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tRNA Genes in Mycobacteria: Organization and Molecular Cloning

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DNAs from nine mycobacteria cleaved with restriction endonucleases were hybridized with cDNA probes synthesized to tRNAs from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. The tRNA genes are conserved, but their gross genomic organization has diverged in six of the nine species examined. Organisms of the *M. tuberculosis* H37Ra and H37Rv-*M. bovis* BCG complex appeared to have identical tRNA gene organization and were indistinguishable from each other. *M. tuberculosis* and *M. smegmatis* tRNA-derived cDNA probes hybridized differentially to tRNA-coding DNA segments in five of the species examined, suggesting the existence of qualitatively different tRNA pools in these slow- and fast-growing species. Mycobacterial DNAs hybridized with cDNA synthesized to 23S plus 16S rRNAs from *Escherichia coli*, and the data suggested that the tRNA genes map close to the rRNA genes. A gene bank of *M. tuberculosis* H37Rv DNA was constructed, and a recombinant plasmid, pSB2, coding for tRNA(s) and rRNA(s) was partially characterized. Plasmid pSB2 recognized a *Sal*I restriction fragment length polymorphism (RFLP) in *M. tuberculosis* H37Rv and H37Ra; however, the RFLP is not linked to the tRNA-coding region. To the best of our knowledge, this is the first report of an RFLP which distinguishes the pathogenic strain *M. tuberculosis* H37Rv from its avirulent derivative H37Ra.

The structural genes for tRNAs constitute a unique class of genetic material. tRNAs being one of the central components of the protein-synthesizing machinery, the tRNA genes provide an attractive system for studying the relationship among genetic makeup, gene expression, and growth rate of an organism. In *Escherichia coli*, with an estimated 60 tRNA species, the genes are distributed among at least 26 different *Eco*RI restriction fragments, with only a few tRNA genes being closely associated with rRNA genes. In contrast, the majority of tRNA genes in *Bacillus subtilis* map on five *Eco*RI fragments which also encode rRNA genes (5, 17). No systematic investigation of mycobacterial tRNA genes has been reported to date. Studies were initiated to examine the gross organization of tRNA genes in the following mycobacterial species: *M. tuberculosis* H37Rv and H37Ra-*M. bovis* BCG (MTB complex), *M. avium-M. intracellulare-M. scrofulaceum* (MAIS complex), and *M. gordonae* (slow growers) and *M. smegmatis* and *M. phlei* (fast growers). Here we report the organization of mycobacterial tRNA genes deciphered by using tRNA probes derived from *M. tuberculosis* and *M. smegmatis*. We also describe the partial characterization of a tRNA- and rRNA-coding recombinant plasmid isolated from a gene bank of *M. tuberculosis*.

MATERIALS AND METHODS

Chemicals. Enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Promega Biotec (Madison, Wis.), and CSIR Centre for Biochemicals (Dehli, India). Chemicals were from Sigma Chemical Co. (St. Louis, Mo.) and Miles Laboratories, Inc. (Elkhart, Ind.) and Glaxo Laboratories and E. Merck (Bombay, India). [α-^{32}P]dATP and [α-^{32}P]dGTP (specific activity, 3,000 Ci/mmol) were purchased from Amersham Corp. (Amersham, United Kingdom) or Board of Radiation and Isotope Technology (Bombay, India).

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Bacterial culture. Strain descriptions are mentioned in Table 1. Cells were grown at 37°C in Youmans and Karlson medium (19) either as surface cultures (for DNA isolations) or as shake cultures (for RNA isolations).

Extraction and purification of mycobacterial RNA and DNA. Mycobacterial cells from the log phase of growth were harvested and RNA was isolated and purified essentially as described previously (18). High-pressure liquid chromatographic analysis (LKB Ultropac TSK G 3000 SW column, 7.5 by 600 mm) confirmed that 23S and 16S rRNAs were absent. A total of 2% of the RNA had the retention time of 5S rRNA (data not shown). The tRNA and the 5S rRNA fractions were resolved by electrophoresis on a 12% denaturing formamide-polyacrylamide gel (6). These were eluted from the gel (11), phenol extracted, chloroform extracted, ethanol precipitated, dissolved in water, and stored at −20°C.

