JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 645-651
Vol. 113, No. 2
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Relationships Among Mycobacteria and Nocardiae Based upon Deoxyribonucleic Acid Reassociation

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Received for publication 16 October 1972

The degree of renaturation between *Nocardia farcinica* 330 deoxyribonucleic acid (DNA) and DNA from 17 nocardial strains and 11 mycobacterial strains, and between *Mycobacterium smegmatis* 405 DNA and DNA from 11 mycobacterial strains and 14 nocardial strains was determined by using the nitrocellulose membrane filter technique. These results indicated that some cultures designated *N. farcinica* were identical to some cultures designated *N. asteroides* but that other strains called *N. asteroides* were distinctly different. The species *M. smegmatis* was homogeneous and distinct from the other species examined. Data comparing the extent of nucleotide sequences shared by *M. smegmatis* 405 DNA and DNA from nine other mycobacterial strains, as determined by the membrane filter technique and by monitoring DNA reassociation optically, were similar. The extent of reassociation between *M. tuberculosis* H37Ra DNA and DNA from 14 mycobacterial strains was determined optically. DNA from *M. bovis* BCG was 86% homologous with *M. tuberculosis* H37Ra DNA. The other mycobacterial DNA preparations examined were less than 40% homologous with *M. tuberculosis* H37Ra DNA. The genome sizes of the mycobacteria examined ranged between 2.5×10^9 daltons and 4.5×10^9 daltons.

The systematics of the genera *Mycobacterium* and *Nocardia* are beset with many difficulties, both at the level of delineating species and differentiating between the two genera (2). The nomenclature and taxonomic status of the type species of the genus *Nocardia* are in dispute (11, 14, 20), and many cultures designated as *Nocardia farcinica* have been reassigned to the genus *Mycobacterium* (2, 14). In addition, the cultures designated as *Mycobacterium rhodochrous* have been returned to the genus *Nocardia* (2, 7, 14). Gordon and Mihm (11) have reduced many putative species to synonymy with *N. asteroides*, but Tsukamura (23) has divided these cultures into two phenons.

Phenetic classifications of mycobacteria tend to lump slow-growing strains into one cluster and the rapidly growing strains into another cluster (2, 12). Accordingly, deoxyribonucleic acid (DNA) reassociation assays seem to provide the most promising means to resolve these taxonomic dilemmas. Gross and Wayne (12) have undertaken such a study. They have measured the capability of 12 mycobacterial DNA samples to bind reference DNA from *M. tuberculosis* and *M. kansasii*. Each species showed extensive nucleotide homology with other members of its taxon but little homology with *M. smegmatis* and *M. phlei*. Nevertheless, their progress has been retarded by the low specific activity of the radiolabeled reference DNA; therefore, new methods are needed to facilitate these studies.

In this report, the capability of selected mycobacterial and nocardial DNA preparations to bind ^{14}C-labeled reference DNA from *N. farcinica* 330 and *M. smegmatis* 405 has been determined. In addition, the extent of reassociation between selected mycobacterial DNA samples and *M. smegmatis* 405 or *M. tuberculosis* H37Ra has been measured optically. Optical reassociation is a powerful tool for estimating the extent of nucleotide sequences shared by DNA extracted from fastidious bacteria.

MATERIALS AND METHODS

The sources of the mycobacteria and nocardiae examined have been reported previously (2). *Mycobacterium bovis* BCG, *M. intracellulare* 475, *M.*

kansasii 476, and *M. tuberculosis* H37Ra were grown at 37 C on Middlebrook 7H9 medium with OADC enrichment (Difco, Detroit, Mich.). The other mycobacteria, nocardiae, and additional selected actinomycetes were grown in peptone-yeast extract (0.05 and 0.3%, respectively) broth at 30 or 37 C, whichever supported better mycelial yields. Cells and mycelia for DNA extraction were incubated until the cultures reached the early stationary phase of growth and were harvested and lysed by methods reported earlier (22). After digestion with 4 mg of lysozyme/ml for 1 hr at 42 C, Pronase was added to a concentration of 2 mg/ml, and this mixture was incubated an additional 1 hr at 42 C. After lysis, DNA was extracted by the procedure of Marmur (16). Stock DNA preparations were stored at 5 C in 0.15 M NaCl containing 0.015 M sodium citrate.

