Isolation and Restriction Endonuclease Analysis of Mycobacterial DNA

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A method for the isolation of DNA from mycobacteria propagated *in vitro* is described that utilizes organic solvents to extract lipoidal components from the outer membrane, and digestion with a protease (nagarse) and lysozyme to penetrate the cell wall. The mycobacterial cells were lysed by the addition of detergent and the DNA was purified by digestion with pronase, sequential phenol and chloroform extractions, and digestion with RNAase A. The isolated DNA, which was obtained in good yields, was of a relatively high M_r and could be readily digested by restriction endonucleases. By this method, the genomes of *Mycobacterium avium*, *M. intracellulare*, *M. lepraemurium*, '*M. lufu*', *M. marinum*, *M. phlei*, *M. scrofulaceum*, *M. smegmatis* and *M. tuberculosis* were isolated and the restriction endonuclease digestion patterns analysed. Each species could be distinguished by the digestion patterns, indicating that this approach can be used for identifying mycobacterial species. This approach is also sufficiently sensitive to differentiate strains since we were able to distinguish two independently isolated strains of *M. tuberculosis*, H37 and H4. In addition, no evidence was obtained for the presence of methylcytosine residues in the sequences 5'.CCGG.3', 5'.CCCGGG.3', 5'.CC(A/T)GG.3' or for methyladenine at 5'.GATC.3' in the DNA of the nine mycobacterial species examined using pairs of restriction enzymes that recognize and cleave at the same nucleotide sequence but differ in their sensitivity to 5-methylcytosine or ⁶N-methyladenine.

INTRODUCTION

Members of the genus *Mycobacterium* are widespread in nature and range from soil-dwelling saprophytes to pathogens of man and animals. Mycobacteria are defined on the basis of impermeability to basic dyes, but once stained, they retain dye even in the presence of acidified organic solvents (Vestal, 1975). This characteristic of being acid-fast is presumably a result of their lipid-rich cell wall. The identification of clinical and environmental isolates is usually based on analysis of phenotypic characters (for reviews see Barksdale & Kim, 1977; Wolinsky, 1979). Since it is possible that the morphology and biosynthetic capabilities of mycobacteria may vary depending on culture conditions and strain differences within a species, the most reliable method of identification would be at the DNA level. Previous studies have examined the guanine plus cytosine content of mycobacterial DNA (Hill, 1966; Imaeda *et al.*, 1982; Wayne & Gross, 1968*b*) and DNA hybridization in solution (Bradley *et al.*, 1973; Gross & Wayne, 1970; Imaeda *et al.*, 1982) as a means of identifying and differentiating species. It is now feasible to characterize strains of micro-organisms at the genomic level by the unique restriction endonuclease digestion patterns of their DNA. This technology requires the isolation of high M_r DNA from mycobacteria, which is difficult because of the impermeable cell wall. Various

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methods of isolating DNA from mycobacteria have been reported (Baess, 1974; Mizuguchi & Tokunaga, 1970; Wayne & Gross, 1968*a*) which generally utilize procedures of cell disruption which may shear DNA. Therefore, we have developed a method for extracting DNA in good yields from mycobacteria cultivated *in vitro* which is of relatively high M_r and can be digested by restriction endonucleases. We report here the isolation and restriction enzyme cleavage analysis of DNA from *Mycobacterium avium*, a member of the *M. avium*-*intracellulare*-*scrofulaceum* (MAIS) complex which causes tuberculosis in birds and has been included as an opportunistic pathogen in patients with acquired immune deficiency (Macher *et al.*, 1983; Zakowski *et al.*, 1982); *M. intracellulare*, which is a rare pathogen for chickens but also causes chronic, and sometimes fatal, pulmonary disease in humans; *M. lepraemurium*, which is the aetiological agent of rodent leprosy; *M. marinum*, which causes chronic skin lesions in humans; *M. scrofulaceum*, a common cause of lymphadenitis in children; *M. tuberculosis*, the cause of human tuberculosis; and three species of soil saprophyte, *M. phlei*, *M. smegmatis* and '*M. lufu*'. These nine species could be distinguished by the restriction endonuclease digestion patterns of purified DNA as visualized after agarose gel electrophoresis. Using eight different restriction enzymes we could not detect differences between avirulent and virulent strains of *M. tuberculosis* H37 but the independent isolates H37 and H4 could be distinguished.

