Numerical Analysis of Relationships among Rapidly Growing, Scotochromogenic Mycobacteria

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SUMMARY

Numerical analysis of the relationships among rapidly growing, scotochromogenic mycobacteria was carried out; a dendrogram was prepared by a single linkage method and the separation between species was tested. The following were distinct species: *Mycobacterium aichiense*; *Mycobacterium rhodesiae*; *Mycobacterium chubuense*; *Mycobacterium obuense*; *Mycobacterium gilvum*; *Mycobacterium duvalii*; *Mycobacterium flavescens*; and *Mycobacterium vaccae*. *Mycobacterium tokaiense* was reduced to a synonym of *M. chubuense*. *Mycobacterium parafortuitum*, *Mycobacterium aurum* and *Mycobacterium neoaurum* were considered to be subspecies of *M. parafortuitum* or part of a *M. parafortuitum* complex.

The separation between species was tested by comparing the extent of overlap of one species with that of another. When two species showed no such overlap, they were regarded as distinct. The results of the test of separation agreed well with taxonomic relationships shown by the dendrogram. Characters useful for differentiating the species were found.

INTRODUCTION

Mycobacterium phlei Lehmann & Neumann 1899 was the first species of rapidly growing scotochromogenic mycobacteria to be described. This organism and *Mycobacterium thermoresistibile* Tsukamura 1966 are the only two rapidly growing mycobacteria that are able to grow at 52 °C. Hence, differentiation of these from other mycobacteria is easy (Tsukamura, 1971) and they were not included in this study. Rapidly growing, scotochromogenic mycobacteria recently described include *Mycobacterium flavescens* Bojalil, Cerbon & Trujillo 1962, with *Mycobacterium acapulcensis* Bojalil *et al.* 1962 being regarded as a synonym of *M. flavescens* (Tsukamura, 1968), *Mycobacterium vaccae* Bönicke & Juhasz 1964, *Mycobacterium parafortuitum* Tsukamura, Toyama & Mizuno 1965, *Mycobacterium aurum* Tsukamura & Tsukamura 1966, *Mycobacterium obuense* Tsukamura & Mizuno 1971, *Mycobacterium rhodesiae* Tsukamura, Mizuno, Gane, Mills & King 1971, *Mycobacterium gilvum* Stanford & Gunthorpe 1971, *Mycobacterium duvalii* Stanford & Gunthorpe 1971, *Mycobacterium neoaurum* Tsukamura 1972, *Mycobacterium aichiense* Tsukamura 1973, *Mycobacterium chubuense* Tsukamura 1973, and *Mycobacterium tokaiense* Tsukamura 1973. All grow rapidly and form yellow- or orange-pigmented, wet and smooth colonies, except for *M. flavescens* which usually forms rough colonies and *M. parafortuitum* which sometimes forms only slightly pigmented, mucoid colonies.

The purposes of this study were to clarify the taxonomic relationships among these species by numerical analysis and to report the characteristics of *M. aichiense*, *M. chubuense* and *M. neoaurum* which have only been described in Japanese. A comparison of the

Table 1. *Strains of rapidly growing, scotochromogenic mycobacteria*

Species	Abbreviation	Strain numbers	Recognized strains and their code numbers†
<i>M. flavescens</i>	FL	01-10	01, ATCCI4474*; 09, ATCCI4473
<i>M. vaccae</i>	VA	01-12	01, ATCCI5483*; 02, ATCCI3034; 03, ATCC23002; 04, ATCC23004; 05, Bönicke2746; 06, Bönicke2722; 07, Bönicke2742-6; 08, Bönicke2743-4; 09, Bönicke2744-6; 10, Bönicke2747; 11, ATCC23014; 12, ATCC23027
<i>M. parafortuitum</i>	PA	01-06	02, ATCCI9688; 05, ATCCI9687 = NCTC10410; 06, ATCC19686 = NCTC10411*
<i>M. aurum</i> (type A)	AA	01-06	04, ATCC25790 = NCTC10440*; 05, ATCC25801; 06, ATCC25802
<i>M. aurum</i> (type B)	AB	01-12	03, ATCC27277
<i>M. chubuense</i>	CH	01-15	13, ATCC27278 = NCTC10819*; 15, ATCC27279
<i>M. obuense</i>	OB	01-05	05, ATCC27023 = NCTC10778*
<i>M. rhodesiae</i>	RH	01-16	06, ATCC27024*
<i>M. gilvum</i>	GI	01-05	01, Stanford392; 02, Stanford35 = NCTC10742*; 03, Stanford132; 04, Stanford205; 05, Stanford391
<i>M. duvalii</i>	DU	01-04	01, Stanford51 = NCTC8645; 02, Stanford70 = NCTC358*; 03, Stanford579 = NCTC509; 04, Stanford580 = NCTC514
<i>M. neoaurum</i>	NE	01-19	01, ATCC25796; 15, ATCC25791; 16, ATCC25795 = NCTC10818*; 17, ATCC25799; 18, ATCC25800; 19, ATCC25798
<i>M. aichiense</i>	AI	01-08	02, ATCC27281; 06, ATCC27280 = NCTC10820*
<i>M. tokaiense</i>	TO	01-03	01, ATCC27282 = NCTC10821*

