Differential Identification of *Mycobacterium szulgai* and Other Scotochromogenic Mycobacteria

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Strains of scotochromogenic mycobacteria were studied by using numerical taxonomy methods in an attempt to more clearly define *Mycobacterium szulgai* and to find tests useful in identifying the species. In this study all strains of *M. szulgai* were strong reducers of nitrate, were slow in hydrolyzing Tween 80, and gave a high semiquantitative catalase reaction. Results obtained indicate that the use of increased pigmentation after 1 h of light exposure at 25 C and that the use of arylsulfatase activity are of questionable diagnostic value in separating the species from other scotochromogenic mycobacteria.

Marks et al. (6) have isolated and described a new species of mycobacterium which is pathogenic for man, having caused such diverse pathology as pulmonary disease, cervical adenitis, and olecranon bursitis. The organism, named *Mycobacterium szulgai*, resembled *M. gordonae* when first examined, but the distinctive thin-layer chromatography pattern of the extractable lipids suggested the unique character of this taxon. Subsequent investigations (1, 6, 7) described the organism as capable of reducing nitrates, of slowly hydrolyzing Tween 80, of producing a strong arylsulfatase reaction, and of being photochromogenic when grown at 25 C. Because the taxonomic status of the species was not clearly outlined, it was the purpose of this study to define *M. szulgai* in relation to other scotochromogens by means of numerical taxonomic methods, and to determine which in vitro tests might be most useful for the identification of this organism by the clinical public health laboratory.

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MATERIALS AND METHODS

Mycobacterial strains. Ninety-eight strains of scotochromogenic mycobacteria assembled for this study were distributed as follows: 12 strains of *M. flavescens*; 31 strains of *M. gordonae*; 37 strains of *M.*

scrofulaceum; 4 strains of *M. szulgai*; and 14 nonspeci-
ciated strains. Eleven strains included in this study were received from outside contributors and, because these cultures have been studied by several other investigators, they are specifically identified in Table 1 and in Fig. 1 and 2. The remaining 87 strains were obtained from cultures submitted for identification to the Mycobacteriology Branch, Center for Disease Control (CDC), Atlanta, Ga. These 87 strains are not specifically identified in any of the accompanying figures or tables.

The four strains of *M. szulgai* were plated first onto oleic acid albumin agar to determine if their colonial morphology differed significantly from that of other scotochromogens. Because some of these cultures yielded colonies with a morphology and consistency that differed from those of the original, they were numbered and studied as separate strains, and their identifying code numbers are listed in Table 1. Because of these five colonial variants, the total number of strains subjected to this taxonomic study was 103.

Numerical taxonomy. The methods and laboratory procedures used have been described previously (2-4, 8), except that the niacin test was performed by the paper strip method (Difco). The coding and processing of data was done by Grady Waters of the Computer Systems Office, CDC, Atlanta, Ga. All the strains were cultured in the Middlebrook and Cohn 7-H9 broth (Difco) before being transferred to specialized media for testing by a total of 52 taxonomic characters.

Photochromogenicity at 25 C. Because *M. szulgai* was later reported to be photochromogenic when tested at 25 C but scotochromogenic when grown at 37 C (7), this reportedly unique property was examined separately after completion of the initial numerical analysis, which had included a test for photochromogenicity at 37 C. Three Lowenstein-Jensen slants were inoculated with diluted suspensions of each strain under test. Two of the tubes were covered with black paper, and all tubes were incubated

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at 25 C. When growth was observed in unshielded cultures, one of the shielded tubes was exposed to light as recommended by Vestal (8).

Accumulation of carotenoid crystals. Schaefer et al. (7) also reported that colonies of *M. szulgai* produced red flecks of carotenoid crystals after prolonged exposure to light at 37 C. Consequently Lowenstein-Jensen slants were inoculated with diluted bacterial suspensions of each strain of *M. szulgai* and incubated at 37 C. When growth was detected, the cultures were exposed to light continuously for 8 weeks at the same temperature. Carotenoid crystals were then searched for by observing the colonies under the microscope.

Temperature relationships. The effect of temperature on growth was studied with a temperature gradient block (Lab Line Instruments, Inc., Melrose Park, Ill.). The temperature ranges were from about 22 to 49 C. The medium used was Middlebrook and

Cohn 7H9 broth (Difco), and the growth density was measured in a Coleman, Jr. model 6/20 spectrophotometer at 650 nm.

