Adansonian Classification of Mycobacteria

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SUMMARY

An Adansonian classification of mycobacteria has been done by using 94 characters. Fifty-nine strains of slowly growing mycobacteria were classified into five groups: (1) *Mycobacterium tuberculosis* and *M. bovis* (2) *M. kansasii*; (3) *M. avium*, 16 strains of non-photochromogens from human sources, 6 strains of non-photochromogenic mycobacteria from soil sources, and scotochromogens from human sources (*M. aquae*); (4) 2 strains of non-photochromogens from human sources; (5) 1 strain of non-photochromogen from human source.

The third group seemed to consist of three subgroups: (3, i) non-chromogens from soil sources; (3, ii) *Mycobacterium avium* and some non-photochromogens, which were inseparable from *M. avium*; (3, iii) some non-photochromogens from human sources resembling *M. avium* (but separable from it) and scotochromogens from human sources.

Slowly growing non-photochromogenic mycobacteria from soil sources (subgroup 3, i) were considered to form a new species, *M. terrae*. A description of this species is given.

Seventy-eight strains of rapidly growing mycobacteria were classified into seven groups: (6) 6 strains of miscellaneous species, *Mycobacterium marinum*, *M. balnei*, *M. platypoecilus*, *M. ranae* and *M. piscium*; (7) *M. thermoresistibile* (sp.nov.); (8) *M. phlei*; (9) *M. aurum* (sp.nov.); (10) *M. fortuitum* and group IV rapid growers; (11) *M. parafortuitum*; (12) *M. smegmatis*. These groups seemed to form independent species. *Mycobacterium thermoresistibile* is a new species capable of growing at 52°. *Mycobacterium aurum* is a new species consisting of rapidly growing scotochromogenic mycobacteria with urease, nicotinamidase and pyrazinamidase and some strains also with acetamidase and allantoinase.

INTRODUCTION

Adansonian classification of bacteria proposed by Sneath (1957*a, b*) was adapted successfully to mycobacteria by Bojalil & Cerbón (1961), Cerbón & Bojalil (1961) and Bojalil, Cerbón & Trujillo (1962). Although much information was obtained by their studies, the taxonomy of Runyon's group II scotochromogens and group III non-photochromogens (Runyon, 1955) was not clarified sufficiently. The purpose of the present paper is to report an Adansonian classification of slow-growing and rapid-growing mycobacteria by using an extended number of features, including a new differentiation system proposed by the present author (Tsukamura, 1965*d*). A new species of slow-growing mycobacteria and two new species of rapid-growing mycobacteria have been recognized.

METHODS

Organisms. The following strains were used: 4 strains of *Mycobacterium tuberculosis*, H37Rv, H37Ra, Aoyama-B, Frankfurt; 5 strains of *M. bovis*, Ravenel, D-4, Denken, Miwa, BCG; 3 strains of *M. kansasii*, Forbes-84*, Bostrum-D-35*, Nagai; 7 strains of *M. avium*, A71, 3717, 4110, 4121, 11755, Kirchberg, Flamingo; 19 strains of group 3 non-photochromogens isolated from human sources, N100616*, N121326*, Ueda (NJ-1), Tagami (NJ-2), Gamoh (NJ-3), Suhara (NJ-6), Hatsuno (NJ-7), Minamisawa (NJ-8), Iijima (NJ-9), Onari (NJ-10), Saito (NJ-16), Hasegawa, Ichihara, Tomida (SJ-7), Sakatani (NJ-17), Niikura, Iwai, Munaishi, Saito-T (NJ-18); 6 strains of non-photochromogenic mycobacteria from soil sources, 301, 315, 317 (NCTC 10424), 318, 361, 362; 15 strains of group 2 scotochromogens from human sources, SP-5*, SP-6*, Matsumoto (SJ-1), Ishii (SJ-2), Watanabe (SJ-3), Okubo (SJ-5), Nagashima (SJ-8), Aruga (SJ-9), Goto (SJ-13), Kubota, Nakagawa, Takahashi, Ezaki, Ito, Tominaga; *M. smegmatis*, SN1, SN2, SN3, SN4, SN5, SN6, SN7, SN8, SN9, SN10 (these ten strains were supplied by Dr R. Bönicke, Forschungsinstitut Borstel, Germany), Wa 63, Wa 290, Wa 236, Wa 237, Wa 402, R 1102, R 1103, CDC, SP-9, Trudeau (these ten strains from the collection of Dr G. P. Kubica, U.S.A., were supplied by Dr H. Saito, Hiroshima University), Denken, Kyushu, Jucho, Takeo (the last two strains were identified by Tsukamura, Toyama & Mizuno (1964)); *M. phlei*, SN101, SN102, SN103, SN104, SN105, SN106, SN107, SN108, SN109, SN110 (these ten strains were supplied by Dr R. Bönicke), 5, Wa 40, Wa 60, Wa 366, Wa 289, Trudeau, SP-11, CDC (these 8 strains were supplied by Dr H. Saito, Hiroshima University, who received them from Dr G. P. Kubica), Denken, Kyushu; *M. fortuitum*, 605*, 606*, 607*, 335, 334, 330, 313, 302, 306, 308 (the latter 7 strains were isolated and identified in this laboratory); Rapid growers (group IV) from human sources, Yamamoto, Nishiwaki, Mimura; *M. marinum*, Ross (Ross, A. J.); *M. balnei*, B916, B913 (Lausanne); *M. ranae*, L-17 (Lausanne); *M. platyopocilus*, Ross; *M. piscium*, Ross (these 6 strains were supplied by Dr K. Konno, Tohoku University); *M. parafortuitum* (Tsukamura, 1966a), 310 (NCTC 10410), 311 (NCTC 10411), 304, 305, 307, 314; *M. thermoresistibile* (sp.nov.), 316 (NCTC 10409), 1005, 1011, 1021, 1025; *M. aurum* (sp.nov.) (rapid-growing scotochromogenic mycobacteria to be presented as a new species in the present study), 358 (NCTC 10437), 312 (NCTC 10438), 303 (NCTC 10439), 309 (NCTC 10440).

Among the above, the non-photochromogens and scotochromogens from human sources, except for the strains with a symbol *, were isolated in Japan from 1948 to 1959 and were considered to be pathogenic for humans (Hibino, 1964). These were collections of Professor S. Hibino (Nagoya University) and were supplied by him in 1961. The strains with symbol * were from the collections of Dr E. H. Runyon and were supplied through Dr K. Takeya (Kyushu University) in 1960. The *Mycobacterium avium* strains were supplied by Dr K. Konno (Tohoku University) in 1964.

The strains had been maintained on Löwenstein-Jensen medium or Ogawa egg medium and were subcultured from single colonies.

The following tests were done in triplicate and, when different results were obtained in three replicates (these occurred rarely), the feature obtained twice was recorded. Data were expressed as either + or -.

(1) Gram reaction (Mackie & McCartney's Handbook, 1960).

(2) Acid-fastness was tested by the Ziehl-Neelsen stain (Mackie & McCartney's *Handbook*, 1960).

(3) Colony morphology was observed on the Sauton agar medium, in which sodium glutamate replaced asparagine, and the results were recorded as 'rough' or 'smooth' (colony morphology was more clearly characterized on the Sauton agar medium than on the Löwenstein-Jensen medium).

(4) Pigmentation of colonies was observed on 2- to 4-week cultures of slow-growing mycobacteria or on 7 day cultures of rapid-growing mycobacteria, growing on the Sauton agar medium (pigmentation on the Sauton agar was more stable than that on egg media).

(5) Reaction to light. Two- to 3-week cultures of slow-growing mycobacteria or 7 day cultures of rapid-growing mycobacteria, growing on the Löwenstein-Jensen medium were exposed to light after replacing a rubber stopper by a loose cotton-wool plug, and allowed to stand at room temperature (20-37°) for 2 days. The appearance of pigmentation was read as a positive reaction to light (photosynthesis of carotenoids).

