INFECTION AND IMMUNITY, May 1989, p. 1535–1541
0019-9567/89/051535-07$02.00/0

Vol. 57, No. 5

Genetic Relationships among *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and Candidate Leprosy Vaccine Strains Determined by DNA Hybridization: Identification of an *M. leprae*-Specific Repetitive Sequence

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Received 16 November 1988/Accepted 20 January 1989

Comparative DNA hybridization studies of genomic DNA indicated that, while different isolates of armadillo-derived *Mycobacterium leprae* have a high degree of homology, binding of *M. leprae* genomic DNA to DNA of other species of mycobacteria or to corynebacteria was low, establishing that *M. leprae* is only remotely genetically related to any of the species examined. Several candidate leprosy vaccine mycobacterial strains were similarly found to have little genetic similarity to *M. leprae*. In contrast, the DNAs of the slow-growing mycobacteria *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. microti* were found to be very closely related. In the course of these studies, an *M. leprae*-specific repetitive sequence, greater than 15-fold per genome equivalent, was identified that might be useful for diagnostic and epidemiological studies.

Mycobacterium leprae, the causative agent of leprosy, remains one of the few human pathogens not cultivable in vitro. This has made its taxonomic relationship vis-à-vis other genera of *Actinomycetales*, especially *Mycobacterium* and *Corynebacterium*, problematic. Elucidation of taxonomic relationships among pathogenic mycobacteria, nonpathogenic species, and possible candidate vaccine strains is crucial for developing effective epidemiological methods and improved strategies for immunization against leprosy.

The discovery that *M. leprae* can be grown in high yield in nine-banded armadillos (*Dasypus novemcinctus*) (18, 29) made it possible to prepare and study the genetic material of the etiologic agent of leprosy. The leprosy bacillus has generally been classified as a *Mycobacterium* species, since its cell wall has been shown to contain mycolic acids (10) and antigens characteristic of mycobacteria (14, 28). Imaeda et al. (17) have compared DNA from *M. leprae* with that of other mycobacteria and corynebacteria by solution hybridization techniques and, surprisingly, found it to be more closely related to corynebacteria isolated from leprosy patients than to any other mycobacterial species tested. Although later experiments by the Imaeda group modified the initial suggestion that *M. leprae* was related to the corynebacteria (1), the implication that *M. leprae* was not a *Mycobacterium* species warranted additional investigation. We reasoned that the filter method of DNA hybridization, combined with thermal elution profiles, might provide additional information on the relatedness of *M. leprae* to other bacterial species. In addition, reports of cultivable mycobacterial strains which have antigenic similarities to *M. leprae* and the capacity to induce sensitization to *M. leprae* in immunologically unresponsive patients with lepromatous leprosy (4, 9; V. Khera, S. Savant, and P. R. Mahadevan, Abstr. 12th Int. Lep. Congr. 1984, no. 223) provided an additional need for comparative studies of hybridization of *M. leprae* DNA to selected species of mycobacteria.

To resolve small differences in DNA binding and to search for small segments of highly homologous regions of generally unrelated DNA samples, we used the method of filter hybridization, in which one single-stranded DNA complement is irreversibly bound to a solid substrate and the other is radioactively labeled, hybridized, and then eluted by temperature-dependent dissociation of double-stranded regions of DNA homology. This method has the advantages of requiring relatively small amounts of DNA, being highly reproducible, and simultaneously furnishing information on the extent of DNA hybridization and thermal stability of hybrids. Clark and Brownell used this technique to investigate genophore homologies among nocardiae (5), and Bradley, after examining genetic relationships among nocardiae and mycobacteria by solution hybridization and filter hybridization, found that both methods yielded comparable results (3). In addition to thermal elution analysis, we analyzed restriction-fragment-length polymorphisms (2) of these various mycobacterial strains by using Southern analyses (27) by probing, with a DNA fragment encoding the highly conserved heat shock gene, the 65-kilodalton (kDa) antigen (21, 34, 35), which revealed a unique repetitive sequence in *M. leprae*.

