**Separation of *Mycobacterium gadium* from Other Rapidly Growing Mycobacteria on the Basis of DNA Homology and Restriction Endonuclease Analysis**

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DNA was isolated from *Mycobacterium gadium* with high purity. Its G + C content was between 64 and 67 mol%. The homology of *M. gadium* DNA with DNA from three other rapidly growing mycobacteria was less than 22%, which indicates that *M. gadium* is a discrete genomic species. Analysis of the DNAs with restriction endonucleases supported this finding.

**INTRODUCTION**

*Mycobacterium gadium* is a rapidly growing species of mycobacterium that was originally isolated from sputum (Casal & Calero, 1974). In 1980, although only one strain (registered as ATCC 27726) had been recognized, *M. gadium* was accepted as a clearly defined species, with ATCC 27726 as the type strain (Skerman et al., 1980). Goodfellow & Wayne (1982) included this species in a group of rapidly growing scotochromogenic mycobacteria, together with *M. gilvum*, *M. duvalii* and *M. komossense*. The similarities or differences between the species of this group (all recently described) have still to be defined. Tsukamura et al. (1981) included *M. gadium* in a numerical taxonomic study of rapidly growing mycobacteria. Only the type strain was studied, but the results indicated the possibility of a clearly defined species. Tsukamura (1983) reported a more detailed study of this species, with a biochemical characterization of four strains (including the type strain). Jenkins (1981) found no specific pattern of lipids for *M. gadium*, but Hall & Ratledge (1984) showed that its mycobactin composition was clearly different from those of other mycobacteria.

Together with numerical taxonomy, DNA:DNA hybridization is an important tool in the characterization of species (Pohl, 1981). However, few DNA:DNA hybridization studies have been done with mycobacterial species, because of difficulties in isolating their DNA (Baess, 1974; Yandell & McCarthy, 1980; Lévy-Frébault et al., 1984). In the present study a highly efficient method was used to isolate DNA from selected mycobacteria, and the DNA was used for DNA:DNA hybridization studies and restriction endonuclease analysis to investigate the relationship of *M. gadium* to other species.

**METHODS**

**Bacterial strains and growth media.** Four species of rapidly growing mycobacteria were studied: *M. fortuitum* ATCC 6841 (type strain), *M. gadium* ATCC 27726 (type strain), *M. gilvum* Stanford 132 and *M. phlei* IMRU 500 were grown in Lowenstein-Jensen medium (Difco) at 37 °C until colonies were observed (ATCC, American Type Culture Collection; IMRU, Institute of Microbiology, Rutgers University).

**Extraction and purification of DNA.** Strains were grown in 200 ml Dubos-albumin medium (Difco) at 37 °C in a gyratory shaker for 3–8 d. The cells were lysed enzymically (Simpson & Wayne, 1977) and DNA was purified by MAK (methylated albumin kieselguhr) chromatography (Tabarés et al., 1973).

**Abbreviation:** MAK, methylated albumin kieselguhr.
Briefly, the lysate was extracted twice with (70 ml m-cresol, 55 ml water and 0.5 g 8-hydroxyquinoline were added to 500 g phenol) (Tabarés et al., 1973) saturated with 0.1 M-NaCl, 0.1 M-Tris/HCl pH 9.0 and 0.5% (w/v) sodium deoxycholate. The mixture was centrifuged (8000 g, 10 min at 4 °C) and the aqueous phase was diluted with 3 vols 0.1 M-NaCl in 0.05 M-phosphate buffer pH 6.8. Pancreatic ribonuclease A (Sigma) was added to give a final concentration of 0.1 mg ml⁻¹, and the mixture was incubated at 37 °C for 30 min. The solution was diluted with 4 vols 0.1 M-NaCl in phosphate buffer pH 6.8 and the DNA was purified on an MAK column at 4 °C. After washing the column with 0.1 M-NaCl and 0.2 M-NaCl, the DNA was eluted with 1 M-NaCl. The DNA was located by absorbance at 260 nm. The solution was dialysed, at 4 °C, in 1 mM-EDTA and 10 mM-Tris/HCl pH 7.5 (TE buffer). The solution was precipitated with ethanol and stored at −20 °C.

About 0.4 mg mycobacterial DNA was obtained per g cells (wet weight). The MAK column chromatography used in the present study improved the yield of DNA over that obtained by other methods, and also permitted the isolation of sufficiently pure preparations (as indicated by A₂₆₀/A₂₈₀ ratios) with high-M₁ DNA.