(6) Growth rate was observed on both the Löwenstein-Jensen medium and the Sauton agar medium. Abundant growth on the egg medium within 3 days or on the Sauton agar within 7 days was recorded as rapid growth, and growth on the former after more than 1 week or on the latter after more than 2 weeks was recorded as slow growth.

(7) Catalase activity was tested by immersing one loopful of the organism into a 30 % aqueous solution of H₂O₂ and observing the occurrence of bubbling.

(8) Peroxidase activity was tested according to the method described by Tiranarayanan & Vischer (1957).

(9) Nitrate reduction. Fifty mg. (moist weight) of the organism were suspended in 5 ml. 0.067 M-phosphate buffer (pH 7.1) containing 0.1 % sodium nitrate, and incubated at 37° for 15-16 hr. Formation of nitrite was examined by adding two drops of 2 % (w/v) *p*-dimethyl-aminobenzaldehyde in 10 % HCl + 1.0 ml. 10 % HCl.

(10) Three-day arylsulphatase test was done according to the method of Wayne (1961).

(11) Two-week arylsulphatase test was done according to the method of Kubica & Vestal (1961) with a final concentration of 0.001 M-tripotassium phenolphthalein disulphate.

(12) Salicylate degradation was observed as a blackening of Sauton agar medium containing 0.1 % (w/v) sodium salicylate, after incubation at 37° for 7 days (Tsukamura, 1965*a*).

(13) Sodium *p*-aminosalicylate (PAS) degradation was observed according to a method described previously (Tsukamura, 1961; Tarshis, 1964; Tsukamura, 1965*a*). Blackening of the Ogawa egg medium or Löwenstein-Jensen medium containing 0.2 % (w/v) PAS after incubation at 37° for 7 days was defined as a positive degradation.

(14) *p*-Aminobenzoate degradation was recorded as a blackening of Sauton agar medium containing 0.2 % (w/v) sodium *p*-aminobenzoate after incubation of 4 weeks (Tsukamura, unpublished test).

(15) Hydroxylamine resistance was defined as an ability of slow-growing mycobacteria to grow on the Löwenstein-Jensen medium or the Ogawa egg medium

containing 0.025 % (w/v) hydroxylamine hydrochloride after incubation for 3 weeks or an ability of rapid-growing mycobacteria to grow on the media containing 0.05 % hydroxylamine after incubation for 1 week (Tsukamura, 1965*b*).

(16) The niacin test was done according to Konno (1963).

(17) Picric acid (0.1 %, w/v) tolerance was defined as ability to grow on the Sauton agar medium containing 0.1 % (w/v) picric acid after incubation for 3 weeks (Tsukamura, 1965*c*).

(18) Picric acid (0.2 %, w/v) tolerance. Ability to grow on Sauton agar containing 0.2 % picric acid after 3 weeks.

(19) Growth at 28°.

(20) Growth at 37°.

(21) Growth at 45°.

(22) Growth at 52° was observed on the Löwenstein-Jensen medium.

(23) Survival at 60° for 1 hr was observed on the Löwenstein-Jensen medium.

(24-33) Bönicke's amidase pattern was tested according to the description of Bönicke (1962). Incubation time was 16 hr. The following amides were used: (24) acetamide; (25) benzamide; (26) urea; (27) isonicotinamide; (28) nicotinamide; (29) pyrazinamide; (30) salicylamide; (31) allantoin; (32) succinamide; (33) malonamide.

(34-41) Utilization of organic acids as sole carbon sources was tested on the following medium: $(\text{NH}_4)\text{SO}_4$, 2.64 g.; KH_2PO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; purified agar (Eiken Co., Tokyo, or Wako Chemical Co., Osaka), 20.0 g.; distilled water, 1000 ml. The medium was adjusted to pH 7.1 by adding 10 % (w/v) KOH. The medium was supplemented with organic acid Na salt to a final concentration 0.02M, and re-adjusted to pH 7.1. The medium was then sterilized by autoclaving at 115° for 30 min. and made up as slopes (sterilization by autoclaving gave the same results as sterilization by heating at 100° for 15 min.). Series of media containing different organic acids and control medium without carbon source were inoculated with one loopful of stock cultures. Growth of slow-growing mycobacteria was observed after incubation for 4 weeks, and growth of rapid-growing mycobacteria after incubation for 2 weeks. For incubation, a loop, 3.5 mm. outside diameter and 2.0 mm. inside diameter, was used throughout. After touching the loop slightly on the growth of test organisms, the inoculum was streaked on the slope of fresh media. Care was taken not to bring any visible bacterial mass on the fresh media. This method of inoculation gave nearly similar results as a semi-quantitative method in which one loopful of 3-day cultures of rapidly growing mycobacteria or 10-day cultures of slow-growing mycobacteria growing in the Dubos liquid medium were used for inoculation. The following organic acids were used for tests: (34) acetate; (35) citrate; (36) succinate; (37) malate; (38) pyruvate; (39) benzoate; (40) malonate; (41) fumarate.

(42-54) Acid from carbohydrates. This was observed on the following medium: $(\text{NH}_4)_2\text{SO}_4$, 2.64 g.; KH_2PO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; yeast extract, 0.2 g.; 0.2 % (w/v) bromthymol-blue in 0.02 M-NaOH, 20 ml.; purified agar, 20.0 g.; distilled water, 980 ml. The medium was adjusted to pH 7.0 and sterilized by autoclaving at 115° for 30 min. Carbohydrates were sterilized separately by heating in solution at 100° for 15 min. and added to the above medium aseptically to a final concentration of 0.5 % (w/v). When necessary the medium was re-adjusted

to pH 7.0 aseptically and poured into tubes. Slopes were inoculated with one loopful of the stock cultures and incubated. Control medium without carbohydrate was always set up. Acid formation was observed after 2 weeks incubation at 28° (rapidly growing mycobacteria) or after 4 weeks incubation at 28° (slowly growing mycobacteria). The following carbohydrates were used: (42) glucose; (43) mannose; (44) galactose; (45) arabinose; (46) xylose; (47) rhamnose; (48) trehalose; (49) maltose; (50) lactose; (51) raffinose; (52) inositol; (53) mannitol; (54) sorbitol.

(55–74) Utilization of carbohydrates (sugars, alcohols, glycols) as sole carbon sources. This was observed by using the same medium (except that yeast extract and bromthymol-blue were omitted) as that used in the tests for acid formation. Sugars were added to a final concentration of 0.5 % (w/v), and alcohols and glycols to a final concentration of 0.1 M. Growth was observed after incubation for 2 weeks (rapidly growing mycobacteria) or after incubation for 4 weeks (slow-growing mycobacteria). The following were used: (55) glycerol; (56) glucose; (57) fructose; (58) sucrose; (59) mannose; (60) galactose; (61) arabinose; (62) xylose; (63) rhamnose; (64) trehalose; (65) raffinose; (66) inositol; (67) mannitol; (68) sorbitol; (69) ethanol; (70) propanol; (71) propyleneglycol; (72) 1,3-butyleneglycol; (73) 1,4-butyleneglycol; (74) 2,3-butyleneglycol.

