Characteristics of the 16S-23S rRNA Intergenic Spacer Region of *Mycoplasma haemomuris*, Previously Classified as '*Haemobartonella muris*'

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ABSTRACT. The intergenic spacer region between the 16S and 23S rRNA genes of *Mycoplasma haemomuris*, previously classified as '*Haemobartonella muris*', was amplified by PCR and sequenced for analysis of the primary and secondary structures of the RNA transcript. The spacer region consisted of 219 base-pairs and lacked the spacer tRNA gene. A hypothetical secondary structure predicted in the RNA transcript of the spacer region was tentatively assigned *boxA* and *boxB* loci peculiar to the members of the *Mycoplasma*. *Mycoplasma haemomuris* and the other species of the genus *Mycoplasma* are consistent with these characteristics of the spacer region.

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

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'*Haemobartonella muris*', an uncultivable haemotrophic bacterial species which infects murine erythrocytes, has long been recognized as a member of the order *Rickettsiales* [8]. It was, however, more than 35 years ago that '*H. muris*' was found to be cell wall-less and indistinguishable from mycoplasmas [19]. Phylogenetic analyses based on the 16S rRNA sequence revealed that '*H. muris*', '*H. felis*' and '*Eperythrozoon suis*' are closely related to *Mycoplasma* species [15]. Similarly, '*E. wenyonii*' was found to be genetically close to the genus *Mycoplasma* [13]. Therefore, it was recently proposed that the two genera, '*Haemobartonella*' and '*Eperythrozoon*', be transferred to the genus *Mycoplasma* [11, 12]. To date no additional genetic comparison except for the 16S rRNA sequence analysis has been made between *M. haemomuris* (synonym of '*H. muris*') and other members of *Mycoplasma* sp. The members of the class *Mollicutes* have only one or two operons for the rRNA genes [1]. The genes coding for rRNA molecules of the genus *Mycoplasma* are organized in operons and arranged in the order of 5'-16S-23S-5S-3', in which the individual rRNA genes are separated by internal transcribed spacer (ITS) regions [14]. The ITS region between the 16S and 23S rRNA genes of mycoplasmas has been shown to lack tRNA genes and to be variable in sequence and length depending on the *Mycoplasma* species [22]. Besides, the ITS region between the 16S and 23S rRNA genes has been used as a genetic marker for comparing phylogenetic relationships of genetically closely related species among not only the mycoplasmas [4, 7, 18], but also other bacterial species [2, 6, 9, 20]. Therefore, analyses of the ITS region may provide useful information for defining the genealogical position of the species of *M. haemomuris*.

In the present study, we sequenced and analyzed the ITS region between the 16S and 23S rRNA genes of *M. haemomuris* and compared it with those of genealogically related *Mycoplasma* species.

Bacterial genome DNA was isolated from the spleen cells

of mice infected with *M. haemomuris* strain Shizuoka. 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'-ACACCATGGGAGYTGGTAAT-3') and reverse (5'-CTTCWTCGACTTYCAGACCCAAGGCAT-3') primers as described previously [3]. A single band of approximately 250 bp was observed for the PCR products in agarose gel electrophoresis. The PCR product was then subjected to direct sequencing by the di-deoxynucleotide chain-termination method [16]. The nucleotide sequence determined in this study has been deposited in the DDBJ, EMBL, GSDB, and NCBI nucleotide sequence databases under the accession number AB080799.

The nucleotide sequence of the 16S-23S rRNA ITS of *M. haemomuris* was compared with those of other *Mycoplasma* species. The *Mycoplasma* species have been clustered into five groups according to the 16S rRNA sequences [23]. Five species, *M. hominis*, *M. arginini*, *M. salivarium*, *M. arthritidis* and *M. orale*, have been included in the *M. hominis* group. *Mycoplasma pneumoniae*, *M. fermentans* and *M. mycoides* subsp. *mycoides* represent the *M. pneumoniae*, *M. fermentans* and *M. mycoides* groups, respectively. *Mycoplasma hyorhinis* and *M. neurolyticum* are from the *M. hyorhinis* group. Three murine *Mycoplasma* species, *M. pulmonis*, *M. arthritidis* and *M. neurolyticum*, were included in this analysis, since *M. haemomuris* is also a murine isolate. *Ureaplasma cati* was included as an out group for phylogenetic analysis. The ITS of *M. haemomuris* was found to be 219 nucleotides. This sequence was aligned with those of the eleven *Mycoplasma* species (Fig. 1). Genealogical relatedness between *M. haemomuris* and other mycoplasmas was obtained by the neighbor-joining method [17]. The tree analysis suggests a monophyletic relationship between *M. pneumoniae* and *M. haemomuris* (Fig. 2). We examined the secondary structure of the ITS of *M. haemomuris*. Computer analysis indicated a hypothetical secondary structure of RNA transcript of the ITS region of *M.*

Fig. 1. Nucleotide sequence alignment of the 13 species including *M. haemomuris*. The 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. *M. mycoides** represents *M. mycoides* subsp. *mycoides*.

haemomuris (Fig. 3). Tentative *boxA* and *boxB* loci [5] were assigned to the secondary structure. The *boxA* and *boxB*, originally found at upstream regions of the *nut* site of the lambda phage genome, are considered to be the p-independent terminators [10]. The function of these sequences remains to be determined. No spacer tRNA gene was found within the ITS, and this is a common feature with those of the other species of the genus *Mycoplasma* [22].

In conclusion, the ITS of *M. haemomuris* lacked spacer tRNA genes and had *boxA* and *boxB* loci which are com-

mon features of the *Mycoplasma* genome. Phylogenetic analysis indicated a monophyletic relationship between *M. haemomuris* and *M. pneumoniae*. These findings on *M. haemomuris* also support its classification as a member of the genus *Mycoplasma*.

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Fig. 2. Phylogenetic tree based on the ITS comparison, suggesting a monophyletic relationship between *M. haemomuris* and *M. pneumoniae* among the twelve *Mycoplasma* species examined. Evolutionary distances were computed with CLUSTAL W [21] using the neighbor-joining method [16]. Numbers at the relevant branches refer to the values of boot-strap analysis of 1,000 replicati ons. Scale bar indicates the evolutionary distance value of 0.1 (ca. 10-nucleotide substitutions per 100 nucleotides). *M. mycoides** represents *M. mycoides* subsp. *mycoides*.

Fig. 3. Computed secondary structure for the ITS of *M. haemomuris*. *BoxA* and *boxB* loci are indicated in the figure. Canonical base-pairing is hyphenated, and uncanonical base-pairing is shown by asterisks.

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