Deoxyribonucleic Acid Relatedness Among *Mycobacterium leprae*, *Mycobacterium lepraemurium*, and Selected Bacteria by Dot Blot and Spectrophotometric Deoxyribonucleic Acid Hybridization Assays

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Deoxyribonucleic acid relatedness between *Mycobacterium leprae* or *Mycobacterium lepraemurium* and other selected bacteria was studied by both dot blot and spectrophotometric deoxyribonucleic acid hybridization assays. The results obtained by the two methods were similar, except for the relatedness values between *M. leprae* and two corynebacterial strains. Among the mycobacterial species examined, acid-fast organisms isolated from armadillos and a mangabey monkey with leprosy-like disease showed 100% relatedness with *M. leprae* grown experimentally in armadillos, suggesting their common origin. In this study we demonstrate the usefulness of the dot blot hybridization technique as a screening method for mycobacterial taxonomy.

The deoxyribonucleic acids (DNAs) of *Mycobacterium leprae* and *Mycobacterium lepraemurium* were analyzed kinetically to determine their genome sizes, base ratios, and genetic relatedness to certain corynebacteria, mycobacteria, and nocardiae (5, 6). *M. leprae* grown in armadillos was found to be a unique organism among the *Corynebacterium-Mycobacterium-Nocardia* group, whereas *M. lepraemurium* grown in mice showed close genetic relatedness to *Mycobacterium avium*. These studies demonstrated the usefulness of DNA relatedness values in taxonomic or phylogenetic studies of the corynebacteria, mycobacteria, and nocardiae. However, one of the disadvantages of the kinetic analysis used in these studies was the requirement for extremely purified DNA samples in amounts of at least 1 mg to determine the DNA relatedness between the test species and 20 other organisms. On the other hand, the dot blot hybridization method (7) requires neither extreme purity nor large amounts of DNA samples. Since the supply of *M. leprae* is seriously limited, this latter method facilitates the study of the DNA relatedness of this organism. In this study we determined the DNA relatedness between *M. leprae* or *M. lepraemurium* and other selected organisms belonging to the *Corynebacterium-Mycobacterium-Nocardia* group by using dot blot hybridization as a screening test in comparison with a spectrophotometric assay.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study and their sources are listed in Tables 1 and 2. The *M. leprae* strain which was used as the standard strain in the kinetic analysis and also as a source of the labeled probe in dot blot hybridization experiments was isolated from cutaneous nodules of armadillo A24 infected with *M. leprae* of human origin. This tissue was prepared by E. E. Storr, Medical Research Institute of Florida Institute of Technology, and was supplied through the National Institute of Allergy and Infectious Diseases. An acid-fast organism, which is listed as *M. leprae* (indigenous) in Table 1, was isolated from spleen tissues of two wild armadillos (armadillos V11 and V69) with leprosy-like disease. These infected tissues were supplied by W. F. Kirchheimer, Na-

tional Hansen's Disease Center. The acid-fast organism listed as *M. leprae* (Mangabey monkey) was isolated from spleen tissue of a mangabey monkey with leprosy-like disease, which was supplied by W. M. Meyers, Armed Forces Institute of Pathology (8). *M. lepraemurium* was isolated from mesenteric lymph nodes of mice infected with *M. lepraemurium* for 4 months. All other mycobacterial and nocardial strains were grown in Middlebrook 7H9 medium containing 1% glycerol, 0.05% Tween 80, and 0.25% bovine serum albumin fraction V (Sigma Chemical Co.) at 37°C with constant shaking. The corynebacteria and *Escherichia coli* were grown in tryptic soy broth (Difco Laboratories) at 37°C with constant shaking.

Preparation of DNA samples. The preparation of DNA samples from bacterial cells grown in vivo or in vitro has been described previously (6). The DNA samples in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) were further sheared with a Heat Systems model W-10 sonicator for 15 s. The fragment size of each sheared DNA was determined by electrophoresis in a 1% agarose gel (type II; Sigma) with 0.04 M tris(hydroxymethyl)aminomethane acetate buffer containing 0.0002 M ethylenediaminetetraacetate (pH 8.0).

