Deoxyribonucleic Acid Relatedness Study of *Mycobacterium fallax*

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Deoxyribonucleic acid (DNA) was extracted from mycobacterial cells by gentle lysis (sodium dodecyl sulfate and pronase treatment) of wall-deficient forms obtained after D-cycloserine and glycine treatment. DNA from *Mycobacterium fallax* CIP 8139^T (= ATCC 35219^T) (T = type strain) was ³H-labeled by nick-translation. DNA relatedness studies (S1 nuclease method) showed that the seven *M. fallax* strains studied form a single DNA hybridization group which is less than 25% related to 16 other species of the genus *Mycobacterium*. The guanine-plus-cytosine content of *M. fallax* DNA was 68 mol%, and the guanine-plus-cytosine contents of *Mycobacterium aurum*, *Mycobacterium chitae*, *Mycobacterium duvalii*, *Mycobacterium flavescens*, *Mycobacterium gilvum*, *Mycobacterium neoaurum*, *Mycobacterium parafortuitum*, *Mycobacterium thermoresistibile*, and *Mycobacterium vaccae* DNAs were 64 to 65 mol%.

A new species, *Mycobacterium fallax*, was recently proposed (18) on the basis of phenotypic characteristics, such as biochemical reactions, physiological properties, and mycolic acid composition.

A taxonomic proposal is hardly complete if it is not supported by deoxyribonucleic acid (DNA) relatedness studies. DNA relatedness has been an invaluable tool for the taxonomy of gram-negative bacteria, especially the *Enterobacteriaceae* (10). Unfortunately, the application of DNA relatedness methods to the gram-positive bacteria and especially the mycobacteria has been limited by the fastidiousness of DNA extraction from these organisms.

Since lysozyme or the usual detergents are inoperative against mycobacterial cell walls, the methods used for the isolation of DNA from mycobacteria have involved either physical disruption of the cells with a French press (1) or autolysis under anaerobic conditions of growth (23). However, lysozyme is able to effect mycobacterial cell lysis when it is used after a treatment in which the cell wall is made fragile with glycine or cycloserine (19). Previous DNA relatedness studies of mycobacteria were done with the spectrophotometric method (2-5, 7, 9, 16, 17), the agar method (14), or the nitrocellulose membrane method (8).

A procedure in which both glycine and D-cycloserine were used to prepare spheroplasts (21) was used with sodium dodecyl sulfate and proteinase treatments to extract DNA. Such extracted DNA could then be purified, labeled (in vitro), and used in DNA relatedness experiments by following the procedures used (with minimal adaptations) with DNAs from the *Enterobacteriaceae* (11-13).

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. The sources of the *M. fallax* strains have been described previously (18).

Extraction of DNA. The following procedure was used for all species studied except *Mycobacterium tuberculosis*. Flasks containing 3 liters of nutrient broth (Difco Laboratories, Detroit, Mich.) were inoculated and incubated at 30°C (*Mycobacterium chelonae*, *M. fallax*, and *Mycobacterium marinum*) or 37°C (all other species) for 15 to 21 days to reach the stationary phase of growth. The bacterial cells were sedimented by centrifugation at 7,000 × g for 30 min,

and the resulting pellets were suspended in 500 ml of fresh nutrient broth supplemented with 100 µg of D-cycloserine per ml, 14 mg of glycine per ml, 6 × 10⁻³ M ethylenediaminetetraacetic acid (EDTA), and 200 µg of lysozyme per ml. The suspensions were incubated at 30°C (*M. chelonae*, *M. fallax*, and *M. marinum*) or 37°C (all other species) for 18 h (allowing about three cell divisions). The wall-deficient cells obtained in this way were harvested by centrifugation at 7,000 × g for 30 min and suspended in 100 ml of tris(hydroxymethyl)aminomethane-EDTA-NaCl buffer [0.05 M tris(hydroxymethyl)aminomethane, 0.05 M EDTA, 0.1 M NaCl, pH 8.0]. The cells were lysed by adding 0.5% (wt/vol) sodium dodecyl sulfate and 60 µg of pronase per ml with incubation for 18 h at 37°C.

