A putative transposase gene in the 16S–23S rRNA intergenic spacer region of *Mycoplasma imitans*

Ryō Harasawa,1 David G. Pitcher,2 Ana S. Ramírez3 and Janet M. Bradbury3

1Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan
2Respiratory and Systemic Infection Laboratory, Health Protection Agency, 61 Colindale Avenue, London NW9 5HT, UK
3Department of Veterinary Pathology, The University of Liverpool, Jordan Bldg, Leahurst, Neston CH64 7TE, UK

Examination of the nucleotide sequences of the 16S–23S intergenic transcribed spacer (ITS) region of *Mycoplasma imitans* and *Mycoplasma gallisepticum* identified a putative transposase gene located only in the ITS of *M. imitans*, which can be used as a genetic marker to distinguish these two species. The relative size of the PCR products of the ITS region allowed a clear distinction to be made between strains of *M. imitans* and *M. gallisepticum*, both of which could be readily discriminated from the type strains of all the other recognized avian *Mycoplasma* species. In addition, the putative transposase gene assigned in the ITS of *M. imitans* was shown to include a sequence homologous to that of the P75 gene of *M. gallisepticum*. This is believed to be the first description of an insertion element in the rRNA operon region of a mycoplasma species.

INTRODUCTION

*Mycoplasma imitans* was first isolated from the turbinates of mule (broiler) ducks in southwest France, and tentatively identified as *Mycoplasma gallisepticum* by immunofluorescence and growth inhibition tests (Dupiellet, 1984). Similar strains were thereafter also isolated from geese in France (Buntz et al., 1986). These duck and goose isolates were found to be closely related to each other, but distinct from *M. gallisepticum*, by additional serological tests (Dupiellet et al., 1988). Further biochemical and molecular analyses, including SDS-PAGE profiles of the cellular proteins, restriction endonuclease cleavage patterns of the genomic DNAs, the G+C contents, and Southern blot hybridization using rRNA and *tuf* gene probes, also supported the observation that duck and goose strains were similar to one another but different from the reference strains of *M. gallisepticum* (Dupiellet, 1988; Yogev et al., 1988a, b). In addition, it was shown by DNA–DNA hybridization that there was a very close genetic relationship between the duck and goose strains but that the levels of homology of these strains with *M. gallisepticum* were only 40–46% (Dupiellet et al., 1988, 1990). Based on the accumulated data, a new species, *M. imitans*, was established for the duck and goose strains (Bradbury et al., 1993) and it was recognized that *M. imitans* and *M. gallisepticum* are distinct species although their rRNA sequences are nearly identical, differing by only two nucleotides (GenBank accession numbers M22441 and L24103). Comparisons of 16S rRNA sequences have been widely used in taxonomic and phylogenetic studies of prokaryotes (Swafford & Olsen, 1990), but 16S rRNA data alone may not be sufficient for defining very recently diverged species (Fox et al., 1992). Therefore in this study the 16S–23S intergenic transcribed spacer (ITS) region of *M. imitans* and *M. gallisepticum* was examined in order to determine species divergence in these sequences.

The ITS region is an important tool for the development of DNA-based classification because it shows a significant degree of variation in length and sequence from one species to another (Gürtler & Stanisich, 1996), and it can also be used to determine the relationships between genetically related bacterial species because of its high rate of divergence (Harasawa et al., 1996). The cistrons for rRNA molecules of most mycoplasmas are organized in an operon and arranged in the order 5’–16S–23S–5S–3’, in which the individual rRNA genes are separated by the ITS regions,
which occupy about 25% of the operon (Razin, 1985). Two copies of the rRNA operon have been detected in \textit{M. imitans} and \textit{M. gallisepticum} (Dupiellet, 1988). The rRNA operon is transcribed in a monocistronic RNA transcript and ITS regions are removed from the primary RNA transcript by a series of co-ordinated nucleolytic events catalysed by RNA-processing enzymes during maturation of the rRNA molecules. In this study, the ITS region between the 16S and 23S rRNA genes of \textit{M. imitans} and \textit{M. gallisepticum} was sequenced and compared. Having determined that the ITS region of these two \textit{Mycoplasma} species was markedly different in size, we compared the PCR products of the ITS region of all the other recognized avian \textit{Mycoplasma} species to see if the size differences had any diagnostic value.