METHODS

Bacterial strains. *Mycobacterium phlei* ATCC 11758, *M. scrofulaceum* ATCC 19981, *M. smegmatis* ATCC 19420, *M. tuberculosis* H37Ra ATCC 25177, and *M. tuberculosis* H37Rv ATCC 25618 were obtained from the American Type Culture Collection. *M. tuberculosis* H4Ra TMC 204 was acquired from the Trudeau Mycobacterial Culture Collection, National Jewish Hospital and Research Center, Denver, Colo., USA. *M. lepraemurium* (Hawaiian) and '*M. lufu*' L-868 were provided through the courtesy of Dr A. Dhople, Florida Institute of Technology, USA. *M. marinum* TMC 1218 and *M. intracellulare* ATCC 13950 were provided through the courtesy of Dr T. Hadfield, Armed Forces Institute of Pathology, Washington, DC, USA. *M. avium* (no. 8063, Pasteur Institute reference strain) and an independent isolate (no. 214) were sent from Belgium by Dr F. Portaels. Stock cultures were maintained at 4 °C on Lowenstein-Jensen slants (BBL). Mycobacteria were grown in 1000 ml Dubos broth base (Difco) containing 0.4% glycerol (Fisher Scientific). The cultures were incubated at 37 °C on a rotary shaker and harvested at late exponential or early stationary phase growth. *M. lepraemurium*, adapted to growth on Lowenstein-Jensen slants by Dr A. Dhople, was harvested by scraping cells from the surface of the medium. *Escherichia coli* MM294 was propagated in Luria broth (Lennox, 1955).

DNA isolations. Mycobacterial cells were recovered by centrifugation at 12000 *g* for 30 min at 4 °C and washed once in 50 mM-Tris/HCl (pH 8.0), 100 mM-EDTA, 150 mM-NaCl (TEN). Bacteria were resuspended in 1 to 5 ml TEN depending on the size of the pellet. Light petroleum (LP; b.p. 35–60 °C) and CHCl_3 were added to the bacterial suspension in a 3:1:1 (LP: CHCl_3 :TEN, by vol.) ratio. After vortex mixing and rocking on a Thermolyne Speci-Mix (Sybron Corporation) for 15 min, the organic phase was centrifuged at 12000 *g*. This extraction was repeated until the organic phase was clear. The extracted cell pellet was resuspended in 1 to 2 ml TEN depending on the size of the cell pellet. Nagarse (protease, type VII, bacterial; Sigma), which was used previously to isolate cell wall components (Takeya & Hisatsune, 1963), was added to the bacterial suspension to a final concentration of 10 mg ml⁻¹ and incubated at 37 °C for 2 to 4 h. Lysozyme (Sigma) was added (50 mg ml⁻¹) and incubated at 50 °C for 2 to 4 h. Bacterial lysis was completed by adding SDS to 1% (w/v) and pronase B (Calbiochem-Behring) to 3 mg ml⁻¹. The lysate was incubated at 37 °C for 12 to 36 h with additions of pronase B (3 mg ml⁻¹) at 12 h intervals. DNA was purified by sequential extractions with equal volumes of phenol (Bethesda Research Laboratories) saturated with TEN, and chloroform/isoamyl alcohol (24:1, v/v). The DNA preparation was dialysed for 48 h against 10 mM-Tris/HCl (pH 7.5), 1 mM-EDTA (pH 7.4), 150 mM-NaCl at 4 °C, digested with RNAase A (20 µg ml⁻¹; Calbiochem-Behring) for 2 h at 37 °C and sequentially extracted with phenol and chloroform/isoamyl alcohol. The DNA was either dialysed exhaustively against 10 mM-Tris/HCl (pH 7.5), 1 mM-EDTA (TE) or was precipitated by adding NaCl to 0.3 M, 2.5 volumes ethanol or 0.56 volume 2-propanol and placed at -20 °C overnight. The DNA was pelleted by centrifugation and redissolved in TE at 4 °C. The concentration and purity of DNA was determined in a Gilford 250 spectrophotometer. If the 280/260 or 240/260 readings were greater than 0.5 ± 0.1, the DNA was treated further with SDS and pronase B, extracted with phenol and chloroform/isoamyl alcohol and precipitated with alcohol or dialysed against TE. DNA was isolated from cultures of *M. avium* and *M. marinum* which were harvested by centrifugation, frozen rapidly in liquid nitrogen, and stored at -70 °C. The pellet was thawed, resuspended in TEN and extracted as described above. *E. coli* cultures were harvested by centrifugation, washed once with TEN, incubated for 10 min at 4 °C with 10 µg

