Evidence for genetic divergence in ribosomal RNA genes in mycobacteria

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DNA was isolated from *Mycobacterium phlei* and from *M. smegmatis*. Each DNA sample was restricted with endonucleases, the fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose film. Fragments of DNA containing rRNA sequences were identified by means of ^{125}I-labelled rRNA of *M. phlei* or of *M. smegmatis*. The distributions of restriction endonuclease sites within the rRNA gene(s) and flanking sequences were found to be characteristic for each of the two species. Hybridizations with heterologous probes indicate that although *M. phlei* rRNA and *M. smegmatis* rRNA share regions of sequence homology, they are probably not identical in primary structure. The results suggest that the rRNA genes might prove to be useful taxonomic markers for mycobacteria.

Mycobacteria *rRNA gene* *Taxonomy* *Restriction-endonuclease site* *Nucleotide-sequence homolog*

1. INTRODUCTION

Classifying and identifying mycobacteria remain problems. Direct measurements of genomic properties provide the most reliable methods for determining relationships between mycobacteria. Information about specific genes, especially genes of pathogenic mycobacteria, could be used to identify, unequivocally, isolates of mycobacteria of clinical interest.

Mycobacterial DNA has been investigated by restriction endonuclease analysis [1] and by the construction of genomic libraries [2–4]. It is possible that rRNA genes might prove to be useful taxonomic markers for mycobacteria. Both prokaryotic and eukaryotic rRNA genes have been widely studied and have been shown to comprise sequences that have been conserved during evolution interspersed with other, divergent (non-conserved) sequences [5]. In eukaryotes, it is the differences in the non-conserved sequences that account for the variations in the nucleotide composition and in the mass of rRNA of different species [6,7].

We report an investigation of the properties of restriction fragments of the rRNA genes of *Mycobacterium phlei* and *M. smegmatis*. rRNA was isolated, made radioactive in vitro and then used to identify restriction fragments of DNA containing rRNA gene sequences. The genomes of these two species of bacteria were readily distinguished by this analysis. The procedures used may be applied to a wide range of mycobacteria.

2. MATERIALS AND METHODS

2.1. *Growth of bacteria*

M. phlei (NCTC 8151) and *M. smegmatis* (NCTC 10265) were grown in nutrient broth (Difco) containing 0.1% (w/v) Tween 80. Late exponential phase cultures were harvested by centrifugation for 10 min at 17000 × *g*.

2.2. *Lysis of cells*

Mycobacteria (1 g) were suspended in 20 ml lysis buffer (6 M guanidinium chloride (Sigma), 15 mM EDTA, 1 mM 2-mercaptoethanol (BDH), 0.1%

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(w/v) Tween 80). The cell suspension was cooled to −15°C and then passed through a French pressure cell (American Instrument, USA) at 850 kg/cm^2 [8]. The supernatants were collected at −15°C.

2.3. *Purification of rRNA*

The supernatant obtained in section 2.2 was gently agitated with poly(U)-Sepharose-4B for 2 h at 4°C. The poly(U)-Sepharose-4B and any associated poly(A)$^+$ RNA were removed by centrifugation for 10 min at 17000 × *g* (see [9]). Ethanol (0.5 or 0.75 vol.) was then added dropwise at 0°C and the solution kept at −20°C overnight. (The solution was kept at −20°C for 2–4 h when 0.75 vol. ethanol was added.) The precipitate of RNA was recovered by centrifuation at 4000 × *g* for 10 min (cf. [10]). The precipitate was dissolved in lysis buffer at 20°C and rRNA again precipitated by the addition of 0.75 vol. ethanol and cooling to −20°C for 2 h. The precipitate was redissolved in TE buffer (10 mM Tris, 2 mM EDTA, pH 7.5) and the solution stored at −20°C.

2.4. *Purification of DNA*

DNA was isolated by a modification of Marmur's [11] procedure [12].

2.5. *Spectrophotometry*

The absorbance of DNA and RNA samples was measured using a Varian 2200 spectrophotometer. E_p values at 260 nm of 6600 and 7700 l·mol^{-1}·cm^{-1}, respectively, were assumed for DNA and RNA.

2.6. *In vitro labelling of rRNA by [^{125}I]iodination*

rRNA was made radioactive with ^{125}I labelling [13] as described in [7]. Radioactivity was measured in a Packard Autogamma spectrometer. The specific activity was at least 2 × 10^6 cpm/μg *M. smegmatis* rRNA and at least 3.5 × 10^6 cpm/μg *M. phlei* rRNA.

