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Molecular-Genetic Evidence for the Relationship of *Mycobacterium leprae* to Slow-growing Pathogenic Mycobacteria[1]

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The placement of *Mycobacterium leprae* in the genus *Mycobacterium* is primarily based on its strong acid-alcohol fastness and the composition of its mycolic acids, i.e., the number of carbons ([5, 13]). Although *M. leprae* is listed as a slow-growing species ([24]), its intrageneric taxonomic position has as yet not been settled and even its membership in the genus *Mycobacterium* has been questioned ([9]). With respect to delayed-type hypersensitivity reactions, lymphocyte transformation tests, and antigenic properties ([12, 14, 28]), *M. leprae* is similar to fast-growing mycobacteria. On the other hand, the low deoxyribonucleic acid (DNA) guanesine plus cytosine (G+C) content ([3, 17]) and the small genome size ([2, 3, 17]) indicate that *M. leprae* may be taxonomically rather isolated. Measurements of genomic relationships by DNA–DNA hybridization between *M. leprae*, a variety of slow- and fast-growing mycobacterial species, and clinical isolates from other genera ([1–3, 17]) failed to resolve the problem because of contradicting results at the inter- and intrageneric levels. Based on DNA homologies ([17]), antigenic reactivity ([14]) and immunological relatedness between ribosomal components ([21]), a possible relationship of *M. leprae* to members of the genus *Corynebacterium* has been suggested.

In this study we compare long, reverse transcriptase generated stretches of the primary structure of the 16S ribosomal (r) ribonucleic acid (RNA) of *M. leprae* to a variety of mycobacterial species and related taxa as defined by Stackebrandt ([26]). This approach is of proven reliability for determining phylogenetic distances at various levels of relationships ([7, 30]).

MATERIALS AND METHODS

Armadillo-derived *M. leprae* have been isolated from the liver and spleen tissues of experimentally infected, nine-banded armadillos held in the Division of Laboratory Animal Science of the Research Institute for Experimental Biology and Medicine, Borstel, Federal Republic of Germany. The animals were kept under conditions at which contamination with other mycobacteria was excluded ([18]). The identity of *M. leprae* was verified by negative growth on synthetic media ([23]), by positive DOPA oxidase reaction ([24]), and by decolorization with pyridine ([22]). Purification was performed by methods described by Draper ([6]). The isolate was positive in an indirect fluorescence technique, using a *M. leprae*-specific monoclonal antibody against the phenolic glycolipid-I antigen of the organism ([19]). For comparison, the following strains were included: *M. tuberculosis* H37Rv, *M. bovis* strain Vallée, *M. avium* TMC 724, *M. scrofulaceum* TMC 1323, *M. phlei* TMC 1516, and *M. fortuitum* TMC 1545. These mycobacteria had been cultivated on Löwenstein-Jensen medium at 37°C.

A total of 2 g (wet weight) of purified *M. leprae* cells was used for the determination of the primary structure of 16S rRNA. Isolation of rRNA was performed as described previously ([7]). The method of Lane, *et al.* ([20]) was used to sequence large overlapping stretches of 16S rRNA from the test strains. Oligonucleotide primers were synthesized using an Applied Biosystems 3801A DNA synthesizer. The chemical composition of these primers, their target sites, and the electrophoretic separation conditions of 35S la-

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THE TABLE. *16S rRNA sequence homologies (lower left triangle) and Knuc values (upper right triangle) between* Mycobacterium *species and reference organisms of the genera* Corynebacterium, Rhodococcus, *and* Nocardia *(calculation based on stretches of 1170 nucleotides).*