**METHODS**

**Strains and culture.** \textit{M. imitans} strains 4229\textsuperscript{T} (same progenitor as NCTC 11733), B2/85 and B35/84 and \textit{M. gallisepticum} strains PG31\textsuperscript{T} (same progenitor as NCTC 10115), S6 (Zander, 1961) and F (Adler, 1960) were cultured in mycoplasma broth or on agar at 37°C. DNA amplifications were performed with a forward primer (5'-GCC TGC CAT ACC CAG-3') and a reverse primer (5'-GTT BCC CAT GCG-3') in a DNA thermal cycler (Perkin-Elmer). Reaction mixtures contained 2 units Taq DNA polymerase (AB Gene), 0.2 mM of each primer, 1x reaction buffer, 1.75 mM MgCl\text{2}, 0.2 mM dNTPs, and water to a volume of 50 μl. DNA amplification was achieved with 5 cycles of denaturation at 94°C for 15 s, renaturation at 60°C for 30 s, and elongation at 72°C for 2 min, followed by 30 cycles with the same parameters, except that there was an extension of 2 s per cycle in the elongation step. A 5 μl volume of each amplification reaction was subjected to electrophoresis in a 1.5% agarose gel. Gels were stained with ethidium bromide (0.3 μg ml\textsuperscript{-1}) and DNA visualized with ultraviolet light.

**Sequencing.** The 16S–23S rRNA intergenic spacer region was amplified as described elsewhere (Harasawa, 1999) using a pair of universal primers, 16S-1359F (5'-GGG TCT GTG ACG CAT CAC GCG-3') and 23S-115R (5'-GGG TBB CCC CAT TCGG-3') (Lane, 1991), with denaturation at 94°C for 30 s, annealing at 55°C for 100 s, and extension at 72°C for 100 s. Amplified DNA products of \textit{M. imitans} strain 4229\textsuperscript{T} and \textit{M. gallisepticum} PG31\textsuperscript{T} were extracted from agarose gels and subjected to direct sequencing twice on each strand in an ABI Prism 310 Genetic Analyser (Perkin Elmer-Cetus) by primer walking.

**RESULTS AND DISCUSSION**

The ITS regions of the 23 type strains of avian \textit{Mycoplasma} species were amplified by PCR (Fig. 1). The nucleotide sequences of the type strains of \textit{M. imitans} and \textit{M. gallisepticum} revealed that their ITS regions are 2488 nt and 645 nt, respectively, in size (Fig. 2). The ITS sequences from the two species were aligned using CLUSTAL X (Thompson et al., 1997). The first 153 nt of the ITS region showed 96% identity between \textit{M. imitans} and \textit{M. gallisepticum} (Fig. 2); this region is believed to form an RNA–RNA double strand with the leader sequence of the 16S rRNA (Chiaruttini & Milet, 1993; Liv et al., 1998). Similarly, the last 138 nt of the ITS region were almost identical (98% identity) between the two species (Fig. 2); this region is believed to form an RNA–RNA double strand with the 3' flanking region of the 23S rRNA. These double-stranded regions contain a recognition site for the rRNA-processing enzyme RNase III (Chiaruttini & Milet, 1993; Liv et al., 1998). Our data suggest that the rRNA processing in \textit{M. imitans} and \textit{M. gallisepticum} is conducted by a similar RNase III, since the two species share common features at the 5' and 3' ends of the ITS region. A putative boxA sequence was found at 90 nt upstream from the 23S rRNA gene of \textit{M. imitans} and \textit{M. gallisepticum}. The boxA sequence is considered to be either an anti-terminator or an internal promoter (Berg et al., 1989). Several palindromic sequences, some of which were nested, were detected in the ITS region of both the species (Fig. 2). These palindromic sequences may be responsible for rearrangement of the ITS region (Gürtler, 1999). The most common insertionless genes found in bacterial ITS regions are tRNA genes, but neither tRNA genes nor their pseudogenes were found in the ITS of these two \textit{Mycoplasma} species. This finding is common to all mycoplasmal ITS regions that have been sequenced (Harasawa, 1999). Instead of tRNA genes, the \textit{M. imitans} genome has an ORF of 1260 nt on the complementary strand of the ITS region. The amino acid sequence predicted from this ORF showed a high similarity score with bacterial transposases of \textit{Yersinia enterocolitica} (72 bits), \textit{Bacillus halodurans} (65 bits), \textit{Escherichia coli} (61 bits) and \textit{Yersinia pestis} (56 bits) using the BLAST algorithms (Altschul et al., 1997). Although other \textit{M. imitans} strains have not yet been sequenced, this is likely to be a common property among \textit{M. imitans} strains, as the PCR products of the three strains all had a similar size by gel electrophoresis (Fig. 3), which was distinct from that of all the \textit{M. gallisepticum} strains tested (Fig. 3). These sizes are relatively large among the \textit{Mycoplasma} species, as most ITS regions are smaller than 500 bp. They are also larger than those of the other avian \textit{Mycoplasma} species examined here. These size differences may prove helpful in discriminating...
Transposase gene in *Mycoplasma imitans*

