Identification of Mycobacteria by Overall Similarity Analysis

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SUMMARY

Morphological, physiological, growth and chemosusceptibility characters were determined for 80 'atypical' acid-fast and 3 non acid-fast strains isolated from tuberculous, or presumed tuberculous, patients, and for 24 control strains belonging to various mycobacterial species. Overall similarity analysis was used for establishing the relationships upon the basis of 60 characters codified by 153 features.

The overall analysis differentiated five clusters having mean matching indices above 80 % (80 phenon = 80P), namely: 80P-I (human and bovine mycobacteria); 80P-II ('atypical' mycobacteria belonging to the I_{st}, II_{nd} and III_{rd} Runyon groups); 80P-III (mycobacteria related to *Mycobacterium fortuitum*); 80P-IV (scotochromogenic mycobacteria with greater metabolic abilities); 80P-V (*M. smegmatis*). Nine strains (among which were three non acid-fast bacilli) were not included in these clusters.

The 'atypical' mycobacteria cluster (80P-II) was formed by three clearly delimited subgroups, which broadly corresponded to Runyon's classification.

A more detailed analysis of certain groups, and the determination of the characters of some 'hypothetical average organisms', allowed the further differentiation of some subgroups and aberrant strains.

The need to standardize the coding methods is stressed. Because of the value of overall similarity in such a variable genus, an international system to classify and identify strains upon a phenetic basis is suggested.

INTRODUCTION

Classification on a phenetic basis (overall similarity analysis, numerical taxonomy, Adansonian classification), using the method suggested by Sneath (1957) and Sneath & Cowan (1958), has found followers in various branches of microbiology: Hill (1959); Gilardi, Hill, Turri & Silvestri (1960); Talbot & Sneath (1960); Hill, Turri, Gilardi & Silvestri (1961); Colwell & Liston (1961); Beers & Lockhart (1962); Beers, Fisher, Megraw & Lockhart (1962); Silvestri, Turri, Hill & Gilardi (1962); Liston, Wiebe & Colwell (1963); Proctor & Kendrick (1963); Colwell & Mandel (1964); Kendrick & Proctor (1964). It was debated at the 17th International Congress of Microbiology at Montreal, at a symposium of the Systematics Association (Heywood & McNeill, 1964), at the Conference at Quebec in 1964; it has been the object of theoretical discussions, e.g. Lysenko & Sneath (1959), Floodgate (1962*a, b*), Sattler (1963), Reynolds (1965), and a book has been published on this subject by Sokal & Sneath (1963).

This shows the interest roused by a method that offers a chance to end much doubt that exists in the systematics of micro-organisms. The correlations between DNA

relationships (Marmur, Falkow & Mandel, 1963) and those obtained by phenetic analysis (Colwell & Mandel, 1964, 1965; Silvestri & Hill, 1965) emphasize the value of the method.

Concerning the mycobacteria, precise studies of the relationship between known species have been carried out with immunological techniques (Siebermann & Barbara, 1964; Koyama, 1964; Kassirskaia & Mencikov, 1965), or with bacteriophages (Manion, Bradley, Zinneman & Hall, 1964). Numerical taxonomy was applied to the study of mycobacteria mainly by Mexican research workers: Bojalil & Cerbón (1961); Cerbón & Bojalil (1961); Bojalil, Cerbón & Trujillo (1962), as well as by Wayne & Doubek (1963), enabling them to systematize the material and to describe new species.

In the pathological material examined in our laboratories mycobacteria of a type other than human or bovine were frequently found (Nasta & Bogdanescu-Cioclov, 1959; Bogdanescu, Bungetianu & Racotta, 1965). Since classification based upon some of the simple characters (Runyon, 1959; Collins, 1962; Marks & Richards, 1962; Buraczewska, 1963; Kagramanov, 1963) was not completely satisfactory, we have tried to classify the strains on a phenetic basis.

METHODS

Strains. Twenty-four previously identified strains (Table 1) and 78 strains isolated from 78 tuberculous, or presumed tuberculous, patients (mainly from sputa) were examined. Variants with different cultural characters, developed spontaneously or obtained by passage through mice from 5 of the 78 strains, have been included as separate strains.

Tests. We studied 60 characters that may be subdivided into 9 groups:

(1) Morphological characters of growth (smooth or rough, eugonic or dysgonic) on Löwenstein-Jensen medium (LJm), after 30 days incubation (examined with a hand lens.)