Spectrophotometric assay of DNA-DNA hybridization. Genome sizes and levels of DNA relatedness were estimated by using the $C_{0t_{0.5}}$ value as a parameter; this value was obtained by spectrophotometric assay of DNA-DNA hybridization with slight modifications of a previously described procedure (6). In this study, two DNA samples were mixed at an equimolar ratio instead of in equal concentrations. The reaction mixture (final concentration, 75 µg/ml) was denatured at 96°C and then reassociated at 68°C, which is the optimal hybridization temperature for mycobacterial DNAs. The degree of hypochromic shift during hybridization of heterologous DNA samples is proportional to the average of the reaction rates of both heterologous and homologous duplex formation (3). Therefore, we hypothesize that the DNA relatedness value between two species (species A and B) implies the average DNA relatedness between species A or B and the presumptive species which has an average genome size of species A and B. In general, the relatedness value (h) between species A (genome size, $A \times 10^9$) and B (genome size, $B \times 10^9$) may indicate that $(A + B)h/2A\%$ of

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TABLE 1. Genome molecular weights and levels of DNA relatedness observed with *M. leprae* and selected bacteria (kinetic analysis)

Organism	Genome mol wt (10 ⁹)	% Relatedness with <i>M. leprae</i>	Ratio of homology (<i>M. leprae</i> /test organism)
<i>M. leprae</i> (experimental)	1.7	100	
<i>M. leprae</i> (indigenous)	1.6	100	
<i>M. leprae</i> (mangabey monkey)	1.8	100	
<i>M. tuberculosis</i> TMC 102 ^a	1.9	21	22/20
<i>M. bovis</i> BCG TMC 1011 ^a	1.9	25	26/23
<i>M. marinum</i> TMC 1218 ^a	2.5	26	32/22
<i>M. intracellulare</i> TMC 1406 ^a	2.0	17	18/16
<i>M. gordonae</i> TMC 1324 ^a	2.8	15	20/12
<i>M. lepraemurium</i> (Hawaiian) ^b	1.8	20	20/19
<i>M. scrofulaceum</i> TMC 1323 ^a	2.4	13	16/11
<i>M. kansasii</i> TMC 1204 ^a	3.0	13	18/10
<i>M. vaccae</i> ATCC 15483 ^{Tc}	2.5	13	16/11
<i>M. avium</i> TMC 706 ^a	2.3	7	8/6
<i>Mycobacterium</i> sp. strain ICRC ^d	3.0	9	12/7
<i>N. otitidis-caviarum</i> ATCC 14629 ^T	3.2	10	14/8
<i>Corynebacterium minutissimum</i> ATCC 23348	1.4	28	25/31
<i>Corynebacterium</i> sp. strain 2628LB ^e	1.1	20	16/25
<i>E. coli</i> K-12 ATCC 23724	2.5	6	7/5

^a TMC, Trudeau Mycobacterial Collection. These strains were supplied by the National Jewish Hospital, Denver, Colo., through the National Institute of Allergy and Infectious Diseases.

^b This organism was grown in mice (5).

^c ATCC, American Type Culture Collection. T = type strain.

^d This organism was isolated from a leprosy patient (1) in the Indian Cancer Research Center and was maintained by L. Barksdale at the New York University Medical Center.

^e This strain of *Corynebacterium* was isolated from a lepromatous leprosy patient (2).

species A and (A + B)/2B% of species B are homologous. For example, 33.3% relatedness between species A (genome size, 1.5 × 10⁹) and species B (genome size, 3.0 × 10⁹) means that one-half of genome A is homologous to one-quarter of genome B.

Dot blot hybridization. Dot blot hybridization was performed by the method of Kafatos et al. (7), with slight

modifications. Namely, DNA samples in 0.1× SSC were denatured with 3 N NaOH for 10 min at room temperature and then neutralized with 3 N HCl. Samples (100, 50, 25, 10, and 1 ng) were filtered through a nitrocellulose sheet placed on a dot blot apparatus (Schleicher & Schuell Inc.) and baked for 2 h at 80°C. ³²P-labeled DNA probes were prepared from 1-μg DNA samples by the method of Rigby et al.