The procedure described above was modified for the extraction of DNA from *M. tuberculosis*. *M. tuberculosis* cells were grown in 3 liters of Proskauer-Beck medium (Difco) supplemented with 1% (wt/vol) Casamino Acids. After 6 weeks of incubation at 37°C, the cells were sedimented by centrifugation, and the pellet was suspended in complete Middlebrook 7H9 medium (Difco) containing D-cycloserine, glycine, EDTA, and lysosyme at the concentrations described above. The suspension was incubated at 37°C for 48 h (assuming a 16-h generation time, about three cell divisions were allowed). The cells were harvested by centrifugation, suspended in tris(hydroxymethyl)aminomethane-EDTA-NaCl buffer, and lysed with sodium dodecyl sulfate and pronase as described above.

Purification of DNA. DNA was purified from the sodium dodecyl sulfate-pronase mixture by using a previously described method (11).

DNA hybridization. DNA from *M. fallax* CIP 8139^T (T = type strain) was labeled in vitro by nick translation as previously described (13), except that 4 µl of DNA (1 mg/ml) and 5 µl of deoxyribonuclease I (10⁻⁸ g/ml) were used.

The S1 nuclease method (12) was used by following the S1 nuclease-trichloroacetic acid procedure described elsewhere (13), except that all volumes were reduced by one-half and the hybridization temperature was 75°C. The temperature (*T_m*) at which 50% of the reassociated DNA became hydrolyzable by the S1 nuclease was determined by using the method of Crosa et al. (12), with one modification. After completion of reassociation, the hybridization mixture was diluted 1:2 with distilled water in order to lower the *T_m* values below 100°C. The difference (Δ*T_m*) between the *T_m* of the homologous reaction and the *T_m* of the heterologous

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TABLE 1. G+C contents and DNA homology between labeled *M. fallax* CIP 8139^T and selected mycobacteria

Source of DNA ^a	G+C content of DNA (mol %) ^b	% Relative binding at 75°C ^c
<i>M. fallax</i>		
CIP 8139 ^T (= ATCC 35219 ^T)	68	100 (0)
Lévy-Frébault 80		91
Lévy-Frébault 191		82 (1.0)
Lévy-Frébault 196		77 (0.5)
Lévy-Frébault 442		87 (1.5)
CIPT 14 139 0001		83
CIPT 14 139 0002		87
<i>M. aurum</i>		
CIPT 14 121 0001 ^T (= ATCC 23366 ^T)	65	18
CIPT 14 121 0002 (= ATCC 23070)		18
<i>M. chelonae</i>	(62) ^d	7
CIPT 14 042 0003 ^T (= NCTC 946 ^T)		
<i>M. chitae</i>		
CIPT 14 116 0001 ^T (= ATCC 19627 ^T)	65	22
CIPT 14 116 0002 (= ATCC 19628)		25 (15.0)
CIPT 14 116 0003 (= ATCC 19629)		20
<i>M. duvalii</i>		
CIPT 14 118 0004 ^T (= NCTC 358 ^T)	65	19
CIPT 14 118 0003		23
<i>M. flavescens</i>		
CIPT 14 023 0001 ^T (= ATCC 14474 ^T)	64, (65) ^e	14
<i>M. fortuitum</i>		
CIPT 14 041 0001 ^T (= ATCC 6841 ^T)	(66) ^e , (68) ^d	18
<i>M. gilvum</i>		
CIPT 14 119 0001 ^T (= NCTC 10742 ^T)	64	21
CIPT 14 119 0002		11
<i>M. marinum</i>		
CIPT 14 012 0001 ^T (= ATCC 927 ^T)	(66) ^f	10
<i>M. neoaurum</i>		
CIPT 14 125 0001 ^T (= ATCC 25795 ^T)	64	19
<i>M. parafortuitum</i>		
CIPT 14 122 0001 ^T (= ATCC 19686 ^T)	65	19
<i>M. phlei</i>		
CIPT 14 130 0001 ^T (= ATCC 11758 ^T)		22
CIPT 14 130 0002		23 (16.0)
CIPT 14 130 0003		21
<i>M. senegalense</i>		
CIPT 14 135 0002 ^T (= NCTC 10956 ^T)	(68) ^g	21
<i>M. smegmatis</i>		
CIPT 14 133 0010 ^T (= ATCC 19420 ^T)		17
CIPT 14 133 0001 (= ATCC 14468)	(68) ^e	16
<i>M. thermoresistibile</i>		
CIPT 14 131 0001 ^T (= ATCC 19527 ^T)	65	20
CIPT 14 131 0004		17
CIPT 14 131 0005		13
<i>M. tuberculosis</i> (H ₃₇ Ra)		
CIPT 14 001 0002 (= ATCC 25177)	(63) ^d	11
<i>M. vaccae</i>		
CIPT 14 120 0006 ^T (= ATCC 15483 ^T)	65, (68) ^d	12

