Examination of the 16S-23S rRNA Intergenic Spacer Sequences of ‘*Candidatus Mycoplasma haemobos*’ and *Mycoplasma haemofelis*

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ABSTRACT. The intergenic spacer region between the 16S and 23S rRNA genes of mycoplasmas has been used for a genetic marker for identification of the species. Here we show the intergenic spacer regions of two hemotropic mycoplasmas, *Mycoplasma haemofelis* and ‘*Candidatus Mycoplasma haemobos*’ (synonym: ‘*C. M. haemobovis*’) are also useful for classification of this particular group of mycoplasmas. The spacer region of *M. haemofelis* and ‘*C. M. haemobos*’ consisted of 209 and 210 base pairs, respectively, and both lacked the spacer tRNA genes. Phylogenetic analysis suggested a monophyletic relationship among hemoplasmas and *M. fastidiosum*. A hypothetical secondary structure predicted in the spacer regions tentatively assigned the boxA and boxB motifs peculiar to the members of the genus *Mycoplasma*. *M. haemofelis* and ‘*C. M. haemobos*’ possessed a stem-loop structure in common, despite the presence of a palindromic nucleotide substitution in the stem region.

KEY WORDS: 16S-23S rRNA, haemoplasma, mycoplasma.

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Hemoplasmas, hemotropic mycoplasmas, are causative of infectious anemia in animals, but have never been cultured *in vitro* [16, 17]. The members of the genus *Mycoplasma* have only one or two operons for the rRNA gene [1]. The genes encoding for rRNA molecules of the genus *Mycoplasma* are usually organized in the order of 5'-16S-23S-5S-3', in which the individual rRNA genes are separated by internal transcribed spacer (ITS) regions [22], except for *Mycoplasma suis* [14, 19]. The ITS region between the 16S and 23S rRNA genes of the genus *Mycoplasma* has been shown to lack spacer tRNA genes so far [15] and to be variable in sequence and length depending on the species [27]. Thus the ITS region has been used as a genetic marker for comparing phylogenetic relationships of genetically closely related species among not only the mycoplasmas [5], but also other bacterial species [3, 12, 13, 25]. Therefore, analyses of ITS regions may provide useful information for defining the genealogical positions of hemoplasmas. In the present study, we examined the ITS sequences of two hemoplasma species, *Mycoplasma haemofelis* [16, 17] and ‘*Candidatus Mycoplasma haemobos*’ (synonym: ‘*C. M. haemobovis*’) [24], both causative of infectious anemia in cats and cattle, respectively.

Heparin or EDTA-anticoagulated blood samples collected from cats in Morioka and cattle in Miyazaki, Japan, were kept at -20°C prior to analysis. Total DNA was extracted from 200 µl of the anticoagulated whole blood

samples by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, eluting into 200 µl of buffer AE, and stored at -20°C until examination in the PCR assay. Two hemoplasma species, *M. haemofelis* and ‘*C. M. haemobos*’, identified in the present study according to the methods as described previously [2, 18], were further subjected to analysis of the ITS region between the 16S and 23S rRNA genes. No mixed infection was apparent in each animal that these hemoplasmas were detected.

ITS region was amplified from the total DNA extracted from ‘*C. M. haemobos*’ strain Gandai1 and *M. haemofelis* strain Gandai2. PCR amplification of the isolated DNA was carried out at 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min for 30 cycles using forward (5'-GTTCCAGGTCT-TGTACACA-3') and reverse (5'-CAGTACTTGTTCAC-TATCGGTA-3') primers as described previously [4]. The PCR products were fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, Me., U.S.A.) in TAE (40 mM Tris, pH8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetraacetate) buffer at 50 volts for 60 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 µg/ml) for 15 min and visualized under UV transilluminator. ITS regions of only *M. haemofelis* and ‘*C. M. haemobos*’ were successfully amplified by this PCR. DNA in a clearly visible band was extracted by using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, Calif., U.S.A.). The GenBank/EMBL/DBJ accession numbers for the 16S-23S rRNA intergenic spacer sequence of ‘*C. M. haemobos*’ strain Gandai1 and *M. haemofelis* strain Gandai2 are AB638407 and AB638408,

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respectively. Reason for unsuccessful amplification of ITS of other hemoplasma species by this PCR remains unidentified. Mismatches in PCR primers were most likely in these cases.