MATERIALS AND METHODS

Bacterial strains and growth. Most of the mycobacteria used in this study were obtained from the Mycobacterial Culture Collection at the National Jewish Hospital and Research Center in Denver, Colo. Bacterial samples were inoculated onto Lowenstein-Jensen slants and transferred to Dubos broth (Difco Laboratories, Detroit, Mich.) supplemented with Tween 80, glycerol (0.5%), and Dubos Medium Albumin (Difco). The following isolates were treated differently. The ICRC bacillus and *Mycobacterium* sp. strain w (R1-7) were generously provided by C. V. Bapat and M. G. Deo at the Indian Cancer Research Center, Bombay, India, and by P. Talwar, National Institute of Immunology, New Delhi, India, respectively. The organisms were grown in

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Middlebrook 7H9 medium supplemented with oleic acid-dextrose complex (OADC; Difco). *Corynebacterium diphtheriae* type gravis S1016 (ATCC 19409) and *C. diphtheriae* type mitis C7 (ATCC 27010) were obtained from N. Groman, University of Washington. *Corynebacterium* sp. strain 2628 L.B. and *Corynebacterium* sp. strain Shep IV were obtained from L. Barksdale, New York University. All corynebacteria were grown in brain heart infusion broth as described previously (12, 25). FMR 306 was obtained from P. R. Mahadevan at the Foundation for Medical Research in Madras, India, and grown in Dubos broth supplemented with 20% human serum. *M. microti* TMC 1601 and *M. tuberculosis* Erdman TMC 107 were obtained from F. Collins at the Trudeau Institute, Saranac Lake, N.Y., and grown in Middlebrook 7H9 medium supplemented with OADC.

Armadillo liver infected with *M. leprae* was obtained from C. Shepard at the Centers for Disease Control in Atlanta, Ga. Two different preparations were made: one from an armadillo which had been inoculated with leprosy bacilli from several patients and another which had been infected with a single isolate. Unless otherwise stated, the latter preparation was used throughout the experiments described here. *M. leprae* cells were purified from the liver tissue as described previously (7).

Large cultures of bacteria were obtained by inoculating bacterial cells in 200 ml of media in roller bottles that were rotated at 1 rpm and at 37 or 31°C, whichever afforded faster growth. When late exponential phase was reached, bacteria were harvested, washed, and suspended in 0.1 M NaCl–0.03 M Tris–0.006 M EDTA.

DNA isolation. For most of the strains, 0.2 ml of packed bacterial cells was disrupted in the presence of acid-washed glass beads (diameter, 0.45 mm) with a cell homogenizer (MSK; B. Braun Biotech, Inc., Allentown, Pa.) at high speed for 2 min at 4°C. This method led to the release of approximately 80% of all releasable DNA. The homogenate was twice extracted with chloroform-phenol (1:1), and the aqueous phase was then extracted with chloroform-isoamyl alcohol (24:1). Sodium acetate was added to 0.3 M, and DNA was precipitated with ethanol, dried, suspended in 0.1 M sodium acetate at pH 5.5, and digested with boiled RNase at 0.1 mg/ml for 1 h at 37°C. Chloroform-phenol extraction and ethanol precipitation were repeated, and DNA samples were suspended in 0.1 M NaCl–0.02 M Tris–0.001 M EDTA. UV light absorption ratios of aqueous DNA samples at 260 and 280 nm were between 1.8 and 2.0.

M. leprae and "*Mycobacterium lufu*" DNAs were isolated as described previously (7). A sample of normal armadillo liver was kindly provided by R. Rees (National Institute for Medical Research, London, England), and DNA was prepared for control purposes by following a protocol published by Maniatis et al. (19).

Purified DNA from *Caulobacter crescentus* CB 13 was kindly provided by L. Shapiro and B. Alexander.

DNA membrane hybridizations. Chromosomal DNAs were bound to membrane filters by using a modification of the procedure of Palmiter et al. (22). The protocol was modified and adapted in the following way. Either 1 μg (low-stringency experiments) or 3 μg (high-stringency experiments) of sample DNA was suspended in 5 μl of a solution containing 2 M NaCl and 0.1 M NaOH and boiled for 2 min. The DNA was then spotted onto prewetted squares (7 mm by 7 mm) of Zetabind (AMF) which had been placed onto Parafilm. During pilot studies, we encountered difficulties with sample DNA retention on membrane filters, especially at higher temperatures. Subsequently, we tested several products (nitrocellulose filters [Schleicher & Schuell, Inc., Keene, N.H.], GeneScreen [Dupont, NEN Research Products, Boston, Mass.], and Zetabind [AMF]), and we found that under the experimental conditions used here, Zetabind retained by far the most sample DNA (^{3}H-labeled DNA from *Escherichia coli*), with approximately 1% of radioactivity being lost between 70 and 99°C. After being air dried, filter squares were floated on, and then submerged in, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (19) with 0.05 M Tris. Filters were then air dried and baked in a vacuum oven at 80°C for 2 h.