(2) Texture characters of growth after 8–15 incubation days on LJm (Marks & Richards, 1962): adherence to the medium, viscosity, texture (granular or butyrous), emulsibility in distilled water.

(3) Microscopic characters after 30 days on LJm: true branching (slide culture in Youman medium), cording, shape (bacilli, coccobacilli), length, thickness (measured with the ocular micrometer), percentage of acid-fast micro-organisms, depth of staining.

(4) Ability and rate of growth on two media (LJm and 7 % glycerol agar) at three different temperatures (25°, 37° and 45°) after loop inoculation (3 mm diam.) of a 10^{-2} mg./ml. bacillary dilution.

(5) Pigmentation in light and dark (Juhlin, 1960).

(6) Drug resistance (percentage method of Canetti, Rist & Grosset, 1963): INH (0.2, 1 and 100 µg./ml.); PAS-acid (1 and 10 µg./ml.); ethionamide (20 µg./ml.); cycloserine (25 µg./ml.); thiosemicarbazone (10 and 40 µg./ml.).

(7) Presence of certain enzymes and vitamins: catalase (Peizer & Widelock, 1955); nicotinamide (Konno, 1960); nitrate reductase (Virtanen, 1960); acetamidase, benzamidase, urease, nicotinamidase, salicilamidase, succinamidase (after 24 hr, Bönicke, 1960).

(8) Ability and rate of utilization of certain amino acids and organic salts: DL-

alanine (Käppler & Janowiec, 1963); sodium acetate, benzoate, citrate, lactate, oxalate, pyruvate, propionate, succinate and tartrate (Gordon & Smith, 1953), after incubation of 0.2 ml. from a 1 mg./ml. bacillary dilution.

Table 1. *List of strains from culture collections or previously identified*

No. and name	Source, etc.	Symbol in diagrams
1 H 37 RV	Human virulent collection strain*	H-HS
2 H 37 RV resistant to 10 µg. INH/ml.	Human collection strain grown <i>in vitro</i> on INH media*	H-HR
3 Strain 1020	Chemosusceptible strain with characters of human tubercle bacilli, isolated from a phthisical patient	H-1020
4 Strain 1325	INH-resistant strain with characters of human tubercle bacilli, isolated from a phthisical patient	H-1325
5 BCG	Bovine attenuated collection strain*	B-BCG
6 Vallée	Bovine virulent collection strain*	B-V
7 Strain B-7	Strain isolated from tuberculous cow with characters of bovine tubercle bacilli†	B-7
8 <i>M. avium</i>	Avian collection strain*	A
9 Strain A-1	Strain isolated from tuberculous fowl†	A-1
10 Strain A-2	As above	A-2
11 Strain A-3	As above	A-3
12 AF 526/59	Pathogenic strains, isolated in England from cattle and classified in the 1st group of Marks & Richards†	2 M
13 AF 281/60		6 M
14 AF 1445/61		5 M
15 AF 226/62		7 M
16 AF 179/63	As above, classified in the IVth group of Marks & Richards	1 M
17 AF 283/63	As above, classified in the Vth group of Marks & Richards	8 M
18 AF 1400/61	As above, classified in the VIth group of Marks & Richards	4 M
19 AF 1156/62		3 M
20 <i>M. fortuitum</i>	Collection strain§	MF
21 <i>M. phlei</i>	As above	MP
22 <i>M. smegmatis</i> (Washington)		MSW
23 <i>M. smegmatis</i> (607)	As above	MS-607
24 Tuberkelährlicher Bacillen of Rabinowitsch (1897)	As above	MR

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(9) Ability and rate of fermentation of certain sugars and higher alcohols (Gordon & Smith, 1953): arabinose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, trehalose, xylose, dulcitol, inositol, mannitol and sorbitol (incubation as for 8).

In order to estimate growth rate, the importance of which in the genus *Mycobacterium* was emphasized by Wayne (1964), we recorded the time growth became visible in the 4th group of tests (2-28 days incubation), and the time the medium changed colour in the 8th and 9th groups of tests (up to 60 days incubation).