RESULTS

Numerical taxonomy. The numerical taxonomy based on the 52 characters used in this investigation separated 103 strains of mycobacteria into seven clusters, identified by letters A to G in Fig. 1 and 2. Figure 1 shows that these seven clusters segregated at correlation coefficients (4) between 65 and 85%. The evolution of the clusters at the 80, 85, and 90% levels of matching can be studied in the nxn matching matrix (4, 5) (Fig. 2).

Cluster A. Cluster A, formed by 39 strains, contained most of the representative isolates

TABLE 1. Source and identifying numbers of cultures received from outside contributors

Strain no.	Species	Contributor ^a	Contributors no.
17	<i>Mycobacterium flavescens</i>	Wayne	272
20	<i>M. flavescens</i>	Wayne	273
21	<i>M. flavescens</i>	Wayne	246
22	<i>M. flavescens</i>	Wayne	109
23	<i>M. flavescens</i>	Wayne	140
58	<i>M. scrofulaceum</i>	Pattyn	677 Sm Dy
93	<i>M. scrofulaceum</i>	Bonicke	SN 801
95, 96, 97, 98	<i>M. szulgai</i>	Wolinsky	954 ^b
99	<i>M. szulgai</i>	Wolinsky	996 ^c
100, 101	<i>M. szulgai</i>	Wolinsky	995 ^b
102, 103	<i>M. szulgai</i>	Wolinsky	1005 ^b

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^b From cultures 954 and 995 distinct colony types were isolated, numbered, and studied as independent strains. Strain 95 represents the parent culture 954 and strains 96 through 98 were colonial variants therefrom; strain 100 is the parent culture 995 and 101 was a colonial variant; strain 102 is the parent culture 1005 and 103 was a colonial variant.

^c Strain 99 (Wolinsky's 996) was derived from the type strain of *M. szulgai* NCTC 10831.

FIG. 1. Correlation coefficient dendrogram of 101 scotochromogenic strains and two nonchromogenic strains of mycobacteria selected for taxonomic analysis. Letters A through G identify the seven recognized clusters described in the text; the numbers 17, 20, 21, 23, 58, 93, and 99 refer to specific cultures identified in Table 1 as representative of the major clusters.

FIG. 2. Matching matrix of 103 strains of mycobacteria based on 52 test characters. The letters A through G and numbers listed on the vertical axis are explained in Fig. 1. The inserts for 80 and 90% levels of matching are included only to show that clusters are poorly defined at 80%, and, except for A, C, and a portion of D, are poorly organized at the 90% level.

previously identified as *M. gordonae* in the CDC culture collection.

Cluster B. Cluster B was a poorly defined cluster formed by two strains labeled as unidentified scotochromogenic mycobacteria in the CDC collection, and one strain identified as *M. gordonae* in the same collection.

Cluster C. The *M. szulgai* strain NCTC 10831 (identified as number 99 in Fig. 1 and 2) and all the other strains originally identified as *M. szulgai* were included in cluster C. Also included were two additional strains from the CDC collection which were previously classified as *M. gordonae*; one was isolated from sputum and the other from a diseased tropical fish.

Cluster D. Cluster D consisted of 29 *M. scrofulaceum* strains. These included strain 677 Sm Dy from S. R. Pattyn's collection and strain SN 801 from R. Bönicke's collection, identified in Fig. 1 and 2 by the numbers 58 and 93, respectively.

Cluster E. Two nonpigmented strains formed cluster E. The strains were not completely identified when this study was started and were included in it because some of their biochemical reactions resembled those described for *M. szulgai*. Further study of these strains led to their identification as *M. terrae*.

Cluster F. Cluster F is a well-defined cluster formed by four strains. Three of them were previously classified as *M. gordonae* and one as *M. scrofulaceum*. These strains were isolated from sputum, and although the properties of the strains included in cluster F are similar to those of *M. scrofulaceum*, they show special

characteristics which separate them from strains in cluster D and others (see Tables 2 and 3).

Cluster G. Cluster G was formed by 15 strains. All 12 strains of *M. flavescens* studied were included in it. The five strains received from L. G. Wayne are specifically identified in both Fig. 1 and 2.

