Cloning the ribosomal RNA operons of *Mycoplasma flocculare* and comparison with those of *Mycoplasma hyopneumoniae*

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In contrast to other mycoplasma species the 16S/23S rRNA and 5S rRNA operons of *Mycoplasma flocculare* and *Mycoplasma hyopneumoniae* map at least 150 kb apart (20% of the genome). Both operons from *M. flocculare* have been cloned and sequenced. The 23S rRNA gene sequence showed 96.7% homology with the corresponding gene of *M. hyopneumoniae*, equalling that found earlier for 16S rRNA and confirming the close phylogenetic relationships of these organisms. A possible upstream promoter was identified. Sequence elements upstream and downstream from each structural gene could form a stem needed for maturation of the immature rRNA transcript to mature 16S and 23S rRNA. We also identified two possible stem-and-loop sequences 3' to the 23S rRNA gene. The 5S rRNA gene itself also showed high homology with the corresponding structural gene of *M. hyopneumoniae*, although the upstream and downstream sequences were highly heterologous.

Keywords: *Mycoplasma flocculare*, *Mycoplasma hyopneumoniae*, rRNA operons, 23S

Because the rRNA genes are among the most conserved genetic elements, comparisons of these sequences have become important phylogenetic tools (Olsen & Woese, 1993). In a recent report we used 16S rRNA sequence comparisons to analyse phylogenetic relationships between three porcine mycoplasmas: *Mycoplasma hyopneumoniae*, *M. flocculare* and *M. hyorhinis*. We demonstrated that these mycoplasmas were closely related, particularly *M. hyopneumoniae* and *M. flocculare* (Stemke *et al.*, 1992). Another common property of these two organisms was that, unlike other mycoplasma species, the 16S/23S rRNA region was separated from the 5S rRNA gene (Taschke *et al.*, 1986). The physical maps of the genomes of both organisms have been established and the 16S/23S and 5S rRNA genes located (Huang & Stemke, 1992). Here we report the sequence of the 16S/23S region as well as the 5S rRNA region of *M. flocculare* and compare these to their counterparts in *M. hyopneumoniae*.

M. flocculare strain Ms42 (ATCC 27716) was grown in modified Friis broth (Friis, 1975) and harvested as previously described (Stemke *et al.*, 1992). DNA was prepared from the washed cell pellet using the method of Gross-Bellard *et al.* (1973).

The M13mp19 vector containing the 5S rRNA gene of *M. flocculare* was selected by probing with a *M. hyopneumoniae* 5S-rRNA-specific oligonucleotide (5'-TGAATCCATT-CCGAAT-3'). The replicative form of the recombinant phage containing a 665 bp *Xba*I fragment was sequenced using the Pharmacia dideoxy sequencing kit following the double-strand sequencing protocol.

The 16S/23S rDNA sequences were obtained from an *Eco*RI partial digest fragment of *M. flocculare* DNA cloned into pBlueScript as previously described (Stemke *et al.*, 1992). A portion of the sequence of the 12 kb insert was determined as above. Primer extension was employed to extend the *M. flocculare* 16S rRNA sequence.

The sequence for the 23S rRNA of *M. hyopneumoniae* was obtained from GenBank as accession number X68421. The sequences for the *M. hyopneumoniae* 5S rRNA gene and 16S/23S rRNA intergenic region were taken directly from Taschke & Herrmann (1986). The sequences for the 16S/23S rRNA genes and the 5S rRNA gene of *M. flocculare* have been deposited as GenBank accession numbers L22210 and U01070, respectively.

The 665 bp sequence of the cloned *Xba*I fragment was determined and compared with the corresponding *M. hyopneumoniae* 5S rRNA sequence. A 106 bp sequence representing the 5S rRNA structural gene was found to have 95.3% homology with the *M. hyopneumoniae* gene. Fig. 1 shows this homology and the possible promoters

The GenBank accession numbers for the sequences reported in this paper are L22210 and U01070.

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MF 1  AAAATTTAATTTCTCTGTTTTCTTGTAAATTATAGCTAAAAAATCCGGTATTTTGGCATAAATATAGGAAAGCCAAATTTTGGGACAAATTTATGC
MF 101  TAAAAAAGTTGTTAAATAGTTGCAAAACTTTTTTTTTTTTTTTTACATTTTCATATATTTTATGCTATATAATTGTTTCACAGAGTAACAATAG
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 1  AAAAAAAAAAAAAAAAAAAAAA-----AAAAA-----AAAAAAAAAAAAAAAAATATATGCTATATAATTGTTTAAATTATAGAGTAACAATAG
MF 201  CAAAAGGATCACCTGATACCATTCGAACTCAGTAGTTAAGCCTTTTACGCCGAAATACTAGAAATAGGAATAGGAATGACTCTTTTTTTAGTT
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 79  CAAAAGGATCACCTGAATCCATTCCGAACTCAGTAGTTAAGCCTTTTACGCCGAAATACTAGAAATAGGAATAGGAATGACTCTTTTTTTT-----TT
MF 301  TTCTGAAATGTTTAAATAATATATCTTTTACCAATAGTTCTACTCTTTTAAAGTATCCTATTTATAGTCTCTTTTAAATAGAGTCCGATAA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 177  TTTT-----TTTTTTTATATATATAA
MF 401  ACAAACCCCGGAAAGAGGAGAGAGTATGAAATTTACTTAAAAATTTACAAGCCCTTCCTTTTTCGCCCTTTCGCCCTTCGATATATTTTCTC
MF 501  TAGTAATGAGATCTCTCAAAATGAGGAGCTGTTTATTAATAAAACAGCAGAAATTTGAATTAAGATCGTAAATTCATATAAATCGCCAGAGT
MF 601  TACCTCAGTTTCTATCTATGCTAATTAATTCCAATCTGTTCTAGAGAGCTGCGGCGAT