Mycobacterial DNA was prepared from spheroplasts (13) essentially as described previously (12).

Plasmid DNA from *E. coli* was isolated as described previously (11).

Preparation of DNA probes. ^{32}P-labeled cDNA probes (10^7 cpm/μg of RNA) to total tRNA were prepared with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The recombinant plasmid pSB2 was labeled by nick translation (11) to a specific activity of 10^7 cpm/μg of DNA.

Gel electrophoresis and Southern hybridization. DNAs digested by restriction enzymes were electrophoresed on 0.8% agarose gels, transferred to nitrocellulose paper, and hybridized at 42°C for 16 h (11). Low-stringency hybridizations were performed as above but at 32°C and in 40% formamide. Hybridizations were performed to tRNA-derived probes in the presence of 50 μg of unlabeled rRNA and to rRNA-specific probes in the presence of 50 μg of unlabeled tRNA. To confirm the authenticity of the tRNA-specific hybridization signals, we hybridized mycobacterial *Pst*I digests at low stringency to cDNA probes derived from *E. coli* $tRNA^{Glu}$ and $tRNA^{Val}$. Fragments which hybridized

TABLE 1. Sizes of mycobacterial DNA fragments hybridizing to tRNA-derived [^{32}P]cDNA probes[a]

Restriction enzyme	Fragment(s) (kilobase pairs)								
	M. smegmatis ATCC 27204	*M. phlei* 141300001	*M. tuberculosis* H37Ra	*M. tuberculosis* H37Rv	*M. bovis* BCG (Guindy)	*M. intracellulare* TMC1406	*M. scrofulaceum* TMC1302	*M. gordonae* TMC 1324	*M. avium* NCTC 8562
PstI[b]	16.0, 11.0, 5.8,[c], 1.8	6.5, 5.3, 1.8, 1.3	7.1, 1.3	7.1, 1.3	7.1, 1.3	5.5, 1.4	9.0, 1.1	11.0, 6.8	ND[e]
PstI[d]	16.0, 11.0	6.5, 5.3, 1.8, 1.3	7.1	7.1	7.1	5.5, 1.4	6.5, 3.9, 2.7	NSD[f]	ND
PvuI[b]	8.5,[c] 4.8, 1.4	9.4, 5.6	7.0	7.0	ND	9.5	9.5, 7.0, 6.0	ND	8.5, 7.5, 5.5
AvaII[b]	2.5	2.6	1.2	1.2	ND	1.9	0.85	ND	2.1, 0.7
SalI[d]	18.0–14.0, 9.5, 4.3, 3.9	7.5, 6.7	6.3	6.3	ND	ND	ND	7.0	9.5, 8.0, 7.0

[a] Data are the average of at least two experiments. Input counts per blot ranged from 2×10^6 to 5×10^6 cpm. Underlined numbers denote fragments hybridizing most strongly in a particular species.
[b] Hybridized with purified tRNA from *M. tuberculosis* H37Ra.
[c] Doublet signal.
[d] Hybridized with purified tRNA from *M. smegmatis*.
[e] ND, Not done.
[f] NSD, No signal detected.

TABLE 2. Sizes of mycobacterial DNA fragments hybridizing to rRNA[a]-derived [^{32}P]cDNA probes[b]

Restriction enzyme	Fragment(s) (kilobase pairs)								
	M. smegmatis	*M. phlei*	*M. tuberculosis* H37Ra	*M. tuberculosis* H37Rv	*M. bovis* BCG	*M. intracellulare*	*M. scrofulaceum*	*M. gordonae*	*M. avium*
PstI	16.0, 11.0, 5.8,[c] 5.0, 3.0, 2.7, 2.4	6.5, 5.3, 1.8, 1.3	13.0, 7.1, 1.3	7.1, 1.3	7.1, 1.3	5.5, 1.4	13.0, 6.5, 1.1	11.0, 6.8, 1.4, 0.7	13.0, 3.9, 3.2
PvuI	8.5,[c] 5.5, 4.8, 1.4	9.4, 5.6, 1.3	7.0, 1.9	7.0, 1.9	ND[d]	9.5	9.5, 7.0	ND	8.5, 7.5, 5.5
AvaII	2.5, 1.2, 0.3	2.6	1.2	1.2	ND	1.9	0.85, 0.3	ND	2.1, 0.7

[a] 16S and 23S rRNA from *E. coli*.
[b] Data are the average of at least two experiments. Input counts ranged between 2×10^6 and 5×10^6 cpm per blot.
[c] Doublet signal.
[d] ND, Not done.