Coding methods. Methods very similar to those suggested by Silvestri *et al.* (1962) were used as follows:

(1) Quantitative data:

(a) Silvestri *et al.* 1962:

Degree of the quantitative character	Computed features		
	A	B	C
0	—	—	—
1	+	—	—
2	+	+	—
3	+	+	+

i.e. 'additive coding' (Sneath, 1962). In this way we codified, for example, the percentage of acid-fast organisms or the drug-resistance.

(b) For the growth rate and growth ability the following coding variant was used (see Discussion):

	A	B	C	D
0 No growth in 28 days on X medium	—	—	NC	NC
1 Growth in 15–28 days	+	—	—	—
2 Growth in 8–14 days	+	+	—	—
3 Growth in 4–7 days	+	+	+	—
4 Growth in –3 days	+	+	+	+

NC = no comparison.

(2) Characters subordinated to other characters (Silvestri *et al.* 1962), for instance: emulsibility 'yes' or 'no'; if 'yes', high or low:

	A	B
Yes, high	+	+
Yes, low	+	—
No	—	NC

(3) For pigmentation in the dark we used a special coding:

Colour of the culture	Presence of pigment (A)	Red pigment (B)	Bright pigment (C)
1 White-grey	—	—	—
2 Yellowish	+	—	—
3 Lemon-yellow	+	—	+
4 Dull-orange	+	+	—
5 Bright-orange	+	+	+

Table 2 shows the characters examined, the number of features given for each character and the coding method (according to the system used above).

The formation of clusters. The comparison of the strains was made without a computer, determining the matching index between each couple of strains according to:

$$\% M = n_c / (n_c + n_d) \times 100,$$

where n_c = the number of concordances (between + and +, and — and — features) and n_d = the number of discordances (between + and — features) (Silvestri *et al.* 1962). For grouping the strains we used a modified Single Link Method (Sneath,

1962). Each new strain joined one or other end of the nucleus according to its higher % *M* with one or other half-cluster. In order to make clustering cleaner it has been necessary to change the position of some strains in the alignment (Sneath & Cowan, 1958). We also calculated the arithmetic means of the indices within and between the groups.

Table 2. Coding of the characters analysed

Group of characters	Character no.	Characters	Nos. of features	Coding method
I	1	Smooth or rough culture	1+	.
	2	Eugonic or dysgonic growth	1+	.
II	3	Adherence to the medium	2	1a
	4	Viscosity of the culture	1+	.
	5	Texture of the culture (granular or butyrous)	2	2
III	6	Emulsibility in distilled water	2	2
	7	True branching	1+	.
	8	Cording	2	2
	9	Shape and length of the bacilli	3	1a
	10	Thickness of the bacilli	1+	.
	11	Percentage of acid-fast organisms	3	1a
IV	12	Depth of staining	1+	.
	13	Growth rate on LJ medium at 37°	3++	1b
	14-18	Growth rate on LJ medium at 25° and 45° and on glycerol agar medium at 25°, 37°, and 45°	4(× 5) = 20	1b
V	19	Pigmentation in the dark	3	3
	20	Pigmentation in the light	1+	.
VI	21-23	INH-, PAS-, and TB ₁ -resistance	3(× 3) = 9	1a
VI	24-26	Streptomycin-, cycloserine-, and ethionamide-resistance	2(× 3) = 6	1a
VII	27	Catalase activity	3	1a
VII	28-35	Nicotinamide presence; nitrate reductase and 6 amidase activities	2(× 8) = 16	1a
VIII	36-46	Rate of utilization of alanine and 9 organic salts	3(× 10) = 30	1b
IX	47-60	Rate of fermentation of 14 sugars and higher alcohols	3(× 14) = 42	1b
Total	60 characters	.	153 features	

+ Coded (+) or (-).

++ The feature 'growth or no growth' was eliminated since all the strains grew on LJ medium at 37°.

Cluster analysis. From clusters having more than 15 strains and showing a % $\bar{M} \geq 80$ we eliminated the features common to all the strains and proceeded to make a new comparison of the strains based on the remaining features (Hill *et al.* 1961).

RESULTS

General Survey

The grouping of the strains in accordance with the *M* indices is shown in Fig. 1. Five clusters were recognized at the 80-phenon level:

(I) A cluster of 19 strains (80P-I), including the human and bovine control strains (1-7 of Table 1) and 12 strains having some atypical 'key characters' (negative to the

Konno test, PAS-resistance, dysgonic growth, INH-resistance associated with positive catalase). The mean intragroup index, $\bar{M}\%$, was 85.7.