TABLE 2. Genome molecular weight and levels of DNA relatedness observed with *M. lepraemurium* and selected bacteria (kinetic analysis)

Organism	Genome mol wt (10 ⁹)	% Relatedness with <i>M. lepraemurium</i>	Ratio of homology (<i>M. lepraemurium</i> /test organism)
<i>M. avium</i> TMC 701	2.7	87	100/73
<i>M. avium</i> TMC 706	2.3	87	99/78
<i>M. avium</i> TMC 721	2.6	85	100/72
<i>M. microti</i> TMC 1601	2.1	87	94/81
<i>M. intracellulare</i> TMC 1403	2.4	47	55/41
<i>M. intracellulare</i> TMC 1406	2.0	27	29/26
<i>M. kansasii</i> TMC 1201	2.6	40	49/34
<i>M. vaccae</i> ATCC 15483 ^T	2.5	35	42/30
<i>M. scrofulaceum</i> TMC 1323	2.4	23	27/20
<i>M. gordonae</i> TMC 1324	2.8	25	32/21
<i>M. bovis</i> ATCC 19210 ^T	1.7	27	26/28
<i>M. tuberculosis</i> TMC 102	1.9	14	14/14
<i>M. marinum</i> TMC 1218	2.5	19	23/16
<i>M. fortuitum</i> TMC 1529	2.0	25	26/24
<i>M. microti</i> TMC 1608	1.9	9	10/8
<i>M. smegmatis</i> TMC 1515	3.0	11	15/9
<i>N. otitidis-caviarum</i> ATCC 14629 ^T	3.2	10	13/8
<i>C. minutissimum</i> ATCC 23348	1.4	11	10/12
<i>E. coli</i> K-12 ATCC 23724	2.5	3	4/3

FIG. 1. Dot blot hybridization on a nitrocellulose filter. Denatured DNA samples (100, 50, 25, 10, and 1 ng from left to right) were dotted onto a nitrocellulose filter and hybridized with an *M. leprae* DNA probe, as described in the text. Lane A, *M. lepraemurium*; lane B, *M. avium* TMC 706; lane C, *M. intracellulare* TMC 1406; lane D, *Mycobacterium* sp. strain ICRC; lane E, *M. scrofulaceum* TMC 1323; lane F, *M. tuberculosis* TMC 102; lane G, *M. gordonae* TMC 1324; lane H, *M. marinum* TMC 1218; lane I, *M. smegmatis* TMC 1515; lane J, *M. kansasii* TMC 1204; lane K, *M. leprae*; lane L, *M. fortuitum* TMC 1529; lane M, *M. vaccae* ATCC 15483^T; lane N, *C. minutissimum* ATCC 23348; lane O, *Corynebacterium* sp. strain 2628LB; lane P, *E. coli* ATCC 23724. The X-ray film was exposed for 7 days.

(9). The filter was hybridized with a ^{32}P -labeled probe derived from *M. leprae* or *M. lepraemurium* (5×10^4 cpm/cm² of filter surface) in a solution containing 50% deionized formamide (Eastman Kodak Co.), 0.01% bovine serum albumin fraction V (Sigma), 0.01% Ficoll (Sigma), 0.01% polyvinylpyrrolidone (Sigma), $5 \times \text{SSC}$, 0.02 M sodium phosphate buffer (pH 6.5), and 10% dextran sulfate (Sigma) for 18 h at 42°C. This hybridization temperature is slightly less stringent to mycobacterial DNAs. After hybridization the filter was rinsed with $2 \times \text{SSC}$ and washed for 1 to 2 h in three changes of $0.2 \times \text{SSC}$ containing 0.1% sodium dodecyl sulfate (Sigma) at 50°C. The washed filter was air dried and then exposed to X-ray film for 2 to 7 days at -70°C . The degree of hybridization on the nitrocellulose filter was estimated by comparing the intensities of signals of lower concentrations of samples, such as 25-, 10-, and 1-ng dots, since the densities observed under our experimental conditions were not quantitative, as discussed below.