(75–86) Utilization for growth of nitrogen compounds as sole nitrogen sources (a selected series from Tsukamura, 1965*d*), where this method was shown to be useful. The tests were carried out in the following medium: glycerol, 30 ml.; KH_2PO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; sodium citrate, 1.0 g.; purified agar, 20.0 g.; distilled water, 970 ml. Nitrogen compounds were added to the above medium to a final concentration of 0.02 M, and the medium was adjusted to pH 7.0. The medium was sterilized by autoclaving at 115° for 30 min. and sloped. Only nitrite medium was sterilized by heating at 100° for 5 min. or else nitrite solution was sterilized by Seitz-filtration and added to medium aseptically. The medium, together with control medium without nitrogen source, was inoculated with one loopful of the stock cultures and incubated. Growth was scored in comparison with growth on the control medium after incubation for 2 weeks (rapid-growing mycobacteria) or after incubation for 4 weeks (slow-growing mycobacteria). The following were used for the tests: (75) sodium L-glutamate; (76) L-serine; (77) L-methionine; (78) acetamide; (79) benzamide; (80) urea; (81) pyrazinamide; (82) isonicotinamide; (83) nicotinamide; (84) succinamide; (85) NaNO_3 ; (86) NaNO_2 (Tsukamura & Tsukamura, 1965).

(87–94) Utilization for growth of nitrogen compounds as sole nitrogen and carbon sources. This was shown recently to be very useful for classification of rapidly growing mycobacteria (Tsukamura, 1965*d*). The tests were carried out on the following medium: KH_2PO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; purified agar, 20.0 g.; distilled water, 1000 ml. The medium was supplemented with a nitrogen compound to a final concentration of 0.02 M, and the medium was adjusted to pH 7.0 and sterilized by autoclaving at 115° for 30 min. After sloping, the medium was inoculated with one loopful of the stock cultures and incubated. Growth was read after incubation for 2 weeks (rapid-growing mycobacteria) or after incubation for 4 weeks (slow-growing mycobacteria). The results were insensitive to inoculum size and the readings were clear. The following compounds were used: (87) sodium L-glutamate; (88) L-serine; (89) glucosamine-HCl; (90) acetamide; (91) benzamide; (92) nicotinamide; (93) monoethanolamine (0.1 M); (94) trimethylene diamine (0.1 M).

Incubation was at 37° unless otherwise noted. As the source of inoculation for tests 3- to 4-week-old cultures of slow-growing mycobacteria or 1-week-old cultures of rapid-growing mycobacteria grown on Löwenstein-Jensen medium were used.

Similarity value. Similarity value (S-value) was expressed as a percentage = {(the number of similar features)/(the number of characters tested)} × 100. A set of cards were prepared, in which the name of 94 characters (94 tests) each bearing symbols + and - were printed. Results obtained on each strain were recorded on a card by checking the symbol + or - with a circle. For comparison between two strains, two cards were overlapped and the number of the same features (or different features) was read by bringing a light behind the cards. If two strains gave the same features (positive or negative results in both strains), one could see only one circle at a given test (character). On the other hand, if two strains gave different features, one could see two circles at a given test. Thus, the number of similar features (or different characters) was counted easily by counting the number of single circles (or the number of pairs of circles).

When this work was done, the number of different (or similar) features was counted by eye. However, if the features are recorded in thick, non-transparent, hard cards or tapes by punching the circles, it would not be difficult to prepare photoelectric equipment suitable for counting the number of similar features (two similar features permit the passage of light).

RESULTS AND DISCUSSION

Adansonian classification of slowly growing mycobacteria.

Grouping of slowly growing mycobacteria

The results are summarized in Fig. 1, which is a diagrammatic representation of the S-value table, prepared by shading squares according to the S-values between the strains of slow-growing mycobacteria. Figure 1 shows the results of 55 strains. Since strains H37Rv and Aoyama-B gave the same results as the H37Ra and Frankfurt strains, and strains Ravenel and D-4 gave the same results as the Denken and BCG strains, these latter four strains were omitted from Fig. 1. The S-values ranged from 72 to 100 %. These relatively high S-values seem to be due to many characters in which all the slow-growing mycobacteria gave negative results. These characters were included as they were useful for differentiation from rapid-growing mycobacteria. Slow-growing mycobacteria were classified into five groups:

Group 1 consisted of *Mycobacterium tuberculosis* and *M. bovis*.

Group 2 of *M. kansasii*.

Group 3 of *M. avium*, most non-photochromogens and scotochromogens.

Group 4 of the non-photochromogens N100616 and N121326.

Group 5 of the non-photochromogen Tomida. The last strain was slightly coloured, like *M. phlei*.

Group 3 could be divided into three subgroups: (3, i) non-photochromogenic mycobacteria from soil sources (6 strains); (3, ii) *Mycobacterium avium* (7 strains) and non-photochromogens from human sources inseparable from *M. avium* (8 strains); (3, iii) non-photochromogens from human sources resembling *M. avium* but separable from it (8 strains) and scotochromogens from human sources (15 strains). In Fig. 1 (3, a), (3, b + 3, c) and 3, d + 3, e) correspond to these three groups, respectively.

The subgroup 3, i (soil isolates) seemed to be a new species and is named *Mycobacterium terrae*. The subgroup 3, iii may be identified with *M. aquae*, since the scotochromogens from human sources had been named *M. aquae* by Bönicke (1962).

Fig. 1. Diagrammatic representation of the S-value table of slow growing mycobacteria, prepared by shading the squares according to the S-values between the strains. The order of strains given by the sorting procedure was as follows (from left to right or from top to bottom): Group 1: *Mycobacterium tuberculosis*, H37 Rv, Aoyama-B, and *M. bovis*, Ravenel, D-4, Miwa; Group 2: *M. kansasii*, Forbes-84, Bostrum-D-35, Nagai; Group 3: (3, a) non-photochromogens from soil sources, (*M. terrae*) 301, 315, 317, 318, 361, 362; (3, b) *M. avium*, A71, 3717, 4110, 4121, 11755, Kirchberg, Flamingo; (3, c) non-photochromogens from human sources, Hatsuno, Minamisawa, Onari, Saito, Sakatani, Hasegawa, Ichihara, Niikura; (3, d) non-photochromogens from human sources, Iwai, Gamoh, Iijima, Saito-T, Munaishi, Ueda, Tagami, Suhara; (3, e) scotochromogens from human sources, (*M. aquae*) Ishii, Nagashima, Kubota, Nakagawa, Okubo, Aruga, Goto, Takahashi, Ezaki, Watanabe, Ito, Matsumoto, Tominaga, SP-5, SP-6; Group 4: non-photochromogens from human sources, N100616, N121326; Group 5: non-photochromogen from human source, Tomida.

Tests for the homogeneity of a group

Intermediate types would be omitted from a group by a diagrammatic representation of the S-values, but it is desirable to confirm 'homogeneity' of a group by a statistical method. If a group contained any strain which was markedly aberrant, that strain must have low S-values to other strains of the group. If the S-values are significantly low, the mean of the S-values for such a strain would be significantly lower than the mean of the S-values for all strains of the group. This comparison is done by the 't'-test.

Since groups 1, 2, 4 and 5 are apparently homogenous (S-values 97–100 %), the test for homogeneity was made on the subgroups of group 3. Examples of these tests are shown in Tables 1–3. These subgroups shown in the tables correspond to the groups 3, a, b, c, d, and e in Fig. 1 and do not always correspond to the taxonomic subgroups obtained by the Adansonian classification. An example on a taxonomic group will be shown later in the section on rapidly growing mycobacteria.

Table 1 shows the homogeneity test on the non-photochromogenic mycobacteria from soil, *Mycobacterium terrae*. If the 6 strains of this group were equally similar to each other for every strain, the mean S-values between that strain and the rest of the group should not differ significantly from the mean of all the S-values between the members of the group. However, when the mean for the whole group was compared with the mean for each strain by the 't'-test, the mean for strain 361 differed significantly from the mean for the group ($P = 0.05$).

