Numerical Analysis of the Relationship Between *Mycobacterium*, Rhodochrous Group, and *Nocardia* by Use of Hypothetical Median Organisms

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A total of 1,054 strains was allocated to species of the genera *Mycobacterium*, *Nocardia*, and *Gordona* (the rhodochrous group) on the basis of 88 characters, and hypothetical median organisms (HMOs) were derived for each species. Numerical analysis was carried out, treating these HMOs as operational taxonomic units. The dendrogram obtained showed that strains received as members of the rhodochrous group, including "*Mycobacterium*" *rhodochrous*, and strains named previously by the present author as *Gordona*, constituted a taxon comparable to the genus *Nocardia*. The analysis showed two separable groups at a 70% *M* value (matching coefficient) level. One consisted of slowly growing mycobacteria (*Mycobacterium* subgenus *Mycobacterium* Tsukamura) and the second consisted of rapidly growing mycobacteria (*Mycobacterium* subgenus *Mycomycobacterium* Tsukamura), the genus *Gordona* (including the rhodochrous group) and *Nocardia*. Diagrammatic representation of the taxonomic relationships by taxonomic distances showed the same result. Four taxa, which might be regarded as separate genera, separated at higher *M* values. These included the slowly growing mycobacteria, the rapidly growing mycobacteria, the genus *Gordona* (rhodochrous group), and the genus *Nocardia*, respectively. The slowly growing mycobacteria seem to be only distantly related to the other three, which bear a close relationship with each other.

Gordon and Mihm (5) named some strains received as nocardiae "*Mycobacterium*" *rhodochrous*. Later, Gordon (4) regarded this taxon as an intermediate between the genera *Mycobacterium* and *Nocardia*. Tsukamura (16) isolated a number of slightly acid-fast organisms from sputa of patients with lung disease and from soil and classified them into three species of a new genus, *Gordona*. Thereafter, strains received as *M. rhodochrous* were shown to belong to the same genus, and the majority of the strains received were identified as belonging to the species *Gordona rhodochroa* of this genus (17). Independently of these studies, Goodfellow (2) and Goodfellow et al. (3) reported that strains of *M. rhodochrous* could be differentiated from nocardiae and rapidly growing mycobacteria, respectively.

Previously, studies on the relationships between rapidly growing mycobacteria, *Gordona* (rhodochrous group), and *Nocardia* were carried out by Tsukamura (15, 16, 18). However, he did not consider the slowly growing mycobacteria. The present study is concerned with the relationships between *Mycobacterium*, including slowly growing mycobacteria, the rhodochrous group, and *Nocardia*.

MATERIALS AND METHODS

A total of 1,054 strains of *Mycobacterium*, the rhodochrous group, and *Nocardia* was allocated to species by comparing their characters with those of reference strains (14).

The characters tested are listed in Table 1. Most of the methods used were described previously (11-14, 16, 20). Amidase activity, catalase activity, Tween hydrolysis activity, esterase activity, β -galactosidase activity, and acid phosphatase activity were tested by the methods of Bönicke (1), Kubica et al. (8), Wayne et al. (23), Kämpfer (6), Tsukamura (19), and Tsukamura et al. (22), respectively. Of these characters, 85 were effective for differentiating the strains. Only three characters (growth at 37°C, permanent mycelium, and weak acid fastness) were ineffective, showing positive or negative matches in all test strains.

The species identified are listed in Table 2.

In employing numerical methods to compare the species, comparisons were made between hypothetical median organisms (HMOs), one HMO first being derived, using the method of Liston et al. (9), for each species by analysis of the characters of the strains which had been allocated to it.