Median reaction patterns. The median reaction patterns (2, 3) of the organisms included in the seven clusters identified above are shown in Table 2. These median reaction patterns were used to select those tests most useful in the differential identification of the species shown in Table 3.

Photochromogenicity at 25 C. Most of the strains tested formed yellow to orange pigment when grown at 25 C in the dark. However, some of them either failed to produce pigment or produced it only slightly. In some cases, pigment formation was affected by exposure to light at 25 C for 1 h, but the property was not unique for *M. szulgai* (Table 4). Table 5 shows the percentage of strains in each cluster that showed changes in pigmentation after 1 h of light exposure at 25 C.

Accumulation of carotenoid crystals. None of the 103 strains tested formed detectable carotenoid crystals after continuous exposure to light at 37 C for 8 weeks.

Growth and the effect of temperature. The optimal temperatures for growth (Fig. 3) of *M. flavescens*, *M. gordonae*, *M. scrofulaceum*, and *M. szulgai* were 33 to 35 C, 38 C, 37 to 38 C, and 36 to 37 C, respectively, and the maximal tem-

TABLE 2. *Percentage of positive results exhibited by seven groups of scotochromogenic mycobacteria in the indicated tests*

Tests	Mycobacterial cluster						
	A	B	C	D	E	F	G
Niacin	0	0	0		0	0	0
Nitrate reduction							
1+ or greater	0	0	100	11	100	0	73
3+ or greater	0	0	100	0	0	0	47
Catalase							
40 mm of bubbles or less	100	100	100	100	100	100	100
50 mm of bubbles or greater	82	67	100	75	100	75	83
68 C, 20 min	97	100	100	79	100	100	100
Tween hydrolysis							
Within 5 days	82	100	0	4	100	0	80
Within 10 days	92	100	55	4	100	0	80
Tween opacity							
After 1 week	0	0	0	0	50	0	87
After 5 weeks	18	33	82	46	100	100	93
Arylsulfatase							
3-Day reaction 1+ or greater	5	0	0	0	0	0	7
2-Week reaction $\pm$ or greater	97	67	73	54	100	100	87
2-Week reaction $\mp$ 3+ or greater	15	0	27	0	0	0	80
Pigmentation at 37 C, in the dark	100	100	100	89	0	100	80
Pigment increased after							
1 h of light exposure	0	67	0	11	0	0	13
2 Weeks of light exposure	82	67	82	43	0	25	33
Growth at 25 C	100	100	100	100	100	75	87
Growth at 37 C	100	100	100	100	100	100	100
Growth at 40 C	97	100	73	100	100	100	100
Growth in presence of: ^a							
CN (10 μ g/ml)	100	100	100	68	100	0	87
TCH (10 μ g/ml)	100	100	100	96	100	100	100
INH (0.2 μ g/ml)	100	100	100	100	100	100	100
INH (1.0 μ g/ml)	100	100	100	100	100	100	27
INH (5.0 μ g/ml)	13	67	27	86	100	100	27
SM (2.0 μ g/ml)	95	33	100	89	50	0	7
SM (10.0 μ g/ml)	59	0	0	14	0	0	7
PAS (2.0 μ g/ml)	64	100	9	57	100	25	87
Tellurite reduction							
Within 3 days	5	67	0	57	0	0	0
Within 6 days	18	67	9	68	0	0	60
Within 10 days	54	67	36	75	100	50	87
5% NaCl tolerance	3	0	0	0	0	0	47
Tolerance to oleate							
0.025%	100	100	100	100	100	100	100
0.050%	100	67	100	100	100	75	100
0.10%	90	67	100	96	100	50	87
Growth on:							
0.5% Nicotinamide	97	67	100	100	100	75	100
MacConkey agar	3	0	0	4	0	0	0
Malachite green agar	100	100	82	100	100	50	33
Methyl violet agar	79	67	91	100	100	0	20
Pryonin B agar	100	100	100	100	100	75	33
Eosin Y agar	100	100	100	100	100	100	100
Biebrich scarlet agar	100	100	100	100	100	100	100
Nitrite inhibition							
M/80	100	100	91	75	100	0	100
M/40	74	33	64	18	100	0	93
Growth on CMA	100	100	100	100	100	100	97

