Partial Nucleotide Sequence of 16S Ribosomal RNA Isolated from Armadillo-grown *Mycobacterium leprae*

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Ribosomal RNA (rRNA) was isolated from *Mycobacterium leprae* recovered from infected tissue of the Nine-banded Armadillo, and nucleotide sequences near the 3' end of the 16S species were determined by primer extension in the presence of dideoxynucleotides. Previously published data for bacterial 16S rRNAs show a pattern of conserved and non-conserved sequences that fit a common secondary structure. Our data for *M. leprae* fit this general pattern.

INTRODUCTION

Little is known about the genome of *Mycobacterium leprae* because of the difficulties in culturing and manipulating this organism. However, some progress has recently been made in cloning and expressing *M. leprae* DNA (Clark-Curtis *et al.*, 1985; Young *et al.*, 1985), and information about specific mycobacterial gene sequences is becoming available (Shinnick, 1987). Such information might prove useful for unequivocally identifying *M. leprae* in isolates of clinical interest or laboratory cultures. We have focussed on rRNA because it is present in several thousand copies in each bacterium and hence probes directed towards rRNA should have a much greater sensitivity than probes directed towards genomic sequences.

We now report the isolation of RNA from *M. leprae* recovered from infected armadillo tissue, and the identification of the nucleotide sequence near to the 3' end of 16S rRNA by the method of primer extension (Lane *et al.*, 1985).

METHODS

Materials. The following items were purchased from the sources indicated: lysozyme, lipase, dithiothreitol (DTT), TEMED, xylene cyanol FF, 2-mercaptoethanol, Tris, EDTA and boric acid from Sigma; Tween 80 from Bio-Rad; ammonium persulphate, glacial acetic acid, methanol, Aristar guanidinium chloride, bromophenol blue and formamide from BDH; AMV reverse transcriptase (FPLC pure), Sephadex G-50, Sephadex G-200, and deoxy- and dideoxyribonucleotide triphosphates from Pharmacia; T4 polynucleotide kinase and oligodeoxyribonucleotides from Anglian Biotechnology; deoxyadenosine 5'- α -[35 S]thiotriphosphate ([α - 35 S]dATP; 600 Ci mmol⁻¹, 22.2 TBq mmol⁻¹), [γ - 32 P]ATP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹) and placental RNAase inhibitor from Amersham; X-ray film from Kodak and Fuji.

Media for bacterial cultures. LB medium (l⁻¹): Bactotryptone (10 g), Bacto-yeast extract (5 g), NaCl (10 g), adjusted to pH 7.5. YEME medium (l⁻¹): Difco yeast extract (3 g), Difco bacto-peptone (5 g), Oxoid malt extract (3 g), glucose (10 g), sucrose (340 g), 1 M-MgCl₂ (5 ml). Nutrient Tween (l⁻¹): Difco nutrient broth (10 g), Tween 80 (6 ml). Modified Dubos medium, part A (l⁻¹): KH₂PO₄ (1 g), K₂HPO₄·12H₂O (6.25 g), sodium citrate (1.25 g), MgSO₄·7H₂O (0.6 g), asparagine (2 g), 10% (v/v) Tween 80 (5 ml), 20% (w/v) Casamino acids (10 ml), adjusted to pH 7.2. Modified Dubos medium, part B (l⁻¹): 10% (w/v) bovine serum albumin fraction V. Immediately before use, 4 ml part B is mixed with 100 ml part A. Tween/glutamate medium, part A (in 0.9 l): sodium glutamate (8 g), Bactocastone (1 g), KH₂PO₄ (1 g), Na₂HPO₄ (2.5 g), Tween 80 (0.5 ml), CaCl₂·2H₂O (1 mg), CuSO₄·5H₂O (0.5 mg), ZnSO₄·7H₂O (0.5 mg). Tween/glutamate medium, part B (in 0.1 l): bovine serum albumin (5 g), ferric ammonium citrate (0.1 g). Immediately before use, 900 ml part A is mixed with 100 ml part B.

