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FEMS Microbiology Letters 185 (2000) 189–192

FEMS MICROBIOLOGY Letters

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The PCR amplification of non-tuberculous mycobacterial 16S rRNA sequences from soil

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Received 6 January 2000; received in revised form 7 February 2000; accepted 23 February 2000

Abstract

Non-tuberculous mycobacteria are free living saprophytic organisms commonly found in soil and water. Some are major causes of opportunistic infection, particularly in immuno-compromised patients, and may influence the efficacy of bacille Calmette-Guérin vaccinations. Many of these organisms are not amenable to culture, so information about their distribution is limited. PCR primers designed to amplify part of the mycobacterial 16S rRNA gene were applied to DNA extracted from cultured organisms and soil. The PCR products from soil contained sequences with similarity to slow growing mycobacteria similar to *Mycobacterium lentiflavum*, and to fast growing mycobacteria such as the xenobiotic degraders PYR-I and RJGII. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Non-tuberculous mycobacterium; Soil; 16S rRNA; PCR

1. Introduction

Non-tuberculous mycobacteria (NTM) are a group of saprophytic organisms that have been isolated from a range of environments including soils [1–3], rivers [1], lakes and water supplies [4,5]. Some of these environmental NTM are opportunistic pathogens, capable of infecting patients who are immuno-compromised or have a pre-disposing lung disease [6]. With the advent of the AIDS epidemic, NTM have become a more serious clinical problem with patients becoming infected predominantly from the environment rather than from people [2,5,7].

In addition to causing infection, exposure to environmental NTM may also be responsible for regional variation in the efficacy of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine. [8]. Exposure of animals to certain environmental NTM can cause sensitisation to mycobacteria [9] and lead to levels of protection against virulent strains similar to that conferred by BCG [10]. The effectiveness of subsequent BCG vaccinations can be masked or modulated by such prior exposure to NTM [11]. Thus, variation in the efficacy of the BCG vaccination may be associated with the regional differences in diversity and prevalence of environmental NTM. Soil is potentially a major reserve, and thus a source of exposure to environmental NTM. Therefore, an improved understanding of the distribution of environmental NTM within soils is important.

Many mycobacteria have been cultured from soils using methods designed to isolate virulent mycobacteria from clinical samples. Typically these studies isolate a variety of species including fast growing organisms such as *Mycobacterium fortuitum* [12] and putative pathogens including organisms from the *Mycobacterium avium* complex [2]. However, these methods are not suitable for detecting all environmental NTM [13] and so cannot fully describe the mycobacterial diversity within soils. The use of PCR to amplify 16S rRNA sequences directly from the soil overcomes the need to culture soil organisms, and so avoids the bias inherent in classical microbiological isolation methods [14]. PCR primers specific for mycobacterial 16S rRNA genes have been designed to amplify sequences from a wide range of species [15,16]. Such primers have not previously been used to amplify soil DNA. In this study, primers specific for mycobacterial 16S rRNA genes were developed and applied to DNA extracted directly from soil to examine mycobacterial diversity.

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PII: S0378-1097(00)00101-4

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2. Materials and methods

Ten soil samples were taken from arable plots at Rothamsted Experimental Station, which have been under a 5-year wheat rotation. Samples were mixed, sieved and DNA extracted from a 1-g subsample using the method described by Cullen [17]. DNA was extracted as described by van Soolingen et al. [18] from cultured isolates of *Mycobacterium chelonae*, *Mycobacterium xenopi*, *M. avium/intracellulare*, *Mycobacterium mucogenicum*, *Mycobacterium kansasii* and several unnamed mycobacteria all originally from Malawi, held at the *Mycobacterium* Reference Unit, Public Health Laboratory Service, Dulwich, UK.