	1	2	3	4	5	6	7	8	9	10
1. *Mycobacterium leprae*	—	0.041	0.037	0.042	0.056	0.084	0.089	0.133	0.128	0.148
2. *M. tuberculosis* strain H37Rv	96.3	—	0.018	0.044	0.052	0.086	0.085	0.130	0.131	0.148
3. *M. bovis* Vallée	96.4	98.2	—	0.041	0.049	0.081	0.080	0.130	0.126	0.145
4. *M. avium* strain 724	95.9	95.7	96.3	—	0.058	0.088	0.087	0.149	0.139	0.154
5. *M. scrofulaceum* TMC 1323	94.6	95.0	95.3	94.4	—	0.071	0.071	0.113	0.095	0.143
6. *M. phlei* TMC 1516	92.0	91.9	92.3	91.7	93.2	—	0.045	0.113	0.088	0.139
7. *M. fortuitum* TMC 1545	91.6	92.0	92.4	91.8	93.2	95.6	—	0.099	0.099	0.131
8. *Nocardia asteroides* DSM 43005	87.8	88.0	88.0	86.5	89.5	89.5	90.7	—	0.073	0.151
9. *Rhodococcus erythropolis* DSM 43188	88.2	88.0	88.4	87.3	89.6	91.7	91.6	93.0	—	0.118
10. *Corynebacterium glutamicum* DSM 20300	86.6	86.6	86.8	86.1	87.0	87.3	88.0	86.3	89.1	—

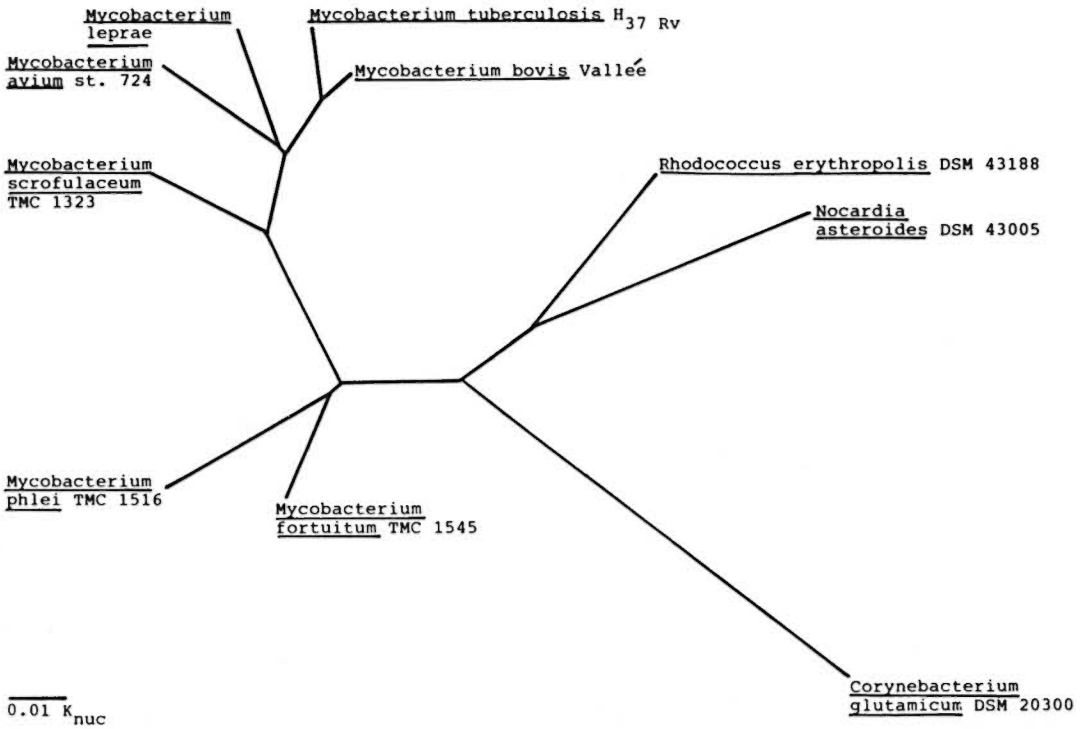
beled complementary DNA (cDNA) have been published ([7]). The sequence data thus obtained were aligned to 16S rRNA stretches of more than 40 actinomycetes ([7] and Smida and Stackebrandt, unpublished) and the homology values were computed ([25]). These values were transformed into estimated evolutionary distance values, Knuc ([15]). Knuc = $-\frac{3}{4} \ln(\frac{4}{3}(S - \frac{1}{4}))$, where S is the observed homology, and ln is the natural logarithm. Knuc transforms homologies to pairwise evolutionary distance values which, because they are only estimates of the evolutionary distances, will not precisely fit any additive tree. The algorithm of Fitch and Margoliash ([10]) contained in the PHYLIP version 2 program of Felsenstein ([8]) was then used for construction of an unrooted phylogenetic tree.