Mean for the group = 94.2 S.D. = 1.41 % ($n_1 = 15$). Mean for strain 361 = 92.0 S.D. = 2.00 % ($n_2 = 5$). d (difference) = 94.2–92.0 = 2.2

$$s^2 = \frac{(n_1 - 1)(s_1)^2 + (n_2 - 1)(s_2)^2}{n_1 + n_2 - 2} = \frac{(15 - 1)(1.41)^2 + (5 - 1)(2.00)^2}{15 + 5 - 2},$$

$$= 2.44$$

$$s = 1.56,$$

where s_1 and s_2 are the standard deviations for the group and for the strain.

$$d \pm t_{n_1+n_2-2, 0.05} s \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} = 2.2 \pm 2.10 \times 1.56 \times \sqrt{\left(\frac{1}{15} + \frac{1}{5}\right)}$$

$$= 2.2 \pm 1.30 \text{ which is } > 0$$

The difference d is therefore significant at a level of $P = 0.05$ (Bennett & Franklin, 1956).

Table 1. *Test for homogeneity of strains of Mycobacterium terrae (subgroup 3, a)*

Strain	Mean S-value for strain ($n = 5$)*	Mean S-value for strain ($n = 4$)†
301	94.0 ± 2.40	95.0 ± 1.83
315	94.0 ± 0.87	94.0 ± 1.00
317	95.0 ± 3.04	96.0 ± 2.22
318	95.0 ± 2.82	97.5 ± 2.77
361	92.0 ± 2.00‡	
362	95.0 ± 0.71	94.5 ± 0.52
	Mean S-value for group ($n = 15$)§	Mean S-value for group ($n = 10$)
	94.2 ± 1.41	95.0 ± 1.71

* Mean of the S-values to other five strains.

† Mean of the S-values to other four strains after strain 361 was omitted.

‡ A significant difference between the mean S-value for this strain and the mean S-value for the group ($P = 0.05$).

§ The number of S-values in the group, (5 values) × (6 strains) = 30. This was divided by 2, as one half of values are a reflected figure. The number of 'n' in the group was calculated similarly also in other cases.

The other strains showed no significant difference from the mean for the group (Table 1). Thus, it was considered that strain 361 should be omitted from the group. After omitting strain 361, mean S-values for the remaining strains were calculated afresh and the same test was carried out. This time, there was no significant difference between the mean for the group and each mean for strains (Table 1). The five strains, 301, 315, 317, 318 and 362 were considered to form a 'homogeneous' group.

Table 2. *Test for homogeneity of strains of Mycobacterium avium (subgroup 3, b)*

Strain	Mean S-value for strain (<i>n</i> = 6)*	Strain	Mean S-value for strain (<i>n</i> = 6)*
A 71	96.0 ± 1.17	11755	96.0 ± 1.34
3717	96.2 ± 1.34	Kirchberg	96.6 ± 1.48
4110	96.2 ± 1.42	Flamingo	95.0 ± 0.78
4121	97.1 ± 0.78		

Mean S-value for group (*n* = 21): 96.2 ± 1.21

* Mean of S-values to other six strains.

No significant difference between the mean for each strain and the mean for group.

Table 3. *Test for homogeneity of strains of the non-photochromogens inseparable from Mycobacterium avium (subgroup 3, c)*

Strain	Mean S-value for strain (<i>n</i> = 7)*	Strain	Mean S-value for strain (<i>n</i> = 7)*
Hatsuno	96.5 ± 1.91	Sakatani	97.0 ± 1.29
Minamisawa	95.0 ± 1.08	Hasegawa	96.0 ± 1.58
Onari	95.0 ± 1.64	Ichihara	94.6 ± 1.15
Saito	96.5 ± 1.29	Niikura	96.4 ± 2.00

Mean S-value for group (*n* = 28): 96.0 ± 1.64

No significant difference between the mean for each strain and the mean for group.

* Mean of S-values to other seven strains.

Table 4. *Test for homogeneity of strains of the non-photochromogens separable from Mycobacterium avium (subgroup 3, d)*

Strain	Mean S-value for strain (<i>n</i> = 7)*	Strain	Mean S-value for strain (<i>n</i> = 7)*
Gamoh	91.9 ± 4.14	Ueda	92.9 ± 9.58
Iijima	92.9 ± 3.24	Tagami	92.3 ± 2.24
Saito-T	92.9 ± 1.63	Suhara	92.2 ± 2.38
Munaishi	92.6 ± 2.45	Iwai	92.2 ± 2.45

Mean S-value for group (*n* = 28): 92.5 ± 2.59

No significant difference between the mean for each strain and the mean for group.

* Mean of S-values to other seven strains.

Similarly, 7 strains of *Mycobacterium avium* seemed to be homogeneous (Table 2). These may be considered as standard *M. avium* strains. The group of eight strains of non-photochromogens inseparable from *M. avium* was also considered to be homogeneous (Table 3). Non-photochromogens separable from *M. avium* were also homogeneous (Table 4). However, the mean value of the group was 92.5 % which was lower than the values of the other subgroups. This might indicate that this consisted of intermediate types. When the scotochromogens were subjected to the tests, strains SP-5 and Goto were first distinguished by their low S-values, and, secondly, strains SP-6 and Ezaki were omitted. The remaining eleven strains were shown to be homogeneous (Table 5).

Table 5. *Test for homogeneity of strains of Mycobacterium aquae*
(subgroup 3, e)

Strain	Mean S-value for strain ($n = 14$)*	Mean S-value for strain ($n = 12$)†	Mean S-value for strain ($n = 10$)‡
SP-5	90.0 $\pm$ 3.30§	.	.
SP-6	92.8 $\pm$ 2.64	93.2 $\pm$ 1.88§	.
Ishii	93.5 $\pm$ 2.97	94.3 $\pm$ 2.36	94.9 $\pm$ 1.38
Nagashima	94.0 $\pm$ 2.57	94.6 $\pm$ 2.13	94.9 $\pm$ 2.03
Kubota	93.5 $\pm$ 2.67	94.0 $\pm$ 2.35	94.4 $\pm$ 2.16
Nakagawa	94.0 $\pm$ 2.08	94.5 $\pm$ 1.57	94.3 $\pm$ 1.60
Okubo	95.5 $\pm$ 1.94	96.0 $\pm$ 1.62	96.2 $\pm$ 1.63
Aruga	95.6 $\pm$ 2.52	96.0 $\pm$ 2.30	96.6 $\pm$ 2.11
Goto	91.0 $\pm$ 3.09§	.	.
Takahashi	94.8 $\pm$ 2.29	96.0 $\pm$ 1.65	95.5 $\pm$ 1.38
Ezaki	92.5 $\pm$ 2.39	93.0 $\pm$ 1.63§	.
Watanabe	95.0 $\pm$ 2.42	96.0 $\pm$ 1.73	95.6 $\pm$ 1.76
Ito	93.5 $\pm$ 2.85	94.0 $\pm$ 2.09	94.7 $\pm$ 1.97
Matsumoto	95.5 $\pm$ 2.40	96.0 $\pm$ 1.76	96.5 $\pm$ 1.53
Tominaga	95.6 $\pm$ 2.64	96.0 $\pm$ 2.59	96.1 $\pm$ 2.85
Mean S-value for group	($n = 105$) 93.8 $\pm$ 2.61	($n = 78$) 95.0 $\pm$ 2.00	($n = 55$) 95.4 $\pm$ 1.99

* Mean of S-values to other 14 strains.

† Mean of S-values to other 12 strains, after strains SP-5 and Goto were omitted.

‡ Mean of S-values to other 10 strains, after strains SP-6 and Ezaki were omitted.

§ Significant difference between the mean for strain and the mean for group ($P = 0.05$).

To find the taxonomic distance from standard *Mycobacterium avium* strains, mean S-values were calculated for non-photochromogens and scotochromogens compared to the standard *M. avium* strains. These values are shown in Table 6. The eight strains of subgroup 3, c found inseparable from standard *M. avium* strains showed no significant difference from the mean S-value of *M. avium*. On the other hand, all of the other strains showed significant difference. Thus, the results of the statistical method agreed with the results of the diagrammatic representation.