Matching coefficients (*M* values) were calculated by the following equation: $M \text{ value} = [n_s / (n_s + n_d)] \times 100\%$, where n_s is the number of characters which show similar code symbols (+ + or - -) and n_d is

TABLE 1. *List of characters used for tests*

Acid fastness (strong)	Growth at 45 C
Acid fastness (weak or partial)	Growth at 52 C
Shape (rod form)	Glucose (C source)
Cord formation	Mannose (C source)
Fragmenting mycelium	Sucrose (C source)
Permanent mycelium	<i>n</i> -Propanol (C source)
Colony (rough)	<i>n</i> -Butanol (C source)
Colony (pigment)	iso-Butanol (C source)
Photochromogenicity	Propylene glycol (C source)
Rapid growth (growth at 3 days)	Galactose (C source)
Tolerance to nitrite (0.1%)	Arabinose (C source)
Tolerance to picric acid (0.2%)	Xylose (C source)
Rifampin (25 µg/ml)	Rhamnose (C source)
PAS (0.2%) tolerance	Trehalose (C source)
NH ₂ OH·HCl (0.125 mg/ml)	Inositol (C source)
NH ₂ OH·HCl (0.25 mg/ml)	Mannitol (C source)
NH ₂ OH·HCl (0.5 mg/ml)	Sorbitol (C source)
TCH (10 µg/ml)	Acetate (C source)
Tolerance to salicylate (0.5 mg/ml)	Citrate (C source)
<i>p</i> -Nitrobenzoic acid (0.5 mg/ml)	Succinate (C source)
Niacin production	Malate (C source)
Tween hydrolysis (7 days)	Pyruvate (C source)
Tween hydrolysis (14 days)	Benzoate (C source)
α-Esterase	Malonate (C source)
β-Esterase	Fumarate (C source)
Acid phosphatase	Acid from glucose
Catalase (foam more than 45 mm)	Acid from mannose
β-Galactosidase	Glucose as C source (glutamate-N)
Nitrate reduction	Acetate as C source (glutamate-N)
3-day arylsulfatase	Succinate as C source (glutamate-N)
2-week arylsulfatase	Pyruvate as C source (glutamate-N)
Salicylate degradation	Glycerol as C source (glutamate-N)
PAS degradation	Glutamate as N-C source
Acetamidase	Serine as N-C source
Benzamidase	Glucosamine as N-C source
Urease	Acetamide as N-C source
Isonicotinamidase	Benzamide as N-C source
Nicotinamidase	Monoethanolamine as N-C source
Pyrazinamidase	Trimethylene diamine as N-C source
Salicylamidase	Benzamide as N source
Allantoinase	Nicotinamide as N source
Succinamidase	Nitrate (N source)
Growth at 28 C	Nitrite (N source)
Growth at 37 C	Ethambutol tolerance (5 µg/ml)

the number of characters which show different code symbols (+ -). Clustering was made by the single-linkage method.

Relationships between the taxa were estimated by taxonomic distance (10). The taxonomic distance (d) was expressed by the following equation: $d = (100 - x)/x$, where x is a mean M value (%) between the taxa. The dimension of a taxon was expressed by a sphere or circle, the radius (r) of which was calculated by the following equation: $r = (100 - y)/y$, where y is a mean M value (%) within a taxon.

RESULTS

Dendrogram. A dendrogram was prepared by comparing the HMOs (Fig. 1). The species of the genus *Nocardia* formed a cluster at the 84%

level, and the species of *Gordona* formed a cluster at the 85% level. The cluster of *Gordona* was linked with several rapidly growing mycobacteria, *M. aichiense*, *M. chubuense*, *M. tokaiense*, *M. aurum*, *M. neoaurum*, *M. parafortuitum*, *M. rhodesiae*, and *M. obuense*, at the 85% level. These rapidly growing mycobacteria are scotochromogenic.

