ELSEVIER

Molecular and Cellular Probes 19 (2005) 371–384

Molecular and Cellular Probes

www.elsevier.com/locate/ymcpr

Deletion of an *mmpL* gene and multiple associated genes from the genome of the S strain of *Mycobacterium avium* subsp. *paratuberculosis* identified by representational difference analysis and in silico analysis

I.B. Marsh*,[1], R.J. Whittington

Faculty of Veterinary Science, University of Sydney, Private Bag 3, Camden, NSW 2570, Australia

Received 15 April 2005; accepted for publication 17 June 2005

Abstract

Mycobacterium avium subsp. *paratuberculosis* (*M. a. paratuberculosis*) can be divided into two major strains, sheep (S) and cattle (C), based on cultural requirements, host specificity, degree of clumping of cells in suspension and minor genomic differences including copy number of insertion elements and point mutations. Representational difference analysis (RDA) with S strain as driver and C strain as tester was used to identify unique genomic regions. Three sequences (RDA1, RDA3 and RDA4) were identified. RDA1 (229 bp) contained a single base difference between S and C strains. RDA4 (163 bp) was an artefact. RDA3 (206 bp) was similar to several sequences in the incomplete genome sequences of *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *avium* 104. In silico analysis led to the identification of a deletion that may be as large as 17 kb in the sheep strain of *M. a. paratuberculosis*. PCR analysis of this region confirmed the deletion of 11, 584 bp that included 10 genes (MAP1734 to MAP1743c) of the *M. a. paratuberculosis* K10 genome. This included the loss of *mmpL5* and *mmpS5* genes and homologues of the *M. tuberculosis* genes: Rv2002 (*fabG3*), Rv2017c (*lipW*), Rv3132c (*devS*), Rv2032 (*acg*) and the conserved hypothetical genes Rv2005c and Rv2026c. PCR reactions designed to detect the single nucleotide polymorphism in RDA1 and the deletion in the *mmpL* region can be used to distinguish these strains. *MmpL* genes, found in *M. tuberculosis* and other mycobacteria are part of the resistance-nodulation-division (RND) family but contain domains unique to mycobacteria thought to play a role in cell wall biogenesis, virulence and other phenotypic characteristics. Absence of *mmpL5* in the S strain of *M. a. paratuberculosis* is unlikely to account for the difference in clumping in suspension but may explain the difference in cultural requirements and host specificity compared to the C strain but the impact of the remainder of the deletion is yet to be ascertained.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Johne's disease; representational difference analysis; *mmpL*; in silico; genome comparison

1. Introduction

Johne's disease (JD) is a chronic enteric disease affecting many ruminant species that is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*). *M. a. paratuberculosis* strains can be divided into two groups using restriction fragment length polymorphism analysis (RFLP) and cultural characteristics. These are known as sheep (S) and cattle (C) strains [1]. The host range for the C strain is quite broad and includes cattle, goats, sheep and man [1,2] as well as a number of wild ruminants [3,4]. The S strain primarily affects sheep and to a lesser degree goats and deer but has been shown to occasionally cause JD in cattle [5]. In Australia, these host specificities have been shown to be strong [6,7]. In addition, S and C strains have different cultural requirements [8], ease of emulsification of colonies in saline and tendency for cells to clump together when in suspension [9]. Management of JD in Australia assumes that cattle are not readily susceptible to infection with the S strain and can safely graze on pasture with or after the removal of sheep with JD. However, these assumptions are based on epidemiological observations of the differences between ovine JD (OJD) and bovine JD (BJD). Additional microbiological evidence is required because little is known about the mechanisms of host

* Corresponding author.

[1] Present address: Elizabeth Macarthur Agricultural Institute, PMB 8 Camden, NSW 2570, Australia.

0890-8508/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2005.06.005

specificity and pathogenicity of the S and C strains of *M. a. paratuberculosis*.

Subtractive DNA hybridisation techniques were originally developed to identify large differences at the DNA level between biological samples that were phenotypically and genotypically very similar. For example Lamar and Palmer [10] used a subtractive hybridisation technique to identify differences between the X and Y chromosomes. The DNA from one sample (the driver) was used to subtract the DNA it had in common with another sample (the tester) leaving only the unique tester DNA. While these early techniques were satisfactory for identifying large genomic differences they did not work well when samples were more highly related. Straus and Ausubel [11] used a modification termed genomic subtraction to identify differences between wild type and deletion mutant strains of yeast, but the physical separation techniques were laborious. Representational difference analysis (RDA) [12] overcame these issues and led to the identification of differences between closely related viral pathogens. Subtractive nucleic acid techniques are now routinely used to identify genomic variability between highly related and complex biological entities and to find differentially expressed genes. Unlike microarray analysis, no prior genome sequence information is required to undertake RDA and nucleic acids are identified in a form available immediately for cloning.