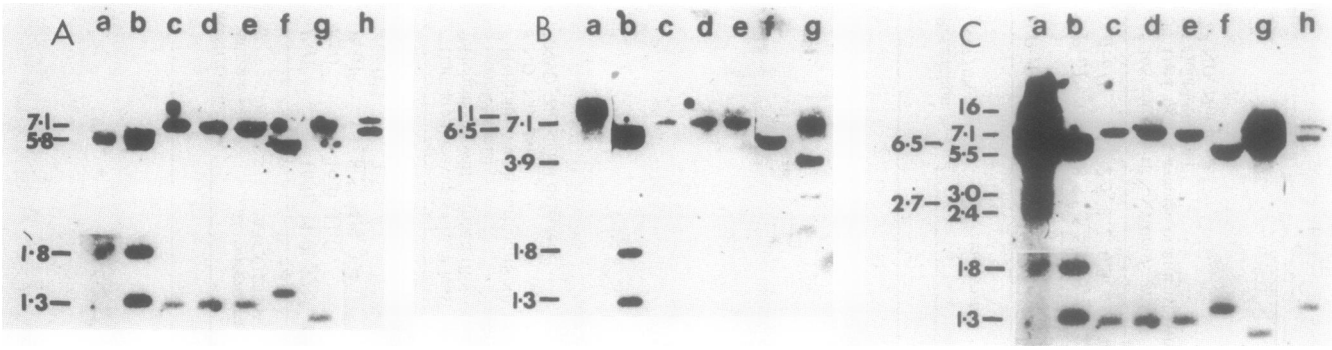

FIG. 1. Hybridization of *Pst*I digests of mycobacterial DNAs to ^{32}P-labeled cDNAs of *M. tuberculosis* H37Ra tRNA (A), *M. smegmatis* tRNA (B), or 23S plus 16S rRNA from *E. coli* (C). Lanes: a, *M. smegmatis*; b, *M. phlei*; c, *M. bovis* BCG; d, *M. tuberculosis* H37Ra; e, *M. tuberculosis* H37Rv; f, *M. intracellulare*; g, *M. scrofulaceum*; and h, *M. gordonae*. Lower portions of panels A and C represent fourfold-longer exposures than the top portions. Numbers indicate fragment sizes in kilobase pairs.

to mycobacterial tRNA probes also hybridized to *E. coli* tRNA probes (data not shown).

Construction of *M. tuberculosis* H37Rv gene bank. A library with a 98 to 99% probability of completely representing the *M. tuberculosis* H37Rv genome was constructed. Mycobacterial DNA subjected to partial digestion with *Sau*3A was fractionated on a 0.8% agarose gel, and fragments 3 to 6 kilobase (kb) long were electroeluted and purified by DEAE-Sephacel chromatography (11). Dephosphorylated *Bam*HI-cleaved pUC8 vector was ligated to insert DNAs at a molar ratio of 1:2, respectively, and introduced into *E. coli* DH1 (8).

RESULTS AND DISCUSSION

Southern hybridization of *Pst*I-digested mycobacterial DNA to tRNA and rRNA probes. Hybridization of mycobacterial tRNA-derived probes with the mycobacterial DNAs revealed distinct patterns, with the exception of the MTB complex (Fig. 1A and B). The hybridization signals observed with tRNA-derived probes from slow- and fast-growing mycobacterial species are summarized in Table 1. To examine tRNA gene organization in depth, we also performed hybridizations with *Pvu*I-, *Ava*II-, and *Sal*I-digested mycobacterial DNAs (Table 1); none of these enzymes were able to distinguish between the tRNA-coding regions of the genomes of *M. tuberculosis* H37Rv and H37Ra. The mycobacterial species examined in this study exhibit considerable variation in genome size and DNA relatedness. *M. tuberculosis* and members of the MTB complex share 90 to 100% DNA homology, while *M. intracellulare*, *M. avium*, *M. scrofulaceum*, *M. smegmatis*, and *M. phlei* show 48, 30, 22, 12, and 7% DNA homology, respectively, with *M. tuberculosis* (2, 9, 10). Hybridization occurred to heterologous mycobacterial tRNA probes, suggesting that the tRNA genes are conserved in the nine mycobacterial species examined. However, the gross tRNA gene organization has diverged to permit the identification of six of the nine mycobacterial species examined. More species and strains need to be examined to evaluate the utility of tRNA probes in epidemiological studies. Organisms of the MTB complex appear to possess apparently identical tRNA gene organization. The data also indicated that the majority of mycobacterial tRNA genes in slow-growing species except in *M. scrofulaceum* are located on the large *Pst*I fragment (7.1-kb major signal in MTB complex, for example), while those from rapidly growing mycobacteria are present on two or more large fragments.