RESULTS AND DISCUSSION

The reverse transcriptase sequences of the eight strains investigated (data not shown) were aligned, and the homologous regions of 1170 nucleotides (79% of the total 16S rRNA) were used for the calculation of the homology values and, derived therefrom, the Knuc values (evolutionary distances) (The Table). A phylogenetic tree based on the latter parameter is shown in The Figure. All slow-growing mycobacteria are highly related, forming a phylogenetically tight cluster apart from the two representatives of fast-growing mycobacteria, *M. phlei* and *M. fortuitum.* While *M. tuberculosis* and *M. bovis* Vallée are phylogenetically virtually indistinguishable, *M. leprae* and *M. avium* form two slightly more ancient lines of descent. With homology values ranging between 94.4% and 95.3% with 16S rRNAs from these four species, *M. scrofulaceum* defines the depth of this very shallow phylogenetic unit.

Slight discrepancies between the pairwise homology/Knuc values and the position of the respective organisms in the tree are obvious. This is the result of the treeing program which produces a pattern in which the distance between any two sequences (replaced in the tree by the respective organisms) best match the estimates (Knuc values) of their evolutionary separations. The tree minimizes the weighted mean square difference between the Knuc values (evolutionary distance estimates) and the corresponding pairwise distances in the tree ([8, 15]).

Representatives of *Nocardia, Rhodococcus,* and *Corynebacterium* are more remotely related, constituting two separate lines of descent, one embracing *N. asteroides* and *R. erythropolis,* the other harboring *C. glutamicum.* More comprehensive outlines of the phylogenetic relationships of



THE FIGURE. Unrooted phylogenetic tree displaying the relationship of *M. leprae* with other slow- and fast-growing mycobacteria and representatives of the genera *Nocardia, Rhodococcus,* and *Corynebacterium.* The branching pattern was derived from Knuc values contained in The Table, upper right triangle.

members of the order *Actinomycetales,* including spore-forming and non-mycelium-forming taxa, have been published ([7, 26]).

The clustering of *M. leprae* within the confines of *M. tuberculosis, M. avium,* and related species clearly indicates the membership of this species not only to the genus *Mycobacterium* but to the subgroup of slow-growing species. This result is surprising considering the low DNA G+C content of only 54–56 mol% ([3]) and the low genome size of 1.3×10^9 ([17]). Comparative values on other mycobacterial species range between 65–72 mol% and $1.8–3.8 \times 10^9$ ([2, 17]), respectively. However, similar broad DNA G+C ranges within groups of closely related organisms has been found in groups of actinomycetes, e.g., the *Renibacterium/Arthrobacter/Micrococcus* cluster ([27]).

The data presented here partially support previously published results of DNA pairing studies ([3, 17]). The branching pattern of the 16S rRNA (The Figure) cannot claim that it finally resolves all problems concerning the relatedness of these organisms. The primary structure of this molecule is too conserved to allow a resolution of very high relationships ([11, 26]) which, in principle, are optimally investigated by DNA-DNA pairing. However, published hybridization data on the intergroup relationships of this subgroup are a moot point. Conflicting results have been published for the relationship of *M. leprae* to other *Mycobacterium* species as well as for slow-growing mycobacteria among themselves. For *M. leprae,* the data may be interpreted in terms of low relatedness to *M. tuberculosis, M. avium,* and *M. scrofulaceum* ([1]); moderate relatedness to *M. tuberculosis* and *M. scrofulaceum* ([17]); or high relatedness to *M. tuberculosis* ([3]). These discrepancies could be explained by differences in the methods used (spectrophotometric and nuclease S1 analyses), in the selection of strains, and in the hybridization temperature. Unfortunately, none of these studies included a complete matrix of reciprocal experiments which could have led to an explanation of these discrepancies. In addition, the low DNA

G+C content and the low genome size of *M. leprae* DNA as compared to other slow-growing mycobacteria make it almost impossible to optimally determine hybridization conditions that match the whole range of DNA used in the pairing studies ([4, 10]).