TABLE 2—Continued

Tests	Mycobacterial cluster						
	A	B	C	D	E	F	G
Colony morphology on oleic acid-albumin agar							
Smooth, S, T, D, or I type	100	100	73	96	0	100	80
Rough R type	0	0	18	18	100	0	60
Smooth K type	3	0	18	11	0	0	0
50% Acid-fast by Ziehl-Neelsen	100	100	100	100	100	100	100
Cellular morphology							
Short rods to coccoid	51	100	82	100	100	100	100
Medium to long rods	87	67	45	21	0	25	7
Growth rate at 37 C							
At 7 days or less	0	0	0	0	0	0	80
At 11 days or more	100	100	100	100	100	100	20

^a Abbreviations: CN, Amithiazone; TCH, thiophen-2-carboxylic acid hydrazide; INH, isoniazid; SM, streptomycin; PAS, *para*-aminosalicylic acid; CMA, corn meal agar.

TABLE 3. Reactions of the five major clusters of mycobacteria in nine selected tests^a

Mycobacterial cluster	Laboratory tests ^a								
	Growth at 7 days or less	Nitro-reductase 3+ or greater	Catalase 50 mm or greater	Tween 80 hydrolysis at 5 days	Growth in presence of INH (5 µg/ml)	Growth in presence of SM (2 µg/ml)	Growth on methylviolet agar	Growth on presence of M/80 NO ₂	Growth in presence of CN (10 µg/ml)
G (<i>M. flavescens</i>)	(+)80	±47	(+)83	(+)80	±27	-7	-20	+100	+87
C (<i>M. szulgai</i>)	-0	(+)100	+100	-0	±27	+100	+91	+91	+100
A (<i>M. gordonae</i>)	-0	-0	+81	(+)83	(-)17	+90	+79	+100	+100
D (<i>M. scrofulaceum</i>)	-0	-0	±75	-4	+86	+89	+100	±75	±68
F (<i>Mycobacterium</i> spp.)	-0	-0	±75	-0	+100	(-)0	(-)0	(-)0	(-)0

^a Abbreviations: INH, Isoniazid; SM, streptomycin; CN, amithiazone.

^b Symbols: -, 0 to 25% positive results; ±, 26 to 50% positive results; ±, 51 to 75% positive results; +, 76 to 100% positive results. Parentheses indicate key reactions for separation, arabic numbers following the + or - designations indicate actual percentage of strains positive in the tests.

TABLE 4. Colony pigmentation of selected strains from five clusters grown at 25 C

Strain no.	Mycobacterial cluster	Colony pigment at 25 C	
		In the dark	After 1 h of light exposure
1	A (<i>M. gordonae</i>)	Buff	Yellow
6	A (<i>M. gordonae</i>)	Buff	Yellow
32	A (<i>M. gordonae</i>)	Buff	Yellow
38	C (<i>M. szulgai</i>)	Buff	Yellow
95	C (<i>M. szulgai</i>)	Pale yellow	Yellow
96	C (<i>M. szulgai</i>)	Pale yellow	Yellow
99	C (<i>M. szulgai</i>)	Pale yellow	Yellow
100	C (<i>M. szulgai</i>)	Pale yellow	Yellow
101	C (<i>M. szulgai</i>)	Pale yellow	Yellow
48	D (<i>M. scrofulaceum</i>)	Yellowish	Yellow
60	D (<i>M. scrofulaceum</i>)	Pale yellow	Yellow
61	D (<i>M. scrofulaceum</i>)	Buff	Yellow
77	D (<i>M. scrofulaceum</i>)	Pale yellow	Yellow
78	D (<i>M. scrofulaceum</i>)	Pale yellow	Yellow
80	D (<i>M. scrofulaceum</i>)	Pale yellow	Yellow
73	F (<i>Mycobacterium</i> spp.)	Pale yellow	Yellow
23	G (<i>M. flavescens</i>)	Buff	Yellow

TABLE 5. Percentage of strains from the five major clusters that changed pigmentation after 1 h of light exposure at 25 C

Cluster	No. of strains in cluster	No. that changed pigmentation after exposure to light	Percentage changing after exposure to light
A (<i>M. gordonae</i>)	39	3	8.0
C (<i>M. szulgai</i>)	11	6	55.0
D (<i>M. scrofulaceum</i>)	28	6	21.0
F (<i>Mycobacterium</i> spp.)	4	1	25.0
G (<i>M. flavescens</i>)	15	1	7.0

peratures at which growth was observed for these same four species were 38.5, 39, 43.5, and 39 C.