* Type (paratype or co-type).

† ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; NCTC, National Collection of Type Cultures, London; Bönicke, Dr R. Bönicke, Forschungsinstitut Borstel, Borstel, West Germany; Stanford, Dr J. L. Stanford, Middlesex Hospital Medical School, London. Other numbers are laboratory codes.

classification by cluster analysis and by the 'separation between species' test (Tsukamura, 1976) was also attempted.

METHODS

Strains. The original designations and sources of the 121 strains of rapidly growing, scotochromogenic mycobacteria which were studied are given in Table 1.

Characters. A total of 80 characters were tested in all the strains. Of the tests previously described (Tsukamura, 1967), tests 59 to 68, 76 to 87, 92 and 93 were omitted and the following nine tests added: (1) α -esterase; (2) β -esterase; (3) resistance to 5 μ g ethambutol per ml Ogawa egg medium; (4, 5) tolerance to 0.1 % and 0.2 % (w/v) NaNO₂ in Sauton agar; (6) tolerance to 1 % (v/v) Tween 80 in Sauton agar; (7) cord formation; (8) utilization of raffinose as sole carbon source; (9) utilization of nicotinamide as dual nitrogen and carbon sources. The methods used were described previously (Tsukamura, 1967, 1969). The esterase activities were tested according to a modification (Tsukamura, Mizuno & Murata, 1972) of the method of Kppler (1965). The characters were tested three times in each strain; if the results of all three replicates were not the same, the results shown by two of three were recorded.

Numerical analysis. Matching coefficients (M values) were calculated using the following equation:

$$M (\%) = [n_s / (n_s + n_d)] \times 100$$

in which n_s is the number of characters in which two test strains show similar code symbols and n_d is the number of characters in which two strains show different code symbols. The M values among the strains were calculated using a NEAC 2200 computer (Nippon

Electric Company, Tokyo, Japan). Clustering was achieved by a single linkage method (Sokal & Sneath, 1963).

The method described by Tsukamura (1976) was used to test whether or not the species formed by cluster analysis were clearly separable from each other. A 'hypothetical median organism' (HMO) (Liston, Wiebe & Colwell, 1963) was prepared for each species, and matching M values were estimated for the strains of each species to the HMO of their own species and to the HMO patterns of other species. The mean of M values ($\bar{M}$) and standard deviation (s) were calculated for the strains of every species to all HMO patterns. The range of a species was expressed as $(\bar{M} \pm 2s)$.

RESULTS

Cluster analysis

In the dendrogram prepared by analysis of 80 characters, *M. aichiense* (2), *M. rhodesiae* (3), *M. chubuense* (4), *M. obuense* (5), *M. gilvum* (6), *M. duvalii* (7), *M. flavescens* (8) and *M. vaccae* (9) appear as separate clusters (Fig. 1). Most of the clusters were separated at the 92 % similarity level, and the clusters of *M. aichiense* and *M. rhodesiae* at the 94 % level. *Mycobacterium tokaiense* was included in the cluster of *M. chubuense*. A strain of *M. gilvum* (G104) was placed in the cluster of *M. aichiense*. *Mycobacterium parafortuitum*, *M. aurum* (both types) and *M. neoaurum* were combined into one cluster at the 92 % level. Within this cluster, 11 strains of *M. neoaurum* formed a subcluster. All strains of *M. parafortuitum*, except for one, were placed outside the cluster consisting of *M. aurum* and *M. neoaurum*. Two types of *M. aurum* which differed in their reactions in the nitrate reduction test were not separated in the dendrogram.