The degree of reassociation between selected DNA preparations was assessed by measuring the extent of binding of mobile, denatured and sheared ^{14}C-DNA and denatured DNA immobilized on nitrocellulose membrane filters (2, 8). *M. smegmatis* 405 DNA and *N. farcinica* 330 DNA were labeled with uracil-2-^{14}C as described previously (21). The ^{14}C-labeled DNA was sheared by passing the stock DNA solution (>100 μg DNA/ml) through a French pressure cell at 20,000 psi. The specific activity of the ^{14}C-labeled *M. smegmatis* 405 DNA was ca. 27,000 counts per min per μg and that of the *N. farcinica* 330 DNA was ca. 26,000 counts per min per μg. The general procedure for preparing the DNA samples for these reassociation assays and for measuring the amount of ^{14}C-DNA bound has been described by Monson et al. (17) except that the filters charged with 50 μg of denatured DNA were incubated with 0.5 μg of sheared and denatured ^{14}C-labeled DNA. Clark and Brownell (5) have described and used the same experimental protocol.

To determine the kinetics of the reassociation process optically, DNA dissolved in 1.2 M NaCl containing 0.12 M sodium citrate (pH 7) was sheared by sonic oscillation (3-min treatment with Biosonik III, Bronwill Scientific, Rochester, N.Y.). The vessel containing the DNA sample was immersed in ice; the DNA was subjected to ultrasonic vibrations for 10-sec intervals with intervening rest periods of 20 sec. Formamide was added to the sheared DNA sample, giving a final solvent concentration of 25% (v/v) formamide, 0.9 M NaCl, and 0.09 M sodium citrate (18). The DNA (40 μg/ml) was heat-denatured in a recording spectrophotometer with an attached temperature programmer (Gilford Instruments Lab., Oberlin, Ohio). The denaturing temperature was maintained for 20 min after the maximal hyperchromicity at 270 nm had been achieved (a 34–38% rise), and then the temperature was rapidly adjusted to 25 C below the mid-point of the hyperchromic shift (T_m). The time corresponding to a temperature of T_m + 10 C was used as the origin or starting time for reassociation. This represents a period of ca. 5 min after resetting the temperature program, and no reassociation occurred during this period. Because formamide absorbs at 260 nm, denaturation and reassociation were monitored optically at 270 nm where DNA still has a large absorption (80% of that at 260 nm) but the absorption by formamide is low. The absorbance at 270 nm was monitored until the denatured DNA was more than 75% reassociated, at which time the T_m of the annealed sample was determined. Genome sizes were calculated using the statement $cot_{½}$ *E. coli*/$cot_{½}$ unknown-2.5 × 10^9/molecular weight of unknown (3, 10). No correction factor to compensate for purported differences in the rate of reassociation due to DNA base compositional differences has been included in the genome size calculations.

The following tests were employed to assess the purity of the DNA preparations: Lowry's protein determination (15), the orcinol reaction to detect the presence of ribonucleic acid (13), and the diphenylamine reaction for DNA determination (4).

RESULTS

Unlabeled and labeled DNA preparations contained no ribonucleic acid detectable by the orcinol reaction (13) nor protein detectable by Lowry's determination (15). The amount of DNA in a sample determined by the diphenylamine reaction (4) was in close agreement (±3%) with that calculated from the absorption at 260 nm.