Determination of DNA base composition. Two methods were used.

(i) Buoyant density in caesium chloride (Mandel et al., 1968). A 30–40 μg sample of DNA was dissolved in 1 ml 0.1 M-Tris/HCl (pH 7.2); the initial density was 1.7220 g ml⁻¹, and the mixture was incubated at 37 °C for 30 min. The solution was diluted with 4 vols 0.1 M-NaCl in phosphate buffer pH 6.8 and the DNA was purified on an MAK column at 4 °C. After washing the column with 0.1 M-NaCl and 0.2 M-NaCl, the DNA was eluted with 1 M-NaCl. The DNA was located by absorbance at 260 nm. The solution was dialysed, at 4 °C, in 1 mM-EDTA and 10 mM-Tris/HCl pH 7.5 (TE buffer). The solution was precipitated with ethanol and stored at −20 °C.

(ii) Melting point of DNA (Mandel & Marmur, 1968). Thermal denaturation of DNA (50 μg ml⁻¹) was measured in 0.1 M-Tris/HCl (pH 7.2); the initial density was 1.7220 g ml⁻¹, calculated from the refractive index of the solution. The DNA was centrifuged for 62 h at 98500 g, 25 °C, in a Beckman T-50 Ti fixed-angle rotor. The gradients were fractionated by withdrawal from the bottom of the tube. The density of each fraction (0.25 ml) was measured in 0.1 M-NaCl and 0.4 M-NaCl, the DNA was eluted with 1 M-NaCl. The DNA was located by absorbance at 260 nm. Three determinations were performed for each DNA. The G + C content (mol%) was calculated from the buoyant density of the DNA by the formula of Mandel et al. (1968), using herpes simplex DNA (70 mol% G + C) as standard.

Radioisotopic DNA. DNA was labelled with 32P by the nick translation procedure of Rigby et al. (1977), using the BRL nick translation reagent kit. Specific activities of approximately 2–3 × 10⁶ c.p.m. (μg DNA)⁻¹ were obtained.

DNA: DNA hybridization. Hybridization was done in modified Denhart’s medium (Post et al., 1980) with nitrocellulose filters (Schleicher & Schuell BA85) as support. Mycobacterial DNA solutions in TE buffer were fixed to the nitrocellulose filters (10 mm diameter) by the method of Post et al. (1980) (about 10 μg DNA per filter), except that the filters were kept in the denaturation and neutralization solutions for 2 min. Under our conditions fixation to the filters was 60–70%. Calf thymus DNA (Sigma) fixed, and filters without DNA were used as controls. Filters containing denatured DNA were kept in a vacuum desicator at 80 °C for 4 h.

The hybridization was performed for 40 h at 55 °C in vials containing 1 ml Denhart’s medium and 0.5 μg mycobacterial [³²P]DNA. Two determinations were made for each assay. Each vial contained six filters: four with DNA from different mycobacteria, one with calf thymus DNA and one without DNA. The ratio labelled DNA:unlabelled DNA was approximately 1:60. After incubation, the filters were washed three times with 2 × SSC at room temperature, and again incubated with modified Denhart’s medium at 55 °C for 15 min. This process was repeated twice. The filters were dried at 80 °C for 15–20 min and the radioactivity was measured with a Packard Tri-Carb 300 liquid scintillation counter. The radioactivity measured on the filters with attached DNA homologous to the labelled DNA in solution was taken as 100%.

Restriction endonuclease analysis. Samples of mycobacterial DNA (2 μg) were digested with about 20 U of each of the following type II restriction endonucleases (Roberts, 1983), using the temperature and buffer specified by the supplier: BglII, Clal EcoRI (all BRL), and HindIII (New England Biolabs). Digests were analysed by gel electrophoresis on 25 cm horizontal slabs of 0.5% (w/v) agarose in Loening’s buffer (Maizel, 1969). Herpes simplex type 2 DNA digested with BglII and HindIII was used as electrophoretic standard (Morse et al., 1977). After electrophoresis (20 h at 50 V), the gels were stained for 30 min in ethidium bromide (1 mg l⁻¹) and photographed under UV at 300 nm.

RESULTS AND DISCUSSION

The preparations of DNA from the mycobacteria exhibited A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios of 1.8–2.0 and 1.7–2.2, respectively, and hyperchromicities (increases in absorbance at 260 nm) between 31% and 33%.

