pettersson *et al.* (1996), who studied the heterogeneity of the 16S rRNA genes within the *Mycoplasma mycoides* cluster. In this previous study, the phylogeny of the six species in the cluster was investigated. The 16S rRNA genes from both operons were amplified by PCR and sequenced. From some of the species, several strains were included. When comparing differences between genes from homologous operons, the largest number of nucleotides differing between two strains from one species was four, and the lowest number was zero. When comparing these results with the results obtained in this study, where the largest number of differences is two and the lowest is zero, it is obvious that the degree of intraspecies heterogeneity in the 16S rRNA genes is of the same order of magnitude in *M. hominis* and in the members of the *M. mycoides* cluster. It seems that, although *M. hominis* is a heterogeneous species, only limited variation of the 16S rRNA genes is permitted, as seen in most other organisms. This is reasonable if it is considered that a certain structure of the 16S rRNA has to be maintained in order for it to take part in protein synthesis.

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References

- Amikam, D., Glaser, G. & Razin, S. (1984). Mycoplasmas (Mollicutes) have a low number of rRNA genes. *J Bacteriol* **158**, 376–378.
- Blanchard, A., Yáñez, A., Dybvig, K., Watson, H. L., Griffiths, G. & Cassell, G. H. (1993). Evaluation of intraspecies genetic variation within the 16S rRNA gene of *Mycoplasma hominis* and detection by polymerase chain reaction. *J Clin Microbiol* **31**, 1358–1361.
- Christiansen, G. & Andersen, H. (1988). Heterogeneity among *Mycoplasma hominis* strains as detected by probes containing parts of ribosomal ribonucleic acid genes. *Int J Syst Bacteriol* **38**, 108–115.
- Christiansen, G., Ladefoged, S., Hauge, S., Birkelund, S. & Andersen, H. (1990). Use of monoclonal antibodies for detection of gene and antigen variation in *Mycoplasma hominis*. *Zentbl Bakteriol Suppl* **20**, 535–545.
- Grau, O., Laigret, F., Carle, P., Tully, J. G., Rose, D. L. & Bove, J. M. (1991). Identification of a plant-derived mollicute as a strain of an avian pathogen, *Mycoplasma iowae*, and its implication for mollicute taxonomy. *Int J Syst Bacteriol* **41**, 473–478.
- Hillier, S. L., Nugent, R. P., Eschenbach, D. A. & 11 other authors (1995). Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The Vaginal Infections and Prematurity Study Group. *N Engl J Med* **333**, 1737–1742.
- Hollingdale, M. R. & Lemcke, R. M. (1969). The antigens of *M. hominis*. *J Hyg* **67**, 585–602.
- Jensen, L. T., Ladefoged, S., Birkelund, S. & Christiansen, G. (1995). Selection of *Mycoplasma hominis* PG21 deletion mutants by cultivation in presence of monoclonal antibody 552. *Infect Immun* **63**, 3336–3347.
- Ladefoged, S. A., Birkelund, S., Hauge, S., Brock, B., Jensen, L. T. & Christiansen, G. (1995). A 135-kilodalton surface antigen of *Mycoplasma hominis* PG21 contains multiple directly repeated sequences. *Infect Immun* **63**, 212–223.
- Ladefoged, S. A., Jensen, L. T., Brock, B., Birkelund, S. & Christiansen, G. (1996). Analysis of 0.5 kilobase-pair repeats in the *Mycoplasma hominis* Imp gene system and identification of gene products. *J Bacteriol* **178**, 2775–2784.
- Lin, J. S. & Kass, E. H. (1974). Serological reactions of *Mycoplasma hominis*: differences among mycoplasmacidal, metabolic inhibition and growth agglutination tests. *Infect Immun* **10**, 535–540.
- McClenaghan, M., Herring, A. J. & Aitken, I. D. (1984). Comparison of *Chlamydia psittaci* isolates by DNA restriction endonuclease analysis. *Infect Immun* **45**, 384–389.
- Nicol, C. S. & Edward, D. (1953). Role of organisms of the Pleuropneumoniae group in human genital infections. *Br J Vener Dis* **29**, 141–150.
- Nyvold, C., Birkelund, S. & Christiansen, G. (1997). The *Mycoplasma hominis* P120 membrane protein contains a 216 amino acid hypervariable domain that is recognized by the human humoral immune response. *Microbiology* **143**, 675–688.
- Olson, L. D., Shane, S. W., Karpas, A. A., Cunningham, T. M., Probst, P. S. & Barile, M. F. (1991). Monoclonal antibodies to surface antigens of a pathogenic *Mycoplasma hominis* strain. *Infect Immun* **59**, 1683–1689.
- Özkökmen, D., Birkelund, S. & Christiansen, G. (1994). Charac-

terization of a *Mycoplasma hominis* gene encoding lysyl-tRNA synthetase (LysRS). *FEMS Microbiol Lett* **116**, 277–282.

Pettersson, B., Leitner, T., Ronaghi, M., Bölske, G., Uhlén, M. & Johansson, K. E. (1996). Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. *J Bacteriol* **178**, 4131–4142.

Quinn, P. A., Butany, J. & Chipman, M. (1985). A prospective study of microbial infection in stillbirth and early neonatal death. *Am J Obstet Gynecol* **151**, 238–249.

Razin, S. & Freundt, E. A. (1984). The mycoplasmas. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 740–793. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Su, C. J. & Baseman, J. B. (1990). Genome size of *Mycoplasma genitalium*. *J Bacteriol* **172**, 4705–4707.

Taylor-Robinson, D., Somersom, N. L., Turner, H. C. & Chanock, R. M. (1963). Serological relationships among human mycoplasmas as shown by complement fixation and gel diffusion. *J Bacteriol* **85**, 1261–1273.

Thomsen, A. C. (1978). Mycoplasmas in human pyelonephritis: demonstration of antibodies in serum and urine. *J Clin Microbiol* **8**, 197–202.

Weisburg, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.

Zhang, Q. & Wise, K. S. (1997). Localized frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. *Mol Microbiol* **25**, 859–869.