Sneath's Adansonian classification (Sneath, 1957 *a, b*) made it possible to indicate the correlation among bacterial strains. In this method, grouping was made by a diagrammatic representation of the S-values. A method has been presented in this paper for testing the homogeneity of a group formed by the Adansonian method. If there is no significant difference at a level of $P = 0.05$, the group may be considered a homogeneous one. If there is a significant difference, strains with significant difference are omitted from the group, and the same test is made

Table 6. Comparison of non-photochromogens and scotochromogens with *Mycobacterium avium*

Strain	Mean S-value to <i>M. avium</i> (<i>n</i> = 7)*	Strain	Mean S-value to <i>M. avium</i> (<i>n</i> = 7)*
N 100616	87.5 ± 2.4	SP-5	84.0 ± 1.4
N 121326	87.5 ± 2.4	SP-6	87.0 ± 1.7
Gamoh	94.2 ± 1.6	Ishii	89.6 ± 1.6
Iijima	93.5 ± 2.0	Nagashima	91.5 ± 1.5
Saito-T	91.5 ± 0.8	Kubota	88.0 ± 2.1
Munaishi	93.5 ± 0.8	Nakagawa	88.6 ± 2.3
Tomida	88.0 ± 1.8	Okubo	90.5 ± 1.7
Ueda	93.5 ± 1.4	Aruga	91.6 ± 1.9
Tagami	92.0 ± 1.5	Goto	89.5 ± 2.1
Suhara	91.5 ± 1.7	Takahashi	90.8 ± 2.1
Hatsuno	95.0 ± 1.6†	Ezaki	85.6 ± 1.6
Minamisawa	95.0 ± 1.7†	Watanabe	88.7 ± 1.9
Onari	95.0 ± 2.0†	Ito	88.5 ± 2.2
Saito	95.5 ± 1.4†	Matsumoto	90.6 ± 1.8
Sakatani	95.5 ± 1.1†	Tominaga	91.5 ± 1.8
Hasegawa	95.0 ± 1.2†	301	91.7 ± 1.7
Ichihara	95.5 ± 1.6	315	88.5 ± 2.3
Niikura	95.0 ± 1.6†	317	91.5 ± 1.1
Iwai	91.7 ± 1.3	318	90.0 ± 1.1
<i>M. phlei</i> Kyu	59.8 ± 1.7	361	86.9 ± 1.8
<i>M. smegma</i> K	37.2 ± 1.9	362	87.7 ± 2.2

* Mean of S-values to 7 standard *M. avium* strains.

† No significant difference between the mean for a test strain and the mean S-value for the *M. avium* strains (96.2 ± 1.72 ; $P = 0.05$ by the 't'-test; see Table 2).

successively on the residual group. The homogeneous group is a unit of classification though it is not always identical with a species. The homogeneity test would be useful for two other purposes.

Species description. Unusual strains might often be grouped erroneously. To decrease the chance of mistakes, it is better to omit unusual strains from species descriptions. Description of the species should preferably be done on the homogeneous group and type strains should be selected from it. If one wishes to make descriptions from a small number of strains, exclusion of unusual strains is needed in order to avoid overestimation of rare characters.

Application to identification. Identification according to Adansonian classification principles would follow the following steps: (i) S-values between the test strain and type strains *a*, *b*, *c*, of various species *A*, *B*, *C*, are measured and the type strain *f* showing the highest S-value is found. The test strain most probably belongs to this species, *F*; (ii) S-values of the test strain to the other type strains are compared with those between this type strain *f* and the other type strains. The former values should not differ significantly from the latter; (iii) A homogeneity test is done, the test strain being supposed to belong to the proposed species *F*. The test strain is incorporated into a homogeneous group of standard strains of the proposed species. If the group thus formed proves to be homogeneous by the 't'-test, the test strain would belong to species *F*.

Description of species (slow-growing mycobacteria)

Biological and biochemical characters of the slow-growing mycobacterial species are shown in Table 7. In this table are shown the characters of the species which were shown to be homogeneous.

Table 7. *Characters of slowly growing mycobacteria*

Typical strain and group ...	<i>M. tuberculosis</i> H 37 Rv	<i>M. bovis</i> D-4	<i>M. kansasii</i> Forbes-84	<i>M. avium</i> 4121	<i>M. aquae</i> Matsumoto	<i>M. terrae</i> 317	Non-photochromogens	
	Gr. 1	Gr. 1	Gr. 2	Gr. 3, ii	Gr. 3, iii	Gr. 3, i	N 100616	Tomida
Characters†								
(3) Colony morphology	R	R	R	S	S*	S	S	S
(4) Pigmentation	—	—	+	—	+	—	—	±‡
(5) Light reaction	—	—	+	—	—	—	—	—
(6) Growth rate	Slow	Slow	Slow	Slow	Slow	Slow	Slow	Slow
(7) Catalase	+	+	+	+	+	+	+	+
(8) Peroxidase	+	+	—	—	—	—	—	—
(9) Nitrate reduction	—	—	+	—	—	—	—	—
(11) Two-week arylsulphatase	—	—	+	—*	+	+	+	+
(15) NH ₄ OH resistance	—	—	—	+	+	+	+	+
(16) Niacin	+	—*	—	—	—	—	—	—
(17) Picric acid resistance (0.1%)	—	—	—	—	—	+	+	+
(19) Growth at 28°	—	—	+	+	+	+	+	+
(20) Growth at 37°	+	+	—	+	—	+	+	—
(21) Growth at 45°	—	—	—	—	—*	—*	—	—
(26) Urease	+	+	+	—	—*	—	—	—
(28) Nicotinamidase	+	—	—	+	—	—	—	—
(29) Pyrazinamidase	—	—	—	+	—	—	+	—
(34) Acetate as C	—	—	—	+	—	+	+	+
(36) Succinate as C	—	—	—	—	+	+	+	—
(37) Malate as C	—	—	—	—	—	—	+	—
(38) Pyruvate as C	—	—	—	—*	+	+	+	+
(55) Glycerol as C	+	+	—	+	+	+	+	+
(56) Glucose as C	—	—	+	+	+	—	+	+
(58) Sucrose as C	—	—	+	—*	—	—	—	+
(69) Ethanol as C	—	—	—	—*	+	—	—	+
(70) Propanol as C	—	—	—	+	—*	—	—	+
(75) Glutamate as N	+	+	+	+	+	+	+	+
(76) Serine as N	—	—	+	—	+	+	+	+
(77) Methionine as N	—	—	—	—	—	—	—	+
(80) Urea as N	—	—	—	—	—	—	—	+
(81) Pyrazinamide as N	—	—	+	+	+	+	+	+
(82) Isonicotinamide as N	—	—	—	—	—	—	+	—
(83) Nicotinamide as N	—	—	—	+	+	+	+	—
(84) Succinamide as N	—	—	—	—	—	—	+	+
(85) Nitrate as N	—	—	+	+	+	+	+	+
(86) Nitrite as N	—	—	—	+	+	+	+	+
(87) Glutamate as N and C	—	—	—	—	—	+	—	+

* One or more strains gave different results

† The number shown here is equal to the number in the section 'Methods'. All strains were Gram-positive and acid-fast. Characters in which all strains gave negative results are not shown.

‡ Strain Tomida is non-photochromogenic in young cultures but orange-coloured in old cultures.

A group of non-photochromogenic mycobacteria isolated from soil are presented as a new species, *Mycobacterium terrae*, of which no previous description has been found. Differentiation of this species from other species can be done from Table 7.

Description of Mycobacterium terrae (sp.nov.):

Holotype: strain 317 (NCTC 10424).

Colonial morphology: white, wet, smooth on Löwenstein-Jensen medium and on Sauton agar. Non-photochromogenic.

Growth rate: visible growth at 7 days on Löwenstein-Jensen medium and at 7-14 days on Sauton agar. Growth after 2 weeks as single colonies on Löwenstein-Jensen medium.