Culture of bacteria. *Escherichia coli* (Y1090) was grown in LB medium at 37 °C overnight on a rotary shaker. *Streptomyces lividans* (TK64) was grown in YEME medium at 30 °C for 48 h on a rotary shaker. *Mycobacterium*

smegmatis (ATCC 607) and *Mycobacterium vaccae* (given by Dr J. L. Stanford, Middlesex Hospital Medical School, London) were grown in nutrient Tween medium at 37 °C for 3 d on a rotary shaker. *Mycobacterium tuberculosis* (H₃₇Rv) was grown in modified Dubos medium at 37 °C: glycine was added in exponential phase to a concentration of 0.2 M and incubation was continued for 48 h. *Mycobacterium microti* (OV254) was grown in Tween/glutamate medium at 37 °C. *M. leprae* was isolated from the skin nodules of a Nine-banded Armadillo according to the most recent IMMLEP procedure (World Health Organization, 1980).

Isolation of RNA. Cultures of *E. coli*, *S. lividans*, *M. smegmatis*, *M. vaccae* and *M. microti* were harvested by centrifugation, resuspended in lysis buffer (6 M-guanidinium chloride, 0.1%, v/v, Tween 80, 1 mM-2-mercaptoethanol, 10 mM-EDTA, pH 7.0) and passed through a French pressure cell (Aminco) at 18000 lbf in⁻² at -10 °C. Insoluble debris was removed by centrifugation at 5000 g for 10 min and the supernatant was deproteinized by repeated extraction with chloroform/octanol (24:1, v/v). RNA was precipitated by the dropwise addition of a 0.5 volume of ethanol (Cox, 1968; Katoch & Cox, 1986).

M. tuberculosis cultures were harvested and resuspended in 10 mM-Tris/HCl (pH 8.0), 1 mM-EDTA, 0.1% (v/v) Tween 80 containing lysozyme and lipase (2 mg ml⁻¹ each) and shaken at 37 °C for 2 h. Cells were pelleted and resuspended in lysis buffer containing 0.1% (v/v) Triton X-100 and shaken at room temperature for 15 min to induce lysis. Cell debris was removed and RNA was precipitated as above.

M. leprae (3 × 10¹⁰ cells) was treated with lysozyme and lipase as above, resuspended in lysis buffer and passed through a French pressure cell at 18000 lbf in⁻² three times. Debris was removed and RNA was precipitated as above.

5' End labelling of oligonucleotide primer. The reaction mixture contained 75 pmol primer, 150 µCi [γ-³²P]ATP and 30 units of polynucleotide kinase in 50 µl 50 mM-Tris/HCl (pH 7.6), 10 mM-MgCl₂, 5 mM-DTT, 0.1 mM-spermidine. After incubation at 37 °C for 30 min, 2 µl 0.5 M-EDTA was added and the reaction was heated to 70 °C for 10 min. Labelled primer was recovered by gel filtration on Sephadex G-50.

Hybridization of primer to RNA. The reaction mixtures contained 0.3 pmol ³²P-labelled primer and 20 µg RNA in 10 µl 50 mM-Tris/HCl (pH 8.0), 6 mM-MgCl₂, 40 mM-KCl (annealing buffer), and was incubated at 70 °C for 10 min and cooled on ice. The 16S rRNA-primer complex was isolated by gel filtration on Sephadex G-200 (Amicon-Wright column, 0.8 cm diameter × 5 cm) run in 10 mM-Tris/HCl (pH 8.0), 200 mM-NaCl, 6 mM-MgCl₂, 1 mM-EDTA at 35 °C.

The equilibrium constant (*K*) for the association of primer I with 16S rRNA was calculated for the reaction given in equation 1 by equation 2.

$$K = \frac{xp^0}{(1-x)p^0(r^0 - xp^0)} \approx \frac{x}{(1-x)r^0} \quad (2)$$

*r*⁰ and *p*⁰ are the initial concentrations, respectively, of RNA and primer; *x* is the fraction of primer bound to 16S rRNA at equilibrium. When ³²P-labelled primer was used *r*⁰ ≫ *xp*⁰, so that *r*⁰ - *xp*⁰ ≈ *r*⁰. On gel filtration the primer bound to 16S rRNA (*xp*⁰) is detected in the void volume, whereas the non-bound primer [(1 - *x*)*p*⁰] is retarded. Hence *K* may be evaluated by measuring the radioactivity (c.p.m.) of the void volume and of the fractions retarded by the column (see equation 3).

$$K \approx \frac{\text{c.p.m. (void volume)}}{\text{c.p.m. (retarded)} r^0} \quad (3)$$

The concentration of RNA was measured by spectrophotometry by means of the relation that an absorbance of 1.0 at 260 nm is equivalent to 40 µg RNA ml⁻¹ [i.e. ε 7750 l⁻¹ (mol PO₄⁻²)⁻¹ cm⁻¹] and that 16S rRNA is approximately one-third of the total RNA assuming that 16S and 23S rRNA are present in equimolar amounts and the ratio of *M_r* values is 1:2.