M. leprae or *M. tuberculosis* probe DNA was labeled with [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) by using a nick translation system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). At the end of the labeling period, a small amount of yeast tRNA was added as a carrier, and the whole mixture was extracted with phenol. The aqueous phase was precipitated twice with ethanol and suspended each time in 0.4 M ammonium acetate. Probe DNA was then sheared by sonication, using a Braun Sonicator, to an average length of 600 base pairs (as determined by gel electrophoresis), precipitated with ethanol, and suspended in 1 mM Tricine (Sigma Chemical Co., St. Louis, Mo.). A sample was coprecipitated with trichloroacetic acid (10%) in the presence of carrier bovine serum albumin. The specific activity of precipitable ^{32}P-labeled DNA was between 0.5×10^8 cpm and 1×10^8 cpm for each microgram of starting DNA.

Membrane filters with baked-on sample DNA were placed individually into 5-ml Wheaton scintillation vials and incubated for 4 h at 60°C in 0.5 ml of prehybridization buffer (4× SSC, 4× Denhardt solution [19], 0.2% sodium dodecyl sulfate [SDS], 0.02 M Tris hydrochloride [pH 8.0], 200 μg of sonicated and heat-denatured salmon sperm DNA [19] per ml). After prehybridization, filters were washed three times at room temperature with the same buffer, excluding Denhardt solution and salmon sperm DNA, and then placed in 0.5 ml of hybridization buffer composed of 4× SSC, 4× Denhardt solution, 0.2% SDS, 0.04 M Tris, and 0.01 M EDTA. Concentrated radioactively labeled probe DNA (1×10^6 to 5×10^6 cpm/ml of hybridization buffer) was then mixed with concentrated sonicated salmon sperm DNA (100 μg/ml of hybridization buffer), and the mixture was boiled for 2 min. Appropriate samples of denatured probe and salmon sperm DNA were then added to membrane filters suspended in hybridization buffer and were preheated to 60°C (low-stringency conditions of hybridization) or 75°C (high-stringency conditions of hybridization). After 16 h of incubation with gentle agitation, filters were washed five times with either high-stringency elution buffer (0.3× SSC, 0.1% SDS, 0.02 M Tris) or low-stringency elution buffer (3× SSC, 0.1% SDS, 0.02 M Tris) at room temperature and then incubated in 5 ml of elution buffer for 1 h at 60 or 75°C to remove nonspecific binding. Supernatant buffer was then replaced with 0.5 ml of fresh elution buffer. The total amount of bound probe DNA was ascertained by raising the temperature, in 8°C steps, to the boiling point and removing the eluate (5). Radioactivities of eluates were determined by liquid scintillation counting. Eluates were mixed with an equal volume of Liquiscint (National Diagnostics, Manville, N.J.), and the resulting gel was analyzed by using an LS5000 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). After subtraction of appropriate background values, cumulative releases (in counts per minute) were calculated and normalized. Binding of probe DNA to homologous sample DNA was between 8 and 14%, depending on the

hybridization temperature, buffer concentration, and relative amounts of sample and probe DNA used.

Southern analyses. Recombinant DNA manipulations were performed as described previously (19, 27). Various mycobacterial DNAs were digested with restriction enzymes and electrophoresed in 0.7% agarose gels. The resulting gels were blotted onto Biotrans nylon membranes (ICN Biomedicals, Irvine, Calif.) and probed by following the manufacturer's recommendations. The 65-kDa antigen gene probes were prepared by subcloning the *Eco*RI inserts from the lambda *gt*11 clones Y3178 (35) and Y3141 (16) for *M. leprae* and *M. tuberculosis*, respectively, into pUC18 (33). Radiolabeled probes were prepared from plasmid DNA of these resulting subclones by nick translation as described above. The 1.6-kilobase (kb) *Xho*I-*Eco*RI *M. leprae* DNA fragment that corresponds to the sequence from nucleotides 2010 to 3610 of the sequenced Y3178 *Eco*RI insert (21) was isolated from an agarose gel and subsequently nick translated. This DNA fragment is located outside of the coding region of the *M. leprae* 65-kDa antigen.