(II) A cluster of 45 strains (80P-II) with very inhomogenous pigmentation, including 12 control strains (8-19 of Table 1), i.e. 4 avian strains and 8 strains which were classified in England as belonging to the Ist, IVth, Vth and VIth groups of Marks & Richards (1962), and 33 newly isolated strains, 3 non-photochromogens and 30 scotochromogens. $\bar{M}\% = 84.5$.

In this last cluster there were 3 subgroups and 1 aberrant strain (174):

80P-IIA: 4 strains received from England included in the Ist group of Marks & Richards, with $\bar{M}\% = 91.8$;

80P-IIB: 29 newly isolated strains with orange-red pigmentation ($\bar{M}\% = 88.2$);

80P-IIC: 4 avian control strains, 4 strains received from England included in the IVth, Vth and VIth groups of Marks & Richards, and 3 newly isolated strains ($\bar{M}\% = 85.8$).

The strain 174, not comprised in these subgroups, is a scotochromogen too, but has some peculiar characters.

(III) A cluster of 28 strains (80P-III), including one control strain, *Mycobacterium fortuitum* (strain 20 of Table 1). The majority of the strains are without pigment and are rapid growers ($\bar{M}\% = 80.1$).

(IV) A cluster (80P-IV) of 3 newly isolated scotochromogenic strains, with are $\bar{M}\% = 81.0$.

(V) A cluster (80P-V) of 3 control strains belonging to *Mycobacterium smegmatis* (strains 22-24 of Table 1); $\bar{M}\% = 90.3$.

The five clusters comprise 98 strains. Nine strains could not be included: one control strain (*Mycobacterium phlei*—strain 21 of Table 1, MP in Fig. 1); 5 newly isolated acid-fast strains and 3 non-acid-fast strains.

The table below shows intra- and inter-cluster mean $\bar{M}\%$ values:

	I	II			III	IV	<i>M. phlei</i>	V
		A	B	C				
I	85.7	.	.	.	.	.	.	.
IIA	79.5	91.8	.	.	.	.	.	.
IIB	72.7	80.5	88.2	.	.	.	.	.
IIC	73.4	78.6	82.5	85.8	.	.	.	.
III	56.9	62.1	65.9	67.7	80.1	.	.	.
IV	53.8	63.4	64.7	63.3	67.6	81.0	.	.
<i>M. phlei</i>	50.2	58.0	62.6	63.5	71.5	69.7	100.0	.
V	32.9	35.9	37.7	39.5	59.8	57.1	71.0	90.3

Thus 80P-I and 80P-II are representing together a 70-phenon, while *Mycobacterium fortuitum* group (80P-III) as well as 80P-IV and *M. phlei* are obviously different. The *M. smegmatis* group (80P-V) showed a very low similarity with the human, bovine, atypical and avian mycobacteria and a higher one with 80P-III and 80P-IV.

Further analysis of the clusters

The analysis was carried out as mentioned (see Methods), by recomparing the strains after removing the common features within each group and, when it was possible, by comparing them with the 'hypothetical average organism' (HAO) of the cluster. For rearrangements of the strains according to their similarities, Sneath's

technique (Sneath, 1962) was completed with Silvestri's (Silvestri *et al.* 1962). The characters of a cluster HAO were simply established by allotting to each feature the majority sign (+ or -) in all the strains of that cluster.

The removal of the common features has differentially reduced the number of variable features within each group. The decreasing of n_e (in numerators and denominators) lowers the value of M , and hence the differences between the strains are accentuated mainly for low values of M . At the same time, the percentage of common features is an indication of the homogeneity of the group.

Fig. 2. % M value diagram of 80P-I group after removing the 95 common features, showing two subgroups and three 'aberrant' strains, nos. 143, 9 and 125. % M : ■, 95-100; ▨, 90-94.9; ▩, 85-89.9; ▪, 80-84.9; ▧, 75-79.9; ▦, 70-74.9; ░, 60-69.9; ▤, 50-59.9; □, 0-49.9.

80P-I includes 19 strains, drug-sensitive or with acquired chemoresistance, grouped together with control strains of *Mycobacterium tuberculosis* of human and bovine type.