lysozyme ml⁻¹ and lysed by the addition of SDS to one-tenth volume of 10% (w/v) SDS. The DNA was purified as described above.

Restriction endonuclease digestion and gel electrophoresis. Using conditions specified by the vendor, DNA was digested with restriction endonucleases purchased from Bethesda Research Laboratories and New England Biolabs. Digestion was terminated by heating at 65 °C for 5 min or by adding SDS to 0.05%. Bacteriophage λ c1857 (Bethesda Research Laboratories) digested with restriction endonucleases as indicated was used as size markers (Szybalski & Szybalski, 1979; Thomas & Davis, 1975). The digested DNA was size-fractionated by agarose gel electrophoresis, in 40 mM-Tris/HCl (pH 7.5), 20 mM-sodium acetate, 1 mM-EDTA. The gel was stained in ethidium bromide solution (0.5 µg ml⁻¹) and photographed under ultraviolet light with Polaroid type 57 film.

Removal of polysaccharides. The procedure described by Hill *et al.* (1972) was followed, with minor modifications. Cetyltrimethylammonium bromide (CTAB; Sigma) was dissolved in 0.4 M-NaCl to give a 5% (w/v) solution, and added to the DNA solution (2:5). After allowing for precipitation of DNA-CTAB for 15 min at room temperature, a few drops of the CTAB solution were added to ensure complete precipitation. After centrifugation, the pellet was washed twice in 0.4 M-NaCl and dissolved in 1 M-NaCl. The DNA solution was extracted with chloroform/isoamyl alcohol until no precipitate was visible at the interface. DNA was precipitated by adding 3 volumes 95% (v/v) ethanol and redissolved in 0.7 M-NaCl. CTAB in 0.7 M-NaCl was added to 1% and precipitation was allowed for 30 min at room temperature. After centrifugation, 3 volumes 95% ethanol were added to the supernatant fluid. The DNA was redissolved in TE and dialysed exhaustively against TE at 4 °C.

RESULTS

DNA yield

Typical results of the yield of DNA from 12 mycobacterial strains, isolated as described in Methods, are presented in Table 1. Using our method, DNA was obtained reproducibly in sufficient quantities from the nine species tested. Variation in the quantity of DNA obtained per ml of culture medium may be a consequence of cell density at the time of harvest. The yield [mg DNA (g wet wt cells)⁻¹] from '*M. lufu*' was also lower than for the other mycobacteria. The size of the purified DNA was determined by agarose gel electrophoresis. Mycobacterial DNA (1 µg) and digested bacteriophage λ (49 kb), which was used as a size marker, were electrophoresed on a 1% agarose gel as described in Methods. If there was evidence of extensive degradation, i.e. fluorescence seen throughout the track, the DNA preparation was discarded. Degradation, presumably by endogenous nucleases, was only an occasional problem. Undigested DNA of *M. tuberculosis* H37Ra, *M. smegmatis* and *M. phlei* is shown in Fig. 5, lanes *b*, *f* and *j*, respectively.