Separation between species by a statistical method

The separation of species was tested by comparing the ranges ($\bar{M} \pm 2s$) of various species with each other. Mean M values of members of various species and their standard deviations are shown in Table 2. The range of *M. aurum* (type A) overlapped with the range of *M. aurum* (type B), *M. neoaurum* and *M. parafortuitum* but was separated from all others. *Mycobacterium aurum* (type B) was not separated from *M. aurum* (type A), *M. neoaurum* and *M. parafortuitum*. *Mycobacterium neoaurum* was not separated from *M. aurum* (both types), but it was separated from all other species including *M. parafortuitum*. *Mycobacterium parafortuitum* was not separated from *M. aurum* (both types), *M. neoaurum* and *M. rhodesiae*, but it was separated from all others. *Mycobacterium aichiense* was not separated from *M. gilvum*. *Mycobacterium chubuense* was not separated from *M. tokaiense*. *Mycobacterium tokaiense* was not separated from *M. chubuense*. *Mycobacterium gilvum* was not separated from *M. aichiense*, *M. chubuense*, *M. tokaiense*, *M. duvalii* and *M. flavescens*. *Mycobacterium obuense*, *M. rhodesiae*, *M. duvalii*, *M. vaccae* and *M. flavescens* were separated from all other species.

Characters useful for distinguishing the species

Characters useful for differentiating the species are shown in Table 3.

The characters common to all species are: Gram-positive; acid-fast; growth at 28 and 37 °C; occur as rods, short rods and coccoids; form yellow or orange scotochromogenic colonies (*M. parafortuitum* shows pale yellow rather than the typical yellow colonies); growth within 3 days; tolerant to 0.2 % picric acid or 0.1 % NaNO₂ in Sauton agar, 1 % Tween 80 in Sauton agar, 0.05 % sodium salicylate in Ogawa egg medium, and 10 µg thiophene-2-carboxylic acid hydrazide per ml Ogawa egg medium; utilize acetate, pyruvate,

Table 2. Mean ($\bar{M}$) and standard deviation (s) of M values to their own HMO and other HMO patterns of various rapidly growing, scotochromogenic mycobacteria

