Deoxyribonucleic Acid Relatedness Among Selected Strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*

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Deoxyribonucleic acids (DNAs) of selected strains of the *Mycobacterium tuberculosis* complex, including strains of *M. tuberculosis*, *Mycobacterium bovis*, *M. bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*, were isolated and analyzed spectrophotometrically. The genome molecular weight and the guanine-plus-cytosine content of the DNA samples were approximately 1.9×10^9 and 62.9 mol%, respectively. DNA relatedness among the strains was studied by both spectrophotometric DNA hybridization and restriction endonuclease cleavage methods. All strains of the four species showed more than 90% DNA relatedness, except for slightly lower levels (85 to 89%) of relatedness between *M. microti* and some strains of *M. bovis*, *M. bovis* BCG, or *M. africanum*. The distribution patterns of the DNA fragments produced with restriction enzymes, such as *EcoRI*, *EcoRV*, *BglII*, *BclI*, and *KpnI*, were indistinguishable upon agarose gel electrophoresis. In contrast, the levels of DNA relatedness between *M. tuberculosis* and other slowly growing mycobacteria, including *Mycobacterium nonchromogenicum*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium avium*, *Mycobacterium scrofulaceum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, and *Mycobacterium asiaticum*, were significantly heterogeneous, ranging from 9 to 53%. My data indicate the extremely close evolutionary relationship among four species of the *M. tuberculosis* complex.

Mycobacterium tuberculosis, *Mycobacterium bovis*, *M. bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum* are grouped as the *M. tuberculosis* complex based upon their phenetic similarities (25, 26). Distinctions among these species are defined on the basis of biochemical (9, 21, 25, 26), bacteriological (7, 13, 21, 26), serological (8, 15, 19, 22) and chemical (23, 27) properties, their levels of pathogenicity for experimental animals (10, 13, 20, 21), and their levels of susceptibility to some mycobacteriophages (6, 28). A thorough review of these taxonomic findings has been published by Wayne (26).

However, any of the extensive phenotypic analyses available measure less than 20% of the total genomic capability of bacteria (4). Consequently, phenotypic differences should not be emphasized as absolute criteria for definition of species. On the other hand, examination of essential properties of chromosomes, such as genome molecular weight, guanine-plus-cytosine content, and deoxyribonucleic acid (DNA) relatedness, provides a more practical and reliable method to determine the genetic relationships among phenetically similar species. In fact, Bradley (3) and Baess (2) reported 100% DNA homology between *M. tuberculosis* and *M. bovis*, including *M. bovis* BCG strains, showing that these organisms belong to the same species.

In this report I provide additional information concerning the genome molecular weights, base ratios, and extents of DNA relatedness among selected strains of the species of the *M. tuberculosis* complex. A total of 13 *M. bovis* BCG strains commonly used for vaccination in the world were also examined to clarify their genetic relatedness within the same species. Furthermore, selected strains of eight established species of slowly growing mycobacteria were analyzed for their DNA relatedness to *M. tuberculosis*.

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. *Mycobacterium avium* TMC 706 was used in place of the type strain, since this strain has been suggested as the working type by the International Working Group on Mycobacterial Taxonomy (18). All strains were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) supplemented with either 1% glucose (for *M. bovis* strains) or 1% glycerol (for other strains) and 0.25% bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) at 37°C with constant shaking at 125 rpm, until the optical density at 560 nm became approximately 1.5.

Spectrophotometric assay of DNA-DNA hybridization. The methods used for preparation of DNA samples from mycobacteria and determination of melting temperatures for calculation of guanine-plus-cytosine contents and the conditions used for DNA-DNA hybridization for determination of $C_{0t0.5}$ values as parameters for estimation of genome molecular weights and levels of DNA relatedness have been described previously (1, 17); 100% homology between samples A and B was confirmed by calculating the $C_{0t0.5}$ values of mixtures of samples A and B at different ratios. If two samples were homologous, the $C_{0t0.5}$ values derived from reaction mixtures at different ratios had to be the same.