Catalase positive. Peroxidase negative. Nitrate not reduced. No salicylate degradation. No PAS degradation. Grows on hydroxylamine medium.

Three-day arylsulphatase activity negative (a few strains were weakly positive);

2-week-arylsulphatase activity positive.

Tolerance to 0.1 % picric acid positive; tolerance to 0.2 % picric acid negative.

Growth temperature: growth at 28° and 37°; no growth at 45°.

Amidase activity for Bönicke's series of ten amides all negative.

Acetate and pyruvate utilized as sole carbon source; citrate, succinate, malate, benzoate, malonate, fumarate not utilized. No acid from glucose, mannose, galactose, arabinose, xylose, rhamnose, trehalose, maltose, lactose, raffinose, inositol, mannitol and sorbitol. None of the above sugars and alcohols utilized as sole carbon source. Fructose, sucrose, ethanol, propanol, propylene glycol, 1,3-butylene-glycol, 1,4-butyleneglycol, 2,3-butyleneglycol not utilized as sole carbon source.

Only L-glutamate utilized as sole simultaneous nitrogen and carbon source; L-serine, glucosamine, acetamide, benzamide, monoethanolamine and trimethylene-diamine not utilized as such source.

L-Glutamate, L-serine, urea, pyrazinamide, nicotinamide, succinamide and nitrate utilized as sole nitrogen source; L-methionine, acetamide, benzamide, isonicotinamide and nitrite not utilized as sole nitrogen source.

Differentiation of this species from *Mycobacterium avium* is done as follows:

- (1) *M. avium* is sensitive to 0.1 % picric acid, but *M. terrae* is tolerant to it; (2) *M. avium* grows at 45°, but *M. terrae* does not; (3) *M. avium* usually possesses nicotinamidase and pyrazinamidase, but *M. terrae* lacks them; (4) *M. avium* usually gives a negative 2-week arylsulphatase, but *M. terrae* gives an intensive reaction; (5) *M. avium* does not utilize L-glutamate as sole simultaneous nitrogen and carbon source, but *M. terrae* usually does.

Differentiation of this species from *Mycobacterium aquae* is done as follows:

- (1) *M. aquae* has a marked orange colour, but *M. terrae* is colourless; (2) *M. aquae* is sensitive to 0.1 % picric acid, but *M. terrae* is tolerant to it; (3) some strains of *M. aquae* give a positive urease, but *M. terrae* does not; (4) *M. aquae* does not utilize L-glutamate as sole nitrogen and carbon source, but *M. terrae* usually utilizes it.

Bönicke (1962) proposed a species name, *Mycobacterium aquae*, for the Runyon's group II scotochromogens, as these were distinguished from other species by the amidase pattern. In the present study, it was noted that some of the non-photochromogens (Runyon's group III) could not be differentiated from *M. aquae* by the Adansonian classification. Characters of *M. aquae* were studied more extensively in the present study. The results are shown in Table 7.

Non-photochromogens N 100616 and N 121326 and non-photochromogen Tomida were distinguished from other mycobacteria by the Adansonian classification. These may form new species, but at present, no conclusions can be drawn as only one or two isolates were studied.

It has been reported by a number of investigators that the non-photochromogens resembled *Mycobacterium avium*. Bojalil & Cerbón (1960) and Meissner (1960) stated that these two organisms were similar in their physiological and morphological characters. Palmer, Edwards, Hopwood & Edwards (1959), Takeya, Zinnaka, Yamaura & Toda (1960), and Smith & Johnstone (1964) stated that they showed a cross-tuberculin reaction. Murohashi, Tokunaga, Mizuguchi & Maruyama (1963)

stated that they could not be separated by phage typing. Furthermore, Bönicke (1962) reported that they had the same amidase pattern, and Bojalil *et al.* (1962) stated that they were not separable by their biochemical characters even though the similarity was based mainly on negative data. On the other hand, there are a few papers in which differences between these two organisms are described. Differences were reported in arylsulphatase activity (Kubica & Beam, 1961), acid phosphatase activity (Urabe, Saito, Tasaka & Matsubayashi, 1965) and virulence to chickens (Durr, Smith & Altman, 1959). Meissner (1960) also observed a difference in virulence to fowls, but she preferred, considering this was the only difference, to include the non-photochromogens in *M. avium*.

The results of the present study showed that 8 of 19 strains of non-photochromogens were inseparable from *Mycobacterium avium* and may be included in that species. It was noticed that another 8 strains also had an S-value of more than 91 % to the standard *M. avium* strains, and formed part of a large group, Group 3, in which *M. avium* was included.

Adansonian classification of rapid-growing mycobacteria

Grouping of rapidly growing mycobacteria

By arranging strains with more negative features to left and those with more positive features to right, an S-value table was prepared, by which 78 strains could be divided clearly into seven groups (Fig. 2): (6) Six strains of various named species; (7) *Mycobacterium thermoresistibile* (sp.nov.); (8) *M. phlei*; (9) rapidly growing scotochromogens (*M. aurum* (sp.nov.)); (10) *M. fortuitum* and rapid growers (Runyons group IV); (11) *M. parafortuitum*; (12) *M. smegmatis*.

Mycobacterium parafortuitum, a new species described by Tsukamura (1966), occupied an independent position.

Mycobacterium thermoresistibile sp.nov. (holotype: strain 316, NCTC 10409) was distinguished from other species by the Adansonian classification and was considered to form a new species. It can grow at 52°, although *M. phlei* is the only mycobacterium known hitherto that is capable of growing at this temperature. Thirty-nine isolates were obtained from soil and formed an homogeneous group. Further details of this species will be described in another paper so only one strain is shown in Fig. 2 for comparison. It was noticed that most soil isolates growing at 52° obtained around Nagoya belonged to this type. Strains of this type grew more slowly than *M. phlei*, and their characters resembled those of slowly growing mycobacteria (compare Tables 7 and 10).

Rapid-growing scotochromogenic mycobacteria from soil sources were recognized as a new species (Fig. 2). This was named *Mycobacterium aurum*, sp.nov. (holotype: strain 358, NCTC 10437). Fourteen isolates of this species were obtained from the soil. The author had considered that this type of scotochromogenic mycobacterium might be a variety of *M. parafortuitum* (Tsukamura, 1966), but the results of the present study show that the species are differentiated by the following characters: (1) *M. aurum* is markedly orange-coloured, but *M. parafortuitum* is non-pigmented or slightly orange or brownish coloured; (2) *M. aurum* grows at 37° but does not grow at 45°, whereas *M. parafortuitum* grows at 45°; (3) *M. aurum* does not utilize malonate as sole carbon source, but *M. parafortuitum* does;

(4) *M. aurum* utilizes monoethanolamine as sole simultaneous nitrogen and carbon source, but *M. parafortuitum* does not; (5) *M. aurum* usually does not utilize pentoses, but *M. parafortuitum* utilizes them. *Mycobacterium aurum* is differentiated by the amidase pattern from *M. acapulcensis*, a rapidly growing scotochromogenic species (Cerbón & Trujillo, 1963). A fuller description of this species will be made elsewhere.

Fig. 2. Diagrammatic representation of the S-value table of rapid-growing mycobacteria. From left to right: (6) *Mycobacterium platypocilus* Ross, *M. piscium* Ross, *M. ranae* L-17, *M. marinum* Ross, *M. balnei* B916, B913; (7) *M. thermoresistibile* 316 (NCTC 10409); (8) *M. phlei* SN101, SN102, SN103, SN104, SN105, SN106, SN107, SN108, SN109, SN110, Kyushu, 5, Wa40, Wa60, Wa366, Wa289, Trudeau, SP-11, CDC, Denken; (9) *M. aurum* 303 (NCTC 10439), 309 (NCTC 10440); (10, a) *M. fortuitum* 606, 607, 605, 335, 334, 330, 313, 302, 306, 308; (10, b) Rapid growers Yamamoto, Nishiwaki, Mimura; (11) *M. parafortuitum* 310 (NCTC 10410), 311 (NCTC 10411), 304, 307, 305, 314; (12) *M. smegmatis* SN1, SN2, SN3, SN4, SN5, SN6, SN7, SN8, SN9, SN10, Wa 63, Wa 290, Wa 236, Wa 237, Wa 402, R1102, R1103, CDC, SP-9, Trudeau, Denken, Kyushu, Jucho, Takeo.