Mean M values to own HMO and other HMO patterns

HMO of:	<i>M. aurum</i> (A)	<i>M. aurum</i> (B)	<i>M. neoaurum</i>	<i>M. parafortuitum</i>	<i>M. aitchiense</i>	<i>M. rhodesiae</i>	<i>M. chubuense</i>	<i>M. tokatense</i>	<i>M. obuense</i>	<i>M. gilvum</i>	<i>M. duvalii</i>	<i>M. flavescens</i>	<i>M. vaccae</i>
	n = 6	n = 12	n = 19	n = 9	n = 8	n = 16	n = 15	n = 3	n = 5	n = 5	n = 4	n = 10	n = 12
<i>M. aurum</i> (A)	95.5 ± 2.9	92.1 ± 3.4*	90.8 ± 2.3*	87.0 ± 2.7*	78.0 ± 1.6	83.7 ± 1.7	78.1 ± 1.6	78.7 ± 4.0	84.0 ± 2.7	78.5 ± 1.8	75.3 ± 1.0	69.5 ± 2.1	79.6 ± 3.0
<i>M. aurum</i> (B)	93.0 ± 3.0*	95.3 ± 2.2	88.7 ± 2.6*	87.7 ± 2.1*	80.4 ± 1.6	84.1 ± 1.5	80.5 ± 1.5	78.7 ± 4.0	81.4 ± 2.5	78.6 ± 1.8	75.8 ± 1.0	71.5 ± 1.9	80.5 ± 2.8
<i>M. neoaurum</i>	90.3 ± 2.8*	87.3 ± 2.8*	95.8 ± 1.8	84.2 ± 2.8	76.3 ± 1.9	81.3 ± 1.9	78.7 ± 1.6	79.7 ± 4.0	84.6 ± 1.3	76.4 ± 2.5	76.3 ± 1.5	68.1 ± 2.4	77.4 ± 2.1
<i>M. parafortuitum</i>	87.5 ± 1.6*	88.7 ± 1.8*	87.6 ± 2.4*	93.3 ± 2.6	82.8 ± 1.2	87.1 ± 2.2*	83.3 ± 1.4	80.3 ± 2.5	84.4 ± 0.9	79.6 ± 2.9	80.3 ± 1.0	74.2 ± 2.4	79.2 ± 2.4
<i>M. aitchiense</i>	80.7 ± 2.8	81.7 ± 2.8	77.3 ± 1.8	81.5 ± 3.2	97.0 ± 2.1	86.4 ± 1.5	85.1 ± 1.2	87.0 ± 1.7	78.4 ± 2.3	84.8 ± 5.5*	88.0 ± 1.4	85.5 ± 1.6	78.9 ± 2.5
<i>M. rhodesiae</i>	85.0 ± 1.8	84.5 ± 2.3	82.3 ± 2.5	87.5 ± 2.9	87.6 ± 1.5	97.0 ± 1.6	84.8 ± 1.6	82.0 ± 1.7	80.4 ± 0.9	81.4 ± 3.9	77.5 ± 1.0	74.8 ± 0.8	76.9 ± 2.2
<i>M. chubuense</i>	79.2 ± 1.5	81.1 ± 1.4	79.9 ± 2.1	83.3 ± 1.2	84.5 ± 1.2	83.6 ± 1.5	97.2 ± 2.1	90.3 ± 2.5*	83.6 ± 0.9	84.0 ± 1.9	85.8 ± 0.5	78.9 ± 1.8	76.2 ± 2.7
<i>M. tokatense</i>	79.0 ± 4.2	80.7 ± 3.1	79.7 ± 2.3	79.7 ± 2.0	88.1 ± 1.4	83.8 ± 1.3	90.1 ± 2.4*	97.0 ± 1.7	82.0 ± 1.4	80.8 ± 3.6	80.8 ± 0.5	77.9 ± 1.7	74.4 ± 2.5
<i>M. obuense</i>	84.8 ± 1.2	83.5 ± 2.0	85.5 ± 2.7	83.2 ± 2.8	77.0 ± 1.4	79.6 ± 1.9	82.0 ± 2.3	83.7 ± 4.0	97.6 ± 2.2	78.6 ± 1.8	78.0 ± 1.4	69.2 ± 2.4	73.4 ± 1.6
<i>M. gilvum</i>	80.8 ± 1.8	80.2 ± 2.3	77.1 ± 1.8	80.7 ± 1.5	84.3 ± 1.0*	80.6 ± 1.4	85.0 ± 2.3*	83.7 ± 4.0*	79.8 ± 1.1	94.6 ± 5.3	88.8 ± 0.5*	81.7 ± 1.7*	77.7 ± 1.9
<i>M. duvalii</i>	76.2 ± 1.6	76.6 ± 1.9	77.2 ± 2.0	79.8 ± 1.0	84.9 ± 1.6	76.5 ± 1.8	85.0 ± 2.7	81.0 ± 1.7	78.4 ± 2.3	88.2 ± 1.3	99.3 ± 0.5	84.2 ± 2.4	74.7 ± 2.2
<i>M. flavescens</i>	72.5 ± 3.3	74.0 ± 2.9	69.6 ± 2.1	75.0 ± 1.7	84.8 ± 1.0	75.3 ± 1.3	79.0 ± 1.5	78.7 ± 2.5	70.0 ± 2.7	81.4 ± 1.5	83.8 ± 1.0	97.6 ± 1.9	73.0 ± 2.3
<i>M. vaccae</i>	81.5 ± 2.2	79.9 ± 3.5	79.1 ± 2.3	82.0 ± 2.5	80.4 ± 1.6	78.8 ± 2.1	77.3 ± 1.9	77.7 ± 2.5	76.0 ± 2.5	74.4 ± 2.3	75.8 ± 0.5	71.5 ± 1.9	94.8 ± 3.2

n, Number of strains. Bold numbers show the mean M value to its own HMO.

* The range of ($\bar{M} \pm 2s$) of these species overlaps with the range of the species in the first column.