Sheared, denatured ^{14}C-labeled DNA isolated from *N. farcinica* 330 was annealed with denatured DNA immobilized on nitrocellulose membrane filters at various incubation temperatures. In the homologous system (*N. farcinica* 330 DNA × *N. farcinica* 330 DNA), more binding of the mobile ^{14}C-labeled DNA occurred at 80 C than at incubation temperatures between 55 and 75 C. The thermal stability of the renatured duplexes formed at both 75 and 80 C was the same as that of native duplexes. Hybrid duplexes formed between *N. farcinica* 330 ^{14}C-labeled DNA and immobilized *N. asteroides* 334 DNA were not as heat stable as native duplexes; their thermal stability was correlated with the annealing temperature.

It is significant that only 4% ± 0.8% of the immobilized DNA leached from the membrane filters during this series of experiments. The DNA immobilized on nitrocellulose membranes was poorly retained, however, when the denatured DNA sample was from an old DNA stock solution or had been extensively sheared during the isolation procedure. The immobilized DNA also leached from the membrane if the DNA-laden filters were not heated at 80 C in vacuo for several hours.

Of the 17 strains of *Nocardia* tested, only the DNA from *N. asteroides* 333 bound the reference *N. farcinica* 330 DNA extensively at 75 C. Strains designated *N. brasiliensis*, *N. caviae*, and *N. transvaliensis* and other strains desig-

nated as *N. asteroides* bound significant amounts of *N. farcinica* 330 DNA (15 to 38% relative binding at 75 C). DNA from *N. erythropolis* and *N. rubra* bound little *N. farcinica* DNA (less than 13% relative binding); neither did DNA from 11 mycobacteria, 2 *Actinoplanes*, 1 *Micromospora*, 1 streptomycete, nor *Streptosporangium* anneal appreciably with *N. farcinica* DNA (Table 1).

Prior to conducting a survey of the capability of DNA from selected actinomycetes to bind *M. smegmatis* 405 reference DNA, the effect of incubation temperature on the extent and specificity of the reassociation reaction was assessed. Although more reference DNA bound at 60 C than at 50, 70, or 80 C, only the homologous duplexes annealed at 70 or 80 C were as thermally stable as native *M. smegmatis* DNA. In this series of experiments only 6% ± 0.8% of the immobilized DNA leached from the membrane filters during the reassociation incubation period. The hybrid duplexes formed during incubation at 80 C included some mismatched sequences because their thermal stability was less than that of the annealed homologous duplexes and that of the native DNA. Because the purpose of this survey was to detect similar, as well as identical, nucleotide sequences, the capability of test DNA from 31 actinomycete strains to bind *M. smegmatis* 405 reference DNA at 70 C was measured.

Four strains identified as *M. smegmatis* (including the reference strain) bound the reference DNA equally at 70 C, and the duplexes were thermally stable. DNA from a fifth strain, designated *M. smegmatis* 433, was similar but not identical to the reference DNA. *M. bovis*, *M. fortuitum*, *M. phlei*, and *M. stercoides* bound appreciable amounts of the reference *M. smegmatis* DNA (31 to 48% relative binding), but these hybrid duplexes had substantially reduced thermal stabilities compared with native duplexes. Of the other 22 actinomycete DNA samples, only DNA from *N. farcinica* 330 annealed appreciably with *M. smegmatis* 405 DNA at 70 C (Table 2). In addition, nine mycobacterial DNA samples were annealed with *M. smegmatis* 405 DNA at 80 C. The relative amount of test DNA bound at 80 C (Table 3) was the same as that bound at 70 C (Table 2) except for *M. bovis* BCG DNA. At 70 C *M. bovis* BCG DNA formed extensive thermally labile duplexes with *M. smegmatis* 405 DNA.