between *M. imitans* and *M. gallisepticum* and also for distinguishing between these two species and all the other avian species.

Nucleotide sequences of a number of putative transposase genes in mycoplasma genomes have been deposited in GenBank, including insertion sequences in *M. pulmonis* strain KD735-26 [1203 bp of IS1138, GenBank accession Z16416] (Bhugra & Dybvig, 1993), *M. agalactiae* strain 3990 [1029 bp, GenBank accession AJ311887] (Pilo et al., 2003), *M. fermentans* strain PG18 [1101 bp of IS1630, GenBank accession AF100324] (Calcutt et al., 1999), *M. mycoides* subsp. *mycoides* SC strain Afade [1602 bp of IS1634, GenBank accession AF062493] (Vilei et al., 1999), *M. hypopneumoniae* [1659 bp, GenBank accession AF272977] (M.J. Calcutt & E. M. Wise, unpublished), *M. hyorhinis* strain GDL-1 [1425 bp of IS1221, GenBank accession U01217] (Zheng & McIntosh, 1995) and *M. penetrans* strain HF-2 [402 aa, GenBank accession AP004174-71] (Sasaki et al., 2002). A partial sequence of a putative insertion sequence-like transposase gene has been reported in *M. orale* strain ATCC 23714 [GenBank accession AY084048] (S. E. Ditty, B. Li, S. Zhang, N. Zou & S. C. Lo, unpublished). Of all the putative transposase genes reported in mycoplasmas thus far, the *M. imitans* transposase is the first to be discovered in an rRNA operon. Although no typical or consensus motif has been found in the transposase protein sequences thus far, the amino acid sequence of the *M. imitans* transposase was found to be most similar to that of *M. penetrans*. Recently sequences more similar to the *M. imitans* transposase have been deposited in the GenBank database under accession numbers AE016967–AE016970 (Geary et al., 2003).

![Image](http://mic.sgmjournals.org)

**Fig. 1.** Comparison of the ITS regions from 23 type strains of avian *Mycoplasma* species by gel electrophoresis of the PCR products. The product includes 180 bp at the 3′ end of the 16S rDNA and 115 bp at the 5′ end of the 23S rDNA. Markers A and B are a 100 bp ladder (Amersham Pharmacia Biotech) and a mixture of λDNA/HindIII and φX174 RF DNA/HaeIII fragments (Invitrogen), respectively. The arrow indicates 800 bp. Lanes: 1, *M. anatis* 13400; 2, *M. anseris* 12190; 3, *M. buteonis* Bb/T2g; 4, *M. cloacae* 383; 5, *M. columbinasale* 694; 6, *M. columbinum* MMP1; 7, *M. columborale* MMP4; 8, *M. corogpys* BV1; 9, *M. falconis* H/T1; 10, *M. gallinaeum* DD; 11, *M. gallinarum* PG16; 12, *M. gallisepticum* PG31; 13, *M. gallopavonis* WR1; 14, *M. gyrophilum* 486; 15, *M. gypis* B1/T1; 16, *M. imitans* 4229; 17, *M. iners* PG30; 18, *M. iowae* 695; 19, *M. lipofaciens* R171; 20, *M. melagrigis* 17529; 21, *M. pellorum* CKK; 22, *M. stuni* UCMF; 23, *M. synoviae* WVU 1853.

