TAXONOMY OF MYCOBACTERIA STUDIED BY POLY-ACRYLAMIDE-GEL ELECTROPHORESIS OF CELL PROTEINS

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PLATES II-V

RECOGNITION of the fact that mycobacteria other than *Mycobacterium tuberculosis* can cause disease in man has led to intensive studies of methods of identifying and defining this group of organisms.

Runyon (1959) was the first to suggest a classification of the so-called atypical mycobacteria. He divided them into four groups on the basis of pigment production, growth rate and colonial morphology. An array of biochemical and other tests has subsequently been introduced (Bönicke, 1962; Kestle, Abbott and Kubica, 1967), and, by means of an Adansonian or numerical analysis of the characteristics of the genus, Wayne (1967) was able to recognise a number of species and subgroups within Runyon's original four large groups. On the basis of these numerical analyses Wayne and Doubek (1968) have provided a simple diagnostic key for the identification of mycobacteria encountered in clinical laboratories.

Genetic relatedness, as demonstrated by gene compatibility or DNA base-sequence analyses, has been suggested as a basis for bacterial taxonomy. Polyacrylamide-gel electrophoresis of cell proteins has been shown to reflect such genetic relatedness and to be of taxonomic value in the classification and identification of mycoplasmas (Razin, 1968) and of enterobacteriaceae (Sacks, Haas and Razin, 1969). Since the present classification of mycobacteria still seems to be rather empirical, we decided to examine the polyacrylamide-gel (PAG) electrophoretic patterns of the cell proteins of various mycobacteria in the hope of demonstrating evidence of genetic similarities or differences between different strains. PAG electrophoresis has been used to study the fractionation of mycobacterial protein components (Affronti, Parlett and Cornesky, 1965; Roszman *et al.*, 1968; Castelnuevo *et al.*, 1969; Dietz, Runyon and Marcus, 1969), but as far as we are aware there have been no previous reports of the use of this method for purposes of classification or identification.

MATERIALS AND METHODS

Organisms. Sixteen strains of atypical mycobacteria isolated from clinical material and 13 type strains were used. The type strains were: *Myco. tuberculosis* H37Rv, BCG, *Myco. kansasii* ATCC 12478, *Myco. marinum* ATCC 927, *Myco. intracellulare* ATCC 13950, *Myco. avium* ATCC 19421, and from the Trudeau Institute, Saranac Lake, N.Y., *Myco. balnei* TMC 1604, *Myco. scrofulaceum* TMC 1312, tap-water type TMC 1318, *Myco. terrae* TMC 1450, *Myco. fortuitum* TMC 1529, *Myco. phlei* TMC 1516, and *Myco. smegmatis* TMC

Received 15 Mar. 1971; accepted 12 July 1971.

1515. All the cultures were maintained on Lowenstein-Jensen medium. The strains isolated from clinical material were identified from growth rate, pigment production and, as suggested by Wayne and Doubek, the results of the niacin test, Tween 80 hydrolysis, catalase production, iron uptake and arysulphatase reaction. In some cases nitrate reduction was tested.

Preparation of cell-free protein extract. All the strains were grown on Lowenstein-Jensen medium. When heavy growth appeared, the colonies were transferred into 600 ml of Dubos liquid medium enriched with albumin, and incubated at 37°C for 3–5 wk. The cultures were harvested by centrifugation at 13,000*g* for 30 min. at 4°C. The supernatant was discarded and the cells were washed three times in normal saline. They were then exposed to ultrasonic disruption at an amplitude of 5 μ m for 15 min. in an MSE 100-watt ultrasonic disintegrator. The tube was packed in ice during the ultrasonic treatment. The disrupted cells were centrifuged and the supernatant fluid was passed through an asbestos filter (Carlson-Ford, grade EKS). The protein content of the filtrate was determined according to Lowry *et al.* (1951). At least two filtrates, prepared from different cultures, were examined from each strain.