RNA sequencing. This was done by the dideoxynucleotide termination method of Sanger *et al.* (1977) modified for the use of reverse transcriptase and RNA templates by Lane *et al.* (1985). RNA (7 µg) was mixed with 0.15 µg primer in 7.5 µl 50 mM-Tris/HCl (pH 8.3), 50 mM-KCl, and 30 µCi dried [³²S]dATP was dissolved in 6.5 µl of this mix. To this were added 6.5 µl 250 mM-Tris/HCl (pH 8.3), 250 mM-KCl, 50 mM-DTT, 50 mM-MgCl₂, and 6.5 µl reverse transcriptase diluted to 1 unit µl⁻¹ in 50 mM-Tris/HCl (pH 8.3), 2 mM-DTT, 50% (v/v) glycerol. Portions (3 µl) were dispensed to each of five tubes, and 2 µl of nucleotide mix (250 µM each of dTTP, dCTP and dGTP in 10 mM-Tris/HCl, pH 8.3) lacking or containing one of the dideoxynucleotides (1.25 µM-ddATP, 30 µM-ddTTP, 30 µM-ddCTP or 19 µM-ddGTP) were added. The reactions were incubated at room temperature for 5 min, and then at 37 °C for 30 min; 1 µl of chase mix (all four dNTPs at 1 mM) was added and incubation was continued for a further 15 min. Reactions were cooled on ice and 6 µl of stop mix (90%, v/v, deionized formamide, 10 mM-EDTA, pH 8.0, 0.01%, v/v, bromophenol blue, 0.1%, v/v, xylene cyanol FF) were added. After heating to 90 °C for 2 min the reactions were analysed on sequencing gels.

Sequencing gels. These were 0.3 mm thick and 38 cm long (Sanger & Coulson, 1978); they consisted of 6% (w/v)

acrylamide and 0.3% (w/v) bisacrylamide in TBE buffer (89 mM-Tris base, 89 mM-boric acid, 20 mM-EDTA), polymerized by the addition of 40 µl TEMED and 25 µl 25% (w/v) ammonium persulphate per 50 ml. Electrophoresis was done at 1500 V for 1–4 h in TBE buffer. Gels were fixed in 10% (w/v) acetic acid for 10 min at room temperature prior to exposure to X-ray film.

Formamide-agarose gel electrophoresis. The molecular size distribution of RNA samples was analysed on 1% (w/v) agarose gels in 50% (v/v) deionized formamide, 0.1 × E buffer (3.6 mM-Tris base, 3.0 mM-Na₂HPO₄, 0.2 mM-EDTA). Samples were made up to 50% formamide, 0.1 × E buffer, 5% glycerol and 0.05% bromophenol blue and heated to 60 °C for 3 min prior to running at 100 V (25 mA) for 2 h. *E. coli* rRNA was used as a standard.

RESULTS AND DISCUSSION

RNA isolated from armadillo-grown *M. leprae* was found to consist of two principal components, which co-migrate with the 16S and 23S rRNAs of *E. coli*. *M. tuberculosis* (Worcel *et al.*, 1968) and *Mycobacterium bovis* (Suzuki *et al.*, 1987) have also been shown to contain rRNAs of these sizes. The yield of rRNA (175 µg from 3×10^{10} *M. leprae*), allowing for a 50% loss during the isolation and purification procedure, suggests that there are approximately 4000 ribosomes per mycobacterium, compared with approximately 20000 ribosomes per bacterium found by Tissières *et al.* (1959) in stationary phase *E. coli*. However, since *M. leprae* harvested from infected tissue is known to include a large proportion of non-viable bacilli, the number of ribosomes per viable bacillus could be much higher than this figure.