In mycobacteriology, subtractive hybridisation techniques have been used in expression studies of *M. avium* subsp *avium* (*M. a. avium*) and *M. bovis* by comparing mRNA from organisms isolated from macrophages and cultured organisms [13–15], and genomes of closely related entities such as *M. a. paratuberculosis* and *M. a. avium* [16–19], *M. a. avium* and *M. intracellulare* [20], virulent and non-virulent *M. bovis* [21] and strains of the same subspecies of *M. avium* [22,23].

The aim of this study was to use a modified RDA method to compare the genomes of the S and C strains of *M. a. paratuberculosis* to understand differences in host specificity and other phenotypic characteristics.

2. Materials and methods

2.1. M. a. paratuberculosis isolates

DNA from one S strain (Telford 9.2) and one C strain (CM00/416) of *M. a. paratuberculosis* were used for RDA. DNA from a second C strain (316v, laboratory adapted) was used as a positive control in PCR experiments. The former isolates were grown on modified 7H10 plus mycobactin J slopes at 37 °C for up to 3–4 months [8] while 316v was grown in modified Watson-Reid medium with mycobactin J [24] at 37 °C for 1–2 months. The cultures were harvested using sterile disposable inoculating loops for slopes or by centrifugation at 2250 *g* at 4 °C for 30 min for broth cultures. The pellets were washed three times with sterile PBS and the resulting cell pellet was stored at −70 °C until required. Strain identity of each DNA preparation (see below) was confirmed by IS*1311* PCR/REA as described [25] and IS*900* RFLP [26].

2.2. DNA extraction

To prevent cross contamination of DNA samples, all work undertaken to extract DNA from the S and C strains of *M. a. paratuberculosis* was performed separately. DNA extraction was performed as described by Choy et al. [26]. The bacterial pellet, 0.4–0.5 g (wet weight), was re-suspended in 1 mL of TE in a sterile 1.5 mL centrifuge tube. The pellets were vigorously mixed using a sterile inoculation loop followed by vortexing (1–2 min) to break up any clumps of aggregated bacteria. The bacteria were then killed by incubation at 80 °C in a hybridisation oven for 30 min after which they were allowed to cool to room temperature for 10 min. DNA extraction was commenced by adding 120 μL of lysozyme solution (200 mg/mL in 10 mM Tris) and 200 units of mutanolysin (20 μL of a 10,000 units per mL stock) to the cell pellets. The cells were gently mixed and incubated overnight at 37 °C with very gentle end over end mixing on a blood mixer (Ratek). The cells were transferred to a 10 mL centrifuge tube followed by the addition of 70 μL of proteinase K solution (10 mg/mL), 210 μL of 10% w/v SDS and incubated at 65 °C (hybridisation oven) for 20 min with gentle mixing (by hand) every 5 min. One hundred and ninety five microlitres of 5 M NaCl and 165 μL CTAB/NaCl, both pre-warmed to 65 °C, were added and gently mixed (by hand) until 'milky' followed by incubation at 65 °C (hybridisation oven) for 10 min. The DNA was extracted and purified using a modified chloroform/isoamyl alcohol technique. An equal volume of 24:1 chloroform/isoamyl alcohol was added and mixed gently, end over end, for 10 s or until an emulsion formed. DNA in the aqueous phase was separated using a phase lock gel system (Eppendorf Cat No. 0032.005.250) according to the manufacturer's instructions. The aqueous phase was poured off into a 10 mL centrifuge tube and 0.6 times the volume of isopropanol was added. This was mixed by inversion and incubated at −20 °C for 2 h. The DNA was transferred, to a new 10 mL centrifuge tube containing ice cold 70% ethanol, using a sterile plastic inoculation loop. The DNA was centrifuged at 2250 *g* at 4 °C for 30 min. The supernatant was removed and the DNA was dried at room temperature with the lid slightly off. The purified DNA was resuspended in sterile TE (pH 8.0) by gentle end over end mixing at 37 °C for 1 h. If the DNA had not fully dissolved this was continued over night at 4 °C. DNA samples were then stored at 4 °C. The concentration of the DNA was calculated by spectrophotometry (Pharmacia GeneQuant II) using the formula ($\mu g/mL = A_{260} \times D.F. \times 50$) where D.F. is the dilution factor.