RESULTS

Hybridization of *M. leprae* DNA. Under conditions of high stringency, hybridizations revealed little homology of *M. leprae* DNA with DNA from 20 different species of mycobacteria (Table 1). Negligible homology was also observed with DNA from putative leprosy-associated corynebacteria or other corynebacteria under similar conditions. However, a very high degree of homology between two independent *M. leprae* preparations was found. One had been prepared from an armadillo inoculated with a pool of human isolates; the other originated from an armadillo infected with a single human isolate. The ability of the hybridization methods used to demonstrate significant homology relationships was further confirmed by the high degree of homology observed for mycobacteria related to *M. tuberculosis*, an observation consistent with results previously reported from the use of other techniques (3, 13, 30). It is perhaps worth noting that *M. tuberculosis* DNA shares significant homologies with DNA from "*M. lufu*," the ICRC bacillus, and *Mycobacterium* strain w, a relationship that had not been previously examined.

Hybridizations performed at conditions of lower stringency demonstrated that most of the mycobacterial and the corynebacterial DNAs do share a low degree of homology (5 to 12%) with *M. leprae* DNA. This level of homology is likely to be significant, however, as the DNA from *Caulobacter crescentus*, which had been selected because it has a G+C content (G+C = 67% [32]) close to that of mycobacteria, and from *E. coli* (G+C = 51% [24]) bound barely above background levels of radioactive label.

Restriction-fragment-length polymorphisms of the *M. leprae* gene encoding the 65-kDa antigen: identification of an *M. leprae*-specific repetitive sequence. A large number of both specific and cross-reactive monoclonal antibodies to antigens and epitopes of *M. leprae* and *M. tuberculosis* have been developed (H. D. Engers, Letter, Infect. Immun. **48**:603–605, 1985; H. D. Engers, Letter, Infect. Immun. **51**:718–720, 1986). By using these antibodies, recombinant lambda *gt*11 phages expressing mycobacterial antigens have been isolated (16, 35). Several of these antigens appear to be stress-induced or heat shock proteins that are conserved (34). We reasoned that Southern analyses using DNA probes for such antigens might reveal restriction-fragment-length polymorphisms that could provide another level of comparative analysis. We compared Southern analyses of DNA from *M. leprae* strains, *M. tuberculosis*-related strains, and the candidate leprosy vaccine strains with that of a DNA fragment containing the entire coding region for the 65-kDa antigen. Surprisingly, the *M. leprae* DNA fragment encoding the 65-kDa antigen hybridized to more than 17 DNA fragments from *M. leprae* DNA digested with *Pst*I, *Bst*EII (Fig. 1b), or *Eco*RI or *Bam*HI (data not shown). Many of these fragments were greater in size than the original probe, suggesting that the probe was hybridizing to sequences of which there were multiple copies throughout the *M. leprae* genome. In contrast, this probe hybridized to no more than two to three DNA fragments of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, or the three candidate leprosy vaccine strains. In parallel experiments, similar Southern blots were probed with a DNA fragment encoding the 65-kDa antigen of *M. tuberculosis* (Fig. 1c). As expected, when the *M. tuberculosis* 65-kDa antigen gene probe was used, no differences were observed among *M. tuberculosis*, *M. bovis*, and BCG. However, restriction-fragment-length polymorphisms were detected in the DNAs of *M. leprae*, the *M. tuberculosis* strains, and the candidate leprosy vaccine strains. Unlike the *M. leprae* probe, the *M. tuberculosis* probe hybridized to only one or two DNA fragments, suggesting that the 65-kDa antigen gene was single copy in the mycobacterial chromosomes. To determine whether the DNA sequence encoding the 65-kDa antigen was present in multiple copies throughout the *M. leprae* genome, we probed Southern blots with various DNA fragments prepared from the original 3.6-kb *Eco*RI fragment that had been isolated from a lambda *gt*11 clone expressing the 65-kDa antigen. As shown in Fig. 1d, the repetitive sequence is present in a DNA fragment that is located adjacent to and outside of the carboxy-terminal end of the DNA sequence encoding the 65-kDa antigen. Clearly, since it fails to hybridize to the other mycobacterial DNAs, this 1.6-kb DNA repetitive fragment is *M. leprae* specific.