Table 3. Comparison of characters among species of rapidly growing, scotochromogenic mycobacteria

Character	Percentage of strains showing positive character												
	<i>M. aurum</i> (A)	<i>M. aurum</i> (B)	<i>M. neoaurum</i>	<i>M. parafortuitum</i>	<i>M. aichiense</i>	<i>M. chubuense</i>	<i>M. tokaiense</i>	<i>M. duvalii</i>	<i>M. flavescens</i>	<i>M. gilvum</i>	<i>M. obuense</i>	<i>M. rhodesiae</i>	<i>M. vaccae</i>
No. of strains ...	6	12	19	6	8	15	3	4	10	5	5	16	12
Nitrate reduction	0	100	5	67	0	100	0	100	100	80	0	0	0
Arylsulphatase (3 day)	0	59	0	50	100	7	0	0	0	0	0	100	67
Arylsulphatase (2 week)	100	100	21	100	100	100	100	0	100	50	100	100	100
α -Esterase	0	0	21	0	0	0	0	100	100	40	0	0	0
β -Esterase	100	100	100	100	50	0	33	100	100	100	100	0	100
Acetamidase	100	75	90	83	0	0	0	0	0	0	0	0	83
Benzamidase	0	8	0	0	0	0	0	0	0	0	0	0	75
Isonicotinamidase	0	0	0	0	0	0	0	0	0	0	0	0	92
Nicotinamidase	83	67	100	100	0	73	33	100	20*	80	100	0	92
Pyrazinamidase	83	67	100	100	0	87	33	100	20*	100	100	100	92
Allantoinase	83	67	74	17	0	0	33	0	0	60	100	0	100
PAS degradation†	0	0	0	0	0	0	0	0	0	0	100	0	0
Sole source of carbon:													
Galactose	17	25	53	0	0	0	0	0	0	0	100	0	8
Arabinose	67	92	100	67	0	0	100	0	0	0	100	0	8
Xylose	67	92	100	100	0	93	100	0	0	0	80	100	0
Rhamnose	0	0	84	17	0	87	100	0	0	0	0	0	100
Trehalose	50	25	79	50	0	13	0	75	10	0	100	0	8
Inositol	100	100	100	67	38	13	67	0	0	100	60	100	0
Sorbitol	0	0	0	0	0	93	100	0	100	0	100	0	0
Malate	100	100	100	100	100	100	100	100	0	20	100	100	100
Malonate	0	0	0	0	0	0	67	0	0	0	0	0	0
Fumarate	100	100	100	100	100	100	100	100	0	20	100	100	83
Citrate	0	0	0	33	0	0	0	0	0	0	0	100	8
Propylene glycol	100	100	95	100	38	100	100	25	0	80	100	100	100
Sucrose	0	17	5	33	13	100	100	100	0	80	100	0	83
Sole sources of carbon and nitrogen:													
Serine	0	0	0	0	0	53	0	0	0	0	0	0	0
Glucosamine	83	100	100	100	75	100	67	100	0	100	100	100	0
Acetamide	67	50	95	0	0	0	0	0	0	0	0	0	0
Monoethanolamine	100	100	68	0	0	0	0	0	0	0	60	0	0
Trimethylenediamine	100	100	100	100	0	100	0	0	0	60	100	100	0
Resistance to													
NH ₂ OH, HCl:													
0.125 mg ml ⁻¹	100	100	100	100	38	20	0	0	100	0	20	100	100
0.25 mg ml ⁻¹	100	100	69	33	13	0	0	0	0	0	0	44	100
0.5 mg ml ⁻¹	50	67	5	0	0	0	0	0	0	0	0	25	100
Resistance to													
ethambutol (5 μ g ml ⁻¹)	100	100	47	0	13	13	0	0	0	0	100	100	92

* The eight strains isolated in Japan showed a negative reaction.

† *p*-Aminosalicylate degradation.

glycerol, glucose, fructose, mannose and mannitol as the sole carbon source in the presence of NH_4^+ ; utilize glutamate as dual nitrogen and carbon sources; urease-positive; no growth at 45 and 52 °C (50 % of the strains of *M. parafortuitum* grow at 45 °C when isolated, but do not grow after a few transfers); do not utilize raffinose or 1,4-butylene glycol as the sole carbon source; do not utilize benzamide and nicotinamide as dual nitrogen and carbon sources; niacin is not formed.

DISCUSSION

The results of both methods of analysis suggest that *M. parafortuitum*, *M. aurum* and *M. neoaurum* may be regarded as a complex i.e. the *M. parafortuitum* complex, or as three subspecies of the species *M. parafortuitum*. *Mycobacterium chubuense* and *M. tokaiense* were not separated from each other by the test of separation and were in the same cluster in the dendrogram and hence these two should be regarded as one species, namely *M. chubuense*. The following species are considered as distinct: *M. parafortuitum*, *M. aichiense*, *M. rhodesiae*, *M. chubuense*, *M. obuense*, *M. gilvum*, *M. duvalii*, *M. flavescens* and *M. vaccae*.

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