Primers for the mycobacterial 16S rRNA gene were designed using the GCG8 suite of programs (Genetics Computer Group, Madison, WI, USA). Sequences representative of all the major clades of the mycobacterial 16S rRNA phylogenetic tree [19] were aligned using the program LOCALPILEUP. Regions of similarity were identified and prospective primers compared to the GenEmbl and Ribosomal Database Project II (RDPII) [19] databases using the programs FASTA and PROBE [19], respectively. Primers with maximal similarity to mycobacterial sequences, but minimal similarity to the 16S rRNA genes of other organisms, were selected. The primer sequences were 5′-ATGCACCACCTGCACACAGG and 5′-GGTGGTTTGTCGCGTTGTTC. PCR reactions contained 10 mM Tris–HCl, pH 8.3, 100 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.8 μM primers, 0.04 U μl^{-1} Taq (Roche Diagnostics, Lewes, UK) and soil DNA, equivalent to 50 μg fresh soil. PCR cycle conditions were 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 45 cycles were used for soil DNA and 25 cycles for DNA from cultured isolates. Each PCR was finished with a final extension at 72°C for 5 min. Agarose gel-purified PCR products were cloned using TA cloning kits® (Invitrogen, Groningen, The Netherlands). Plasmids were prepared with Qiagen Mini kits (Qiagen, Crawley, UK) and sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer, Warrington, UK). To confirm that PCR products from cultured isolates were mycobacterial, they were restriction-digested by adding 2 μl×10 ‘O’ reaction buffer (500 mM Tris, pH 7.5, 100 mM $MgCl_2$, 1 M NaCl, 1 mg ml^{-1} BSA) and 10 U *Eco*130I (Helena Bioscience, Sunderland, UK) to 20 μl of PCR product and incubating at 37°C for 18 h.

Cloned sequences from PCR amplifications of soil DNA were compared to the GenEmbl and RDPII databases using the programs FASTA and Sequence Match [19], respectively, and aligned with the most closely related sequences using LOCALPILEUP and CLUSTALW [20]. Transition to transversion ratios was estimated using the program PUZZLE [21]. Ambiguous positions in the alignments were removed and distance matrices and trees generated using the PHYLIP V3.57 suite of programs [22] using DNADIST (Kimura-2-parameter method), DNAML and NEIGHBOUR (neighbour joining).

The 16S rRNA sequences from this study have been deposited in the GenEmbl databases under accession numbers AJ269557–AJ269566, named myco 1–myco 10.

3. Results and discussion

The forward and reverse primers were identical to 50% and 82%, respectively, of the *Mycobacterium* sequences in the RDPII database [19] and had single mismatches with the majority of the other mycobacterial sequences. Non-mycobacterial sequences had significantly less similarity to the primers. Thus, although not inclusive, the primers were predicted to be specific for mycobacterial 16S rRNA genes. Mycobacterial 16S rRNA gene product of the predicted 470-bp size was amplified from all the cultured isolates tested and from soil DNA. Digestion of PCR products from the cultured isolates with *Eco*130I to give 261- and 208-bp fragments was a convenient method of confirming the identities of the products. The product from *M. xenopi* was an exception: it was not predicted to, and did not, digest with *Eco*130I.

All of the 10 16S rRNA sequences isolated from the soil contained the *Eco*130I restriction site, were homologous to other mycobacterial 16S rRNA sequences and could be placed phylogenetically within the *Mycobacterium* clade. Most of the variable positions in regions encoding 16S rRNA stem structures contained complementary substitutions. The sequences formed three groups (Fig. 1). Group A contained sequences myco 1, myco 2 and myco 3 which were most similar, and phylogenetically closest to the fast growing xenobiotic degrading soil mycobacteria PYR-I [23] and the related environmental isolate *Mycobacterium austroafricanum* [24]. These sequences may be from similar pollutant degrading organisms, although the site is not contaminated. Alternatively, they may be representative of a more extensive and perhaps widespread group of mycobacteria, from which xenobiotic degrading strains have been selected for by the soil contaminants or by the procedures used to isolate them. Group B contained sequences myco 4, myco 5 and myco 6 which were most similar to, and phylogenetically associated with, the slow growing *Mycobacterium heidelbergense* and *Mycobacterium lentiflavum* [25]. The isolation of a sequence similar to myco 4 by McVeigh et al. [14] when examining the diversity of actinomycetes in woodland soils suggests that these organisms are not uncommon in the environment. Group C contained the sequences myco 7, myco 8, myco 9 and myco 10, which could not be reliably associated with any particular mycobacterial group. They may represent a novel group of mycobacteria, or may be an artefact of the PCR and cloning processes. They do show some indications of being chimeric although it would be unusual to generate such a large proportion of chimeras