Rapidly growing mycobacteria (Table 2) were placed in dispersed positions. Slowly growing mycobacteria (Table 2) formed a distinct cluster at the 86% level, with the exception of *M. tuberculosis* and *M. bovis*. On the basis of the data presented, these two organisms, *M. tuberculosis* and *M. bovis*, were more sharply separated from the other slowly growing mycobacteria

TABLE 2. *List of species tested*

<i>Mycobacterium</i> subgenus <i>Mycobacterium</i> (slowly growing mycobacteria) (12)	
<i>M. tuberculosis</i> (Zopf) Lehmann and Neumann 1896 (90 strains)	
<i>M. bovis</i> Bergey, Breed, Hammer, Huntoon, Murray and Harrison 1934 (20 strains)	
<i>M. kansasii</i> Hauduroy 1955 (12 strains)	
<i>M. marinum</i> Aronson 1926 (10 strains)	
<i>M. marianum</i> Suzanne and Penso 1953 (<i>M. scrofulaceum</i> Prissick and Masson 1956) (26 strains)	
<i>M. gordonae</i> Bojalil, Cerbón and Trujillo 1962 (50 strains)	
<i>M. szulgai</i> Marks, Jenkins and Tsukamura 1972 (4 strains)	
<i>M. xenopi</i> Schwabacher 1959 (4 strains)	
<i>M. avium</i> Chester 1901 (26 strains)	
<i>M. intracellulare</i> (Cuttino and McCabe) Runyon 1966 (76 strains)	
<i>M. nonchromogenicum</i> Tsukamura 1965 (93 strains)	
<i>M. terrae</i> Wayne 1966 (15 strains)	
<i>M. novum</i> Tsukamura 1967 (26 strains)	
<i>M. triviale</i> Kubica, Silcox, Kilburn, Smithwick, Beam, Jones and Stottmeier 1970 (22 strains)	
<i>M. gastri</i> Wayne 1966 (8 strains)	
<i>Mycobacterium</i> subgenus <i>Mycobacterium</i> (rapidly growing mycobacteria) (12)	
<i>M. flavescentis</i> Bojalil, Cerbón and Trujillo 1962 (10 strains)	
<i>M. thermoresistibile</i> Tsukamura 1966 (39 strains)	
<i>M. fortuitum</i> Da Costa Cruz 1938 (39 strains)	
<i>M. fortuitum</i> subsp. <i>thermophilum</i> Tsukamura 1967 (63 strains)	
<i>M. chelonae</i> subsp. <i>chelonae</i> Bergey, Harrison, Breed, Hammer and Huntoon 1923 (24 strains)	
<i>M. chelonae</i> subsp. <i>abscessus</i> Moore and Frerichs 1953 (26 strains)	
<i>M. phlei</i> Lehmann and Neumann 1899 (21 strains)	
<i>M. smegmatis</i> Lehmann and Neumann 1899 (26 strains)	
<i>M. vaccae</i> Bönicke and Juhász 1964 (12 strains)	
<i>M. parafortuitum</i> Tsukamura, Toyama and Mizuno 1965 (6 strains)	
<i>M. aurum</i> Tsukamura 1966 (34 strains)	
<i>M. chitae</i> Tsukamura 1967 (6 strains)	
<i>M. agri</i> Tsukamura 1972 (31 strains)	
<i>M. obuense</i> Tsukamura and Mizuno 1971 (5 strains)	
<i>M. rhodesiae</i> Tsukamura, Mizuno, Gane, Mills and King 1971 (16 strains)	
<i>M. neoaurum</i> Tsukamura 1972 (19 strains)	
<i>M. chubuense</i> Tsukamura 1973 (15 strains)	
<i>M. aichiense</i> Tsukamura 1973 (8 strains)	
<i>M. tokaiense</i> Tsukamura 1973 (3 strains)	
<i>Gordona</i> (Rhodochrous group)	
<i>G. bronchialis</i> Tsukamura 1971 (41 strains)	
<i>G. rubropertincta</i> (Grotenfelt) Tsukamura 1971 (14 strains)	
<i>G. terrae</i> Tsukamura 1971 (20 strains)	
<i>G. rhodochroa</i> (Zopf) Tsukamura 1973 (10 strains)	
<i>G. aurantiaca</i> Tsukamura and Mizuno 1971 (6 strains)	
<i>Nocardia</i>	
<i>N. asteroides</i> (Eppinger) Blanchard 1896 (21 strains)	
<i>N. brasiliensis</i> (Lindenberg) Pinoy 1913 (12 strains)	
<i>N. caviae</i> (Erikson) Erikson 1935 (8 strains)	
<i>N. farcinica</i> Trevisan 1889 (32 strains) ^a	
<i>N. rubra</i> (Kruse) Chalmers and Christopherson 1916 (5 strains) ^b	

^a This species was separated from *N. asteroides* by Tsukamura (14), who regarded it as a distinct species. The name is based on the fact that the group contains *N. farcinica* ATCC 3318.