Restriction endonuclease cleavage analysis. DNA samples of selected strains of mycobacteria were digested with restriction enzymes *EcoRI*, *EcoRV*, *BglII*, *BclI*, and *KpnI* (Bethesda Research Laboratories, Bethesda, Md.) according to the instructions of the supplier. The DNA samples were then separated by electrophoresis through agarose gels [1% in 0.04 M tris(hydroxymethyl)aminomethane acetate buffer containing 0.002 M ethylenediaminetetraacetate, pH 8.0], stained with ethidium bromide, and photographed with Polaroid type 55 film.

RESULTS

Four strains of *M. tuberculosis*, 5 strains of *M. bovis*, 13 strains of *M. bovis* BCG, 2 strains of *M. microti*, and 1 strain of *M. africanum* contained chromosomal DNAs with almost

identical genome molecular weights and base ratios ($1.9 \times 10^9 \pm 0.1 \times 10^9$ and 62.9 ± 0.3 mol%, respectively) (Table 1). The genome of *M. bovis* BCG TMC 1010 was slightly larger than the other genomes. Among selected strains of slowly growing mycobacteria that did not belong to the *M. tuberculosis* complex, the DNAs of *Mycobacterium asiaticum* and *Mycobacterium kansasii* showed characteristics similar to those of *M. tuberculosis*, whereas *Mycobacterium nonchromogenicum*, *Mycobacterium intracellulare*, *M. avium*, *Mycobacterium scrofulaceum*, *Mycobacterium haemophilum*, and *Mycobacterium marinum* contained DNAs which differed from the DNA of *M. tuberculosis* with respect to genome molecular weight, base ratio, or both (Table 2).

In the spectrophotometric assay of DNA-DNA hybridization, *M. tuberculosis* showed 9 to 53% DNA relatedness to *M. asiaticum*, *M. marinum*, *M. haemophilum*, *M. scrofulaceum*, *M. avium*, *M. kansasii*, *M. intracellulare*, and *M. nonchromogenicum*, (Table 2).

The extent of DNA relatedness among strains of the *M. tuberculosis* complex was examined by using two strains of each species as references (Table 3). *M. microti* TMC 1619^T (T = type strain) was not used in this study, because this strain appeared to be heterogeneous even after several single-colony transfers on Middlebrook 7H11 agar medium. The heterogeneity of the population was evidenced by the

TABLE 1. Origins, genome molecular weights, and base ratios of strains of the *M. tuberculosis* complex

Strain	Genome mol wt (10^9) ^a	Guanine-plus-cytosine content (mol%) ^b
<i>M. tuberculosis</i>		
TMC 102 ^T (= H37Rv) ^c	2.0	63.3
TMC 201 (= H37Ra)	1.9	63.1
TMC 327	2.0	63.3
MH 1160 ^d	2.0	62.7
<i>M. bovis</i>		
ATCC 19210 ^{Te}	1.8	62.7
TMC 401 (Ravenel)	1.9	62.9
TMC 405	1.8	63.3
TMC 407 (Branch)	1.9	62.9
TMC 409 (Vallee)	1.9	63.1
<i>M. bovis</i> BCG		
TMC 1010 (Danish)	2.3	62.9
TMC 1011 (Pasteur)	1.9	63.3
TMC 1012 (Montreal)	1.9	62.8
TMC 1013 (Brazilian)	2.0	62.5
TMC 1019 (Japanese)	1.8	62.9
TMC 1020 (Mexican)	2.0	62.9
TMC 1021 (Australian)	1.9	62.9
TMC 1022 (Russian)	2.0	62.9
TMC 1024 (Glaxo)	1.8	62.7
TMC 1025 (Prague)	1.9	62.9
TMC 1028 (Tice)	2.1	62.7
TMC 1029 (Phipps)	1.8	62.9
TMC 1030 (Connaught)	1.9	62.7
<i>M. africanum</i> TMC 5122	2.0	62.7
<i>M. microti</i>		
TMC 1608	1.9	62.3
26003 ^f	1.9	63.3

^a Average of six assays.

^b Average of three to six assays.

^c TMC, Trudeau Mycobacterial Culture Collection (strains supplied by the National Jewish Hospital through the National Institute of Allergy and Infectious Diseases).

^d Isolated from a patient with pulmonary tuberculosis in the University of Medicine and Dentistry of New Jersey Hospital, Newark.

^e ATCC, American Type Culture Collection, Rockville, Md.

^f Supplied by M. Tsukamura, National Chubu Hospital, Japan.