DISCUSSION

The present study was undertaken to investigate genetic relationships among *M. leprae*, mycobacterial isolates that represent candidate vaccine strains against leprosy, other pathogenic and nonpathogenic mycobacteria, and related genera. Mycobacterial taxonomy has been based on both phenotypic and genetic analyses. Gross and Wayne (13) compared parameters of phenotypic similarity, DNA hybridization, and thermal stability of hybridized DNA within the genus *Mycobacterium* and found a high degree of correlation between these taxonomic methods. The study of *M. leprae* has been hindered by the fact that it remains one of the few bacteria that have not been grown successfully in vitro. Many of the scientific limitations were alleviated when it was discovered that leprosy bacilli could be grown in the mouse footpad (26) and grew in high yields in the armadillo (18, 29). In addition to its unique antigens and epitopes (15; Engers, Letter, Infect. Immun., 1985), *M. leprae* differs from other members at the DNA level in having a low G+C ratio (56% versus 60 to 70%) (3, 7, 31) and a small genome size similar to that of *M. tuberculosis* (7). Imaeda and colleagues (17) also investigated DNA homology between *M. leprae* and related bacteria by optical DNA-DNA reassociation in solution. Their findings indicated a 68% sequence homology with a corynebacterial isolate (2628 L.B.) from a leprosy patient, considerably higher than that found with any of the 10 mycobacterial species tested in the present study.

In the selection of bacterial test species to be compared with *M. leprae*, we endeavored to cover a representative

TABLE 1. Hybridization analysis of *M. leprae* DNA to other mycobacterial DNAs and leprosy-associated corynebacteria[a]

Sample DNA	Percent binding of probe DNA		
	M. leprae		*M. tuberculosis*, high stringency
	Low stringency	High stringency	
M. leprae grown in armadillos			
Single inoculum	100	100	1.5
Mixed inoculum	ND	92 ± 6	ND
Leprosy-associated *Corynebacterium* strains			
2628 L.B.	<1	<1	<1
Shep IV	<1	<1	ND
Other *Corynebacterium* strains			
C. diphtheriae type gravis	6.4 ± 1.3	<1	<1
C. diphtheriae type mitis	8.7 ± 0.3	<1	1.3
Corynebacterium sp. strain 813	<1	<1	ND
Candidate leprosy vaccine strains			
ICRC bacillus	10.5 ± 3.5	2.9	30.9
Mycobacterium strain w R1-7	10.5 ± 2.8	3.9	20.0
FMR bacillus	7.9 ± 0.8	1.4	6.4 ± 0.8
M. tuberculosis complex strains			
M. tuberculosis (Erdman)	ND	1.2	100
M. tuberculosis H37Rv	8.2 ± 3.0	ND	94.8 ± 8.6
M. bovis (TMC 409)	8.3 ± 1.1	<1	75.1 ± 6.9
M. bovis BCG (Pasteur)	6.7 ± 0.9	1.1	96.7 ± 9.8
M. africanum (TMC 5122)	12.3 ± 0.2	<1	77.6 ± 5.0
M. microti (TMC 1601)	3.3	1.1	88.0 ± 4.9
Other mycobacteria			
M. avium (TMC 724)	10.8 ± 1.2	1.3	8.0
M. chelonei (TMC 1544)	9.0 ± 0.3	<1	1.4
M. fortuitum (TMC 1529)	5.7 ± 0.1	<1	4.3
M. gordonae (TMC 1324)	5.0 ± 0.2	ND	ND
M. intracellulare (TMC 1406)	6.6 ± 0.3	2.2	26.0 ± 4.0
M. kansasii (TMC 1204)	9.0 ± 0.1	1.5	23.5 ± 1.8
"*M. lufu*"	12.2 ± 2.2	2.6	48.2 ± 7.1
M. marinum (TMC 1218)	5.8 ± 0.3	<1	6.1
M. nonchromojenicum (TMC 1481)	6.1 ± 0.2	1.0	8.7
M. phlei (TMC 1548)	10.3 ± 1.9	1.2	9.6
M. scrofulaceum (TMC 1320)	7.2 ± 0.4	2.2	15.6
M. smegmatis (TMC 1515)	6.8 ± 0.7	<1	4.3
M. vaccae (TMC 1526)	<1	<1	<1
Other DNAs			
Caulobacter crescentus	1.7 ± 0.1	ND	ND
Dasypus novemcinctus	ND	<1	ND
Escherichia coli K-12	2.0 ± 0.2	ND	ND