2.7. *Hybridization with ^{125}I-labelled rRNA*

Nitrocellulose film was soaked in 2 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% (w/v) SDS for 10 min and then kept in prehybridization buffer supplemented with tRNA (10 μg/ml) at 42°C for 2 h. ^{125}I-labelled rRNA (5 × 10^4 cpm/ml) was then added and the incubation continued for a further 16 h. The nitrocellulose film was then washed with 4 × SSC, 0.1% (w/v) SDS for 1 h at 42°C, and then with 0.1 × SSC, 0.1% (w/v) SDS for 1 h at 50°C, dried and subjected to autoradiography at −70°C.

2.8. *Microdensitometry*

The absorbance of the photographic film was measured using a Joyce-Loebl Chromoscan microdensitometer. Several films which had been exposed for different periods of time were examined to ensure that the absorbance lay in the linear region of the dose-response curve.

3. RESULTS

3.1. *Identification of restriction enzyme generated fragments of rRNA genes*

Mycobacterial DNA was treated with restriction endonucleases. The fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose film [14]. The immobilised DNA was hybridized with ^{125}I-labelled rRNA probe and restriction fragments containing rRNA sequences were identified by autoradiography. The relative amount of ^{125}I-labelled probe hybridizing to each fragment was estimated by measuring the absorbance of the photographic film. The principal results are presented in fig.1 and tables 1 and 2.

3.2. *rRNA genes of* M. phlei

At least 2 *Eco*RI sites are located within the coding region of the rRNA genes and generate a 3 kbp fragment. Two *Eco*RI sites in DNA sequences flanking the rRNA genes give rise to the fragments of 6.4 and 16 kbp.

Five *Bam*HI fragments of *M. phlei* DNA hybridized with ^{125}I-labelled *M. phlei* rRNA judged by the number of bands on the autoradiograph (fig.1). Two fragments of 5.8 and 6.4 kbp which hybridize weakly with ^{125}I-labelled probe clearly contain flanking sequences as well as sequences coding for rRNA. Compared with the others, the band at 2.3 kbp is very intense (table 1) because 2 different fragments each of 2.3 kbp hybridized with the probe but were not separated by electrophoresis. These fragments were distinguished when a *Bam*HI/*Eco*RI double digest of *M. phlei* DNA was probed with ^{125}I-labelled *M. phlei* rRNA, and the autoradiograph revealed that

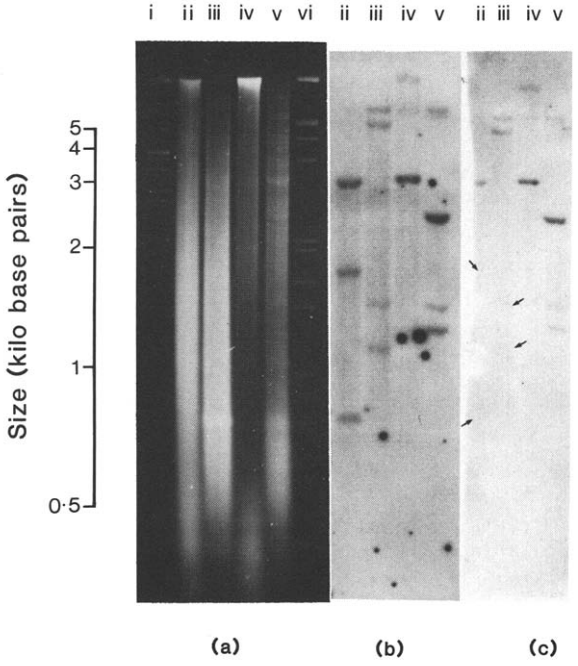

Fig.1. Hybridization of restricted mycobacterial DNA with ^{125}I-labelled mycobacterial rRNA. DNA was isolated from *M. phlei* and *M. smegmatis*. The DNA samples were treated with either *Eco*RI or *Bam*HI endonucleases, the digestion products separated by electrophoresis through 1% (w/v)-agarose gels and then transferred to nitrocellulose film [14]. DNA immobilised on nitrocellulose was then hybridized with ^{125}I-labelled rRNA isolated from *M. phlei* or *M. smegmatis* (see section 2). The location of radioactive RNA·DNA hybrids was established by autoradiography. (a) Ethidium bromide-stained gel. (b) Autoradiograph of nitrocellulose film after hybridization with ^{125}I-labelled rRNA from *M. smegmatis*. (c) Autoradiograph of nitrocellulose film after hybridization with ^{125}I-labelled rRNA from *M. phlei*. Tracks: i, phage lambda DNA restricted with both *Eco*RI and *Bam*HI endonucleases; ii, *M. smegmatis* DNA restricted with *Eco*RI endonuclease; iii, *M. smegmatis* DNA restricted with *Bam*HI endonuclease; iv, *M. phlei* DNA restricted with *Eco*RI endonuclease; v, *M. phlei* DNA restricted with *Bam*HI endonuclease; vi, phage lambda DNA restricted with both *Eco*RI and *Hin*dIII endonuclease. ⟶, location of bands discernible on the negative.