TABLE 2. Genome molecular weights, base ratios, and levels of DNA relatedness observed between *M. tuberculosis* and other mycobacterial species

Strain	Genome mol wt (10^9) ^a	Guanine-plus-cytosine content (mol%) ^b	% Relatedness with <i>M. tuberculosis</i> TMC 102 ^{Tc}
<i>M. nonchromogenicum</i> TMC 1481 ^T	1.8	64.7	53
<i>M. intracellulare</i> TMC 1406 ^T	2.0	65.7	48
<i>M. kansasii</i> TMC 1204 ^T	2.2	63.3	32
<i>M. avium</i> TMC 706	2.3	66.7	27
<i>M. scrofulaceum</i> TMC 1323 ^T	2.4	65.8	22
<i>M. haemophilum</i> TMC 804 ^T	1.8	61.5	17
<i>M. marinum</i> TMC 1218 ^T	2.5	63.1	11
<i>M. asiaticum</i> TMC 803 ^T	2.0	63.1	9

^a Average of six assays.

^b Average of three to six assays.

^c Average of six assays.

fact that the DNA sample prepared from this strain showed about 90% DNA relatedness to both *M. avium* and *M. tuberculosis*, which are distantly related (Table 2). Similarly, *M. microti* TMC 1601 was not included, because of its high level of DNA relatedness with *M. avium* or *Mycobacterium lepraemurium*, as reported previously (1).

The levels of DNA relatedness between *M. tuberculosis* and other members of the *M. tuberculosis* complex ranged from 90 to 100% (Table 3). *M. bovis* and *M. bovis* BCG

TABLE 3. Levels of DNA relatedness observed among strains of the *M. tuberculosis* complex

Strain	% Relatedness with: ^a					
	<i>M. tuberculosis</i>		<i>M. bovis</i>	<i>M. bovis</i>	<i>M. microti</i>	
	TMC 102 ^T	TMC 201	ATCC 19210 ^T	BCG TMC 1011	TMC 1608	26003
<i>M. tuberculosis</i>						
TMC 102	Ref ^b	100	99	98	100	97
TMC 201	100	Ref	99	100	90	90
TMC 327	100	100	98	94	ND ^c	ND
MH 1160	100	99	100	97	96	100
<i>M. bovis</i>						
ATCC 19210	99	99	Ref	100	100	100
TMC 401	97	100	100	100	91	87
TMC 405	98	100	100	100	96	100
TMC 407	100	97	100	100	96	98
TMC 409	100	100	100	95	96	99
<i>M. bovis</i> BCG						
TMC 1010	100	96	100	98	100	100
TMC 1011	98	100	100	Ref	100	100
TMC 1012	97	100	97	100	90	89
TMC 1013	100	100	ND	100	ND	ND
TMC 1019	100	100	97	100	96	100
TMC 1020	100	100	100	100	85	89
TMC 1021	100	98	100	100	97	99
TMC 1022	100	97	100	96	95	100
TMC 1024	100	100	100	100	98	100
TMC 1025	100	100	100	100	100	100
TMC 1028	98	100	97	100	ND	ND
TMC 1029	100	99	100	100	100	100
TMC 1030	100	100	100	100	98	99
<i>M. africanum</i>						
TMC 5122	100	100	96	95	98	89

^a Average of six assays.

^b Ref, Reference.

^c ND, Not done.

FIG. 1. Agarose gel electrophoresis of various DNAs digested with both *EcoRI* and *EcoRV*. Lane A, *M. tuberculosis* TMC 102^T; lane B, *M. bovis* ATCC 19210^T; lane C, *M. bovis* BCG TMC 1011; lane D, *M. microti* TMC 1608; lane E, *M. africanum* TMC 5122; lane F, *M. nonchromogenicum* TMC 1481^T; lane G, *M. intracellulare* TMC 1406^T; lane H, *M. kansasii* TMC 1204^T; lane I, *M. avium* TMC 706; lane J, *M. scrofulaceum* TMC 1323^T; lane K, *M. haemophilum* TMC 804^T; lane L, *M. marinum* TMC 1218^T; lane M, *M. asiaticum* TMC 803^T.

demonstrated 95 to 100% relatedness with representative strains of *M. tuberculosis* and *M. bovis*. Two strains of *M. microti* also showed 90 to 100% relatedness with most strains of *M. tuberculosis*, *M. bovis*, and *M. africanum*. However, these strains of *M. microti* contained DNAs with slightly lower levels of relatedness (85 to 89%) to some strains of *M. bovis* and *M. bovis* BCG. *M. africanum* showed slightly closer relatedness with *M. tuberculosis* than with other members of the *M. tuberculosis* complex, although only one strain of *M. africanum* was examined in this study.