^a CIP, Collection Institut Pasteur, Paris, France; CIPT, Collection Institut Pasteur Tuberculose, Paris, France; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England.

^b Previously published G+C contents are given in parentheses. Other G+C contents are results from this study.

^c ΔT_m values (in degrees Celsius) are given in parentheses.

^d Data from reference 16.

^e Data from reference 22.

^f Data from reference 15.

^g Data from reference 6.

reaction was an estimate of divergence between two DNAs (10).

Determination of DNA base composition. The melting temperatures of 50- μ g/ml DNA solutions in $0.1 \times$ SSC buffer (1

$\times$ SSC is 0.15 M NaCl plus 0.015 M trisodium citrate) were measured with a Gilford spectrophotometer. The guanine-plus-cytosine (G+C) contents of DNAs were determined from melting temperatures by the equation of Owen et al. (20). The DNA of *Escherichia coli* K-12 was used as standard; this DNA was taken to have a G+C content of 50.6 mol%.

RESULTS

The DNA preparations exhibited ratios of absorbance at 260 nm (A_{260}) to A_{280} and A_{260} to A_{230} of 1.7 to 1.8 and 1.7 to 2.1, respectively, and hyperchromicities (increases in absorbance at 260 nm) between 25 and 29%.

The DNA relatedness results with labeled reference DNA from *M. fallax* CIP 8139^T are shown in Table 1. The S1 nuclease-resistant core (in the incubated control tubes containing only denatured labeled DNA) was calculated to be $18 \pm 5\%$ (mean $\pm$ standard deviation) at 75°C. Six strains of *M. fallax* were 77 to 91% related to strain CIP 8139^T, with ΔT_m values below 1.5°C. Thus, the seven strains of *M. fallax* constitute a homogeneous DNA relatedness group. *M. fallax* CIP 8139^T was only 7 to 25% related to 26 strains belonging to 16 *Mycobacterium* species. The species closest to *M. fallax* were *Mycobacterium chitae* (20 to 25% related to *M. fallax*, with a ΔT_m value of 15.0°C) and *Mycobacterium phlei* (21 to 23% related to *M. fallax* with a ΔT_m value of 16.0°C).

The G+C content of *M. fallax* CIP 8139^T DNA was calculated to be 68 ± 1 mol%, whereas the calculated G+C contents of DNAs from *Mycobacterium aurum*, *Mycobacterium chitae*, *Mycobacterium duvalii*, *Mycobacterium flavescens*, *Mycobacterium gilvum*, *Mycobacterium neoaurum*, *Mycobacterium parafortuitum*, *Mycobacterium thermoresistibile*, and *Mycobacterium vaccae* were 64 to 65 mol%.

DISCUSSION

The use of DNA-DNA hybridization in mycobacterial taxonomy has been limited by the difficulty of DNA extraction from mycobacterial cells (1, 19, 23). In this study, DNA was extracted from spheroplasts, and good yields were obtained. DNA preparations seemed pure enough (as judged by ratios of A_{260} to A_{280} and A_{230} to A_{260}) although labeling by nick translation yielded DNA samples that were only 82% hydrolyzable by S1 nuclease after heat denaturation.

The G+C content of *M. fallax* DNA (68 mol%) is within the 60 to 70 mol% range reported for *Mycobacterium* species (22).

The DNA relatedness data provided evidence that *M. fallax* is a discrete genomic species that is distinct from previously published *Mycobacterium* species.

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