MOLECULAR CHARACTERISATION OF BACTERIA

16S rRNA sequence analysis of an isolate of *Mycobacterium haemophilum* from a heart transplant patient

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Summary. Biopsy samples from a heart transplant patient with cellulitis and bursitis yielded an isolate of *Mycobacterium haemophilum*. The isolate was identified on the basis of a growth requirement for haemin or ferric ammonium citrate, growth at 30°C but not at 37°C, negative catalase test, intracellular growth in McCoy fibroblasts and sequence identity with a portion of the 16S rRNA sequence of the type strain. In comparisons with known 16S rRNA sequences, *M. haemophilum* grouped with other pathogenic, slow-growing mycobacteria, showing close sequence similarity to *M. marinum* (98.8%) and lower similarity to *M. ulcerans* and *M. tuberculosis* complex organisms. *M. haemophilum* and *M. marinum* share other features including optimal growth at 30°C and the ability to cause superficial skin lesions in man.

Introduction

Mycobacterium haemophilum is a slow-growing, strongly acid- and alcohol-fast bacillus, first described by Sompolinsky *et al.*,¹ that requires haemin or ferric ammonium citrate for growth,² shows no detectable catalase activity and grows optimally at 30°C. In contrast to other slow-growing mycobacteria that grow on McCoy fibroblasts, *M. haemophilum* grows intracellularly in these cells.⁴ It is an opportunist pathogen most commonly isolated from superficial skin lesions in organ transplant patients.⁵ Infections have also been documented in three AIDS patients, two with disseminated infection and one with tenosynovitis.^{6,7} The true incidence of infection with *M. haemophilum* may be underestimated because of its fastidious growth requirements, but the incidence will probably increase with the rising number of HIV-positive patients.

Wayne and Sramek speculated that *M. haemophilum* could be a biochemically inert or fastidious subspecies of *M. avium*.⁵ In this report, 16S rRNA gene sequence data for an isolate of *M. haemophilum* are presented and compared with known sequences for other mycobacteria.

Materials and methods

Isolation and identification

Skin and cephalic vein biopsy samples from the right arm of a heart transplant patient with cellulitis and bursitis of his right elbow were submitted to the microbiology laboratory, Groote Schuur Hospital, for routine bacterial and mycobacterial culture. Standard bacterial and mycobacterial culture media were inoculated, along with Lowenstein and Jensen slopes (LJ) with and without ferric ammonium citrate (FAC, 15 mg/ml).² The latter were cultured at 30°C.

Identification tests comprised: Gram and Ziehl-Neelsen (ZN) stains; confirmation of growth temperature range and iron dependence by subculture on LJ with and without FAC incubated at 30°C and 37°C; culture on boiled horse blood 5% agar slopes incubated aerobically and in a candle jar at 30°C; culture on a confluent monolayer of McCoy fibroblasts stained for acid- and alcohol-fast bacilli after incubation for 4, 6, 8 and 10 days at 30°C;¹ and tests for niacin production, catalase (semi-quantitative), nitrate reduction, urease, Tween 80 hydrolysis and arylsulphatase activity.⁸

Cloning of the *M. haemophilum* 16S rRNA gene

The *Escherichia coli* pUC19 vector was used in all cloning experiments.⁹ Chromosomal DNA was purified from *M. haemophilum* as described previously.¹⁰

The 16S rRNA gene was amplified by PCR with the bacterial small subunit ribosomal primers pA and pH.¹¹ The reaction mixture contained: 2 mM of each dNTP; 2 units of *Taq* polymerase (Boehringer Mannheim); 100 pmols of each primer; and 10 µl of genomic DNA (*c.* 100 ng) in 50 µl of *Taq* buffer (Boehringer Mannheim) overlaid with 50 µl of mineral oil. This was incubated at 95°C for 5 min, then subjected to 35 cycles of: 93°C for 20 s (denaturation), 60°C for 30 s (annealing) and 72°C for 60 s (primer extension). The amplified product was separated by agarose gel electrophoresis, eluted from the gel¹² and cloned by the T-A cloning method. Briefly, this method exploits the template-independent terminal transferase activity of *Taq* polymerase which results in the addition of a single adenosine, or, in conditions where only thymidine is provided, a single thymidine to the 3' end of the PCR production during amplification.¹³ T-tailed vector was made by use of this activity by incubating blunt-ended pUC19 (1 µg) for 2 h at 70°C with *Taq* polymerase (1 U) and dTTP (2 mM) in 20 µl of *Taq* buffer. Ligation of the PCR products to a T-tailed vector resulted in cloning efficiencies similar to sticky-end cloning.¹⁴

Sequence determination

The cloned 16S rRNA gene was sequenced (Pharmacia ³²P Sequencing Kit) by the dideoxy method.¹⁵ The DNA insert was mapped and restriction fragments were subcloned in pUC19 for sequencing with M13/pUC19 universal primers. Where no suitable restriction sites were available for subcloning, custom primers were synthesised. The gene was sequenced in both orientations.

Data analysis

The sequence was compared with known sequences deposited in the Ribosomal Database Project (RDP) with the SIMILARITY-RANK program which finds similar deposited sequences, and the ALIGN-SEQUENCE program.¹⁶ A mycobacterial phylogenetic tree was generated (courtesy of Ross Overbeek, RDP) and percentage similarities for the organisms clustered with *M. haemophilum* were calculated with the FASTA programme.¹⁷ The *M. haemophilum* sequence was submitted to GenBank, accession number U06638.