Restriction enzyme cleavage analysis showed that the distribution patterns of DNA fragments produced with a single enzyme (either *EcoRI*, *EcoRV*, *BglII*, *BclI*, or *KpnI*) were indistinguishable among representative strains of the *M. tuberculosis* complex (data not shown). When DNAs of the strains of the *M. tuberculosis* complex were digested with two enzymes, such as *EcoRI* and *EcoRV*, which recognize totally different sequences, the patterns of restricted fragments were visually identical (Fig. 1).

In contrast, restricted fragments of DNAs derived from *M. nonchromogenicum*, *M. intracellulare*, *M. kansasii*, *M. avium*, *M. scrofulaceum*, *M. haemophilum*, *M. marinum*, and *M. asiaticum* by digestion with *EcoRI* and *EcoRV* showed various patterns, which differed significantly not only from those of the *M. tuberculosis* complex but also from each other (Fig. 1).

DISCUSSION

A total of 25 strains of the *M. tuberculosis* complex (consisting of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, and *M. africanum*) contained chromosomal DNAs with almost identical basic properties, such as base ratio and

genome molecular weight. A spectrophotometric assay of DNA-DNA hybridization showed more than 90% DNA relatedness among these strains, except for slightly lower levels of relatedness between *M. microti* and some strains of *M. bovis* and *M. bovis* BCG.

The extent of DNA relatedness estimated by using $C_{0t_{0.5}}$ values represents the degree of the stability of both stacking and pairing of bases within hybrid molecules (12). However, unmatched regions due to a deletion, insertion, or substitution of several nucleotides cannot be detected by the spectrophotometric assay employed in this study. Therefore, 100% relatedness does not necessarily mean identical sequences in the total stretch of chromosomal DNAs in two test samples.

In addition to the extremely high levels of DNA relatedness among strains of the *M. tuberculosis* complex as observed by the spectrophotometric assay, the distribution patterns of restriction fragments produced with several endonucleases, individually and in combination, were indistinguishable among selected strains of the complex, indicating almost identical distribution of specific sequences throughout the chromosomes.

The principle of restriction enzyme cleavage analysis is based on the fact that the frequencies and locations of specific DNA sequences in chromosomal DNA are characteristic of individual bacterial strains (11). Therefore, the same distribution pattern of restricted fragments observed among members of the *M. tuberculosis* complex implies that these DNAs are most likely the same. However, alterations in nucleotide sequences between two restriction sites cannot be detected by this method. These undetectable changes in DNA sequences may result in differentiation of five species of the *M. tuberculosis* complex, as reflected by their stable

phenotypic differences. Among various strains of *M. bovis* BCG, the diversity in virulence to animals (5, 10, 13, 20), antigenicity (16, 22, 24), and bacteriological (13) and chemical (23) characteristics can also be explained in terms of undetectable minor alterations in chromosomal sequences which are, as a whole, almost identical among the 13 strains tested in this study.

In contrast to the extremely high levels of DNA relatedness among members of the *M. tuberculosis* complex, the DNAs of other slowly growing mycobacteria, including *M. avium*, *M. kansasii*, *Mycobacterium gordonae*, and *M. intracellulare*, are reportedly heterologous to the DNA of *M. tuberculosis* (2, 14). In addition to these species, significantly lower levels of DNA relatedness between *M. tuberculosis* and *M. nonchromogenicum*, *M. scrofulaceum*, *M. marinum*, *M. haemophilum*, or *M. asiaticum* were found by using spectrophotometric DNA hybridization and restriction endonuclease cleavage analyses in this study.

In conclusion, the experiments described above indicate the close evolutionary relationships among *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, and *M. africanum*, as evidenced by almost identical genomic DNAs. My data also support the reclassification of these species as a single species, as suggested by numerical taxonomy (25, 26).

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