[a] Purified single-stranded sample DNA was baked onto Zetabind, prehybridized, washed, hybridized with 0.5 × 10^6 to 2.5 × 10^6 cpm of single-stranded radiolabeled probe DNA, and washed again. Radiolabeled DNA was then thermally eluted in 0.3× SSC or 3.0× SSC as described in Materials and Methods. Data were normalized with homologous DNA binding expressed as 100%. Statistics (mean ± standard deviation), where given, refer to duplicate experiments. All other data are from single experiments, except with the leprosy-associated corynebacteria, which represent triplicate experiments. ND, Not determined. The TMC numbers are Trudeau Mycobacterial Culture Collection reference numbers.

cross-section of established mycobacteria (20 species) and a number of other organisms of interest. From the (potentially) pathogenic members of the *M. tuberculosis* complex (30), we chose representatives which, within their subgroupings (especially BCG, *M. bovis*, and *M. tuberculosis*), were closely related by DNA hybridization. We also used three Indian isolates of human origin reported to be immunologically related to *M. leprae* and considered candidate vaccines against leprosy (ICRC, FMR 306, and *Mycobacterium* strain w) and suggested by some to represent cultivable forms of *M. leprae*. "*M. lufu*" is noteworthy because it shares the characteristic sensitivity of *M. leprae* to Dapsone, a standard drug in the treatment of leprosy (23). Several species and isolates of corynebacteria were studied because they had previously been reported to be genetically related to *M. leprae*: two well-established strains of *Corynebacterium diphtheriae*, one isolate of an aerobic skin coryneform (*Corynebacterium* strain 813), and two isolates of leprosy-associated corynebacteria (*Corynebacterium* strains 2628 L.B. and Shep IV). *Corynebacterium* strain 2628 L.B. is the organism which Imaeda et al. (17) had found to be most closely related to *M. leprae*. Our thermal elution experiments indicated that *Corynebacterium* strains 2628 L.B. and Shep IV were more closely related to each other (90%) than to any of several mycobacteria tested (data not shown). For control purposes, we also included a species of *Caulobacter*

