Available online at www.sciencedirect.com
ScienceDirect
Veterinary Microbiology 128 (2008) 279–287
veterinary microbiology
www.elsevier.com/locate/vetmic

High inter-species and low intra-species variation in 16S–23S rDNA spacer sequences of pathogenic avian mycoplasmas offers potential use as a diagnostic tool

A.S. Ramírez [a,b], C.J. Naylor [a], D.G. Pitcher [c,1], J.M. Bradbury [a,*]

[a] *Department of Veterinary Pathology, University of Liverpool, Leahurst Veterinary Teaching Hospital, Neston CH64 7TE, UK*
[b] *Unit of Epidemiology and Preventive Medicine, Veterinary Faculty, University of Las Palmas de G.C., Trasmontaña s/n, Arucas, 35416 Las Palmas, Spain*
[c] *Respiratory and Systemic Infection Laboratory, Health Protection Agency, 61 Colindale Avenue, London NW9 5HT, UK*

Received 6 August 2007; received in revised form 19 October 2007; accepted 24 October 2007

Abstract

In order to investigate its value for phylogenetic analysis, species characterisation and diagnosis, the 16S–23S rDNA intergenic spacer regions (ISRs) of the type strain of 23 avian *Mycoplasma* species were amplified and the sequences determined. Also sequenced were the reference strains of *Mycoplasma iowae* serotypes J, K, N, Q and R and a number of field strains of *Mycoplasma synoviae*, *Mycoplasma gallisepticum*, *Mycoplasma meleagridis* and *M. iowae*. The ISRs demonstrated a high level of size variation (178–2488 bp) between species and did not include tRNA genes. Phylogenetic analysis performed using the information conflicted with that based on the 16S rDNA and was therefore not helpful for phylogenetic studies. However, the ISR did appear to be of value for determining species since there was high inter-species variation between all 23 avian *Mycoplasma* species, and in addition there was low intra-species variation, at least in the four pathogenic species. It could also be very useful as additional information in the description of a new species and as a target for species-specific PCRs.

Keywords: Mollicutes; Mycoplasma; Intergenic spacer region; Phylogeny

1. Introduction

There are 23 recognised avian *Mycoplasma* species of which four, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* and *Mycoplasma iowae* are recognised as economically important poultry pathogens. They belong to the class *Mollicutes* and are characterized by a lack of cell wall

* Corresponding author at: Department of Veterinary Pathology, University of Liverpool, Leahurst Veterinary Teaching Hospital, Neston CH64 7TE, UK. Tel.: +44 151 794-6115; fax: +44 151 794 6110.
E-mail address: jmb41@liverpool.ac.uk (J.M. Bradbury).
[1] Deceased.

0378-1135/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2007.10.023

and for being the smallest self-replicating prokaryotes (Bradbury, 2001).

All self-replicating cells contain ribosomes, consisting of ribosomal proteins and ribosomal RNA (rRNA). Bacteria have between one and 15 rRNA operons, the common organization being 16S rRNA–23S rRNA–5S rRNA with an intergenic spacer region (ISR) separating each rRNA gene. Sequence variation in 16S–23S ISRs among relatively close bacterial taxa is high for some bacterial genera (Gütler, 1999).

In the genus *Mycoplasma* knowledge of the number and organization of the rRNA operons is limited but the most common number is one or two (Johansson et al., 1998; Razin et al., 1998) and unlike other bacteria no tRNAs have been found in the 16S–23S rRNA ISR (Harasawa et al., 1992; Johansson et al., 1998; Razin et al., 1998; Gütler, 1999). ISRs from many *Mycoplasma* spp. have been sequenced (Harasawa et al., 1992, 2000, 2004; Harasawa, 1999; Volokov et al., 2006) and although the region was not useful for differentiating between the 'mycoides cluster' mycoplasmas (Harasawa et al., 2000), it was valuable in supporting separation of two biovars of *Ureaplasma urealyticum* into distinct species: *U. urealyticum* and *Ureaplasma parvum* (Harasawa and Kanamoto, 1999; Robertson et al., 2002).

Our goal was to explore the value of the ISR of avian mycoplasmas in phylogenetic studies and for its possible diagnostic use. A similar study was reported by Chalker and Brownlie (2004) for canine mycoplasmas. We designed a primer set to obtain PCR products containing the entire ISR of all the avian *Mycoplasma* type strains. The ISRs were sequenced and a phylogenetic tree constructed. Inter-species differentiation of avian mycoplasmas using the ISR was evaluated and compared with results obtained using 16S rDNA sequences.

2. Materials and methods

2.1. Organisms and culture conditions

The 23 avian *Mycoplasma* species (type strains) used are named in Table 1. They were cultivated in mycoplasma broth and on mycoplasma agar plates (Bradbury, 1977) at 37 °C for between one and 5 days, in a CO_2 rich atmosphere. They had been filter-cloned through membranes of pore size 0.45 μm (Tully, 1983) and their identity and purity confirmed by the indirect immunofluorescence (Rosendal and Black, 1972). In addition to the type strains five other *M. iowae* serotypes (reference strains J, K, N, Q and R), 21 field strains of *M. synoviae*, 11 of *M. gallisepticum*, 16 of *M. meleagridis* and 16 of *M. iowae* were examined.

2.2. DNA preparation and PCR

DNA was extracted from broth cultures using Chelex resin as described elsewhere (Harasawa et al., 2004). To obtain the complete ISR sequence the PCR protocol described by Harasawa et al. (2004) was used in a DNA thermal cycler (Perkin-Elmer) but with different primers. In the present study the forward primer was 5′-CGT TCT CGG GTC TTG TAC AC-3′ and the reverse primer was 5′-CGC AGG TTT GCA CGT CCT TCA TCG-3′. DNA amplification was accomplished with five 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 for an extension of 2 s per cycle in the elongation step.

2.3. Sequencing and sequence analysis

The ISRs of the type strains of 21 species were sequenced and analysed, the type strains of *M. gallisepticum* and *Mycoplasma imitans* having been sequenced in previous work (Harasawa et al., 2004). The relevant accession numbers are in Table 1.

Sequencing was conducted by Imperial College, London (UK) and was determined for each strand of DNA. The resulting chromatograms were examined in Chromas (Version 1.45; School of Health Science, Griffith University, Australia). The forward sequence and reverse complimented sequences of all the type strains were compared to produce a consensus sequence using Generunner (Version 3.05; Hastings Software Inc.). All the sequences were then aligned automatically with Clustal W and then the alignment was completed manually using Bioedit Version 7.0.0 (Hall, 1999).