Group 6, comprised *Mycobacterium platypocilus*, *M. marinum*, *M. balnei*, *M. ranae* and *M. piscium*. These resembled each other closely, irrespective of the presence or absence of photochromogenicity. The identity of *M. marinum* and *M. balnei* was reported by McMillen & Kushner (1959) and Bojalil *et al.* (1962)

Strains of this group grew much more slowly than other rapidly growing mycobacteria and, as shown later, their natural position seemed to be in the group of slowly growing mycobacteria.

Homogeneity tests

Mycobacterium smegmatis: Of 24 strains tested, 9 were omitted successively because of low mean S-values and the remaining fifteen, SN 2, SN 5, SN 6, SN 7, SN 8, SN 10, Wa 63, Wa 290, Wa 237, R 1102, R 1103, CDC, SP-9, Denken, Takeo, formed a homogeneous group. The mean S-value for this group was $96.5 \pm 1.25\%$ ($n = 105$). *M. phlei*: of 20 strains tested, 13 strains, SN 102, SN 103, SN 104, SN 105, SN 106, SN 107, SN 108, SN 109, SN 110, Kyushu, 5, Wa 60, Wa 366, formed a homogeneous group showing a mean value of $94.0 \pm 2.15\%$ ($n = 78$).

Mycobacterium fortuitum. The 13 strains of *M. fortuitum*, included three rapid growers from human sources. These three were omitted by the first test. The similarity value between strains Yamamoto and Nishiwaki was 95 %, and they may form a subgroup (10, b) within the species. Among strains identified as *M. fortuitum*, 8 strains, 606, 335, 334, 330, 313, 302, 306, 308, formed a homogeneous group (Table 8).

Table 8. Homogeneity test for strains identified as *Mycobacterium fortuitum*

Strain	Mean S-value for each strain			
	($n = 12$) First test	($n = 9$) Second test	($n = 8$) Third test	($n = 7$) Fourth test
606	90.2 ± 2.34	91.2 ± 1.80	91.0 ± 1.77	90.8 ± 1.92
335	90.5 ± 3.23	92.0 ± 1.34	91.2 ± 1.85	92.3 ± 1.41
334	91.0 ± 2.84	92.0 ± 1.95	92.5 ± 1.97	93.0 ± 1.36
313	90.2 ± 4.05	91.8 ± 2.00	92.0 ± 2.14	92.2 ± 2.16
302	88.5 ± 5.41	91.2 ± 2.80	91.6 ± 2.56	92.0 ± 2.48
306	88.0 ± 5.24	90.0 ± 3.82	91.0 ± 3.25	92.0 ± 2.34
308	89.4 ± 4.69	91.3 ± 2.82	92.3 ± 2.30	92.7 ± 2.12
330	90.5 ± 2.94	91.0 ± 2.43	91.7 ± 2.24	92.0 ± 2.28
607	88.5 ± 2.57	$88.2 \pm 6.25^*$	.	.
605	88.5 ± 3.20	89.5 ± 2.00	$89.5 \pm 2.27^*$	.
Yamamoto	$85.9 \pm 4.33^*$	.	.	.
Nishiwaki	$85.0 \pm 4.67^*$	.	.	.
Mimura	$86.0 \pm 2.21^*$	.	.	.
Mean S-value for group	89.0 ± 3.98 ($n = 78$)	90.9 ± 2.46 ($n = 45$)	91.5 ± 2.30 ($n = 36$)	92.0 ± 2.05 ($n = 28$)

* Significant difference from the mean S-value for group at $P = 0.05$.

Mycobacterium parafortuitum. The S-value table of this group is shown in Table 9. Six strains formed a homogeneous group by the usual test. However, the mean for the group was low, $88.5 \pm 4.54\%$ ($n = 15$), suggesting that some unusual strains were included in the group. Strains 310, 311, 304 and 305 form a more compact group with a mean of $93.8 \pm 2.19\%$ ($n = 6$). Mean S-values for the strains 307 and 314 were $84.5 \pm 2.39\%$ and $84.5 \pm 1.32\%$ respectively, and they differed significantly from the mean of the group of 4 strains. Thus, the hypothesis that the strains 307 and 314 entered the group was rejected. As typical strains of *M. parafortuitum*, the following 4 strains have therefore been listed: 310 (NCTC 10410), 311 (NCTC 10411), 304 and 305.

Table 9. *S*-value table for strains identified as *Mycobacterium parafortuitum*

Strain	S-value (%)					
	310	311	304	305	307	314
310	100	.	.	.	.	.
311	98	100	.	.	.	.
304	93	93	100	.	.	.
305	92	93	93	100	.	.
307	86	87	83	82	100	.
314	84	86	83	84	85	100

Six miscellaneous strains, *Mycobacterium marinum*, *M. balnei*, *M. piscium*, *M. ranae* and *M. platypocillus*, which were in the past classified as different species, formed a homogeneous group, the mean S-value for the group being $92.5 \pm 2.58\%$ ($n = 15$). The correct name of this group seems to be *M. marinum* Aronson.

Description of species of rapidly growing mycobacteria

Characters of the species are shown in Table 10. As has been stated elsewhere (Tsukamura, 1965*d*), the pattern of utilization of nitrogen compounds as sole (simultaneous) nitrogen and carbon source seemed to be very useful for differentiation of rapid-growing mycobacteria. It was noticed, however, that the ability to utilize an amide as sole nitrogen and carbon source for growth is not always correlated with the amidase activity corresponding to the amide. For example, *Mycobacterium phlei* does not possess detectable acetamidase, but is able to utilize acetamide as sole nitrogen and carbon source. On the other hand, *M. parafortuitum* has acetamidase, but does not utilize acetamide as sole nitrogen and carbon source. However both organisms utilize acetate and ammonia as sole carbon and nitrogen sources. The explanation for the discrepancies is not yet known.

General view of the relationship among mycobacterial species

An S-value table was prepared by sorting one to three representative strains of both slow- and fast-growing species (Fig. 3). The genus *Mycobacterium* seemed to be separated into two large groups; one, A, consisting of *M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. marinum* (*M. balnei*, *M. ranae*, *M. piscium* and *M. platypocillus*), *M. avium*, *M. aquae*, *M. terrae*, *M. thermoresistibile* and strains N100616 and Tomida; another, B, consisting of *M. phlei*, *M. fortuitum*, *M. aurum*, *M. parafortuitum* and *M. smegmatis*.

As seen in the figure, *Mycobacterium thermoresistibile* and strains N100616 and Tomida seem to be intermediate types between the two large groups. *M. marinum* should be placed in the former group.

Strains of group A grow slowly, are not tolerant to 0.1% picric acid, do not utilize L-glutamate as sole nitrogen and carbon source, and do not utilize fumarate, malate and succinate as sole carbon source. On the other hand, strains of group B show the opposite features.

The intermediate form *Mycobacterium thermoresistibile* is tolerant to 0.1% picric acid and utilize L-glutamate as sole nitrogen and carbon source, and even fumarate as sole carbon source.

Table 10. Characters of rapidly growing mycobacteria

Characters in which all test strains gave a negative reaction are omitted from table.