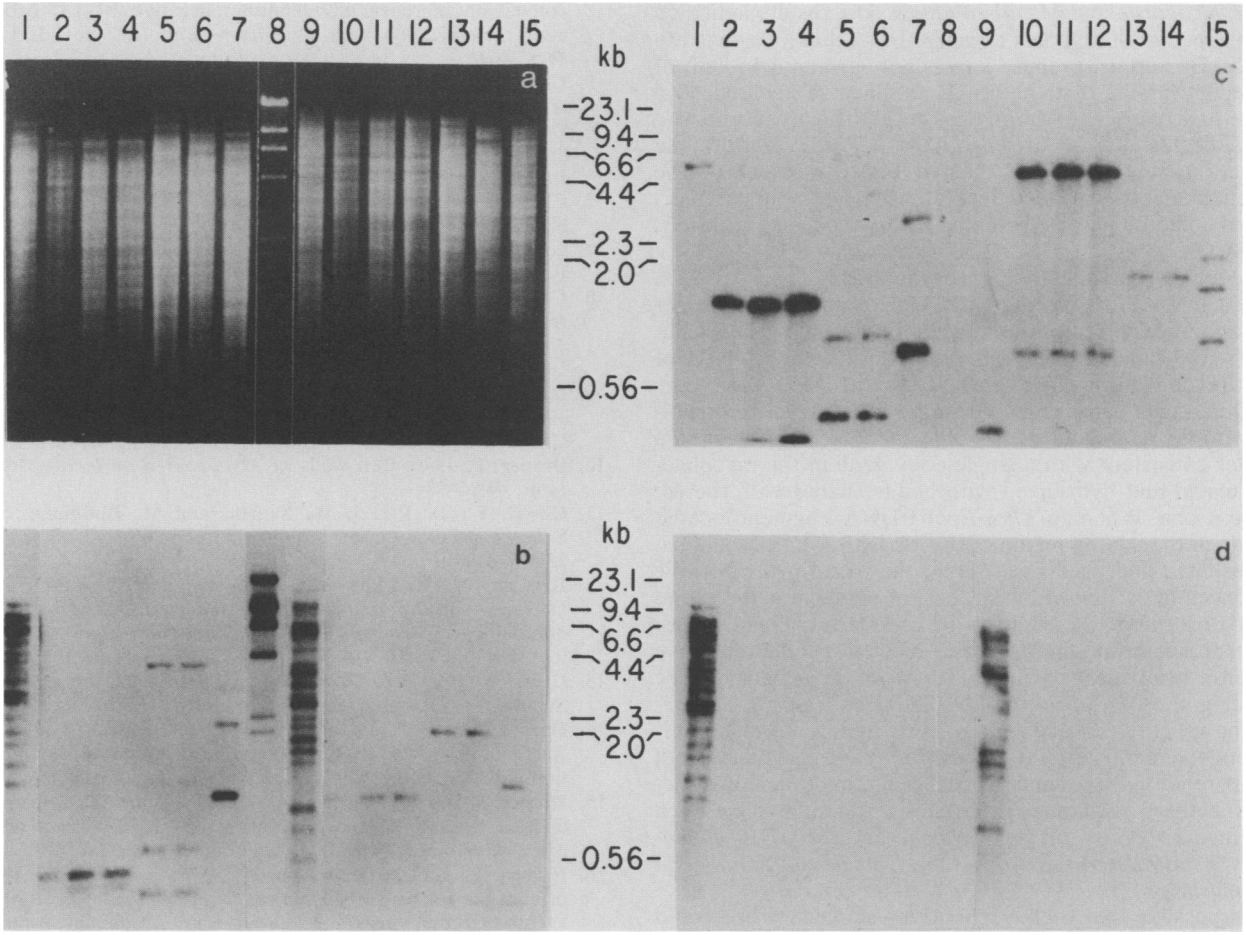

FIG. 1. Southern analyses of mycobacterial DNAs probed with DNA fragments containing the 65-kDa antigen. Mycobacterial chromosomal DNAs were digested with either *Pst*I (lanes 1 to 7) or *Bst*EII (lanes 9 to 15) and electrophoresed on a 0.7% agarose gel. The ethidium-bromide stained gel is shown in panel a with DNAs from *M. leprae* (lanes 1 and 9), *M. tuberculosis* Erdman (lanes 2 and 10), *M. bovis* (lanes 3 and 11), BCG (lanes 4 and 12), the ICRC bacillus (lanes 5 and 13), *Mycobacterium* strain w (lanes 6 and 14), and the FMR bacillus (lanes 7 and 15). Three identical gels were run as shown in panel a, except that gel b had lambda DNA digested with *Hin*dIII and end labeled with α-^{32}P included. Southern analyses were performed on these gels, and the resulting blots were probed with different labeled probes: panel b was probed with the *M. leprae* 3.6-kb *Eco*RI fragment; panel c was probed with the *M. tuberculosis* 65-kDa antigen gene probe; and panel d was probed with the 1.1-kb *M. leprae Xho*I-*Eco*RI DNA fragment that is adjacent to the coding region of the 65-kDa antigen.

(G+C = 67% [32]) and *E. coli* (G+C = 51% [24]). Normal armadillo DNA was used to rule out the contamination of *M. leprae* probe DNA. Overall, the data presented here indicate that *M. leprae* DNA is not closely related to that of any of the bacteria we tested. Even under the least stringent conditions (i.e., a hybridization temperature of 60°C and an elution buffer concentration of 3× SSC), maximum binding of probe DNA to heterologous sample DNA barely exceeded 10% of homologous DNA hybridization (Table 1). In contrast, when we carried out an experiment using nick-translated DNA from *M. tuberculosis* as a probe, we obtained a wide spectrum of homologies.