RESULTS

All of the DNA samples which we prepared in this study consisted of a relatively homogeneous population of fragment sizes, ranging in molecular weight from 3×10^5 to 1×10^6 . Among the mycobacterial and nocardial strains, whose genome molecular weights ranged from 1.6×10^9 to 3.2×10^9 , *M. leprae* had the smallest genome (Tables 1 and 2). The genome molecular weights of the corynebacteria examined were significantly smaller (1.1×10^9 and 1.4×10^9) than those of the mycobacteria and nocardia. The levels of DNA relatedness between *M. leprae* and other selected bacteria as determined by kinetic analysis are shown in Table 1. Acid-fast organisms isolated from wild armadillos and a mangabey monkey with leprosy-like disease contained DNAs which were indistinguishable from the DNA of *M. leprae* grown experimentally in armadillos. Thus, the organisms found in wild animals represent *M. leprae*.

In the kinetic analysis *M. leprae* showed 20 to 30% DNA relatedness with *Mycobacterium tuberculosis*, *Mycobacte-*

rium bovis, *Mycobacterium marinum*, *M. lepraemurium*, and two strains of corynebacteria (Table 1). The DNAs of *Mycobacterium gordonae*, *Mycobacterium scrofulaceum*, *Mycobacterium kansasii*, *Mycobacterium vaccae*, and *Nocardia oitidis-caviarum* were related to the DNA of *M. leprae* at levels of 10 to 20%, whereas *Mycobacterium* sp. strain ICRC and *M. avium* showed less than 10% relatedness to *M. leprae*.

In the dot blot hybridization experiments with *M. leprae* as the probe, the very weak signals observed after 2 days of exposure to X-ray films reflected low degrees of hybridization. One week of exposure (Fig. 1) revealed appreciable intensities, although these intensities were by no means quantitative (4). In this experiment (Fig. 1) the majority of mycobacteria showed similar degrees of hybridization to *M. leprae*. However, *M. tuberculosis* appeared to be slightly more reactive than *Mycobacterium intracellulare*, *M. lepraemurium*, *M. avium*, *Mycobacterium* sp. strain ICRC, *M. scrofulaceum*, and *M. kansasii*. Other mycobacteria showed significantly lower degrees of hybridization. The extents of hybridization between *M. leprae* and two strains of corynebacteria were as low as those of *E. coli* in dot blot hybridization experiments.

Table 2 shows the levels of DNA relatedness between *M. lepraemurium* and selected bacteria. Strains of *M. avium* showed more than 85% DNA relatedness to *M. lepraemurium*. Among the other mycobacterial strains, one strain of *Mycobacterium microti* (strain TMC 1601) showed a very high level of relatedness (87%), whereas another (strain TMC 1608) was significantly less homologous (9%). In this connection, it must be mentioned that the DNAs of strains TMC 1601 and TMC 1608 are more than 95% homologous to those of *M. avium* TMC 706 and *M. tuberculosis* TMC 102, respectively. *M. intracellulare*, *M. kansasii*, and *M. vaccae* showed 30 to 50% DNA relatedness to *M. lepraemurium*, and all of the other strains showed less than 30% DNA relatedness to this species.

The effect of DNA fragment size on the calculated values

FIG. 2. Dot blot hybridization on a nitrocellulose filter. The order of test samples is the same as the order in Fig. 1. *M. lepraemurium* DNA was used as the probe. The X-ray film was exposed for 2 days.

of DNA relatedness was examined. When DNAs of *E. coli* and *M. lepraemurium* with fragment molecular weights between 3×10^5 and 1×10^7 were mixed at equal concentrations, the level of relatedness was calculated to be 36%. On the other hand, sonicated samples with fragment molecular weights of 3×10^5 to 1×10^6 , when mixed in an equimolar ratio, showed only 3% relatedness (Table 2).

In a dot blot hybridization experiment using *M. lepraemurium* DNA as the probe (Fig. 2), *M. avium* showed a significantly higher intensity of signals than other species at the 10- and 25-ng dots. A second group comprised of *M. intracellulare*, *M. scrofulaceum*, *Mycobacterium* sp. strain ICRC, and *M. tuberculosis* showed moderate signals, and all other strains tested revealed low degrees of hybridization. Dots containing DNAs of two strains of corynebacteria and *E. coli* were hardly visualized on this filter after exposure for 2 days to the X-ray film.