Table 1. Yield of DNA from 12 mycobacterial strains

Strain	DNA yield	
	[mg DNA (g wet wt cells) ⁻¹]*	[µg DNA (ml culture) ⁻¹ †]
<i>M. avium</i> (Pasteur no. 8063)	1.6	1.2
<i>M. avium</i> no. 214	1.3	1.0
<i>M. intracellulare</i> ATCC 13950	1.1	2.8
' <i>M. lufu</i> ' L-868	0.3	0.6
<i>M. marinum</i> TMC 1218	1.1	2.5
<i>M. phlei</i> ATCC 11758	1.5	1.3
<i>M. scrofulaceum</i> ATCC 19981	ND	1.4
<i>M. smegmatis</i> ATCC 19420	1.2	1.8
<i>M. tuberculosis</i> H4Ra TMC 204	ND	0.8
<i>M. tuberculosis</i> H37Ra ATCC 25177	1.2	1.5
<i>M. tuberculosis</i> H37Rv ATCC 25618	2.0	1.7
<i>M. lepraemurium</i> (Hawaiian)	ND	20.0‡

ND, Not determined.

* Bacteria were grown to late exponential or early stationary phase in 1000 ml Dubos broth base with 0.4% glycerol. The pellet obtained after harvesting the cells was weighed in a tared tube.

† The DNA concentration was determined spectrophotometrically.

‡ 20 µg per slant. *M. lepraemurium* was grown on 20 Lowenstein-Jensen slants and was harvested by scraping the cells from the surface of the medium.

Fig. 1. Mycobacterial DNA digested with restriction endonuclease *Bam*HI and size-fractionated on a 1% agarose gel. For each digest 3 µg DNA was used. Bacteriophage λ DNA digested sequentially with *Hind*III and *Eco*RI was used as size markers (lane a). Lanes: (b) *E. coli*; (c) *M. smegmatis*; (d) *M. phlei*; (e) *M. scrofulaceum*; (f) *M. marinum*; (g) *M. lepraemurium*; (h) *M. tuberculosis* H37Rv; (i) *M. tuberculosis* H37Ra; (j) *M. tuberculosis* H4Ra; (k) *M. avium* no. 214; (l) *M. avium* no. 8063; (m) *M. intracellulare*; (n) '*M. lufu*'.

Restriction endonuclease analysis

The enzymes *Bam*HI, *Eco*RI, *Hind*III and *Pst*I were used to establish digestion patterns of the mycobacteria. DNA (3 µg) from each strain was incubated with 6 units of enzyme for 2 h and analysed by gel electrophoresis as described above. The four restriction endonucleases allowed for species differentiation at the DNA level. For example, Fig. 1 shows an ethidium bromide stained gel obtained with DNA digested with *Bam*HI. Each species was distinguished by its unique digestion pattern [for example, compare *M. phlei* (lane d) with *M. scrofulaceum* (lane e)]. However, there were no apparent differences between *M. tuberculosis* strains H37Rv (lane h), H37Ra (lane i) and H4Ra (lane j). Similarly, we could not detect any differences between the *Bam*HI digestion patterns of two isolates of *M. avium* (lanes k and l).

*Pst*I cleaved the mycobacterial DNAs (Fig. 2) except for *M. lepraemurium* DNA (lane g). Again, each species of mycobacterium was distinguishable by its digestion pattern but there were no apparent differences among *M. tuberculosis* strains H37Rv (lane h), H37Ra (lane i) and H4Ra (lane j) or between two independent isolates of *M. avium* (lanes k and l). The absence of digestion of *M. lepraemurium* DNA by *Pst*I was not due to inhibition of the enzyme by the DNA preparation, since this DNA was digested by other enzymes (e.g. *Bam*HI; Fig. 1, lane g) and complete digestion was assayed by the addition of bacteriophage λ DNA to the reaction. Although the λ DNA was digested to completion, the *M. lepraemurium* DNA remained undigested (data not shown), demonstrating that the enzyme was not inhibited *in trans*. It seems

Fig. 2. Agarose gel electrophoresis through a 1% gel of 3 µg mycobacterial DNA digested with *Pst*I. Bacteriophage λ DNA digested with *Hind*III and *Eco*RI was used as size markers (lane a). Lanes: (b) *E. coli*; (c) *M. smegmatis*; (d) *M. phlei*; (e) *M. scrofulaceum*; (f) *M. marinum*; (g) *M. lepraemurium*; (h) *M. tuberculosis* H37Rv; (i) *M. tuberculosis* H37Ra; (j) *M. tuberculosis* H4Ra; (k) *M. avium* no. 214; (l) *M. avium* no. 8063; (m) *M. intracellulare*; (n) '*M. lufu*'.

unlikely that the recognition sequence for *Pst*I is absent in *M. lepraemurium* DNA and the absence of enzyme digestion may be due to DNA modification at, or around, the recognition sequence.