By comparing the 58 variable features, left after removing the common ones, two 'nuclei' became evident: (1) the strains 133 and 136; (2) the BCG and Vallée strains. By grouping the other strains around them, two subgroups ensued (Fig. 2). These subgroups do not differ from one another on account of their type (human or bovine) but mainly owing to differences in chemo-susceptibility. Indeed, subgroup *b* includes only strains susceptible to all the tested drugs, while in subgroup *a*, excepting strains 133 and B-7, all the others show different chemoresistance extents to one or another drug. The subgroups do not differ only in chemoresistance: in contrast with the strains of subgroup *b*, most of those belonging to *a* show dysgonic growth, a less granular texture and better emulsibility, a very slow growth rate on LJm, resistance to 10 μ g. INH and they do not utilize acetate and do not ferment glucose.

80P-II. The obvious differences in the three subgroups (Fig. 1) require separate analyses.

In 80P-IIA, after the elimination of the common features, only 19 variable features (out of 153) were left. The homogeneity of the 4 strains confirmed both their classification in group I of Marks & Richards (1962) and the species rank accorded to this group (*Mycobacterium kansasii*).

Fig. 3. % M value diagram of 80P-II B group after removing the 90 common features, showing three subgroups and an 'aberrant' strain, no. 101 (see Table 3). % M: ■, 95-100; ■, 90-94.9; ■, 85-89.9; ■, 80-84.9; ■, 75-79.9; ■, 70-74.9; □, 60-69.9; □, 50-59.9; □, 0-49.9.

80P-II B (29 newly isolated scotochromogenic strains). The review of similarities, based on 63 remaining variable features, has identified a subgroup of 6 strains (subgroup c). The remaining 23 strains make two other subgroups (subgroups a and b in Fig. 3).

The differences between the three subgroups (statistically estimated upon a majority of characters) appear in the extent of streptomycin, PAS and ethionamide resistance, in urease activity, in acetate utilization rate, in lactate utilization and maltose fermentation. The presence of a bright-orange pigment is a very constant character.

We established the characters of the HAO for the 80P-IIB group and compared them with each strain of the group (Table 3). Only strain 101 showed an *M* index of less than 75 % with the HAO. Thus, the group is homogenous, with a relatively low variability.

Fig. 4. % *M* value diagram of 80P-IIC group after removing the 106 common features, showing two subgroups. % *M*: ■, 95-100; ▤, 90-94.9; ▥, 85-89.9; ▦, 80-84.9; ▧, 75-79.9; ▨, 70-74.9; ▩, 60-69.9; □, 50-59.9; □, 0-49.9.

Table 3. The % *M* values of the strains grouped in 80P-IIB with the 'hypothetical average organism' of the same group

Strain	% <i>M</i>	Strain	% <i>M</i>
110	92	5	84
7	92	119	84
106	90	88	84
2	89	147	82
75	89	113	81
89	89	130	81
79	89	160	80
8	89	128	80
156	89	150b	79
139	87	3	79
120	87	132	79
117	87	138	76
127	86	129	75
155	84	101	69
145	84		

80P-IIC (11 strains). After the elimination of the common features, 47 variable features remained, and the comparison based upon them differentiated two subgroups (Fig. 4): one, *a*, included all the avian strains, the control strain 1M (belonging to Marks & Richards IVth group, considered by these authors as including avian type strains) and one newly isolated strain; the other, *b*, included three control strains classified in the Vth and VIth Marks & Richards groups and two newly isolated ones. Only four strains in *a* and two strains in *b* grew on LJm at 45°.

The characters which clearly differentiated subgroup *a* are: a relatively slow growth (at least 15 days on LJm at 25° and 7 days at 37°), Th- and TB₁-resistance, a positive nitrate reductase test, and a more rapid lactate utilization (in most cases in 20 days).

80P-III (28 strains, including the control strain *Mycobacterium fortuitum*) showed

a greater variability. Six strains originated from three dissociated strains by mouse passage (39*a*, *b*; 114*a*, *b*; 148*a*, *b*). Only 53 features were common to all the strains of the group.