Polyacrylamide-gel electrophoresis was performed according to the methods of Davis (1964) and Ornstein (1964) in Tris-glycine buffer at pH 8.3. Phenol-acetic acid extracts, on electrophoresis at an acid pH {the method used for mycoplasmas (Razin), enterobacteriaceae (Sacks *et al.*) and other bacteria (King, Theodore and Cole, 1969)} failed to give adequate separation of mycobacterial proteins, but an alkaline pH gave highly satisfactory results. From 0.05 ml to 0.1 ml of the cell-free extract, containing about 400 mg protein, was placed on the top of the gel and mixed with 0.05 ml of 40 per cent. sucrose solution. Electrophoresis was performed at room temperature with a constant current of 4 mA per tube. The gels were stained with 1 per cent. amidoblack in 7 per cent. acetic acid for 30 min., and excess dye was then removed electrophoretically. Several gels were prepared from each extract. Densitometer tracings of the stained gels were made in a Gilford 2400 spectrophotometer equipped with a Model 2410 scanner.

RESULTS

The duplicate extracts of each strain always gave identical electrophoretic patterns. Similarly, repeat examinations of the same extract were always identical.

Table I shows the results of the morphological and biochemical identification of the 16 strains isolated from clinical material. Most of them were isolated from urine and identified as scotochromogens. Eleven strains belonged to the tap-water subgroup, which has rarely been implicated conclusively as pathogenic (Kestle *et al.*; Wayne, Doubek and Diaz, 1967). Three strains belonged to the scrofula subgroup, which is known to cause cervical lymphadenitis, but has not been implicated aetiologically in renal disease. Two strains identified as *Myco. fortuitum* were isolated, one from sputum and the other from an abscess.

The polyacrylamide-gel electrophoretic patterns of the various mycobacteria were compared. Representative strains from each of Runyon's four groups of atypical mycobacteria all showed different gel patterns (fig. 1). The differences were sometimes striking, and did not always agree with the biochemical and morphological groupings; there was similarity between strains from different groups, and differences between strains from the same group. These are described in detail below.

Runyon's four groups were compared with *Myco. tuberculosis*; the gel patterns and their densitometer tracings (fig. 2*a* and *b*) showed that *Myco. kansasii* and the scotochromogen *Myco. scrofulaceum* were both very similar to

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FIG. 1.—PAG electrophoretic patterns of *Myco. kansasii* (A), *Myco. balnei* (B), *Myco. scrofulaceum* (C), tap-water type (D), *Myco. avium* (E), *Myco. intracellulare* (F), *Myco. fortuitum* (G), *Myco. phlei* (H) and *Myco. smegmatis* (I).

FIG. 2a.—PAG electrophoretic patterns of *Myco. tuberculosis* H37Rv (A), *Myco. kansasii* (B), *Myco. scrofulaceum* (C), *Myco. intracellulare* (D) and *Myco. fortuitum* (E).

FIG. 2b.—Densitometer tracings from the gels shown in fig. 2a.

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FIG. 3a.—PAG electrophoretic patterns of *Myco. kansasii* (A), *Myco. scrofulaceum* (B) and tap-water type (C).

FIG. 3b.—Densitometer tracings from the gels shown in fig. 3a.

FIG. 4a.—PAG electrophoretic patterns of *Myco. avium* (A), *Myco. intracellulare* (B) and *Myco. terrae* (C).

FIG. 4b.—Densitometer tracings from the gels shown in fig. 4a.

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FIG. 5a.—PAG electrophoretic patterns of *Myco. fortuitum* (A), *Myco. phlei* (B) and *Myco. smegmatis* (C).

FIG. 5b.—Densitometer tracings from the gels shown in fig. 5a.

FIG. 6a.—PAG electrophoretic patterns of *Myco. balnei* (A) and *Myco. marinum* (B).

FIG. 6b.—Densitometer tracings from the gels shown in fig. 6a.

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FIG. 7a.—PAG electrophoretic patterns of *Myco. tuberculosis* H37Rv (A) and BCG (B).