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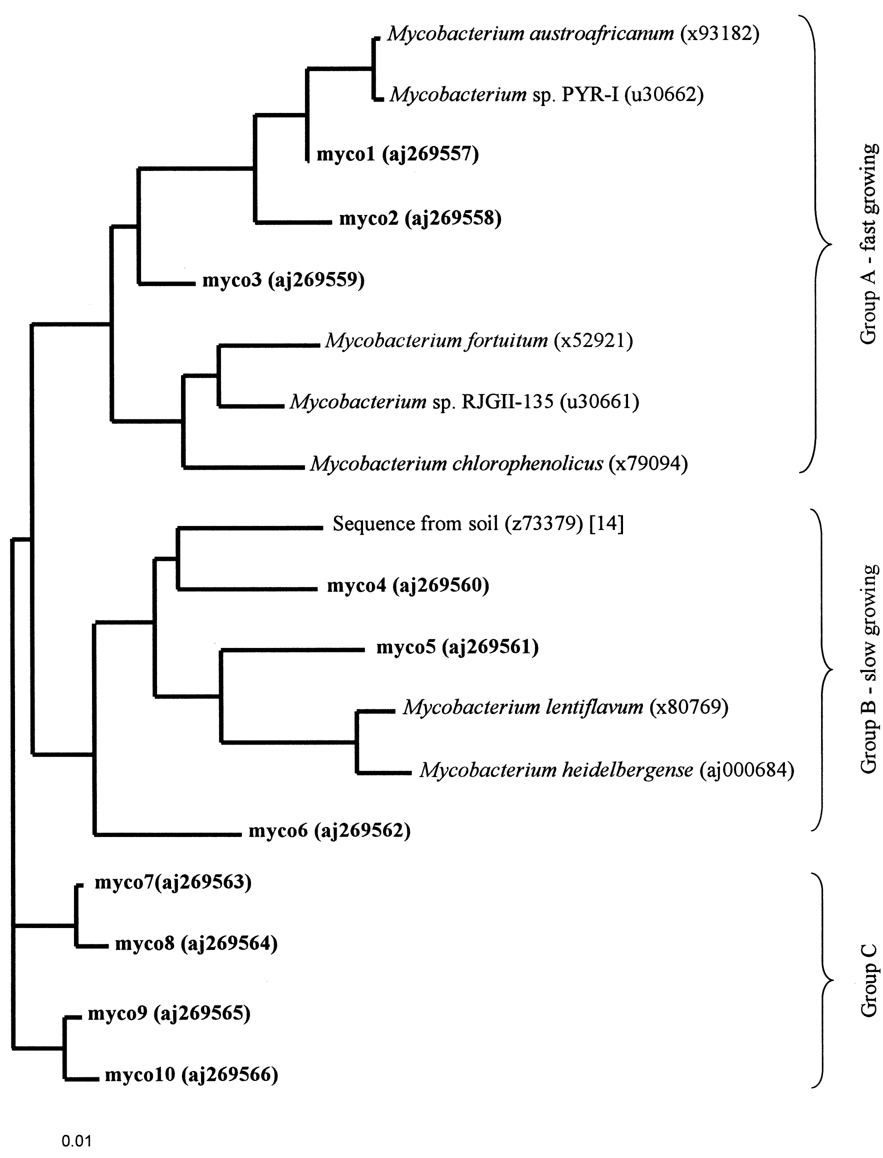

Fig. 1. A phylogenetic tree constructed using DNADIST and NEIGHBOUR [22] showing the relationship between sequences isolated from soil in this study (in bold) and the most similar sequences from the GenEmbl database, accession numbers are shown in brackets.

using these methods. The clinical significance of the sequences from this study cannot be determined, indeed, unless cultured, an organism's properties can only be inferred from its 16S rRNA sequence. However, no sequences similar to those of the virulent mycobacteria were isolated.

This study has demonstrated the utility of these primers in amplifying DNA extracted from cultured isolates, and directly from soil. Although the primers may not fully describe the mycobacterial diversity of this soil, they do demonstrate that both fast and slow growing groups are prevalent. Using these primers to amplify gene sequences directly from environmental samples has considerable potential to further our knowledge of environmental mycobacterial diversity. By examining samples from areas such as Malawi or Southern India, where the variable efficacy of the BCG vaccine has been studied, the relationship between uncultured environmental NTM and the vaccine can be investigated.

Acknowledgements

Ben Chilima is supported by EU Grant ERBIC18TC970254. We thank Koen de Smet, Belgian Free University, for useful advice and Malcolm Yates of the Mycobacterial Reference Unit, Public Health Laboratory Service, for supplying the cultured reference strains. IACR receives grant-aided support from the Biotechnology and Biological Research Council of the UK.

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