We were unable to confirm the initial findings of Imaeda et al. (17) that *M. leprae* was more closely related to the leprosy-associated *Corynebacterium* strain 2628 L.B. than to other mycobacteria. In our experiments, the five corynebacterial species tested bound less *M. leprae* DNA than did most other mycobacteria, and the leprosy-associated *Corynebacterium* strains 2628 L.B. and Shep IV seemed least homologous of all. The initial results of the Imaeda group have been recently revised to indicate only about 20% homology between *M. leprae* and the corynebacteria. The methodology used in the initial work has been critically evaluated (1, 7), but to what extent discrepancies remaining may be related to differences in technique remains unclear.

Since three of the mycobacterial isolates examined have been suggested as possible vaccine strains that might have antigenic and possibly genetic similarities to *M. leprae*, two of which (ICRC and *Mycobacterium* strain w) are in clinical trials, it was of interest to examine the relationship of *Mycobacterium* strain w and the ICRC and FMR bacilli. None of the three had significant genomic similarity to *M. leprae* DNA, but the *Mycobacterium* strain w bacillus showed the highest degree of homology at the lowest stringency.

In an effort to more precisely characterize genetic relationships among the mycobacteria, Southern analyses of digests of DNA from *M. leprae*, the *M. tuberculosis* complex, and the candidate vaccine strains were probed with DNAs encoding a major serologically detected 65-kDa anti-

gen of *M. leprae* and *M. tuberculosis*. The results indicated that all species of mycobacteria had DNA that hybridized to the 65-kDa antigen probes. This is not surprising in light of the recent finding that the 65-kDa antigen of mycobacteria has a high degree of homology to the highly conserved *groEL* heat shock or stress protein in *E. coli* (34). While restriction polymorphisms existed between many of the mycobacterial species tested with these probes, no polymorphisms could be detected among *M. tuberculosis*, *M. bovis*, and BCG.

The most surprising finding was that the *M. leprae* 65-kDa antigen DNA probe consistently gave a pattern of greater than 15 bands in restriction digests of *M. leprae* DNA but only 1 to 3 bands on *M. tuberculosis* DNA and DNAs of other mycobacteria. The probe from the *M. tuberculosis* 65-kDa antigen gene, shown to share 95% sequence homology with the analogous *M. leprae* gene (34), hybridized in a manner consistent with a single-copy gene to the mycobacteria tested and hybridized with bands shared with the *M. leprae* probe. When an *Xho*I-*Eco*RI DNA fragment located outside of the coding region of the *M. leprae* 65-kDa antigen was isolated and used as a probe, the pattern of repetitive sequences in *M. leprae* was identical to that for the entire lambda *gt*11 insert, except for the 65-kDa fragments. These results indicate that an *M. leprae* species-specific repetitive sequence is adjacent to but not a part of the gene sequence encoding the *M. leprae* 65-kDa antigen. This *M. leprae*-specific repetitive sequence has been found and characterized independently by one of us (6). While the function of such a repetitive sequence is not clear, multiple-copy specific repetitive sequences of unknown functions have been described for *Neisseria gonorrhoeae* and *Neisseria meningitidis* (8), *Bordetella pertussis* (20), *E. coli*, and *Salmonella typhimurium* (11).

It is possible that such a repetitive sequence probe could prove to be useful in in situ hybridization for more rapid and definitive diagnosis of leprosy and for epidemiological studies.

ACKNOWLEDGMENTS

We are grateful to M. G. Deo of the Indian Cancer Research Centre; P. R. Mahadevan, in Bombay, India; and G. P. Talwar of the National Institute for Immunology, New Delhi, India, for generously providing cultures of their mycobacterial vaccine strains. We are most grateful to N. Groman for providing the *Corynebacterium diphtheriae* and *Corynebacterium diphtheriae* type mitis strains and to L. Barksdale and the late C. Shepard for providing the leprosy-associated corynebacterial strains.

This work was supported in part by a Victor Heiser Fellowship to C.M.G., by Public Health Service grants AI02111 and AI23545 from the National Institutes of Health, and by the THELEP and IMMLEP components of the United Nations Development Program/World Bank/World Health Organization Programme for Research and Training in Tropical Diseases.

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