DISCUSSION

In this study we found significantly lower levels of DNA relatedness between *M. leprae* and the majority of bacterial strains tested than the levels reported previously (5, 6). In the latter experiments, DNA samples varying in molecular weight from 3×10^5 to 1×10^7 were mixed at equal concentrations for reassociation. In contrast, in this study we used equimolar mixtures of DNA samples which had been sheared into smaller fragments that ranged in molecular weight from 3×10^5 to 1×10^6 . Since DNA relatedness was calculated by using the $C_{0t0.5}$ value as a parameter, the higher relatedness values in the previous reports were most likely due to smaller $C_{0t0.5}$ values derived from shorter times required for 50% reassociation, which occurred as a result of a zippering phenomenon during pairing of larger fragments (4). In fact, the model experiment with *E. coli* (genome molecular weight, 2.5×10^9) and *M. lepraemurium* (genome molecular weight, 1.8×10^9), which are taxonomically unrelated, showed an unrealistically high level of DNA relatedness (36%) when larger DNA fragments were mixed at the same concentration. On the other hand, an equimolar mixture of smaller fragments resulted in a very low degree of relatedness (3%), which was also demonstrated by dot blot

hybridization. The smaller genome size estimated in the previous report may also be explained in terms of smaller $C_{0t0.5}$ values obtained by hybridization of larger fragments. Based on the experiments described here, the importance of using an equimolar mixture of two samples with relatively uniform sizes should be emphasized, especially when the genome sizes differ significantly. The ratios of homology calculated on the basis of genome size, as listed in Tables 1 and 2, facilitate comparisons among organisms having varying genome sizes. However, these relatedness values may not reflect the degrees of exact base pairing of specific chromosomal regions; rather, they should be interpreted as average levels of DNA relatedness between two organisms.

Dot blot hybridization experiments in which we used *M. leprae* DNA as the probe showed low levels of relatedness between *M. leprae* and all of the strains tested. *M. tuberculosis* appeared to be closer to *M. leprae* among these strains. Some disagreements in the order of DNA relatedness between results of dot blot and spectrophotometric DNA hybridization assays can be explained as follows. The intensities of signals shown in Fig. 1 do not reflect quantitative differences because of the long period of exposure to X-ray films needed for visualization of weak reactions (4). In addition, the degree of DNA binding below 20% observed in kinetic analyses has only semiquantitative value (3).

Two corynebacterial strains exhibited very low levels of relatedness with *M. leprae* in dot blot hybridization experiments, whereas kinetic analysis showed relatedness with *M. leprae* at levels comparable to those of *M. tuberculosis* and *M. kansasii*. This disagreement may also be due to the inaccuracy of the spectrophotometric assay of DNA-DNA hybridization between two samples with very low homology, as discussed above.

In this study, the levels of DNA relatedness between *M. lepraemurium* and other bacteria also differed from the levels observed previously (5), as discussed above. Nonetheless, *M. avium* still showed the highest level of DNA relatedness (about 85%) with *M. lepraemurium*, as shown in the dot blot hybridization experiment (Fig. 2), suggesting a close phylogenetic relationship of these species. Among the strains not tested for visually, one strain of *M. microti* (strain

TMC 1691) showed a very high level of relatedness with *M. lepraemurium*, whereas another (strain TMC 1608) was less homologous. In this connection, it must be mentioned that the DNAs of strains TMC 1601 and TMC 1608 are homologous to those of *M. avium* and *M. tuberculosis*, respectively.

In dot blot hybridization experiments using *M. lepraemurium* DNA as the probe, the order of relatedness differed slightly from the order observed in the kinetic analysis, probably due to the reasons discussed above.

In conclusion, in this study we demonstrated the usefulness of the dot blot hybridization method for analysis of DNA relatedness among mycobacteria. This method particularly facilitates the screening of many test organisms for estimation of DNA relatedness with fastidious species, such as *M. leprae* and *M. lepraemurium*, since it requires a very small amount (1 µg) of DNA for the preparation of probes.

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