The degree of reassociation between denatured DNA samples was also determined quantitatively by spectrophotometry. Prior to a survey of the capability of denatured DNA from selected mycobacteria to reassociate in solution, the effect of incubation temperature on the rate of reassociation was determined. The reassociation rate constant was relatively unaffected by incubation temperatures 15 to 35 C below the melting temperature (Table 4). These results are consistent with the findings of

TABLE 1. *Reassociation at 60 and 75 C of DNA samples from various actinomycetes with that of N. farcinica 330*[a]

DNA source	Relative DNA bound[b] (%)	
	60 C	75 C
Nocardia farcinica 330	100 (18)[c]	100 (21)[c]
N. asteroides 333	101	80
N. brasiliensis 301	74	38
N. brasiliensis 473	59	38
N. caviae 421	76	31
Nocardia sp. 304	81	31
N. phenotolerans 514	82	18
N. transvaliensis 516	97	32
N. asteroides 300	41	26
N. asteroides 334	66	15
N. erythropolis 305	43	9
N. erythropolis 340	47	9
N. erythropolis 474	23	8
N. erythropolis 456	22	12
N. globerula 472	29	9
N. rubra 338	70	13
N. rubra 321	70	11
N. rubra 327	42	8
Mycobacterium sp. 436	45	5
M. bovis BCG	25	2
M. fortuitum 471	40	19
M. intracellulare 475	60	14
M. marinum 437	36	17
M. phlei 435	84	14
M. smegmatis 405	54	9
M. smegmatis 461	37	8
M. smegmatis 477	30	6
M. stercoides 406	70	10
M. tuberculosis H37Ra	37	7
Actinoplanes philippinensis 367	40	4
A. utahensis 409	24	1
Streptomyces venezuelae 13	51	9
Streptosporangium roseum 345	61	6
Blank filters	41 count/min	

[a] By the method of Enquist and Bradley (7; also see 5) using nitrocellulose membrane filters. The reaction mixtures contained ca. 13,000 count/min in 0.5 μg of labeled DNA.

[b] Background counts of 21 to 29 count/min were subtracted prior to calculation.

[c] Absolute percent binding of the reference DNA is given in parentheses.

TABLE 2. *Reassociation at 70 C of DNA samples from various actinomycetes with that of M. smegmatis*[a]

DNA source	Relative DNA bound[b] (%)	T_m[c] (C)
Mycobacterium smegmatis 405	100 (22)[d]	79.5
M. smegmatis 477	95	79.0
M. smegmatis 461	94	78.5
M. smegmatis 403	108	80.0
M. smegmatis 433	78	76.0
M. bovis BCG	48	68.5
M. stercoides 406	36	68.0
M. phlei 435	32	70.5
M. fortuitum 471	31	70.0
M. intracellulare 475	18	68.5
Mycobacterium sp. 436	23	69.5
M. marinum 437	10	72.0
Nocardia asteroides 300	8	70.0
N. asteroides 334	17	77.0
N. brasiliensis 301	13	69.0
N. brasiliensis 473	10	70.0
N. caviae 421	18	73.0
N. coeliaca 520	13	70.0
N. erythropolis 305	21	76.0
N. erythropolis 456	10	70.0
N. erythropolis 439	7	66.0
N. farcinica 330	38	73.0
N. madurae 302	7	80.0
N. rubra 327	9	72.0
N. rubra 338	17	77.0
N. rubra 321	13	79.5
Actinoplanes philippinensis 367	9	65.0
Dactylosporangium aurantiacum A63	5	65.0
Micromonospora sp. 401	6	76.0
Mycococcus sp. 354	12	76.0
Streptomyces venezuelae 13	15	76.0
Streptosporangium roseum 345	10	65.0
Escherichia coli CSH-2	4	65.0
Micrococcus lysodeikticus 418	16	65.0
Blank filter	70 count/min	

[a] By the method of Enquist and Bradley (7; also see 5) using nitrocellulose membrane filters. The reaction mixtures contained ca. 13,500 counts/min, in 0.5 μg of labeled DNA.
[b] Background counts of 24 to 32 counts/min were subtracted prior to calculation.
[c] Temperature at which half of the bound reference DNA is eluted with 0.015 M NaCl-0.0015 M sodium citrate.
[d] Absolute percent binding of the reference DNA is given in parentheses.

previous workers (10, 24).