Digestion of mycobacterial DNA with *Bam*HI, *Eco*RI (data not shown) and *Pst*I produced fragments ranging in size from a few hundred bp to more than 20000 bp. In contrast, the size of the DNA fragments produced by digestion with *Hind*III was relatively large, especially in *M. smegmatis* (Fig. 3, lane c), *M. phlei* (lane d), *M. scrofulaceum* (lane e), *M. lepraemurium* (lane g), *M. tuberculosis* H4Ra (lane j) and '*M. lufu*' (lane n). Complete digestion was assayed by the addition of λ DNA to that of *M. tuberculosis* H4Ra, '*M. lufu*' and *M. avium* (Fig. 4, lanes b, e and h, respectively). Although the λ DNA was digested to completion, the mycobacterial DNA remained relatively undigested, demonstrating that the enzyme was not inhibited *in trans* from digesting the DNA. The resistance of DNA from *M. tuberculosis* H4Ra to digestion with *Hind*III (Fig. 3, lane j) distinguished it from that of strain H37Rv (lane h) and strain H37Ra (lane i). These results suggest that DNA from *M. tuberculosis* H4Ra is modified by methylation at, or near, the *Hind*III recognition sequence and therefore is relatively resistant to digestion with *Hind*III.

Previous reports have noted the association of mycobacterial DNA with contaminating polysaccharides (Baess, 1974). The polysaccharide content in our DNA preparations, as

Fig. 3. Restriction endonuclease digests of mycobacterial DNA with *Hind*III, size-fractionated on a 1% agarose gel. Bacteriophage λ DNA digested with *Hind*III and *Eco*RI was used as size markers (lane a). Lanes: (b) *E. coli*; (c) *M. smegmatis*; (d) *M. phlei*; (e) *M. scrofulaceum*; (f) *M. marinum*; (g) *M. lepraemurium*; (h) *M. tuberculosis* H37Rv; (i) *M. tuberculosis* H37Ra; (j) *M. tuberculosis* H4Ra; (k) *M. avium* no. 214; (l) *M. avium* no. 8063; (m) *M. intracellulare*; (n) '*M. lufu*'.

determined by the anthrone hexose assay (Ashwell, 1957), ranged from $0.112 \text{ mg (mg DNA)}^{-1}$ in *M. phlei* to $2.97 \text{ mg (mg DNA)}^{-1}$ in *M. scrofulaceum*. To determine if the polysaccharides affected digestion of DNA with restriction endonucleases, polysaccharides were removed from *M. tuberculosis* H37Ra, *M. smegmatis* and *M. phlei*; before precipitation with CTAB, they contained 1.64 , 0.708 and $0.112 \text{ mg polysaccharide (mg DNA)}^{-1}$, respectively. No polysaccharides could be detected after CTAB precipitation. Degradation of DNA was evident after the elimination of polysaccharides (Fig. 5, lanes c and k). DNA was digested with *Pst*I before and after removal of the polysaccharides (lanes d, e, h, i, l and m). No difference was seen in the restriction patterns. There was no correlation between the quantity of polysaccharides and the pattern of digestion with restriction enzymes.

Methylation in mycobacterial DNA

Methylation of nucleotides can be determined by using pairs of restriction enzymes which recognize the same nucleotide sequence but differ in their sensitivity to methylation at, or near, the recognition site (for review see Roberts, 1983). To examine the quantity and distribution of methylated nucleotides in mycobacterial DNA, $3 \mu\text{g}$ DNA were digested with enzymes which differ in their sensitivity to methylation at a cytosine residue contained in their respective recognition sequences. *Hpa*II and *Msp*I recognize $5'\text{-CCGG-}3'$; *Hpa*II is sensitive to methylation at the internal cytosine residue whereas *Msp*I is not (Roberts, 1983); *Sma*I, sensitive to methylation at CpG, recognizes and cleaves $5'\text{-CCCGGG-}3'$; *Xma*I recognizes the same sequence but cleaves asymmetrically, i.e. $5'\text{-CCCGGG-}3'$ and is not sensitive to