one 2.3 kbp *Bam*HI fragment contained an *Eco*RI site whereas the other did not (not shown).

3.3. *rRNA genes of* M. smegmatis

The rRNA genes of *M. smegmatis* were found to have 4 *Eco*RI sites within or very close to the coding region generating fragments of 0.8, 1.7 and 2.9 kbp (see fig.1). The sum, 5.4 kbp, of the 3 fragments is close to the minimum size (4.6 kbp) of 16 S and 23 S rRNA coding regions. Three *Bam*HI sites were found to be located within the rRNA coding region, generating fragments of 1.1 and 1.4 kbp. The 1.1 kbp fragment hybridized most strongly with probe, suggesting that non-coding sequences are present in the 1.4 kbp fragment, as well as in the 5.3 and 6.4 kbp fragments (see table 2).

Table 1

Comparison of the hybridization of homologous and heterologous ^{125}I-labelled rRNA probes with *M. phlei* DNA restricted with *Bam*HI or *Eco*RI endonucleases

^{125}I-labelled rRNA probe	Relative absorbance of autoradiograph							
	*Bam*HI fragments					*Eco*RI fragments		
	6.4 kbp	5.8 kbp	2.3 kbp	1.35 kbp	1.25 kbp	16 kbp	6.4 kbp	3 kbp
M. phlei	1[a]	0.6	5	0.4	0.6	3.2	1[a]	9.3
M. smegmatis	1[b]		2.62	0.4	1.04	0.6	1[b]	5.4

[ab] The absorbance of the autoradiograph corresponding to this restriction fragment was used as the reference value, the absolute value of absorbance obtained using the homologous probe was not the same as that measured for the heterologous probe. The relative values refer to absorbances measured for the same probe, i.e. either homologous or heterologous rRNA

The absorbance of autoradiographs similar to those presented in fig.1 was measured (see section 2) within the linear region of the dose-response curve of the photographic film. Values are presented as the relative absorbances of the photographic film owing to the hybridization of radioactive probe with the restriction enzyme fragments specified

Table 2

Comparison of the hybridization of homologous and heterologous ^{125}I-labelled rRNA probes with *M. smegmatis* DNA restricted with *Bam*HI or *Eco*RI endonucleases

^{125}I-labelled rRNA probe	Relative absorbance						
	*Bam*HI fragments				*Eco*RI fragments		
	6.4 kbp	5.3 kbp	1.4 kbp	1.1 kbp	1.9 kbp	1.7 kbp	0.75 kbp
M. smegmatis	1.07	1.0[a]	0.8	1.1	1[a]	0.7	0.4
M. phlei	0.8	1.0[b]	0.4	0.09	1[b]	0.45	0.17

[ab] The absorbance of the autoradiograph corresponding to this restriction fragment was used as the reference value; the absolute value of absorbance obtained using the homologous probe was not the same as that measured for the heterologous probe. The relative values refer to absorbances measured for the same probe, i.e. either homologous or heterologous rRNA

The absorbances of autoradiographs similar to those presented in fig.1 were measured within the linear range of the dose-response curve of the photographic film. Values are presented as the relative absorbances of the photographic film owing to the hybridization of the radioactive probe with the restriction enzyme fragments specified