The ITS region between the 16S and 23S rRNA genes of the genus *Mycoplasma* is well conserved within a species and used for a genetic marker for identification and classification of mycoplasmas [5]. ITS regions of *M. haemofelis* and '*C. M. haemobos*' were consisting of 209 and 210 base pairs, respectively. The nucleotide sequences of the ITS in these 2 species showed moderate similarity with that of *M. haemomuris* previously determined [8], and shared a consensus motifs called boxA and boxB in common (Fig. 1). The boxA, originally found at upstream regions of the *nut* site of the lambda phage genome, is considered to be a *cis*-acting element for the *Escherichia coli* NusA protein, a transcription control factor [20]. The conserved nature of the boxA sequence among these different species may implicate an important role in survival mechanisms, though the exact function of this motif in the ITS remains unknown [11]. The boxB sequence previously reported in mycoplasmas [5] was also assigned about ten nucleotides upstream of the boxA sequence of both the hemoplasma species. Presence of these particular motifs suggests that ITS might act as a controlling element for transcription of the rRNA operon in hemoplasmas. No spacer tRNA gene was identified within the ITS of *M. haemofelis* and '*C. M. haemobos*', and this is

a common feature in line with the other species of the genus *Mycoplasma* [27]. Transposase gene has been demonstrated in ITS of *M. imitans* [10], but such feature was not the case in these two hemoplasma species.

The nucleotide sequences of ITS regions of both the hemoplasmas were compared with authentic mycoplasma species in phylogenetic analysis. In the present study, we explored the genealogical relatedness among 20 mycoplasma species including not only ureaplasmas but also three hemoplasma species, *M. haemomuris*, *M. haemofelis*, and '*C. M. haemobos*'. The comprehensive phylogenetic tree suggested the 20 mycoplasma sequences were divided into three clusters consisting of Hominis, Pneumoniae and Fastidiosum groups (Fig. 2). Hemoplasmas formed a monophyletic clade in the Fastidiosum group in the present study, allowing more precise assignment of hemoplasmas as compared with phylogenetic analysis based on the 16S rRNA sequences [16, 24]. Although the Hominis and Pneumoniae groups in mycoplasmas have already been established in the phylogenetic analyses based on the 16S rRNA gene as well as on the RNase P RNA gene [21], we showed a monophyletic relationship among hemoplasmas and *M. fastidiosum* by phylogenetically analyzing ITS regions. Our findings support that given hemoplasmas are members of the class *Mollicutes*.

The secondary structures of the ITS were predicted according to the algorithm of Zuker and Stiegler [28]. Five

Fig. 1. Nucleotide sequence alignment of ITS from the three hemoplasma species, *M. haemofelis*, '*C. M. haemobos*' and *M. haemomuris*. The nucleotide sequence of *M. haemomuris* was obtained from the previous report [8]. Nucleotide sequence numbers are given from a consensus sequence. Homologous nucleotides are shown as inverted characters. Dashes indicate nucleotide gaps between adjacent nucleotides introduced for the alignment. Box A is underlined, and box B is shown by a dotted line.

Fig. 2. Unrooted phylogenetic tree based on the ITS comparison, suggesting a monophyletic relationship among hemoplasmas and *M. fastidiosum*. Following nucleotide sequences were obtained from the DNA databases and accession number is given in parenthesis. They are *M. salivarium* PG20 (X58558), *M. pulmonis* m53 (X58554), *M. neurolyticum* Sabin Type A (X58552), *M. buccale* CH20247 (D89504), *M. primatum* HRC292 (D89509), *M. caviae* G122 (D89505), *M. hominis* PG21 (X58559), *M. fermentans* PG18 (X58553), *M. hyorhinis* BTS-7 (X58555), *M. penetrans* GTU54 (D89508), *M. muris* RIII4 (D89507), *M. pneumoniae* Mac (D14528), *M. genitalium* G37 (D14526), *M. fastidiosum* ATCC33229 (AY781782), *Ureaplasma urealyticum* T960 (X58561), *U. cati* F2 (D636859), *U. canigenitalium* D6P-C (D63684), *M. haemomuris* Shizuoka (AB080799), *M. haemofelis* Gandai2 (AB638408), and 'C. M. haemobos' Gandai1 (AB638407). Scale bar indicates the estimated evolutionary distance that was computed with CLUSTAL W [26] using neighbor-joining method [23]. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications.