Results and Discussion

The patient, a 54-year-old male, was admitted to Groote Schuur Hospital in February 1988 with end-stage cardiac failure after previous myocardial infarctions. He received a heart transplant and was discharged 2 weeks later without any evidence of tissue rejection. Three months later he developed cellulitis over his right elbow while receiving azathioprine 150 mg daily, methyl prednisolone 14 mg twice daily and cyclosporine 1.9 ml twice daily. At biopsy of the infected skin and basilic vein, the vein was found to be

thrombosed and acid-fast bacilli were found in the biopsy specimens. Anti-tuberculous therapy with daily rifampicin 600 mg, isoniazid 400 mg, pyrazinamide 2 g and ethambutol 1.2 g, was started. Due to the difficulty in controlling the infection, and because there was no evidence of tissue rejection, the methyl prednisolone was stopped. The cellulitis steadily improved and within 2 months after initiation of anti-tuberculous therapy, no evidence of inflammation was present. The patient was re-admitted 2 months later with severe shortness of breath and bilateral lung infiltrates due to cytomegalovirus infection. His clinical course was stormy and, after several septicaemic episodes, he died in septicaemic shock. At autopsy no suppurative disease was noted, but no material was submitted for bacterial culture.

The initial skin and vein biopsies contained numerous acid- and alcohol-fast bacilli seen on direct microscopy, but yielded no growth from routine cultures, nor from mycobacterial cultures other than those on LJ with FAC incubated at 30°C. After incubation for 4 weeks on this medium, non-pigmented, buff coloured, smooth colonies were observed. The organism was a weakly staining gram-positive bacillus, strongly acid- and alcohol-fast, with erratic, short cording. Iron dependence and growth temperature range were confirmed by subculture on LJ with and without FAC at 30°C and 37°C. Cultures at 30°C on boiled blood agar yielded rough, waxy colonies with an undulating surface. CO₂ enrichment did not enhance growth, in contrast to earlier descriptions.¹⁸ Culture on McCoy cells showed intracellular acid- and alcohol-fast bacilli, most evident after incubation for 4 days. Biochemical tests gave negative results, with the exception of nitrate reductase, which was weakly positive. These characteristics supported an identification of the isolate as *M. haemophilum*—particularly the negative catalase test which distinguishes *M. haemophilum* from *M. marinum* and *M. ulcerans*.³

The sequence of the cloned 16S rRNA gene was 1489-bp long, excluding the universal primer sequences. Comparison with known 16S rRNA gene sequences placed *M. haemophilum* in a cluster with other slow-growing pathogenic mycobacteria comprising *M. marinum*, *M. ulcerans*, *M. tuberculosis*, *M. bovis* and *M. leprae* (table). *M. marinum* (X52920) showed the closest similarity (98.8%), with sequence divergence mainly in the region between bases 137 and 152 (9 of 16 mismatches; figure). PCR amplification and direct sequencing of this region from the type strain of *M. haemophilum* (ATCC 29548) yielded a sequence identical to that obtained for the clinical isolate. The 16S rRNA sequence for another, otherwise undescribed, *M. haemophilum* isolate (GenBank accession number L24800) showed only 98.8% similarity with our isolate, with differences scattered, apparently randomly, through the sequences. Similarities in the phenolic 1-glycolipid antigens and fatty acid profiles of *M. haemophilum* and *M. leprae* have been noted.^{19,20} A similarity of 98.0% in 16S

Table. Similarities in 16S rRNA gene sequences for the mycobacteria showing closest sequence similarity to *M. haemophilum*

Species	GenBank accession number	Similarity (%) of aligned 16S rRNA sequence with							
		M20940	X55589	L24800	U06638	X55587	X52920	X52917	Z13990
<i>M. bovis</i>	M20940	100.0							
<i>M. bovis</i>	X55589	99.6	100.0						
<i>M. haemophilum</i>	L24800	97.7	97.3	100.0					
<i>M. haemophilum</i>	U06638	98.6	98.2	98.8	100.0				
<i>M. leprae</i>	X55587	96.6	96.3	96.3	98.0	100.0			
<i>M. marinum</i>	X52920	99.1	98.8	97.8	98.8	96.5	100.0		
<i>M. tuberculosis</i>	X52917	99.9	99.6	97.6	98.6	96.6	99.0	100.0	
<i>M. ulcerans</i>	Z13990	98.2	98.0	97.0	97.8	95.6	99.0	98.2	100.0
<i>M. ulcerans</i>	X58954	99.0	98.7	97.7	98.7	96.4	99.9	99.0	99.1

Figure. Alignment of the portion of the 16S rRNA gene sequences showing maximal sequence divergence between the *M. haemophilum* isolate (U06638) and *M. marinum* (X52920).

rRNA sequences for *M. leprae* (X55587) and our *M. haemophilum* isolate was found, making this the closest neighbour of *M. leprae*.

The close similarity of *M. haemophilum* and *M. marinum* sequences was unexpected, as it has been suggested that *M. haemophilum* is related to the *M. avium* complex.⁵ However, *M. marinum* also causes infections that tend to be limited to skin surfaces,⁵ and shows optimum growth at 30°C, which may be the reason for superficial dissemination of these infections.

It may be informative to investigate the host range of *M. haemophilum* in cold-blooded vertebrates, to establish whether these species occupy a similar ecological niche.

We thank Professor A. Forder, Department of Medical Microbiology, Groote Schuur Hospital, for his encouragement and support, the Department of Cardio-thoracic Surgery for clinical information, R. Overbeek for the mycobacterial phylogenetic tree and Dr Gay Elisha for her comments on the manuscript. This work was supported by grants from the Medical Research Council, University of Cape Town and DG Murray Trust.

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