FIG. 7b.—Densitometer tracings from the gels shown in fig. 7a.

FIG. 8a.—PAG electrophoretic patterns of *Myco. scrofulaceum* type strain (A), local isolate 1157 identified as *Myco. scrofulaceum* (B), tap-water type strain (C) and local isolate 778 identified as tap-water type (D).

FIG. 8b.— Densitometer tracings from gels shown in fig. 8a.

Myco. tuberculosis. In all three cases the major protein bands were concentrated in the central portion of the gel. On the other hand, *Myco. intracellulare* and the rapid grower *Myco. fortuitum* had similar gel patterns, which were distinct from those of the chromogenic strains. Here there was a wider scatter of the bands throughout the length of the gel.

The two scotochromogens—the tap-water type and *Myco. scrofulaceum*—had very similar patterns, which were so close to that of *Myco. kansasii* (fig. 3*a* and *b*) as to suggest that the three chromogenic strains form a homogeneous group.

The three non-photochromogenic strains (*Myco. avium*, *Myco. intracellulare* and *Myco. terrae*) each had different electrophoretic patterns, and although there was a certain similarity between *Myco. intracellulare* and *Myco. terrae*,

TABLE I
Source and identification of 16 strains of atypical mycobacteria isolated from clinical material

Source	Number, isolated from this source, of		
	scotochromogens		rapid growers (<i>Myco. fortuitum</i>)
	<i>Myco. scrofulaceum</i>	Tap-water type	
Sputum	0	0	1
Gastric juice	0	1	0
Pus	0	0	1
Urine	3	10	0
All sources	3	11	2

there was none at all between *Myco. intracellulare* and *Myco. avium* (fig. 4*a* and *b*).

The rapid growers (*Myco. fortuitum*, *Myco. phlei* and *Myco. smegmatis*) also differed; there was some resemblance between the patterns of *Myco. fortuitum* and *Myco. phlei*, but neither of them resembled *Myco. smegmatis* (fig. 5*a* and *b*). The latter had a characteristic pattern, which did not resemble that of any other strain used in this study.

Myco. balnei, which is a rapid grower and also a photochromogen, gave an electrophoretic pattern quite different from those of both the other rapid growers and the photochromogen *Myco. kansasii*, but almost identical with that of *Myco. marinum* (fig. 6*a* and *b*).

Myco. tuberculosis and the strain of BCG had almost identical patterns (fig. 7*a* and *b*).

The clinical isolates that had biochemical and morphological characteristics of the tap-water type or of *Myco. scrofulaceum* had the same electrophoretic patterns as their respective type strains (fig. 8*a* and *b*). The clinical isolates of

Myco. fortuitum also had patterns identical with that of the *Myco. fortuitum* type strain.

DISCUSSION

The fact that almost identical gel patterns were obtained with the *Mycobacterium tuberculosis* type strain (H37Rv) and the BCG strain, as well as with the clinical isolates and their corresponding biochemical and morphological type strains, indicates that the PAG electrophoresis method is as reliable a measure

TABLE II
Comparison of Runyon's classification of atypical mycobacteria with grouping based on polyacrylamide gel electrophoresis

Runyon's groups		PAG-electrophoresis groups	
I	<i>Myco. kansasii</i> <i>Myco. marinum</i> (<i>balnei</i>)	1	<i>Myco. kansasii</i>
II	<i>Myco. scrofulaceum</i> Tap-water type		<i>Myco. scrofulaceum</i> Tap-water type
III	<i>Myco. avium</i> <i>Myco. intracellulare</i> (Battey) <i>Myco. terrae</i>	2	<i>Myco. avium</i>
		3	<i>Myco. intracellulare</i> <i>Myco. terrae</i>
IV	<i>Myco. fortuitum</i> <i>Myco. phlei</i> <i>Myco. smegmatis</i>	4	<i>Myco. fortuitum</i> <i>Myco. phlei</i>
		5	<i>Myco. smegmatis</i>
		6	<i>Myco. marinum</i> <i>Myco. balnei</i>

of genetic relatedness in mycobacteria as it appears to be in other organisms (Razin, 1968; King *et al.*, 1969; Sacks *et al.*, 1969). If this basic assumption is correct, then the other results presented can be used to examine the validity of the current classification of the so-called atypical mycobacteria. The findings, in general, agree with the usual classification into four groups (table II), but there are some exceptions and also some interesting implications of the gel pattern grouping.