stem-loop domains were allocated in ITS of *M. haemofelis* and 'C. M. haemobos' (Fig. 3). The boxA was assigned on the stem portion of domain IV, and this was common to the structure of 'C. M. haemomuris' [8]. No identical architecture was evident between these two species. Domain III was seen in both the species in common, despite a single nucleotide substitution that was capable to form a stable secondary structure to minimize free energy, by G-T wobble as well as canonical Watson-Crick base pairings at stem region. Secondary structures in ITS region have sometime provided a key character to distinguish closely related species among mycoplasmas [6, 7, 9].

Here we demonstrated that ITS regions of hemoplasmas possess a feature of the genus *Mycoplasma*, and hemoplasmas form a monophyletic relationship with *M. fastidiosum* in phylogenetic analysis. A common stem-loop structure was identified in *M. haemofelis* and 'C. M. haemobos', though its significance remained unknown.

REFERENCES

- Amikam, D., Glaser, G. and Razin, S. 1984. Mycoplasmas (*Mollicutes*) have a low number of rRNA genes. *J. Bacteriol.* **158**: 376–378.
- Fujihara, M., Watanabe, M., Yamada, T. and Harasawa, R. 2007. Occurrence of 'Candidatus Mycoplasma turicensis' infection in domestic cats in Japan. *J. Vet. Med. Sci.* **69**: 1061–1063.
- Graham, T. A., Golsteyn-Thomas, E. J., Thomas, J. E. and Gannon, V. P. J. 1997. Inter- and intraspecies comparison of the 16S-23S rRNA operon intergenic spacer regions of six *Listeria* spp. *Int. J. Syst. Bacteriol.* **47**: 863–869.
- Harasawa, R. 1996. PCR: application of nested PCR to detection of mycoplasmas. pp.75–79. In: *Molecular and Diagnostic Procedures in Mycoplasma*, vol. 2 (Razin, S. and Tully, J.G. eds.), Academic Press, New York.
- Harasawa, R. 1999. Genetic relationships among mycoplasmas based on the 16S-23S rRNA spacer sequence. *Microbiol. Immunol.* **43**: 127–132.

Fig. 3. Hypothetical secondary structures for the ITS of *M. haemofelis* (top) and '*C. M. haemobos*' (bottom). Canonical Watson-Crick base-pairing is hyphenated, and a wobble base-pairing tolerated in the secondary structure is shown by an asterisk. The boxA was a part of the stem region of domain IV, and boxB was located between domains III and IV.