Calculations of the degree of nucleotide sequence homology and genome size are based upon comparative $cot_{1/2}$ values, which are reliable only when reassociation, as monitored optically, proceeds as a second-order reaction (10, 24). The straight line resulting from a second-order rate plot (24) confirmed that the reassociation of *M. tuberculosis* H37Ra DNA obeyed second-order kinetics (Fig. 1). A more critical test for second-order kinetics involved

TABLE 3. *Reassociation of DNA samples from various mycobacteria with that of M. smegmatis 405*

DNA source	Membrane filters[a]		Optical reassociation homology[b] (%)
	Relative DNA bound (%)	T_m[c] (C)	
M. smegmatis 405	100 (16)[d]	78	100
M. smegmatis 477	102	78	100
M. smegmatis 461	101	78	97
M. smegmatis 433	79	77	83
M. phlei 435	24	73	45
M. fortuitum 471	29	72	37
M. intracellulare 475	19	71	26
Mycobacterium sp. 436	16	76	23
M. marinum 437	12	70	19
M. bovis BCG	7	79	12
Nocardia farcinica 330	11	77	Not done

[a] By the method of Enquist and Bradley (7; also see 5) using nitrocellulose membrane filters. The reaction mixtures containing ca. 13,500 counts/min in 0.5 μg of labeled DNA were incubated at 80 C. Background counts of 26 to 31 counts/min were subtracted prior to calculation.
[b] Percent homology = $100 + 100\,(cot_{1/2}A + cot_{1/2}B - 2\,cot_{1/2}mix)/(cot_{1/2}A + cot_{1/2}B)$ (adapted from equation in reference 19).
[c] Temperature at which half of the bound reference DNA is eluted with 0.015 M NaCl-0.0015 M sodium citrate.
[d] Absolute percent binding of the reference DNA is given in parentheses.

TABLE 4. *Effect of incubation temperature on rate of optimal reassociation*

$T_m - T_r$[a] (C)	k_2 ($M^{-1}sec^{-1}$)[b]		
	BCG[c]	H37Ra[d]	Mixture
15	1.8	2.1	1.6
20	1.9	2.0	1.8
25	2.0	2.1	2.0
30	2.0	2.2	1.9
35	2.0	2.4	1.8

[a] T_m, Melting temperature; T_r, reassociation temperature.
[b] As defined by Wetmur and Davidson (24).
[c] BCG, *M. bovis* BCG.
[d] H37Ra, *M. tuberculosis* H37Ra.

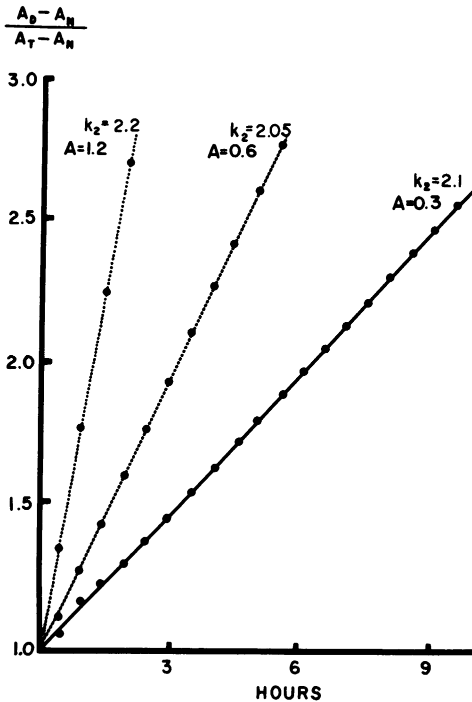

FIG. 1. *Kinetics of DNA reassociation. Sheared and denatured DNA of M. tuberculosis H37Ra renatured according to second-order kinetics. The second-order rate constant (k_2) was 2.1 ± 0.1 liter per mole per sec for DNA samples having initial reactant concentrations of A_{260} = 0.3 (——), 0.6 (-----), and 1.2 (·····). A_D, Absorbance of denatured DNA; A_N, absorbance of native DNA; A_T, absorbance of reassociating DNA at time T.*

calculating the second-order rate constants for several initial reactant concentrations. Over the range tested, the second-order rate constant for reassociation was independent of the initial reactant concentration (Fig. 1).