A difference in tRNA pools in the slow-growing *M. tuberculosis* and the fast-growing *M. smegmatis* was indicated by differential hybridization to tRNA-coding segments of mycobacterial DNA. This was clearly illustrated in *M. scrofulaceum* and *M. smegmatis*, in which entirely different fragments hybridized to tRNA-derived probes (Table 1). Mycobacteria provide a unique system to study tRNA gene expression, with species within a genus exhibiting various generation times.

*Pst*I digests of mycobacterial DNAs were also hybridized to cDNA probes derived from *E. coli* 23S plus 16S rRNAs. The *Pst*I fragments that hybridized to the tRNA-derived probes also hybridized to the rRNA-derived probe. Additional genomic fragments hybridized to the rRNA-derived probe in digests of *M. smegmatis*, *M. scrofulaceum*, and *M. gordonae* (Fig. 1C; Table 2). Upon hybridization of the rRNA-specific probe to *Pvu*I and *Ava*II blots of mycobacterial DNAs, a similar result was obtained—signals obtained were nearly identical to those obtained with tRNA-specific probes (Tables 1 and 2). Thus, the hybridization data with DNAs cleaved with *Pst*I, *Ava*II, and *Pvu*I suggested that the tRNA genes are closely associated with rRNA genes in mycobacteria. Our findings that *E. coli* rRNA-specific probes hybridized well with DNA fragments encoding the corresponding rRNA genes in mycobacteria support the existence of marked sequence homology between rRNAs of *E. coli* and putative rRNA gene sequences of mycobacteria. The nucleotide sequences of the 16S rRNA genes from *M. bovis* BCG and *E. coli* have 75% homology (15). Other workers have used *E. coli*- and *M. bovis* BCG-derived rDNA probes to analyze mycobacterial rDNA organization. By and large, the fragments hybridizing to rRNA-specific probes in our study were similar in size to the reported data (1, 16). The observed differences may be attributable to strain variation. Close association of tRNA and rRNA genes has also been observed in *B. subtilis*, another gram-positive organism (17). Since both tRNA and rRNA genes are structural genes that are vital components of the protein-synthesizing machinery, the location of tRNA genes in the close vicinity of the rRNA genes offers functional advantages in terms of facilitating coordinated regulation of gene expression for growth and for adaptation to the environment.

Isolation of clones containing tRNA genes of *M. tuberculosis*. A total of 1,200 transformants composing 9% of a genomic library of *M. tuberculosis* were screened (7) with ^{32}P-labeled cDNA to tRNA from *M. tuberculosis*. Nine positive signals were obtained (pSB1 to pSB9). Three of the clones hybrid-