**Transposase gene in *Mycoplasma imitans***

- **Fig. 2.** Partial amino acid alignment of transposase sequences from *M. imitans* (GenBank accession AY037872) and *M. gallisepticum* (GenBank accession AF272977) with other putative transposase sequences in GenBank.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>Length (bp)</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. imitans</em></td>
<td>AY037872</td>
<td>315</td>
<td>83</td>
</tr>
<tr>
<td><em>M. gallisepticum</em></td>
<td>AF272977</td>
<td>315</td>
<td>83</td>
</tr>
<tr>
<td><em>M. gallinarum</em></td>
<td>AF062493</td>
<td>315</td>
<td>83</td>
</tr>
<tr>
<td><em>M. gallinaeum</em></td>
<td>DD</td>
<td>315</td>
<td>83</td>
</tr>
<tr>
<td><em>M. gallinaceum</em></td>
<td>PG16</td>
<td>315</td>
<td>83</td>
</tr>
<tr>
<td><em>M. penetrans</em></td>
<td>PG30</td>
<td>315</td>
<td>83</td>
</tr>
<tr>
<td><em>M. iowae</em></td>
<td>695</td>
<td>315</td>
<td>83</td>
</tr>
</tbody>
</table>

The alignment shows that the transposase gene from *M. imitans* is highly conserved with other putative transposase sequences from different mycoplasma species.
ORF (complementary strand in box)

P5 homologue (underlined)
recognized during natural infections (Spencer et al., 2002). A possible explanation for the presence of this inverted gene could be that non-homologous recombination has occurred, followed by reverse transcription of the P75-like mRNA. Reverse transcription has been reported in some bacterial species (Lim & Maas, 1989; Varmus, 1989). Although reverse transcriptase has not been reported in mycoplasmas, prokaryotic DNA polymerases are known to have some reverse transcriptase activity in some species (Lou et al., 1991; Myers & Gelfand, 1991). An alternative explanation is that the transposase gene may have inserted downstream of the P75 gene and been imprecisely excised, leaving behind part of its DNA sequence.

Conclusions
This study reports the discovery of a novel insertion sequence present in the rRNA operon of Mycoplasma imitans. This element, a putative transposase, is the first to be described in this region of the genome of a Mycoplasma species. The presence of an unusually long ITS sequence in this species could be useful in differentiating it from Mycoplasma gallisepticum and other avian mycoplasmas.

ACKNOWLEDGEMENTS
We would like to acknowledge the help and advice of Dr C. J. Naylor.

Fig. 2. Sequence alignment of the ITS regions of M. gallisepticum strain PG31T (upper line) and M. imitans strain 4229T (lower line). The nucleotide sequence numbers are from the consensus alignment. Identical nucleotides between the two species are shown as white letters on a black background. Dashes indicate spacers between adjacent nucleotides introduced for maximum alignment. Palindromic sequences are indicated by arrows pointing in opposite directions. The open reading frame on the complementary strand is shown in a box. The P75 gene homologue is underlined.
Comparision of the ITS regions of M. gallisepticum and M. imitans by gel electrophoresis of PCR products. M. gallisep-
"cium (lanes 2–4) and M. imitans (lanes 5–7) produced bands of 940 bp and 2783 bp, respectively. Lanes: 1, 100 bp ladder (Invitrogen); 2, M. gallisepticum PG31; 3, M. gallisepticum S6; 4, M. gallisepticum MgF; 5, M. imitans 4229; 6, M. imitans B35/84; 7, M. imitans B35/84; 8, 1 kb ladder (Invitrogen).

**Fig. 3.** Comparison of the ITS regions of M. gallisepticum and M. imitans by gel electrophoresis of PCR products. M. gallisep-
cium (lanes 2–4) and M. imitans (lanes 5–7) produced bands of 940 bp and 2783 bp, respectively. Lanes: 1, 100 bp ladder (Invitrogen); 2, M. gallisepticum PG31; 3, M. gallisepticum S6; 4, M. gallisepticum MgF; 5, M. imitans 4229; 6, M. imitans B35/84; 7, M. imitans B35/84; 8, 1 kb ladder (Invitrogen).

**REFERENCES**


Rosendal, S. & Black, F. T. (1972). Direct and indirect immuno-

Sasaki, Y., Ishikawa, L., Yamashita, A. & 8 other authors (2002). The complete genomic sequence of Mycoplasma penetrans, an intra-