The degree of annealing in free solution, as monitored optically, between *M. smegmatis* 405 DNA and DNA from nine selected mycobacteria was compared with the capability of mobile ^{14}C-labeled reference DNA from *M. smegmatis* 405 to bind to test DNA immobilized on nitrocellulose membrane filters. The degree of nucleotide sequence homology estimated by the two methods was similar (Table 3). Optical reassociation was also used as a basis for determining the degree of homology between *M. tuberculosis* H37Ra DNA and selected mycobacterial DNA samples. Only *M. bovis* BCG DNA substantially annealed with *M. tuberculosis* H37Ra DNA. DNA from *M. fortuitum* 471, *M. intracellulare* 475, or *M. kansasii* 476 significantly annealed with *M. tuberculosis* H37Ra DNA (26–38%). *M. smegmatis* DNA and *M. phlei* DNA did not appreciably renature with *M. tuberculosis* H37Ra DNA (Table 5).

DISCUSSION

For a survey of relationships between *N. farcinica* 330 and selected actinomycetes, annealing temperatures of 60 and 75 C were chosen. At 60 C, mismatched hybrid duplexes with decreased thermal stability were formed; at 75 C only well-matched hybrid duplexes which were relatively heat stable were formed. This experimental design differentiates among pairs of DNA which are essentially identical, pairs which although significantly different are related, and pairs which are unrelated or only remotely related.

The culture designated *N. farcinica* 330 is closely related to one designated *N. asteroides* 333 but not to ones designated *N. asteroides* 300 and *N. asteroides* 334. These results indicate that the taxa *N. farcinica* and *N. asteroides* are not adequately defined and that there are at least two genospecies presently included under the epithet *N. asteroides*. Tsukamura (23) also concluded, based upon phenetic analyses, that the cultures received as *N. asteroides* could be divided into two phenons. Although I concur with Lechevalier and co-workers (14) that *N. farcinica* should be considered a nomen dubia, I have generally retained the

TABLE 5. *Optical reassociation of DNA samples from various mycobacteria with that of M. tuberculosis H37Ra*

DNA source	Percent homology[a]	Genome size[b] (daltons)
M. tuberculosis H37Ra	100	2.5×10^9
M. bovis BGC	86	2.8×10^9
M. kansasii 476	38	4.2×10^9
M. stercoides 406	37	3.8×10^9
Mycobacterium sp. 436	33	3.9×10^9
Mycobacterium sp. 378	32	3.4×10^9
M. marinum 437	29	3.8×10^9
M. intracellulare 475	29	3.1×10^9
M. fortuitum 471	26	2.8×10^9
M. smegmatis 433	14	4.2×10^9
M. smegmatis 461	12	4.2×10^9
M. smegmatis 405	11	4.5×10^9
M. smegmatis 403	10	4.5×10^9
M. smegmatis 477	9	4.3×10^9
M. phlei 435	7	3.5×10^9

[a] Percent homology = $100 + 100 (\cot_{1/2}A + \cot_{1/2}B - 2 \cot_{1/2}mix)/(\cot_{1/2}A + \cot_{1/2}B)$ (adapted from equation in reference 19).

[b] Genome size = $2.5 \times 10^9 \cot_{1/2}(unknown)/\cot_{1/2}(E. coli)$ (adapted from equation in reference 3).

designations borne by a culture when it was initially entered into our culture collection. Additional study is required before either the "working type" proposed by Sneath and Skerman (20) or the putative type culture of the genus (11) can be accepted as the correct type culture for *N. asteroides*. Accordingly, it is premature to reassign cultures to or out of this taxon unless they are unquestionably misidentified.