A comparison based upon the 100 variable features gave two subgroups differentiated by culture consistency and adherence to the medium, by the appearance or

Fig. 5. % *M* value diagram of 80P-III group after removing the 53 common features, showing two subgroups and five 'aberrant' strains, nos. 157, 1, 4, 51 and 148*b* (see Table 4).
% *M*: ■, 95-100; ▤, 90-94.9; ▥, 85-89.9; ▦, 80-84.9; ▧, 75-79.9; ▨, 70-74.9; ▩, 60-69.9; □, 50-59.9; □, 0-49.9.

not of a faint yellowish pigmentation, by the growth rate at 25°, by ethionamide- and TB₁-resistance, by acetate and succinate utilization rate, and by glucose, mannose and trehalose fermentation rates (Fig. 5).

Some strains of the group are slow growers (157, 1, 39*a*, 39*b*, 10*a*, 4), and others did not grow on glycerol agar at 37° (1, 114*a*, 6). Strain 74 grew on LJm at 45°.

The peripheral strains of the subgroups showed relatively low similarities with the nuclei. The overall similarity analysis between the strains and the HAO of the group showed the deviation of these strains from this mean value (Table 4). If we consider

as a limit of the 'species' a 75 % *M* value against the HAO, the strains 157, 1, 4, 148*b* and 51 would not be included in the group.

80P-IV included only three newly isolated scotochromogenic strains, dull-orange pigmented (excepting the bright-orange pigmented strain 81), having short rods (less than 1 μ), intermediate growth rates, and greater metabolic abilities. Their number does not allow a more detailed statistical analysis.

80P-V is composed of three *Mycobacterium smegmatis* control strains with no newly isolated strains. Because of the low number of strains and the high $\bar{M}$ of the group, no statistical problems are raised.

Table 4. The %*M* values of the strains grouped in 80P-III with the 'hypothetical average organism' of the same group

Strain	% <i>M</i>	Strain	% <i>M</i>
112	91	74	81
151	91	148 <i>a</i>	80
154	91	146 <i>b</i>	80
115	89	10 <i>a</i>	80
114 <i>a</i>	89	39 <i>a</i>	79
116	88	MF	79
39 <i>b</i>	87	158	79
137	85	123	77
73	85	53	77
114 <i>b</i>	84	1	74
104	83	4	74
134	83	148 <i>b</i>	74
6	83	51	71
59	81	157	69

DISCUSSION

In order to achieve objectivity regarding numerical taxonomy, two conditions are supposed to be fulfilled: (1) the analysis of as many characters as possible, and (2) their comparison based upon a uniform coding method.

It is probable that, in our study, the addition of new mycobacterial characters would define more accurately the relative position of the strains. For instance, the impossibility of distinguishing by the tests used, in the presence of acquired chemoresistance, between the *Mycobacterium tuberculosis* strains and the *M. bovis* strains might raise discussion. Nevertheless, some classifications include both human and bovine tubercle bacilli in one species, *M. tuberculosis* (Topley and Wilson's *Principles*, 1964).

Since the 80P-I group, which includes weakly metabolizing and slowly growing strains, is shown mainly on the basis of negative features, further study using more characters is indicated.

As to the second condition, there have been several suggestions. Thus, the inclusion of negative matches as similarities (Hill *et al.* 1961; Silvestri *et al.* 1962) is considered by Sneath (1962) as distorting the results if the organisms are very diverse. Of course, if the inclusion of negative features increases the similarity percentages, then excluding them will decrease the number of comparisons—a great statistical disadvantage. Moreover, as Beers & Lockhart (1962) showed, the negative score (–) has in many cases a conventional value, not meaning the lack of a definite character but the opposite of a character marked with (+) (for instance, drug resistance *versus* susceptibility).

Sneath (1962) suggests care when comparing negative features, since, for instance, the inability of a given strain to ferment glucose will bring about its inability to ferment any other sugar. This was seen in most cases in our data, but it has underlined the similarity between strains with extremely weak metabolic capacity, increasing the human and bovine group up to 80P value, i.e. to a comparable similarity level with the other groups. It does not seem logical to consider the *Mycobacterium smegmatis* group have a higher similarity level than the *M. tuberculosis* group solely because the comparisons consider only the features marked (+). Moreover, further analysis of the group, involving the elimination of the common features, removes this 'similarity excess'. It must be mentioned that an attempt made to compare the 80P-I strains solely on the basis of the (+) features did not resolve the problems discussed above.

Inclusion of the (–) features leads to an alignment with all the samples of the genus *Mycobacterium* we had at our disposal arranged along a gradient of fermentative ability, beginning with *M. tuberculosis* (human and bovine types) and ending with *M. smegmatis*.