^b Contains strains collected by Tsukamura (14) with different species names.

than the *Gordona* organisms from the nocardiae, etc.

Nocardia rubra was not incorporated into the cluster of *Nocardia* but into the cluster of *Gordona*. Though this organism shows fragmenting (temporary) mycelium, and in this point differs from other hitherto described *Gordona* organisms, it is elsewhere proposed that it be transferred to the genus *Gordona* (21).

Mycobacterium smegmatis was not incorporated into any cluster at a level above 79%. It was linked to all the other strains at the 78% level as was the cluster consisting of *M. bovis* and *M. tuberculosis*. The *M* value between *M. bovis* and *M. smegmatis* was, however, only 23%.

M value table. A diagrammatic representation of the *M* values obtained by comparing the

FIG. 1. Dendrogram showing the relationship of the HMOs of the species studied.

HMOs is shown in Fig. 2. It reflects the relationships noted from the dendrogram and dramatically emphasizes the isolation of the *M. bovis*, *M. tuberculosis* cluster from all other groups even at the 70% level.

Taxonomic distances. Taxonomic distances between slowly growing mycobacteria (*Mycobacterium* subgenus *Mycobacterium*), rapidly growing mycobacteria (*Mycobacterium* subgenus *Mycomycobacterium*), genus *Nocardia*, and the rhodochrous group (genus *Gordona*) are shown in Fig. 3.

The results confirm that slowly growing mycobacteria are distinctly separate from the other three taxa: rapidly growing mycobacteria, *Gordona*, and *Nocardia*. The latter three taxa are near each other. The characters useful for distinguishing these taxa are shown in Table 3.

DISCUSSION

Previously, Tsukamura (12) proposed that the genus *Mycobacterium* be divided into two

subgenera, *Mycobacterium* and *Mycomycobacterium*. These subgenera corresponded approximately to slowly growing mycobacteria and rapidly growing mycobacteria, respectively. The subgenera were separated from each other not only by growth rate but also by several other characters (11, 12, 15).

In the present study, it was confirmed that slowly growing mycobacteria distinctly differ from rapidly growing mycobacteria. Rapidly growing mycobacteria appeared to be similar to *Gordona* and *Nocardia*.

The taxonomic status of *Gordona* is considered to be equivalent to that of *Nocardia*. The recognition of two subgenera of the genus *Mycobacterium* is also strengthened by the data shown in the present study. Should the subgenus *Mycomycobacterium* Tsukamura be raised to the status of a genus, *M. phlei* may be appropriate as the type species. Previously, Tsukamura (15) proposed *M. smegmatis* as the type species of the subgenus *Mycomycobacterium*. In light of the results of the present study,

FIG. 2. Diagrammatic representation of *M*-value table.

however, *M. smegmatis* seems inappropriate as the type species.

Some slight changes in the affinities between taxa are seen in the present study in contrast to the data of previous studies. These are probably due to difference in the characters used for study. In the present study, the number of enzyme tests was increased and the number of tests belonging to the same kind was decreased. For example, previously, 10 tests on acid formation from various carbohydrates were used, but only two tests (acid from glucose and acid from mannose) were used in the present study. Previously, 11 tests on the utilization of nitrogen compounds were used, whereas only 4 tests were used in the present study. Furthermore, a few tests which in the past gave relatively large experimental errors were omitted. These modifications of the set of characters were undertaken with the expected result of a sharper separation between taxa.