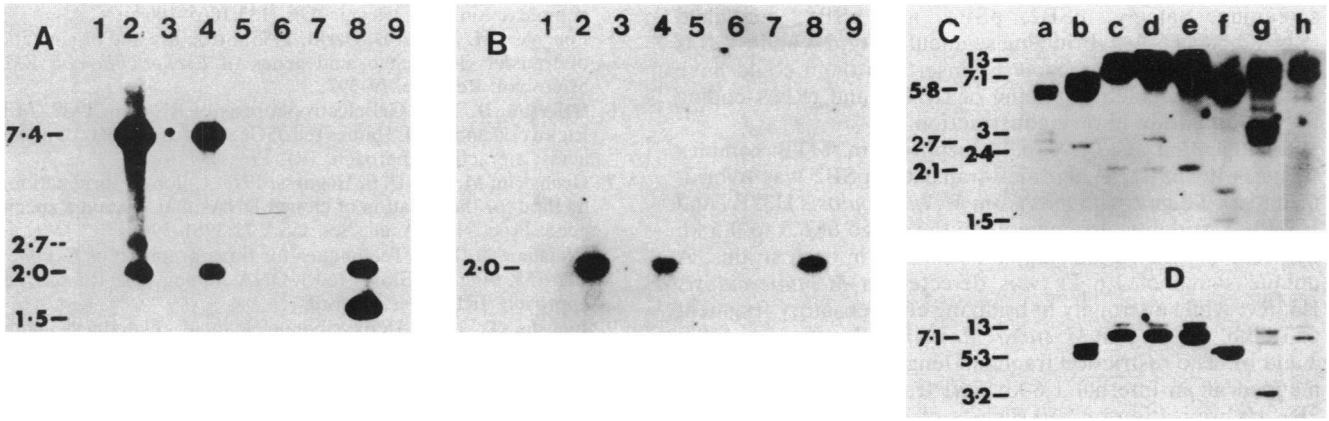

FIG. 2. Identification of tRNA- and rRNA-coding recombinant plasmids. (A) Hybridization of ^{32}P-labeled cDNA to tRNA from *M. tuberculosis* H37Ra with *Eco*RI plus *Hin*dIII double digests of pSB1 to pSB9 is shown in lanes 1 to 9, respectively. (B) Hybridization of ^{32}P-labeled cDNA to 23S plus 16S rRNAs from *E. coli* to the same blot as in panel A. (C) *Pst*I digests of mycobacterial DNAs hybridized with ^{32}P-labeled pSB2 (3 days of exposure); lane a, *M. smegmatis*; lane b, *M. phlei*; lane c, *M. bovis* BCG; lane d, *M. tuberculosis* H37Ra; lane e, *M. tuberculosis* H37Rv; lane f, *M. intracellulare*; lane g, *M. scrofulaceum*; and lane h, *M. gordonae*. (D) A 16-h exposure of panel C. Fragment indicated by dotted line is a partial digestion product. Numbers indicate fragment sizes in kilobase pairs.

ized strongly with tRNA- and rRNA-derived probes (Fig. 2A and B), and one hybridized weakly with a tRNA-derived probe (data not shown). None of the tRNA-coding clones isolated hybridized with the 1.3-kb genomic *Pst*I fragment of *M. tuberculosis* DNA (Fig. 1). The strong signal intensity of the 7.1-kb *Pst*I fragment suggests that the majority of the mycobacterial tRNA genes are located on this fragment. The library was screened with cDNA to total tRNA, and therefore it is understandable that the clones obtained were those that hybridized to the 7.1-kb genomic *Pst*I fragment.

Characterization of pSB2. ^{32}P-labeled pSB2 probe hybridized very strongly with *Pst*I digests of mycobacterial DNAs, and the majority of the signals were identical to those obtained with tRNA- and rRNA-specific cDNA probes. These data confirmed the identity of pSB2 as a recombinant plasmid containing a tRNA gene(s) and an rRNA gene(s). Hybridization to additional fragments was also observed, which probably represents chromosomal segments not coding for tRNA or rRNA (Fig. 2C and D). Plasmid pSB2 was subjected to either single or double digestion with several restriction endonucleases, and Southern hybridizations to the tRNA- and rRNA-derived probes were done. Based on the hybridization data (not shown), a restriction map of pSB2 was constructed (Fig. 3C). The presence of a 10-kb mycobacterial insert in pSB2 suggests that two *Sau*3A fragments were ligated together during library construction. However, the physical proximity of a tRNA gene(s) and an rRNA gene(s) in pSB2 probably represents their true location on the chromosome. This is substantiated by (i) nearly identical hybridization signals obtained in genomic blots of *M. tuberculosis* DNA with tRNA- and rRNA-derived probes with all the enzymes tested (*Pst*I, *Ava*II, and *Pvu*I) and (ii) the fact that three of the four clones identified by library