For a survey of relationships between *M. smegmatis* 405 and selected actinomycetes, annealing temperatures of 70 and 80 C were chosen. Even though 70 C is ca. 25 C below the T_m of *M. smegmatis*, a substantial proportion of the hybrid duplexes formed had reduced thermal stabilities. It should be noted that reassociation occurred in 0.69 M NaCl-0.069 M sodium citrate but that thermal stabilities were ascertained by thermal elution with 0.015 M NaCl-0.0015 M sodium citrate. These results indicate that, of the mycobacteria examined, *M. smegmatis* is more closely related to *M. phlei* and *M. fortuitum* than to *M. intracellulare* or *M. bovis*. *M. bovis* DNA shared almost no exactly matching nucleotide sequences with *M. smegmatis* but did possess a substantial proportion of partly matching sequences.

Okanishi and Gregory (18) have successfully utilized a solvent consisting of 0.9 M NaCl, 0.09 M sodium citrate, and 50% formamide for reassociation of an excess of sheared, denatured ^{14}C-labeled DNA with denatured DNA immobilized on nitrocellulose filters. It should be noted that their method is based upon saturation of the immobilized DNA, where our methods (2, 8, 17) rely upon maximal binding of the DNA in the mobile phase. Accordingly, it is not surprising that Okanishi and Gregory found that leaching of immobilized DNA from the nitrocellulose membrane filters greatly complicated their assays. In my experiments where retention of the immobilized DNA has been measured, little DNA had been lost. Similarly, Gillespie and Gillespie (9) have observed that retention of DNA approaches 100% when appropriate precautions are followed in preparing the DNA-laden membrane filters. The work of Okanishi and Gregory, however, has provided invaluable information that has facilitated development of the protocol for measuring DNA reassociation optically.

Optical reassociation of DNA constitutes a powerful experimental tool for estimating genome sizes (1, 10, 24) and the degree of shared nucleotide sequences (6) in DNA samples from bacteria whose DNA can be isotopically labeled only with great difficulty (12, 19). Genome size values are dependent upon references whose molecular weights are not definitively established. The molecular weight of the genome of *Escherichia coli* is variously reported as 2.2×10^9 (19) to 2.8×10^9 (10). In this study, I have used the value of 2.5×10^9 daltons for *E. coli* K-12 genome size (10). The genome sizes of the mycobacterial DNA samples analyzed range between 2.5×10^9 daltons for *M. tuberculosis* H37Ra to 4.5×10^9 for *M. smegmatis*.

The determinations of the relative capability of two denatured DNA samples to reassociate, as measured optically, are subject to large errors, primarily arising from inaccurate measurement of the reassociation rate constant (6). The homology data presented herein should be considered qualitative rather than absolute. The degree of renaturation of various mycobacterial DNA samples with *M. smegmatis* DNA as determined optically and by the nitrocellulose membrane filter technique were gratifyingly similar. Accordingly, this study was extended to include measurements with *M. tuberculosie* H37Ra DNA as the reference. For species in common with the work of Gross and Wayne, who used agar as the immobile phase, the results are similar except for our lower degree of renaturation between *M. tuberculosis* H37Ra and *M. intracellulare* 475. This difference may reflect a difference in the test strains, but our reassociation results are more consistent with Gross and Wayne's phenetic results than are their reassociation results. The agreement between the ranking of relatedness among these mycobacteria based upon DNA reassociation in free solution and my phenetic study (2) is excellent. Neither study, however, definitively reveals the identity of the cultures designated herein as *Mycobacterium* sp. but which have variously been identified as *M. intracellulare*, *M. fortuitum*, and *N. farcinica*. Optical reassociation now provides the techniques to resolve these issues that would have been exceedingly tedious using other established reassociation techniques.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-09098 from the National Institute of Allergy and Infectious Diseases.

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