Finally, using the same argument as Beers & Lockhart (1962), if one considers that inability to ferment sugars is not due to the lack of genes but to the activity of repressors, theoretically the fermentation inability might be marked with (+), i.e. repressor activity, and the reverse with (–), repressor inactivity.

In subdividing the characters into features we preferred the 'additive codification' (Silvestri *et al.* 1962). Sneath (1962) shows that the non-additive method does not afford any information concerning the degree of difference between different characters. With regard to the genus *Mycobacterium*, not only the metabolic abilities scored as 'yes' or 'no', but also the rate of utilization of the substrate plays a prominent part in differentiation (Wayne, 1964).

We must still give the reasons that led us to use for some characters the coding method we have marked 1b (Methods). It was of the following type:

	A	B	C	D
0 No growth in 28 days on X medium	–	–	NC	NC
1 Growth in 15–28 days	+	–	–	–
2 Growth in 8–14 days	+	+	–	–
3 Growth in 4–7 days	+	+	+	–
4 Growth in 1–3 days	+	+	+	+

Thus, the additive method was used only in comparing variants 1–4. In this way the matching indices (M) between the 0 variant and the variants 2–4 are equal ($M = 0\%$). On the other hand we did not score total incompatibility between variants 0 and 1, since a strain that did not grow in 28 days is potentially still able to grow. Therefore, between a strain with behaviour 0 and a strain with behaviour 1, M will be 50%. $M_{0,1}$ will be hence equal to 50% as well as $M_{1,3}$ or $M_{2,4}$, but having a lower 'weight', since $M_{0,1}$ is codified only by two features while $M_{1,3}$ or $M_{2,4}$ are codified by 4 features.

'Character weight' also introduces a subjective element in weighting of the characters. For instance, we were subjective in giving only one feature to photochromogenicity but three for the scotochromogenicity; we gave two features for streptomycin-, ethionamide-, and cycloserine-resistance but three for INH-, PAS-, and TB-resistance etc. (Table 2).

The pathogenicity of *Mycobacterium tuberculosis* led to the neglect for a long time

of the related species, thus distorting the objective biological study of the whole genus (Wayne, 1964). The increasingly frequent isolation of 'atypical' strains showed the need for more knowledge. Runyon's classification, based upon a few characters, was extremely useful for a rapid and rough identification. Attempts to improve it (Collins, 1962) or to achieve classification according to practical (medical) criteria (Kagramanov, 1963; Buraczewska, 1963) have been made. The tentative classification of Marks & Richards (1962) also aims at a practical purpose, in studying only potentially pathogenic strains. They emphasized that the Vth, VIth and VIIth groups were provisional, and should be studied by overall similarity analysis. The studies of Bönicke (1960), Cerbón & Trujillo (1963), Morellini *et al.* (1964) tried to cover the whole genus, but the differentiation of species was based on key characters.

By using overall similarity analysis the groups of strains fell into a definite order. The extreme position of *Mycobacterium smegmatis* was also pointed out by Bojalil *et al.* (1962) using the same method. *M. tuberculosis*, usually placed in the centre of the genus, becomes, when one considers interspecific relationships, a peripheral species which has peculiar characters because of its specialization.

We have already shown that the two subgroups in 80P-I are differentiated to a great extent by the chemoresistance of the strains, with which other differences are also associated. It seems logical to ask whether difference in pathogenicity, which is the main distinction between the two 'species' *Mycobacterium tuberculosis* and *M. bovis*, is any more important than the character of INH-resistance. The recent research of Cattaneo, De Ritis & Lucchesi (1965) has shown differences between the fatty-acid content of the INH-susceptible and INH-resistant tubercle bacilli.

As to *Mycobacterium kansasii*, the key character of photopigmentation might seem well chosen, but according to Bojalil *et al.* (1962). *M. marinum* also has this character and it is also found in *M. balnei*, *M. scrofulaceum*, *M. marianum*, and sometimes in *M. aquae* type I and *M. battaglini* (Morellini *et al.* 1964). Hauduroy, Hovanessian & Roussianos (1965) show that even in *M. kansasii* photochromogenicity is a secondary and unstable character, which should only be used for a first orientation. Consequently, a photochromogen does not necessarily belong to *M. kansasii* and a strain of this species is not necessarily a photochromogen. The high similarity values between 80P-IIA and 80P-I support the opinion of Weissfeiler, Karassova & Holland (1964) that *M. kansasii* has been derived by mutation from *M. tuberculosis*.