The very similar patterns of the two scotochromogens, the photochromogen *Myco. kansasii* and *Myco. tuberculosis* lend support to the previously expressed belief that the chromogenic strains are mutants (Xalabarder, 1961; Tarshis, 1962; Weissfeiler, Karassova and Holland, 1964) or variants (Reimann and Ma, 1965) of *Myco. tuberculosis*. The similarity that we have demonstrated between *Myco. kansasii* and the scotochromogens is also consistent with the view that photochromogenicity is an unstable property and not suitable for classification

purposes (Hauduroy, Hovanessian and Roussianos, 1965). Scotochromogenic strains of *Myco. kansasii* have been reported (Tacquet, Tison and Devulder, 1965).

The differences between strains that have previously been grouped together are also important. *Myco. marinum* is photochromogenic and for this reason has been included in Runyon's group I (Wayne and Doubek, 1968). The results of PAG electrophoresis do not support this classification. *Myco. balnei* has been regarded as very similar to, if not identical with, *Myco. marinum* in various properties including antigenic structure (Lind and Norlin, 1963). They also have very similar gel patterns, which are sufficiently different from those of all of the other groups to justify their independent grouping—a separation that is further justified by their requirement for a lower growth-temperature than the other mycobacteria.

The differences shown here between *Myco. avium* and the other two non-chromogenic bacteria (*Myco. intracellulare* and *Myco. terrae*) suggest that *Myco. avium* should be placed in a separate group. This supports the view that *Myco. intracellulare* is sufficiently different from *Myco. avium* to justify the creation of two species (Runyon, 1967) rather than the continuation of the use of the epithet "Battey-*avium* complex".

Our results indicate a closer genetic relationship, within the non-chromogenic group III of Runyon, between *Myco. intracellulare* and *Myco. terrae* than between either of them and *Myco. avium*. On the other hand, the relationship between the pathogenic *Myco. intracellulare* and the rapid-growing *Myco. fortuitum*, which has also been implicated in human disease, is closer than that between *Myco. fortuitum* and *Myco. terrae*, which is generally considered to be non-pathogenic. Our results for the rapid growers would place *Myco. fortuitum* and the non-pathogenic *Myco. phlei* in the same group, and the non-pathogenic *Myco. smegmatis* in a separate group. There are thus indications of genetic relationships among the various atypical mycobacteria that are not always correlated with either colonial pigmentation, growth rate or pathogenicity.

We do not suggest that a new classification of the mycobacteria based solely on our results can be introduced at this time. The possible limitations and inaccuracies of the present biochemical and morphological grouping system should, however, be recognised and efforts made to evolve a classification that will reflect genetic relatedness.

SUMMARY

Polyacrylamide-gel electrophoretic patterns of the proteins of type strains of atypical mycobacteria and of clinical isolates were compared. Strains identified according to morphological and biochemical characteristics showed patterns identical with those of their respective type strains.

The various type strains could be grouped according to the degree of similarity shown by their protein-gel patterns. This grouping did not always correspond to the standard classification of Runyon.

The photo- and scoto-chromogens appear to be closely related to each other, and to be closer to *Myco. tuberculosis* than to the other atypical mycobacteria. Within the non-photochromogenic group, *Myco. avium* and *Myco. intracellulare* appear to be sufficiently different to justify their separation into different groups. Similarly, the rapid growers seem to form two distinct groups. It is suggested that the current system of classification of *Mycobacterium* spp. does not accurately reflect genetic relatedness between the different types.

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