Oligonucleotide primers were designed to hybridize with regions of the 16S rRNA sequence which are highly conserved among prokaryotes. The total RNA fraction was used without further purification for the determination of nucleotide sequence by primer extension using reverse transcriptase (Lane *et al.*, 1985). Conditions for binding oligodeoxyribonucleotide primers to mycobacterial rRNA were investigated using ³²P-labelled primer I (5'AAG-GAGGTGATCCA(T/C)CCGC3'), which is complementary to residues 1522–1541 of *E. coli* (Noller, 1984). The *K* values of this primer for 16S rRNAs from three cultivatable mycobacteria were compared with those for *E. coli* and *S. lividans*. The results are summarized in Table 1, which shows that the stability of the 16S rRNA · primer I complex was similar in all five species. However, using this primer, no significant cDNA synthesis was obtained with reverse transcriptase using RNAs from *E. coli*, *M. leprae* or other mycobacterial species. The enzyme is known to stop at methylated adenine (Hagenbuchle *et al.*, 1978), and guanine residues (Youvan & Hearst, 1981); in *E. coli* there are two methylated adenine residues immediately upstream from the primer binding site (bases 1518, 1519; Brosius *et al.*, 1978). We infer that mycobacterial 16S rRNA has a sequence complementary to primer I (with none or very few mismatches) and that there is similar methylation of nucleotides in this region.

When primer II (5'TACGGCTACCTTGTTACGACTT3') was used (see Fig. 1), the sequence of 277 nucleotides was established from residues 1230–1507, using the numbering system for *E. coli*. The 16S rRNA sequences that are conserved between *M. leprae* and nine archaebacteria, 13 eubacteria and four chloroplast species (Huysmans & De Wachter, 1986) are boxed in Fig. 1. The changes in sequence as compared to *E. coli* result in conservation of the

Table 1. *Equilibrium constant (K) for the complex formed between 16S rRNA and ³²P-labelled primer-I*

rRNA species	10 ⁵ × <i>K</i> (l mol ⁻¹)*
<i>E. coli</i>	1.18
<i>S. lividans</i>	1.26
<i>M. smegmatis</i>	0.87
<i>M. vaccae</i>	0.73
<i>M. microti</i>	0.87

* *K* was measured by gel filtration (Sephadex-G200) as described in Methods. The concentration is expressed as mol nucleotide l⁻¹.

Fig. 1. Partial nucleotide sequence of *M. leprae* 16S rRNA. The sequence is presented as the secondary structure proposed for *E. coli* 16S rRNA (for review, see Noller, 1984); the helices 36–40 are indicated. Residues common to *M. leprae* and the nine archaeobacteria, 13 eubacteria and four chloroplast species listed by Huysmans & De Wachter (1986) are boxed; ► indicates difference between *M. leprae* and *E. coli*. The numbering system for *E. coli* is used although there is a deletion of a base pair in helix 36. N indicates that the nucleotide was not identified. The sequence 1229–1488 was measured directly by primer extension. The sequence 1488–1507 is complementary to primer II and the sequence is conserved among the 26 16S rRNA species listed by Huysmans & De Wachter (1986). The sequence 1502–1542 is conjectural; the sequence 1522–1540 is complementary to primer I, and the highly conserved adenine residues at 1518 and 1519 would, if methylated, stop the progress of reverse transcriptase.

secondary structure proposed for *E. coli* (Noller, 1984), suggesting that this scheme is also applicable to *M. leprae*.

These highly conserved residues account for 97 of the nucleotides sequenced. Of the remaining 180 bases the *M. leprae* sequence has a higher proportion of GC base pairs than the *E. coli* sequence, which is consistent with the known high G + C content of mycobacterial DNA (55.8% for *M. leprae*, Imaeda *et al.*, 1982), and differs from the *E. coli* sequence at 62 sites. There are fewer (41) differences between *M. leprae* (Fig. 1) and *S. lividans* (Bibb & Cohen, 1982) sequences, suggesting that *M. leprae* is more closely related to the latter than to *E. coli*.

The sequence data of *M. leprae* 16S rRNA may be extended by the use of other suitable oligodeoxyribonucleotide primers based on rRNA sequences which are widely conserved among prokaryotes. The methods described above are generally applicable, and are suitable for

the study of other mycobacteria. The observation that there are probably several thousand rRNA copies per *M. leprae* bacillus, and that the *M. leprae* 16S rRNA sequence shows differences from other prokaryotic 16S rRNA sequences suggests that it should be possible to design specific oligonucleotide probes capable of detecting *M. leprae* in infected tissue.

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