The 80P-IIB subgroups (Fig. 3) were distinguished neither by their amidase activities nor by their capacity to ferment mannose, by which Bojalil *et al.* (1962) distinguished *Mycobacterium marianum* from *M. gordonae*.

The probability that the Battey strains belong to *Mycobacterium avium* has been emphasized many times. Wayne & Doubek (1963), using overall similarity analysis, differentiated three subgroups in the IIIrd Runyon group, only one of which was potentially pathogenic and which perhaps belonged to the Battey-avian-porcine complex. Since the tests used by them are not the same as ours, we cannot compare them further. On our data the 80P-IIC group, although numerically scanty, could be split into two subgroups, but the key character of growth at 45° seems of little use for differentiating this complex of strains. Not all the strains in the 80P-III group possessed the key characters commonly used for the identification of Runyon's IVth group (rapid growth, growth on glycerol agar at 37°).

There is great similarity between the 80P-II subgroups (A, photochromogens

B, scotochromogens; C, avian complex of strains). The scotochromogenic mycobacteria are generally not pathogenic, but their isolation from human pathological products is very frequent, in contrast with the saprophytic mycobacteria (*Mycobacterium phlei*, *M. smegmatis*). Their relationship with the pathogenic groups (pointed out in recent work: Siebenmann & Barbara, 1964; Koyama, 1964; Kassirskaia & Mencikov, 1965) raises the question of close genetic connexions which would deserve more thorough examination. The scotochromogenic strains in the 80P-IV group could not be identified as belonging to any of the species of Bojalil *et al.* (1962) or Morellini *et al.* (1964). We do not wish to propose a new species for these strains. However our failure to identify them with any of Bojalil's or Morellini's species emphasizes that it is sometimes impossible to identify mycobacteria by key characters.

The strains which could not be grouped raise a similar question. Most likely they would represent unique strains belonging to certain definite groups (species), but they cannot be identified further in the present state of our knowledge. There are also strains that were initially clustered but which were subsequently eliminated by the second analysis. These aberrant strains might be considered either as variants arising as an effect of unknown agents, or as mixtures (either mixed cultures or genetic hybrids) belonging to different groups. The latter explanation is possible since we have five strains which dissociated on culture. These five strains raise genetic problems. Most of them are on the periphery of the group to which they belong: strains 146*a* (Fig. 2); 150*b* (Fig. 3); 39*a*, 39*b*, 146*b*, 10*a*, 148*b* (Fig. 5). They even occur outside any group (strain 10*b*, Fig. 1). The pairs with very similar partners, such as 39*a* and *b*, 114*a* and *b* (Fig. 5), differ by one or two obvious characters (pigmentation, consistency), and thus may give clues about the first steps in the variation of mycobacteria.

In conclusion, from the results obtained both by us and others, overall similarity analysis seems to be the best method, for the time being, for systematizing this group. By this method, both Bojalil *et al.* (1962) and ourselves were able to group mycobacteria according to their relative affinity. This work revealed a great similarity between the slowly growing 'atypical' mycobacteria, and moreover the present study has shown the close relationship between them and *Mycobacterium tuberculosis*. Our work also shows, however, the considerable variability of strains isolated from human pathological material and the difficulty of identifying them.

To produce an adequate taxonomic revision of this genus would require a comprehensive overall similarity analysis of a large number of pure strains (both from collections and newly isolated) from all over the world, and from all the environments where mycobacteria live. Standardized tests and coding methods would be needed. An international reference centre could establish on this basis the groups (phenons, species) and subgroups (phenons, varieties) as well as the characters of the corresponding 'hypothetical average organism' or 'hypothetical median organism' (Liston *et al.* 1963). When comparing an unknown strain with these 'hypothetical organisms' it would be sufficient to use only those characters which proved to be significant as a result of a statistical estimation (Beers & Lockhart, 1962). Thus, newly isolated strains from various countries would be correctly placed by such comparisons. This would solve both the present points of controversy—the theoretical one regarding the classification and the practical one regarding the identification of mycobacterial strains.

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