It may be surprising that *M. smegmatis* has shown such a low affinity to other taxa containing mycobacteria. However, the same finding

had been obtained previously in our study (laboratory H), which was included in the cooperative study of the International Working Group on Mycobacterial Taxonomy (7). It is believed that the greater the number of characters which are effective for separation between strains (or taxa), the sharper will be the separation. *M. smegmatis* is believed to have been distinguished from others in the present study by the presence of many characters effective for separation. The organism is believed to occupy a special position among mycobacteria.

In the present study, *N. rubra* was incorporated into the cluster of the *Gordona* organisms. In fact, mean *M* values of this organism to *Gordona*, *Nocardia*, rapidly growing mycobacteria, and slowly growing mycobacteria were $80.0 \pm 4.3\%$ ($n = 5$), $76.1 \pm 2.9\%$ ($n = 4$), $68.5 \pm 4.1\%$ ($n = 19$), and $54.0 \pm 4.8\%$ ($n = 15$), respectively, where n is the number of *M* values. The similarity to the genus *Gordona* was the highest. Gordon and Mihm (5) included a strain of *N. rubra* (strain McClung-1) in *M. rhodochrous*. The finding of the present study

FIG. 3. Relationships between the taxa expressed by taxonomic distances. Abbreviations: M1, *Mycobacterium* subgenus *Mycobacterium* (slowly growing mycobacteria); M2, *Mycobacterium* subgenus *Mycomycobacterium* (rapidly growing mycobacteria); G, *Gordona*; N, *Nocardia*. The relationships between the taxa are shown by taxonomic distances, and extent of a taxon is expressed by the radius of a sphere. *Nocardia rubra* is incorporated into the genus *Gordona* as *Gordona*.

TABLE 3. Characters useful for distinguishing between *Mycobacterium* subgenus *Mycobacterium*, *Mycobacterium* subgenus *Mycomycobacterium*, *Gordona*, and *Nocardia*

Character	HMOs (species) showing positive reaction (%)			
	<i>Mycobacterium</i>	<i>Mycomycobacterium</i>	<i>Gordona</i>	<i>Nocardia</i>
Strong acid fastness	100	100	0	0
Fragmenting mycelium	0	0	17	100
Growth at 3 days	7	100	100	100
0.1% NaNO ₂ -Sauton agar	0	100	100	100
0.2% Picric acid-Sauton agar	0	95	100	100
β -Galactosidase	0	21	0	100
Nitrate reduction	27	53	83	25
2-Week arylsulfatase	73	89	0	0
Glucose as C source	27	100	100	100
Mannose as C source	0	89	100	50
Sucrose as C source	0	32	100	0
<i>n</i> -Butanol as C source	20	89	100	100
Succinate as C source	0	89	100	100
Malate as C source	0	89	100	100
Fumarate as C source	0	84	100	100
Acid from glucose	0	89	100	100
Acid from mannose	0	74	83	0
Glutamate as N and C source	0	100	100	100
Trimethylene diamine as N and C source	0	58	0	0
NaNO ₃ as N source (glucose-C)	0	84	100	100
Rough colonies	40	26	100	100
Propylene glycol as C source	0	58	83	25
No. of HMOs	15	19	6	4

supported their finding. A proposal on the taxonomic status of *N. rubra* is reported elsewhere in this journal (21).