Species Typical strain and group ...	<i>M. marinum</i> B 916 (6)	<i>M. thermo- resistibile</i> 316 (7)	<i>M. phlei</i> SN 106 (8)	<i>M. fortuitum</i> 334 (10, a)	<i>M. aurum</i> 358 (9)	<i>M. para- fortuitum</i> 310 (11)	<i>M. smegmatis</i> SN 2 (12)
Character†							
Colony morphology	Rough	Smooth	Rough*	Smooth	Smooth	Smooth	Smooth*
Pigmentation	+	—	±	—	+	±	—
Photochromogeneity	—*	—	—	—	+	—	—
Growth rate	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid
Catalase	+	+	+	+	+	+	+
NaNO ₂ reduction	+	+	+	+	—	+	—
Third-day-arylsulphatase	—	—	—	+	—	—	—
Second-week-arylsulphatase	+	—	+	+	+	+	+
Salicylate degradation	—	—	—	+	—	—	—
PAS degradation	—	—	—	—	—	—	—
NH ₄ OH resistance	—	—	—	+	—	—	—
Picric acid tolerance (0.1 %)	—	+	+	+	+	+	+
Picric acid tolerance (0.2 %)	—	+	+	+	+	+	+
Growth at 28°	+	+	+	+	+	+	+
Growth at 37°	+	+	+	+	+	+	+
Growth at 45°	—	+	+	—	—	+	+
Growth at 52°	—	+	+	—	—	—	+
Acetamidase	—	—	—	+	—*	+	+
Benzamidase	—	—	—	—	—	—	—
Urease	+	+	+	+	+	+	+
Isonicotinamidase	—	—	—	—	—	—	—
Nicotinamidase	—	+	+	+	+	+	+
Pyrazinamidase	—	+	+	+	+	+	+
Allantoinase	+	—	—	—	—	—	—
Succinamidase	—	—	—	—	—	—	+
Utilization of organic acids as sole carbon source:							
Acetate	—*	+	+	+	+	+	+
Citrate	—	—	—*	+	—*	+	+
Succinate	—	±	±	+	+	+	+
Malate	—	±	+	+	+	+	+
Pyruvate	—	—	+	+	+	+	+
Benzoate	—	—	—	—	—	+	+
Malonate	—	—	+	—	—	+	+
Fumarate	—	+	+	+	+	+	+
Acid from:							
Glucose	—	—	—*	+	+	+	+
Mannose	—	—	—*	+	+	+	+
Galactose	—	—	—	—	—	—	+
Arabinose	—	—	—*	—	—	+	+
Xylose	—	—	—	—	—	+	+
Rhamnose	—	—	—	—	—	—	+
Trehalose	—	—	—	+	+	+	+
Inositol	—	—	—	—*	—*	+	+
Mannitol	—	—	—*	—*	—*	+	+
Sorbitol	—	—	—*	—	—	—	+
Utilization of carbohydrates as sole carbon source:							
Glycerol	+	+	+	+	+	+	+
Glucose	—	—*	+	+	+	+	+
Fructose	—	—	+	+	+	+	+
Sucrose	—	—	—*	—*	+	+	+
Mannose	—	—	+	+	+	+	+
Galactose	—	—	+	—*	—	+	+
Arabinose	—	—	+	—*	—	+	+
Xylose	—	—	—*	—*	—	+	+
Rhamnose	—	—	—	—*	—	+	+
Trehalose	—	—	+	+	+	—	+
Raffinose	—	—	—	—	+	—*	—
Inositol	—	—	—	—*	+	+	+
Mannitol	—	—	+	+	+	+	+
Sorbitol	—	—	+	+	—	+	+
Ethanol	—	+	+	+	+	+	+
Propanol	—	+	+	+	+	+	+
Propylene glycol	—	—	—*	—*	+	+	+
1, 3-Butylene glycol	—	—	+	+	+	+	+
2, 3-Butylene glycol	—	—	+	+	+	—*	+
Utilization of nitrogen compounds as sole nitrogen source:							
L-Glutamate	+	+	+	+	+	+	+
L-Serine	—*	+	—*	+	+	+	+
L-Methionine	—	—	—*	+	+	—	+
Acetamide	—	—	+	+	+	+	+
Benzamide	—	—	—	—	+	—	+
Urea	+	+	+	+	+	+	+
Pyrazinamide	+	+	+	+	+	+	+
Isonicotinamide	—*	—	—	—	—*	—*	+
Nicotinamide	—*	—*	+	+	+	+	+
Succinamide	+	+	—	+	+	+	+
Nitrate	+	+	+	+	+	+	+
Nitrite	—	—	+	+	—	—	+
Utilization of nitrogen compounds as sole nitrogen and carbon source:							
L-Glutamate	—*	+	+	+	+	+	+
L-Serine	—	—	—*	—*	—*	—	+
Glucosamine	—	—	—*	+	+	+	+
Acetamide	—	—	+	+	—*	—	+
Benzamide	—	—	—	—	—	—	+
Nicotinamide	—	—	—	—	—	—	+
Monoethanolamine	—	—	—	+	+	—	+
Trimethylene diamine	—	—	+	+	+	+	+

* A few or several strains of the homogeneous group showed different reactions.

† Utilization means utilization for growth.

Fig. 3. Diagrammatic representation of the S-value table of both fast- and slow-growing mycobacteria. The groups are numbered as in Figs. 1 and 2.

From left to right: (1a) *Mycobacterium tuberculosis* H37Rv, (1b) *M. bovis* D-4; (2) *M. kansasii* Forbes-84, Bostrum-D-35, Nagai; (6) *M. platypocilus* Ross, *M. balnei* B916, B193, *M. marinum* Ross, *M. ranae* L-17, *M. piscium* Ross; (3, b) *M. avium* 3717, 4121, Kirchberg; (3e) *M. aquae* Okubo, Aruga, Matsumoto; (3,a) *M. terrae* 317 (NCTC10424), 318; (7) *M. thermoresistibile* 316 (NCTC10409); (4) non-photochromogens N100616 (NCTC10425); (5) non-photochromogen Tomida (NCTC10428); (8) *M. phlei* SN103, SN106, SN108; (10,a) *M. fortuitum* 335, 334, 330; (9) *M. aurum* 303 (NCTC10439), 309 (NCTC10440); (11) *M. parafortuitum* 310 (NCTC10410), 311 (NCTC10411), 304; (12) *M. smegmatis* SN2, SN5, SN6.

In this figure, *M. avium*, *M. aquae* and *M. terrae* form one group (group 3). Although these three species form one large group, they are divided into subgroups if the diagrammatic representation is done more finely (at 5% levels; see Fig. 1).

An ideal taxonomy would be based on the similarity of the DNA structure or the gene structure. From this point of view, it is desirable that the characters to be used for the classification show 'one gene-one character' correspondence. If this is the case, all characters would be equivalent. There is no reason for supposing some characters (for example, immunological features) to be more valuable than others. As has been stated by Sneath (1957*a*) only when some characters are associated with several or many other characters, would they be regarded as more important ones.

It should always be remembered that we observe only a small sample of numerous characters. It is most important that the characters to be used for the classification are selected at random. Even if we produced a number of strains (that differed from a known species in several characters) by successive selections, such strains should not be regarded as having changed to another species. In such a case, selection would have been done deliberately to obtain certain characters. These selected characters should not be used as characters for the classification. Even if we observed several differences with respect to the selected characters, it is probable that the organisms have many similar features in characters not tested. Only when the organisms prove to differ significantly from a known species in respect of characters selected at random, should they be regarded as another species. Classification is a matter of statistics and probability.

The following strains are deposited in the National Collection of Type Cultures as type strains: (1) *Mycobacterium terrae* 317 (NCTC 10424); (2) *M. thermoresistibile* 316 (NCTC 10409); (3) *M. aurum* 358 (NCTC 10437), 312 (NCTC 10438), 303 (NCTC 10439) and 309 (NCTC 10440); (4) *M. parafortuitum* 310 (NCTC 10410) and 311 (NCTC 10411).

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