Fig. 4. Assay for *Hind*III activity by incubating mycobacterial and λ DNA. Mycobacterial DNA (1 μ g) was added to 1 μ g λ DNA and digested with *Hind*III. Bacteriophage λ DNA digested with *Hind*III was used as a control (lane a). Lanes: (b) *M. tuberculosis* H4Ra plus λ , *Hind*III; (c) '*M. lufu*' undigested; (d) '*M. lufu*', *Hind*III; (e) '*M. lufu*' plus λ , *Hind*III; (f) *M. avium* no. 8063, *Hind*III; (g) *M. avium* no. 214, *Hind*III; (h) *M. avium* no. 8063 plus λ , *Hind*III. (i) λ , *Hind*III/*Eco*RI.

methylation (Youssoufian & Mulder, 1981). Since identical patterns were observed for each mycobacterial strain (data not shown), it may be assumed that 5-methylcytosine (m^5C) is not present in these sequences. A third pair of enzymes, *Bst*NI and *Eco*RII, recognizes and cleaves the nucleotide sequence 5'.CC(A/T)GG.3' (Roberts, 1983). *Bst*NI is not sensitive to m^5C at this sequence while *Eco*RII is sensitive to methylation at this sequence. For example, *E. coli* DNA was readily digested by *Bst*NI (Fig. 6, lane b) but not by *Eco*RII (lane c) because this strain contains methylated DNA. 5-Methylcytosine could not be detected at this site in the mycobacterial DNA examined and no difference was apparent in the digestion patterns of *M. tuberculosis* H37Ra and H37Rv when digested with *Bst*NI and *Eco*RII, which indicated that m^5C is not contained in this recognition site. However, it was possible to differentiate independently isolated strains of *M. tuberculosis* (H4 and H37) with *Bst*NI. A band of approximately 4.2 kb is present in *M. tuberculosis* H37 digested with *Bst*NI (lane f) or *Eco*RII (lane g) but is absent in *M. tuberculosis* H4 digested with *Bst*NI (lane h). In addition, strain H4 appears to contain some methylation since the DNA is not digested by *Eco*RII (lane i) to the same extent as it is by *Bst*NI (lane h). In order to examine the quantity and distribution of N^6 -methyladenine (m^6A) in the mycobacterial strains in the present study, enzymes were utilized which recognize the same nucleotide sequence but differ in their sensitivity to methylation.

Fig. 5. DNA from *M. tuberculosis* H37Ra, *M. smegmatis* and *M. phlei*, before and after elimination of polysaccharides by precipitation with cetyltrimethylammonium bromide (CTAB). Undigested DNA (2 µg) and 3 µg DNA digested with *Pst*I were electrophoresed through a 1% agarose gel. Bacteriophage λ DNA digested with *Hind*III and *Eco*RI was used as size markers (lane a). Lanes: (b) *M. tuberculosis* H37Ra, before CTAB, undigested; (c) *M. tuberculosis* H37Ra, after CTAB, undigested; (d) *M. tuberculosis* H37Ra, before CTAB, *Pst*I; (e) *M. tuberculosis* H37Ra, after CTAB, *Pst*I; (f) *M. smegmatis*, before CTAB, undigested; (g) *M. smegmatis*, after CTAB, undigested; (h) *M. smegmatis*, before CTAB, *Pst*I; (i) *M. smegmatis*, after CTAB, *Pst*I; (j) *M. phlei*, before CTAB, undigested; (k) *M. phlei*, after CTAB, undigested; (l) *M. phlei*, before CTAB, *Pst*I; (m) *M. phlei*, after CTAB, *Pst*I.

*Sau*3AI and *Mbo*I recognize the sequence 5'.GATC.3'. *Sau*3AI digests regardless of m⁶A, whereas *Mbo*I does not cleave if the A residue is methylated. DNA (3 µg) from each strain was digested with *Sau*3AI or *Mbo*I and loaded on a 2.5% agarose gel. No difference in restriction patterns between *Sau*3AI and *Mbo*I was evident for any of the species, which indicated that m⁶A was not present in this particular sequence of nucleotides (data not shown).