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LITERATURE CITED

1. Bönicke, R. 1962. L'identification des mycobactéries à l'aide de méthodes biochimiques. *Bull. Union Int. Tuberc.* 32:13-76.
2. Goodfellow, M. 1971. Numerical taxonomy of some nocardioform bacteria. *J. Gen. Microbiol.* 69:33-80.
3. Goodfellow, M., A. Fleming, and M. J. Sackin. 1972. Numerical classification of "*Mycobacterium*" *rhodochrous* and Runyon's group IV mycobacteria. *Int. J. Syst. Bacteriol.* 22:81-98.
4. Gordon, R. E. 1966. Some strains in search of a genus—*Corynebacterium*, *Mycobacterium*, *Nocardia* or what? *J. Gen. Microbiol.* 43:329-343.
5. Gordon, R. E., and J. M. Mihm. 1957. A comparative study of some strains received as nocardiae. *J. Bacteriol.* 73:15-27.
6. Käßler, W. 1965. Acetyl-Naphthylamin-Esterasen-Aktivität von Mykobakterien. *Beitr. Klin. Tuberk. Lungenk.* 130:1-4.
7. Kubica, G. P., I. Baess, R. E. Gordon, P. A. Jenkins, J. B. G. Kwapinski, C. McDurmont, S. R. Pattyn, H. Saito, V. Silcox, J. L. Stanford, K. Takeya, and M. Tsukamura. 1972. A co-operative numerical analysis of rapidly growing mycobacteria. *J. Gen. Microbiol.* 73:55-70.
8. Kubica, G. P., W. D. Jones, Jr., V. D. Abbott, R. E. Beam, J. O. Kilburn, and J. C. Cater, Jr. 1966. Differential identification of mycobacteria. I. Tests on catalase activity. *Am. Rev. Resp. Dis.* 94:400-405.
9. Liston, J., W. Wiebe, and R. R. Colwell. 1963. Quantitative approach to the study of bacterial species. *J. Bacteriol.* 85:1061-1070.
10. Lysenko, O., and P. H. A. Sneath. 1959. The use of models in bacterial classification. *J. Gen. Microbiol.* 20:284-299.
11. Tsukamura, M. 1966. Adansonian classification of mycobacteria. *J. Gen. Microbiol.* 45:253-273.
12. Tsukamura, M. 1967. Identification of mycobacteria. *Tubercle* 48:311-338.
13. Tsukamura, M. 1969. Identification of Group II scotochromogens and Group III nonphotochromogens of mycobacteria. *Tubercle* 50:51-60.
14. Tsukamura, M. 1969. Numerical taxonomy of the genus *Nocardia*. *J. Gen. Microbiol.* 56:265-287.
15. Tsukamura, M. 1970. Relationship between *Mycobacterium* and *Nocardia*. *Jpn. J. Microbiol.* 14:187-195.
16. Tsukamura, M. 1971. Proposal of a new genus, *Gordona*, for slightly acid-fast organisms occurring in sputa of patients with pulmonary disease and in soil. *J. Gen. Microbiol.* 68:15-26.
17. Tsukamura, M. 1973. A taxonomic study of strains received as "*Mycobacterium*" *rhodochrous*. Description of *Gordona rhodochroa* (Zopf; Overbeck; Gordon et Mihm) Tsukamura *comb. nov.* *Jpn. J. Microbiol.* 17:189-197.
18. Tsukamura, M. 1974. A further numerical taxonomic study of the Rhodochrous group. *Jpn. J. Microbiol.* 18:37-44.
19. Tsukamura, M. 1974. Differentiation of the '*Mycobacterium*' *rhodochrous*-group from nocardiae by β -galactosidase activity. *J. Gen. Microbiol.* 80:553-555.
20. Tsukamura, M., and S. Mizuno. 1971. Differentiation of *Mycobacterium avium* and *Mycobacterium intracellulare* by utilization of butanols as carbon source. *Kekkaku* 46:197-202.
21. Tsukamura, M., S. Mizuno, and H. Murata. 1975. Numerical taxonomy study of the taxonomic position of *Nocardia rubra* reclassified as *Gordona lentifragmenta* Tsukamura *nom. nov.* *Int. J. Syst. Bacteriol.* 25:377-382.
22. Tsukamura, M., S. Mizuno, H. Murata, and T. Oshima. 1974. Acid phosphatase activity of mycobacteria. *Iryo* 28:11-14.
23. Wayne, L. G., J. R. Doubek, and R. L. Russell. 1964. Classification and identification of mycobacteria. I. Tests employing Tween 80 as substrate. *Am. Rev. Resp. Dis.* 90:588-597.