The high average homology value of 96% for the five slow-growing mycobacteria means that each possible pair of sequences differ in 50 bases only. A high fraction of these mutational events occurs in a region of the primary structure known to be highly variable ([31]), i.e., position 179 to 230 (according to the IUB numbering system for *E. coli*); 15 and 13 differences are present in this region for the *M. leprae*/*M. tuberculosis* and the *M. leprae*/*M. avium* sequences, respectively. The extent of this variation should be sufficient to develop species-specific, nucleic-acid probes which could be used for rapid identification of these highly pathogenic organisms. Such probes (although of unknown sequence) are already commercially available for a number of mycobacterial species, i.e., one for the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum,* and *M. microti*), a second for the *M. avium* complex (*M. avium* and *M. intracellulare*). As far as data are available, members of the first complex show high DNA homologies but share few intercomplex homologies with the two species of the second complex ([1-3, 17]). Since these two complexes are approximately equidistantly related to *M. leprae* (The Table, The Figure), it is highly likely that the sequence peculiarities of the *M. leprae* 16S rRNA will facilitate unambiguous detection of *M. leprae* cells even within tissues containing mixed bacterial populations.

SUMMARY

A total of 1170 nucleotides of the 16S rRNA from *Mycobacterium leprae* were compared to the homologous regions of *M. tuberculosis, M. bovis* Vallée, *M. avium, M. scrofulaceum, M. phlei, M. fortuitum* and one representative each of the genera *Corynebacterium, Nocardia,* and *Rhodococcus.* Homology values were calculated and a phylogenetic tree was constructed from the evolutionary distance values. Despite differences in DNA G+C content and genome size, *M. leprae* is a true member of the slow-growing pathogenic mycobacteria, branching off intermediate to the other members of this subgroup. Slow- and fast-growing mycobacteria are phylogenetically well separated but constitute an individual branch of the actinomycetes proper. Significant structural variation of certain regions of the 16S rRNA may allow construction of *M. leprae*-specific probes used for rapid identification.

RESUMEN

Se compararon un total de 1170 nucleótidos del RNAr 16S del *Mycobacterium leprae,* con las regiones homólogas de *M. tuberculosis, M. bovis* Vallee, *M. avium, M. scrofulaceum, M. phlei, M. fortuitum,* y de un representante de los géneros *Corynebacterium, Nocardia,* y *Rhodococcus.* Se calcularon los valores de homología y se construyó un árbol filogenético con los valores de distancia evolutiva. No obstante las diferencias en el contenido de G+C (DNA) y del tamaño del genoma, *M. leprae* es un verdadero miembro de las microbacterias de crecimiento lento, ramificandose en posición intermedia entre los otros miembros de este subgrupo. Las micobacterias de crecimiento rápido y las de crecimiento lento están bien separadas pero constituyen una rama individual de los actinomicetos. La variación estructural significante de ciertas regiones del RNAr 16S, puede permitir la construcción de sondas específicas para *M. leprae* útiles para su rápida identificación.

RÉSUMÉ

Un total de 1170 nucléotides du rRNA 16S de *Mycobacterium leprae* ont été comparé aux régions homologues de *M. tuberculosis,* de *M. bovis* Vallee, de *M. avium,* de *M. scrofulaceum,* de *M. phlei,* de *M. fortuitum,* et d'un représentant de chacun des genres *Corynobacteries, Nocardia,* et *Rhodococcus.* Les valeurs d'homologie ont été calculées, et un arbre phylogénique a été construit, à partir des divergences au point de vue de l'évolution. Malgré des différences dans le contenu guanine et cytosine (G+C) de l'ADN, et dans la dimension du genome, on a pu constater que *M. leprae* appartient réellement au groupe des mycobactéries pathogéniques à croissance lente, représentant un embranchement intermédiaire par rapport aux autres membres de ce sous-groupe. Les mycobactéries à croissance lente et celles à croissance rapide sont phylogénétiquement bien séparées, mais constituent une branche séparée du groupe proprement dit des actinomycètes. Une variation structurelle significative de certaines régions du rRNA 16S peut permettre de mettre au point des épreuves spécifiques pour *M. leprae,* qui pourraient être utilisées pour une identification rapide.

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