3.4. *Hybridization of rRNA gene sequences with heterologous probes*

The fragments of DNA produced by restriction endonucleases which were found to hybridize with homologous probe were also found to hybridise with heterologous probe (see fig.1). This result shows that *M. phlei* and *M. smegmatis* rRNAs have nucleotide sequences in common. The proportion of sequences present in a fragment of DNA that is common to both *M. phlei* and *M. smegmatis* rRNA can be deduced from the extent of hybridization with homologous and heterologous ^{125}I-labelled probes (see table 1). Although *M. phlei* rRNA and *M. smegmatis* rRNA share regions of sequence homology, they are probably not identical in primary structure. Comparison of the appropriate autoradiographs reveals that hybridisation to the *M. phlei* 2.3 kbp *Bam*HI fragment was less pronounced in comparison with the other fragments when ^{125}I-labelled *M. smegmatis* rRNA was the probe, than when ^{125}I-labelled *M. phlei* rRNA was the probe. Hybridization to the 16 kbp *Eco*RI fragment of *M. phlei* was diminished when heterologous probe rather than homologous probe was used. Similarly, *M. smegmatis* rDNA fragments appeared to hybridize less with heterologous probe than with homologous probe (table 2). This effect was pronounced for 2 *Bam*HI fragments (1.1 and 1.7 kbp) and 2 *Eco*RI fragments (0.75 and 1.7 kbp).

4. DISCUSSION

The procedure described for the lysis of both *M. phlei* and *M. smegmatis* in 6 M guanidinium chloride at −20°C using a French pressure cell, and the methods for isolating rRNA and DNA are all generally applicable to mycobacteria. The method described for labelling rRNA to high specific activity in vitro avoids the constraints of labelling rRNA of slowly growing mycobacteria with radioactivity in the growth medium. Thus the rRNA genes of mycobacteria are open to investigation by means of simple methods. A knowledge of the structure of a particular gene, in this case the rRNA gene cluster, is of taxonomic value. Knowledge of the structure of rRNA genes of pathogenic mycobacteria could be valuable in identifying, unambiguously, particular species or certain strains.

Mycobacterial rRNA, like rRNA of other prokaryotes, comprises 5 S, 16 S and 23 S rRNA [15]. In *Escherichia coli* the genes for these rRNA species are found in a cluster in the order 16 S, 5 S, 23 S rRNA. The rRNA sequences of the genes extend over approx. 5000 basepairs taking into account the non-coding spacer sequences separating one gene from the next. There are 7 rRNA gene clusters in *E. coli* [5]. The nucleotide sequences of the coding regions of the genes are believed to be identical but the sequences flanking the gene

cluster are likely to be different in each case.

The number of rRNA genes present in the mycobacterial genome is not known but, by analogy with most prokaryotes, it is likely that multiple copies are present [16]. Thus fragments of rRNA genes generated by cleavage of the genome with restriction endonucleases will comprise fragments of the coding region of each gene and also fragments with both coding and flanking sequences. The array of fragments derived from rRNA coding sequences might be expected to be characteristic of a particular species because the rRNA sequences themselves are unlikely to differ appreciably between strains. Nucleotide sequences flanking rRNA are more likely to be strain-specific and this will be reflected in the sizes of the fragments containing both rRNA coding sequences and flanking sequences.

Other strains of *M. phlei* and *M. smegmatis* need to be studied in order to establish the range of variations in the array of restriction endonuclease fragments of the rRNA genes that characterises a single mycobacterial species. Current knowledge is confined to the size of the genome and the extent to which nucleotide sequences are shared between strains, as measured by hybridization studies. For example, 4 strains of *M. smegmatis* were investigated by Bradley [17] and the size of the genome was found to range from 4.2×10^9 to 4.5×10^9 Da, and the extent of nucleotide sequence homologies compared to one strain was approx. 100, 97 and 83%.

The genomic properties of different species of mycobacteria show considerable variation in size, ranging from 2.5×10^9 Da (e.g. *M. tuberculosis*) to 4.5×10^9 Da (e.g. *M. smegmatis*), and also in nucleotide sequence [8,17]. The extent to which 2 species may have nucleotide sequences in common ranges from upwards of 7%. *M. phlei* has a genome (3.5×10^9 Da) that is approx. 81% of the size (4.3×10^9 Da) of that of *M. smegmatis* [17]. Approx. 1.5×10^9 Da of each genome are accounted for by nucleotide sequences common to both species [17].

The results presented above (see tables 1 and 2) show that the pattern of restriction enzyme fragments derived from the rRNA genes of *M. phlei* is different from the pattern of fragments derived from the rRNA genes of *M. smegmatis*. We infer that the sequences flanking the rRNA genes are not identical in the 2 genomes and it also appears that the sequences of the rRNA genes themselves are significantly different in the 2 species.

Eventually, it should be possible to construct probes that are more sophisticated than unfractionated rRNA. For example, nucleotide sequences specific to the rRNA gene cluster of a particular species of mycobacteria would be advantageous as a probe.

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