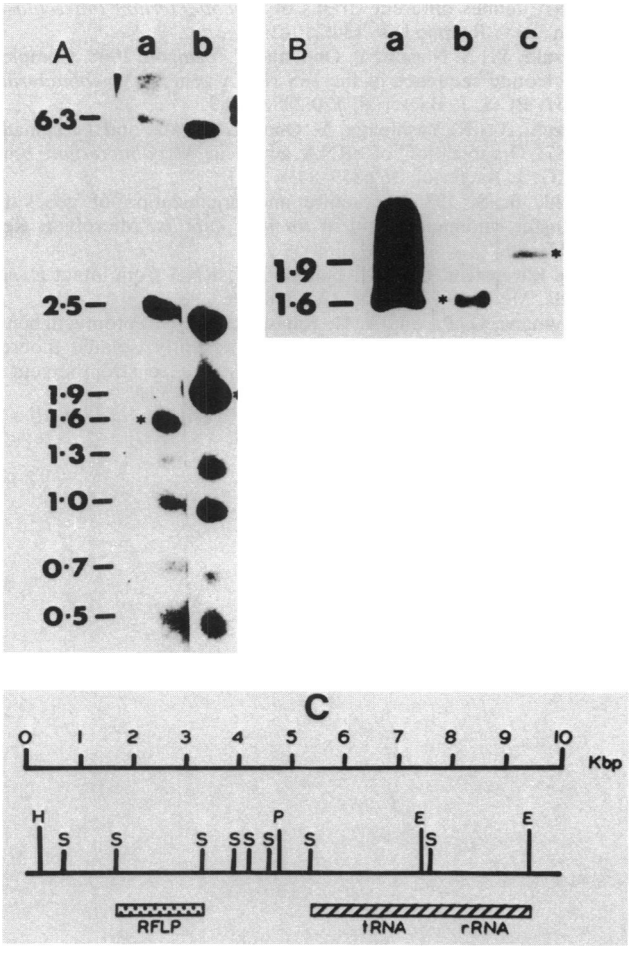

FIG. 3. Identification of restriction fragment length polymorphism in *M. tuberculosis* by pSB2. *Sal*I digests of DNAs hybridized with ^{32}P-labeled nick-translated pSB2 (A) and nick-translated 1.6-kb *Sal*I fragment of pSB2 (B). In panel A, lanes a and b are *M. tuberculosis* H37Rv and H37Ra, respectively. In panel B, lane a is pSB2; lanes b and c are *M. tuberculosis* H37Rv and H37Ra, respectively. Numbers indicate fragment sizes in kilobase pairs (Kbp), and asterisks indicate the restriction fragment length polymorphism. (C) Physical map of plasmid pSB2. Boxes represent the regions hybridizing to the tRNA- and rRNA-derived probes. Abbreviations for restriction enzyme sites are as follows: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; and S, *Sal*I.

screening, namely, pSB2, pSB4, and pSB8, contained tRNA- and rRNA-hybridizing segments (Fig. 2A and B). It is unlikely that the mycobacterial inserts in them could have arisen from accidental joining of tRNA- and rRNA-coding fragments during library construction.

Identification of DNA polymorphism in MTB complex strains with pSB2. When nick-translated pSB2 was hybridized to *SalI* digests of DNA from *M. tuberculosis* H37Rv and H37Ra, six identical fragments in the range of 6.3 to 0.5 kb hybridized with various signal intensities in both strains. A unique signal of 1.6 kb was detected in *M. tuberculosis* H37Rv, while a strongly hybridizing compensatory fragment of 1.9 kb was seen in *M. tuberculosis* H37Ra (Fig. 3A, lanes a and b). The restriction fragment length polymorphism was mapped at an internal 1.6-kb *SalI* fragment of pSB2 (Fig. 3B). Hybridization of *SalI* digests of *M. tuberculosis* DNA with tRNA-derived probes suggested that the restriction fragment length polymorphism is not linked to the segment coding for tRNA(s) and rRNA(s) (Fig. 3C; Table 1). This was confirmed by hybridization with *AvaII* and *PvuI* blots (data not shown). Earlier attempts to distinguish avirulent *M. tuberculosis* H37Ra from virulent *M. tuberculosis* H37Rv on the basis of restriction fragment length patterns (3, 4, 12, 14) have failed, probably because these strains are highly homologous at the DNA level (10).

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