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Fig. 1. Alignment of the 5S rRNA genes of *M. flocculare* and *M. hyopneumoniae*. MF, *M. flocculare*; MH, *M. hyopneumoniae*. (:), Sequence identity. Underlined are possible promoters of each species.

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(a) MF 2524  GCGAGCGAAATGGAATA-GCCCAACCGTTTTTACCGTGTGTAGGACATTTTAACTGAGTTACAAATATATATATAGTAGAATAAGTTGGAAGCTTT
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 250  GCGAGCGAAATGGAAGAGCCCAACCGTTTTTACCGGTTATAGGACATTTTAAATGAGTTAGAAATATATATATAGTAGAATAAGTTGGAAGCTTT

(b) MF 3087  GGACTTGTGA-TAGCGGTGAATC-CATTCGAACCTGGAGATAGCTGTTCTCTTGGAAATAGCTTTAGGCTAGCGTGTAGTATATATTAATGAGGAG
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 800  G-ACTTGTGATAGCGGTGAATTCATTCGAACCTGGAGATAGCTGTTCTCTTGGAAATAGCTTTAGGCTAGCGTGTAGTATATATTAATGAGGAG

MF 3170  TAGAGCACTGAATATCCAAATGCGGACCTAGCTGTACTGACTATATACCAACTCCGAAATCCATTATTTTACCATGCAATCCGAAACGTGGGTGATA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 898  TAGAGCACTGAATATGGAATGCGGACCTAGCTGTACTGACTATTA-ATCAAACTCCGAAATCCATTATTTTACCATGCAATCCGAAACGTGGGTGATA

(c) MF 3468  ACCGGAATTTTTTTTAAATTTGCTTTAGGAGAGCTTTGGATTTCCG-TGAAGCTAGAGGGGACGTACGTAGTGAAGAAATTAAGATGAGAAATGC
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 1186  GCGGTAATATTTTA-----AGTTTACGTAGGAGAGCTTTTAAATTTCCGCTGAAGCTAGAGGTTGACC-AC-TAGTGAAGAAATTAAGATGAGAAATGC

(d) MF 3866  GATCTATGTTTCCAGAAAGCTCTAGTATTAATTTTTTAAACCTGTACCAAGAACGA-CACACGTCCCAAGATGAGTATTTCTAAGGTGAGCGA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
MH 1589  GACTCTCTGTTTCCAGAAAGCTCTAGGCTTAATTCATTA-ACAACCTGTACCAAGAACGACACACGTCCCAAGATGAGTATTTCTAAGGTGAGCGA

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Fig. 2. Alignment of selected regions of *M. flocculare* and *M. hyopneumoniae* 23S rRNA genes with relatively large sequence variation. MF, *M. flocculare*; MH, *M. hyopneumoniae*. (:), Sequence identity. Numbering for MH was taken from GenBank accession number X68421.

for the *M. flocculare* gene. Two upstream TATAA Pribnow box sequences were noted, one directly homologous to the *M. hyopneumoniae* promoter at the -10 position but preceded by another TATAA sequence at the -25 position. Except for the -10 region, the lack of

homology in both the upstream and downstream regions of these genes is very apparent. More sequence information for both species both upstream and downstream of that presently available and determination of other genetic markers above and below these sequences

Fig. 3. Sequence homology comparison of *M. flocculare* and *M. hyopneumoniae* 16S/23S rRNA intergenic regions. MF, *M. flocculare*; MH, *M. hyopneumoniae*. Numbering for MH was taken from Taschke & Herrmann (1986; Fig. 3(B) intergenic region). Underlined regions are possible rRNA maturation stem-forming sequences; the single nucleotide difference is compensated by a corresponding change in the region upstream of the 16S rRNA structural sequence.

will be required to verify these conclusions and judge their significance.