The DNA base compositions (Table 1) were within the range for most mycobacteria (Imaeda et al., 1982). The values obtained for M. fortuitum and M. phlei were in agreement with previously published data (Baess & Mansa, 1978; Lévy-Frêbault et al., 1984). The G + C content
DNA from Mycobacterium gadium

Fig. 1. Agarose gel patterns of mycobacterial DNA digested with restriction endonucleases. (a) Herpes simplex type 2 DNA digested with BglII; fragment sizes (kbp) (Morse et al., 1977) are indicated on the left. (b–d) Mycobacterial DNA digested with BglII (b), CluI (c) and HindIII (d). (e) Undigested mycobacterial DNA. Lanes 1, M. fortuitum ATCC 6841; lanes 2, M. gadium ATCC 27726; lanes 3, M. gilvum Stanford 132; lanes 4, M. phlei IMRU 500.

Table 1. Base composition of genomic DNA from four species of rapidly growing mycobacteria

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Mean $T_m$ (°C)*</th>
<th>Mean $G + C$ †</th>
<th>Mean buoyant density (g ml⁻¹)‡</th>
<th>Mean $G + C$ †</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. fortuitum ATCC 6841</td>
<td>81.8</td>
<td>68 ± 0.3</td>
<td>1.7256</td>
<td>67 ± 0.4</td>
</tr>
<tr>
<td>M. gadium ATCC 27729</td>
<td>81.7</td>
<td>67 ± 0.2</td>
<td>1.7227</td>
<td>64 ± 0.4</td>
</tr>
<tr>
<td>M. gilvum Stanford 132</td>
<td>–</td>
<td>–</td>
<td>1.7227</td>
<td>64 ± 0.7</td>
</tr>
<tr>
<td>M. phlei IMRU 500</td>
<td>80.7</td>
<td>66 ± 0.25</td>
<td>1.7276</td>
<td>69 ± 0.9</td>
</tr>
</tbody>
</table>

* Standard deviations for duplicate determinations were all less than 0.2 °C.
† Mean values ± standard deviations.
‡ Standard deviations for triplicate determinations were all less than 0.0001 g ml⁻¹.

of DNA from M. gilvum, obtained by melting point determination by Lévy-Frébault et al. (1984) is in agreement with the value obtained in the present study by buoyant density analysis in caesium chloride.

The patterns obtained by analysis of mycobacterial DNA with the restriction endonucleases BglII and CluI (Fig. 1) and EcoRI (not shown) were clearly distinguishable from one species to another, and were consistently reproducible. All the DNA fragments obtained by digestion with these three restriction enzymes had molecular masses below 23.2 MDa (35.7 kbp). In contrast,
Table 2. DNA homologies (%) between four species of rapidly growing mycobacteria

The values shown are means ± standard deviations, obtained from eight determinations.

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>M. fortuitum</th>
<th>M. gadium</th>
<th>M. phlei</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. fortuitum</td>
<td>100</td>
<td>11 ± 4</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>M. gadium</td>
<td>9 ± 2</td>
<td>100</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>M. gilcicum</td>
<td>6 ± 1</td>
<td>11 ± 2</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>M. phlei</td>
<td>12 ± 1</td>
<td>22 ± 4</td>
<td>100</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>0.6 ± 0.2</td>
<td>1 ± 0.4</td>
<td>1 ± 0.5</td>
</tr>
</tbody>
</table>

HindIII gave little digestion of the four mycobacterial DNAs (Fig. 1d). This resistance to digestion by HindIII contrasts with the susceptibility of DNA from slow-growing mycobacteria to digestion with this enzyme (Collins & De Lisle, 1984).

The DNA homology studies also provided evidence that M. gadium is a discrete species. The homology between DNA from M. gadium and the other three species studied ranged from 6% to 22% (Table 2). The optical method of measuring DNA:DNA hybridization yields greater homology percentages than the nitrocellulose method (Schleifer & Stackebrandt, 1983). Nevertheless, the values for pairing of DNAs from rapidly growing mycobacteria obtained by the optical method were also generally low (Baess, 1982). These low DNA homology values indicate clearly defined species (Johnson, 1984). Generally, rapidly growing mycobacteria, even though they have many phenotypic properties in common, are not closely related genetically (Grange, 1982; Lévy-Frébault et al., 1984). It now appears that M. gadium, too, is distinct from other rapidly growing scotochromogenic mycobacteria.

REFERENCES


MORSE, L. S., BUCHMAN, T. G., ROIZMAN, B. &
DNA from Mycobacterium gadium