- Harasawa, R., Hotzel, H. and Sachse, K. 2000. Comparison of the 16S-23S rRNA intergenic spacer regions among strains of the *Mycoplasma mycoides* cluster, and reassessment of the taxonomic position of *Mycoplasma* sp. bovine group 7. *Int. J. Syst. Evol. Microbiol.* **50**: 1325–1329.
- Harasawa, R. and Kanamoto, Y. 1999. Differentiation of two biovars of *Ureaplasma urealyticum* based on the 16S-23S rRNA intergenic spacer region. *J. Clin. Microbiol.* **37**: 4135–4138.
- Harasawa, R., Kawahara, M. and Rikihisa, Y. 2002. Characteristics of the 16S-23S rRNA intergenic spacer region of *Mycoplasma haemomuris*, previously classified as '*Haemobartonella muris*'. *J. Vet. Med. Sci.* **64**: 1161–1164.
- Harasawa, R., Lefkowitz, E.J., Glass, J.I. and Cassell, G.H. 1996. Phylogenetic analysis of the 16S-23S rRNA intergenic spacer region of the genus *Ureaplasma*. *J. Vet. Med. Sci.* **58**: 191–195.
- Harasawa, R., Pitcher, D.G., Ramirez, A.S. and Bradbury, J.M. 2004. A putative transposase gene in the 16S-23S rRNA intergenic spacer region of *Mycoplasma imitans*. *Microbiology* **150**: 1023–1029.
- Harasawa, R., Uemori, T., Asada, K., Kato, I. and Shiragami, N. 1992. 'boxA'-like sequence between the 16S/23S spacer in the rRNA operon. *FEBS Lett.* **297**: 209–211.
- Jagoueix, S., Bove, J. M. and Garnier, M. 1997. Comparison of the 16S/23S ribosomal intergenic regions of '*Candidatus Liberobacter asiaticum*' and '*Candidatus Liberobacter africanum*', the two species associated with citrus huang-longbing (greening) disease. *Int. J. Syst. Bacteriol.* **47**: 224–227.
- Leblond-Bourget, N., Philippe, H., Mangin, I. and Decaris, B. 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. *Int. J. Syst. Bacteriol.* **46**: 102–111.
- Messick, J.B., Santos, A.P. and Guimaraes, A.M. 2011. Complete genome sequences of two hemotropic mycoplasmas, *Mycoplasma haemofelis* strain Ohio2 and *Mycoplasma suis* strain Illinois. *J. Bacteriol.* **193**: 2068–2069.
- Nakagawa, T., Uemori, T., Asada, K., Kato, I. and Harasawa, R. 1992. *Acholeplasma laidlawii* has tRNA genes in the 16S-23S spacer of the rRNA operon. *J. Bacteriol.* **174**: 8163–8165.
- Neimark, H., Johansson, K.-E., Rikihisa, Y. and Tully, J.G. 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus Mycoplasma haemofelis*', '*Candidatus Mycoplasma haemomuris*', '*Candidatus Mycoplasma haemosuis*' and '*Candidatus Mycoplasma wenyonii*'. *Int. J. Syst. Evol. Microbiol.* **51**: 891–899.
- Neimark, H., Johansson, K.-E., Rikihisa, Y. and Tully, J.G. 2002. Revision of hemotropic *Mycoplasma* species names. *Int. J. Syst. Evol. Microbiol.* **52**: 683.
- Nishizawa, I., Sato, M., Fujihara, M., Sato, S. and Harasawa, R. 2010. Differential detection of hemotropic *Mycoplasma* species in cattle by melting curve analysis of PCR products. *J. Vet. Med. Sci.* **72**: 77–79. (Erratum, *J. Vet. Med. Sci.* **72**: 1704.)
- Oehlerking, J., Kube, M., Felder, K. M., Matter, D., Wittenbrink, M. M., Schwarzenbach, S., Kramer, M. M., Hoelzle, K. and Hoelzle, L. E. 2011. Complete genome sequence of the hemotropic *Mycoplasma suis* strain KI3806. *J. Bacteriol.* **193**: 2369–2370.
- Olson, E.R., Tomich, C.-S. and Friedman, D.I. 1987. The nusA recognition site. *J. Mol. Biol.* **180**: 1053–1063.
- Peters, I.R., Helps, C.R., McAuliffe, L., Neimark, H., Lappin, M.R., Gruffydd-Jones, T.J., Day, M.J., Hoelzle, L. E., Willi, B., Meli, M., Hofmann-Lehmann, R. and Tasker, S. 2008. RNase P RNA gene (*rnpB*) phylogeny of hemoplasmas and other *Mycoplasma* species. *J. Clin. Microbiol.* **46**: 1873–1877.
- Razin, S. 1985. Molecular biology and genetics of mycoplasmas (Mollicutes). *Microbiol. Rev.* **49**: 419–455.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
- Tagawa, M., Matsumoto, K. and Inokuma, H. 2008. Molecular

- detection of *Mycoplasma wenyonii* and 'Candidatus *Mycoplasma haemobos*' in cattle in Hokkaido, Japan. *Vet. Microbiol.* **132**: 177–180.
25. Thain, T., Ward-Rainey, N., Kroppenstedt, R. M., Stackebrandt, E. and Rainey, F.A. 1977. Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S-23S ribosomal DNA intergenic spacers. *Int. J. Syst. Bacteriol.* **47**: 202–206.
 26. Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.
 27. Uemori, T., Asada, K., Kato, I. and Harasawa, R. 1992. Amplification of the 16S-23S spacer region in rRNA operons of mycoplasmas by the polymerase chain reaction. *Syst. Appl. Microbiol.* **15**: 181–186.
 28. Zuker, M. and Stiegler, P. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary. *Nucleic Acids Res.* **9**: 133–148.