For the 16S/23S region, a total of 5459 nucleotides was determined, including the 16S rRNA sequence already reported as GenBank accession number X63377. Only two other mycoplasma 23S rRNA genes have been totally sequenced: those of *M. pneumoniae* and *M. hyopneumoniae* (Ludwig *et al.*, 1992). Using the Nalign program of PcGene, homology with *M. hyopneumoniae* was 96.7% for the 23S gene, similar to that found with the 16S rRNA sequence comparison (Stemke *et al.*, 1992). Both 5' and 3' ends of this sequence were even more highly conserved for these two species; only four nucleotides in the first 235 5' nucleotides and four in the last 561 nucleotides at the 3' end showed variation. Fig. 2 indicates areas of greater diversity found in interior regions.

The 16S/23S intergenic region of *M. flocculare* (403 bp) was 105 bp shorter than that of *M. hyopneumoniae* (Taschke & Herrmann, 1986). However, the two sequences could be aligned by the introduction of large (greater than four nucleotide) gaps to the *M. flocculare* sequence; after these adjustments, homology was calculated as 82.9%. As seen in Fig. 3, homology was greatest in the 3' half of this region. The rRNA operons of prokaryotes typically contain the 16S, 23S and 5S rRNA genes (in this order) with tRNA genes located in the first intergenic region. All *Mycoplasma* and *Ureaplasma* species (Harasawa *et al.*, 1992) deviate from this organization in that no tRNA genes

occupy the intergenic region. Our data indicate that this is also true for *M. flocculare*.

At least four possible promoter sites occupy the region upstream of the 16S rRNA gene (2, 98, 155 and 323); the AT-rich nature of the mollicute genome makes the random frequency of the TATAA sequence quite high. However, at nucleotide 155 is TATAATG, differing only in its final G from the TATAATA suggested by Taschke & Herrmann (1986) to be one of the promoters for the *M. hyopneumoniae* *rrn* locus.

Upstream of the *M. flocculare* 16S and 23S sequence at positions 311 and 2098, respectively, is the sequence CAAAACT; downstream of the 16S (position 2040) and 23S (position 5226) sequence is the exact inverted complement, AGTTTGT. These are required to form a portion of the stems for the maturation of the RNA transcript to mature 16S and 23S rRNA (Taschke & Herrmann, 1986). As shown in Fig. 3, and marked by underlining, these sequences are highly conserved in the intergenic region between the 16S and 23S rRNA structural genes for *M. flocculare* and *M. hyopneumoniae*. Between positions 5256 and 5299, and between 5341 and 5380, are probable 'hairpins' which may be a portion of the termination signal, although in neither case is the GC content high. Thus, all elements required for transcription and maturation of a separate 16S/23S operon are present in this sequence. This is expected since, as indicated above, the 5S rRNA gene is over 150 kb removed in the

DNA of both species from the 16S/23S rRNA genes (Taschke *et al.*, 1986; Stemke *et al.*, 1992).

REFERENCES

- Friis, N. F. (1975). Some recommendations concerning primary isolation of *Mycoplasma suis* pneumoniae and *Mycoplasma flocculare*. *Nord Veterinaermed* **27**, 337–339.
- Gross-Bellard, M., Oudet, P. & Chambon, P. (1973). Isolation of high molecular weight DNA from mammalian cells. *Eur J Biochem* **36**, 32–38.
- Harasawa, R., Uemori, T., Asada, K., Kato, I. & Shiragami, N. (1992). 'boxA'-like sequence between the 16S/23S spacer in rRNA operon of mycoplasmas. *FEBS Lett* **297**, 209–211.
- Huang, Y. & Stemke, G. W. (1992). Construction of the physical maps of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* and the location of rRNA genes on these maps. *Can J Microbiol* **38**, 659–663.
- Ludwig, W., Kirchhof, G., Klugbauer, N., Weizenegger, M., Bextzl, D., Ehrmann, M., Hertel, C., Jilg, S., Tatzel, R., Zitzelsberger, H., Liebl, S., Hochberger, M., Shah, J., Lane, D., Wallnofer, P. R. & Scheifer, K. H. (1992). Complete 23S ribosomal RNA sequences of Gram-positive bacteria with a low DNA G + C content. *Appl Microbiol* **15**, 487–501.
- Olsen, G. J. & Woese, C. R. (1993). Ribosomal RNA – a key to phylogeny. *FASEB J* **7**, 113–123.
- Stemke, G. W., Laigret, F., Grau, O. & Bovè, J. M. (1992). Phylogenetic relationships of three porcine mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and complete 16S rRNA sequence of *M. flocculare*. *Int J Syst Bacteriol* **42**, 220–225.
- Taschke, C. & Herrmann, R. (1986). Analysis of transcription and processing signals of the 16S–23S rRNA operon of *Mycoplasma hyopneumoniae*. *Mol & Gen Genet* **205**, 434–441.
- Taschke, C., Klinkert, M. Q., Wolters, J. & Herrmann, R. (1986). Organization of the ribosomal RNA genes in *Mycoplasma hyopneumoniae*: the 5S rRNA gene is separated from the 16S and 23S rRNA genes. *Mol & Gen Genet* **205**, 428–433.

Received 14 September 1993; revised 15 November 1993; accepted 17 December 1993.