DISCUSSION

Our results agree with previous numerical taxonomy studies of the scotochromogenic mycobacteria (4, 9), in that *M. flavescens* clearly segregated from clusters of *M. gordonae* and *M. scrofulaceum*, and other groups of scotochromogenic strains. Furthermore, we showed that the newly described species *M. szulgai* (6) is clearly segregated from clusters of other scotochromogenic species (Fig. 1 and 2).

However, not all the properties described previously for *M. szulgai* were confirmed by the methods used in the present investigation. For example, only 9% of the *M. szulgai* strains were found to be resistant to *para*-aminosalicylic acid (Table 2) although these bacteria were reported to be highly resistant to the drug (6, 7). In addition, contrary to reports that *M. szulgai* was a strong arylsulfatase producer (1, 6), the 3-day arylsulfatase test was negative (Table 2), and only 27% of the strains produced enough enzyme for a strong (3+ or greater) reaction in the 2-week test.

M. szulgai was described as capable of hydrolyzing Tween 80 slowly and weakly (6) and capable of hydrolyzing the substrate within 5 days if a large inoculum is used (7). However, when we emulsified a standard 4-mm loopful of culture from solid medium in 2.0 ml of Tween 80 substrate, none of the *M. szulgai* strains studied was able to hydrolyze the reagent within 5 days, and only 55% of them gave a positive reaction within 10 days (Table 2).

The property of photochromogenicity at 25 C attributed to *M. szulgai* was reported to be of great diagnostic significance (7). In our study *M. szulgai* formed less pigment when grown in the dark at 25 C, but was still considered by all three authors to have some pigment. Pigment, especially when pale, is a very subjective thing,

FIG. 3. Effect of temperature on the growth of the indicated mycobacteria. (a) *M. flavescens*; (b) *M. szulgai*; (c) *M. scrofulaceum*; (d) *M. gordonae*.

and this could account for differences from previous reports. Although the pigmentation of some strains was increased by 1 h of light exposure at 25 C, the phenomenon was not exclusive for *M. szulgai* and therefore was of uncertain diagnostic value (Tables 4 and 5). As judged from our data (Tables 2 and 3), the main characteristics that separate *M. szulgai* from other scotochromogens are a reaction of 3+ or more in the nitrate reductase test, failure to hydrolyze Tween 80 within 5 days, and a semi-quantitative catalase reaction of 50 mm or more.

Of the clusters recognized, cluster B is the most difficult to characterize. The median reaction patterns of this cluster was found to be very similar to that of cluster A (*M. gordonae*) except that cluster B organisms had a tendency to increase pigment formation after 1 h of exposure to light at 37 C, and were more susceptible to streptomycin. The failure to precisely characterize this cluster may be due to the fact that only two strains were examined. Cluster F was well defined and clearly segregated from the other clusters (Fig. 1 and 2). The reaction pattern of this group facilitated its separation from other groups (Table 2). Although the median reaction pattern of cluster F was similar to that of cluster D (*M. scrofulaceum*), the two groups were separable since organisms in cluster F failed to grow on methyl violet agar or in the presence of M/80 nitrite and were susceptible to streptomycin and amithiazone in concentrations of 2 and 10 µg/ml, respectively (Table 3).

A comparison of results in a limited number

of tests suggests that cluster B and F are different from "cluster 3" reported by Wayne et al. (9). It was not possible to compare cluster B or F to the IIIB organisms of Kubica et al. (4) since the latter were not completely described. Organisms in cluster F could represent a new species that may eventually be named when more strains exhibiting similar properties are isolated in the future. Conversely, the collection of more "cluster F-type" strains and the use of still more differential taxonomic features could also reveal this group to be only a subspecies of *M. scrofulaceum* differing in only a few characters from the parent taxon.

Since all previously described strains of *M. szulgai* were isolated from humans and were considered the agents of disease, it should be noted that one of the strains we recognized was isolated from tuberculosis-like lesions in a tropical fish.

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