DISCUSSION

We have compared the restriction endonuclease digestion patterns of DNA isolated from nine species of mycobacteria cultured *in vitro*. To facilitate this type of analysis it was necessary to develop a method for the isolation of undegraded DNA in good yields that was susceptible to cleavage by restriction enzymes. As demonstrated in Table 1, the average yield of DNA obtained in this study was comparable to that obtained by Baess (1974) [1 mg DNA (g wet wt cells)⁻¹], Mizuguchi & Tokunaga (1970) (1.34 mg DNA l⁻¹) and Wayne & Gross (1968a) [0.67 mg DNA (g wet wt cells)⁻¹]. Our results indicate that polysaccharides do not interfere with digestion of mycobacterial DNA by restriction endonucleases, thereby allowing the omission of

Fig. 6. *Bst*NI and *Eco*RII digests of DNA from *M. tuberculosis* strains, size-fractionated on a 2% agarose gel. Bacteriophage λ DNA digested with *Hind*III and *Eco*RI was used as size markers (lane a). Lanes: (b) and (c) *E. coli*, *Bst*NI and *Eco*RII, respectively; (d) and (e) *M. tuberculosis* H37Rv, *Bst*NI and *Eco*RII, respectively; (f) and (g) *M. tuberculosis* H37Ra, *Bst*NI and *Eco*RII, respectively; (h) and (i) *M. tuberculosis* H4Ra, *Bst*NI and *Eco*RII, respectively.

procedures for polysaccharide removal which cause a substantial loss in the quantity of DNA recovered. Baess (1974) noted a loss of 57% of BCG DNA after the first CTAB precipitation; in the present studies, 84% of the DNA was lost during removal of polysaccharides.

The restriction endonuclease digestion patterns presented here allow for the differentiation of nine mycobacterial species. This study supports the status of *M. avium*, *M. intracellulare* and *M. scrofulaceum* as independent species because restriction endonuclease digestion patterns of DNA isolated from them were readily distinguishable. Moreover, the restriction enzyme digestion patterns indicated similarities between strains of the same species. The technique, however, is sensitive enough to detect strain differences, as has been reported by others for bacteria (Marshall *et al.*, 1981; Mielenz *et al.*, 1979; Meselson & Yuan, 1968) and protozoans (Morel *et al.*, 1980). Independent isolates of *M. tuberculosis* (H37 and H4) could be differentiated on the basis of their digestion patterns with *Hind*III and *Bst*NI. *M. tuberculosis* H37Ra and H37Rv could not be distinguished. Collins & De Lisle (1984) have also used restriction endonuclease analyses to distinguish different isolates of *M. tuberculosis*.

Methylated nucleotides have been detected in *M. tuberculosis* strains H37Rv and H37Ra, *M. smegmatis* and *M. phlei* (Srivastava *et al.*, 1981; Somogyi *et al.*, 1982). In our studies, we could not demonstrate either m⁵C at three DNA sequences using three matched pairs of enzymes differing in their sensitivity to methylated nucleotides, or m⁶A at one sequence. These results suggest that the appropriate pairs of restriction enzymes to determine the distribution of the methylated nucleotides are not available. The presence of m⁵C and its effects on gene regulation (Razin & Friedman, 1981) and the inclusion of a plasmid (Crawford & Bates, 1979) have been

suggested as possible explanations for the virulence of strain H37Rv. In our electrophoretic analysis of undigested DNA from strain H37Rv, the presence of a plasmid could not be detected.

We are currently modifying our procedure to obtain DNA from *M. lepraemurium* isolated from mouse tissue and *M. leprae* isolated from infected armadillo tissue and human biopsy specimens. Characterization of the genome from *M. leprae* would be particularly useful in establishing the successful *in vitro* cultivation of the aetiologic agent of leprosy. It would also be of value to identify mycobacteria in biopsy material based on the comparison of fragment patterns obtained by digestion of DNA with reference strains. This could be done by extracting DNA directly from biopsy material, assuming an adequate number of bacteria, or by first cultivating the organism *in vitro*. This technology also provides a means for the application of restriction endonuclease technology in the analysis of mycobacterial gene structure and genome organization.

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