Differential Identification of *Mycobacterium kansasii* and *Mycobacterium marinum*

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This report deals with the differential diagnosis between *Mycobacterium marinum* and *M. kansasii*. We found that the two species could be differentiated by using six main tests, namely, the nitrate reduction test, the arylsulfatase test, the ability to grow in the presence of 10.0 μg of amithiazole per ml, the ability to grow in the presence of 5.0 μg of kanamycin per ml, the temperature-ratio test, and the rate of growth on solid medium. In contrast to *M. kansasii*, considerable variation was observed among strains of *M. marinum*. However, the evidence obtained was not considered sufficient to justify the conclusion that more than one species was represented among the strains identified as *M. marinum*.

Isolated cases or epidemics of skin infections by *Mycobacterium marinum* Aronson, 1926 (reference 2; syn. *M. platypoeilus* Baker and Hagan, 1942 (4); *M. balnei* Linell and Norden, 1954 (22)) have been reported in the United States through the years (1, 8, 10, 23, 24, 28, 29, 30, 32, 33, 35, 36, 42). An epidemic of chronic skin granuloma from which *M. marinum* ("swimming-pool" granuloma or "mycobacteriosis balnearia") was cultured prompted a review of the clinical cases and isolates reported to the Center for Disease Control (CDC; Morbidity and Mortality Weekly Report, vol. 18, no. 41, 11 October 1969). Furthermore, several laboratories for which CDC serves as a reference laboratory were having difficulty distinguishing between *M. marinum* and *M. kansasii*. This situation indicated that useful bacteriological procedures needed to be investigated.

Both *M. marinum* and *M. kansasii* are photochromogenic mycobacteria (Runyon's group I) and are isolated in clinical microbiology laboratories, but their clinical significance differs. Whereas *M. kansasii* is chiefly associated with pulmonary disease resembling tuberculosis (41), *M. marinum* is rarely isolated from sputum. However, *M. marinum* has been found to be associated with chronic skin granuloma (3, 7–9, 11–17, 19, 23–25, 27, 29–37, 40, 42–45) which occasionally may resemble a sporotrichosis infection (1, 10, 28).

This report is concerned with the description of photochromogenic mycobacterial strains isolated recently from well-documented cases of chronic skin lesions in humans. The bacteriological study was undertaken (i) to establish the median reaction pattern of these strains by adopting the methods of numerical taxonomy; (ii) to compare the median reaction pattern exhibited by these strains to all others identified in this laboratory as *M. marinum*; (iii) to establish the median reaction pattern of *M. marinum*; (iv) to compare the median reaction patterns of the above with *M. kansasii*; and (v) to find procedures useful in discriminating between *M. marinum* and *M. kansasii*.

Because the epidemiological evidence available indicates that all of these chronic skin infections were acquired under conditions related to an aquatic environment (marine or fresh water environment), the photochromogenic mycobacteria which are the subject of this report will be collectively referred to as aquatic photochromogenic mycobacteria.

**MATERIALS AND METHODS**

*Mycobacterial strains*. The 202 photochromogenic strains used in this investigation were distributed as follows: 139 were strains of *M. kansasii* maintained in the repository culture collection of CDC, 34 were photochromogenic mycobacteria identified previously as *M. marinum* and also maintained in the culture collection, and the remaining 29 strains were recently isolated from cases of chronic skin infections in humans. The last two groups of 63 strains (34 plus 29) were reported by this laboratory as belonging to the species *M. marinum* and their sources are indicated in Table 1.

**Storage of cultures**. All strains in the collection were stored frozen at −20°C. The growth on Lowenstein-Jensen medium was suspended in about 1.0 ml of distilled water dispensed in Wheaton vials, and these were stored in a freezer at the above temperature.
Numerical taxonomy. Fifty-three characters were used and these are listed in Table 2. The methods and laboratory procedures employed in this study were described previously (18, 38).

Growth and the effect of temperature. One strain of *M. marinum* maintained in this laboratory for years, one strain recently isolated, and one strain of *M. kansasii* were inoculated in Middlebrook and Cohn 7H9 medium (Difco) dispensed in screw-capped test tubes (16 by 125 mm). The cultures were incubated at 35 to 36°C until the stationary phase of growth, and then 0.1 ml of culture was inoculated in 5.0 ml of the same medium dispensed in optically matched test tubes. The cultures were incubated at 22, 27, 33, 35, 37, and 40°C for 10 days. At daily intervals, the cultures were stirred in a Vortex, and the cell density was read in a Coleman Jr. spectrophotometer at 650 nm. The growth rate constant (K) was calculated from the slope of a semilogarithmic plot of the cell density against the time of incubation.

Drug susceptibility testing. The drug susceptibility testing was performed as indicated in Procedures for the Isolation and Identification of Mycobacteria (38) except that the working solution of rifampin was made in ethanol to a final concentration of 1.0 µg/ml. The cells were grown in Middlebrook and Cohn 7H9 medium (Difco) for 8 days, and appropriate dilutions of the cultures were plated in control and drug containing 7H10 medium dispensed in disposable quadrant petri dishes. The colony counts were made after 3 weeks of incubation at 35 to 36°C.

RESULTS

Numerical taxonomy: similarity index. The reactions of all strains of *M. kansasii* and *M. marinum* were compared to the median reaction pattern established for *M. kansasii* by finding the percent similarity to the median (S) value for each strain. S value of *M. kansasii* showed a spread from 40 to 100% among all photochromogenic mycobacteria studied (Fig. 1). This frequency distribution reveals two clusters of organisms, one at 40 to 80% and the other at 80 to 100%. The latter group, which contains the *M. kansasii* strains, indicates that the strains represent a homogeneous group in contrast to the other photochromogenic strains investigated. The group with a range of 40 to 80% in Fig. suggests a bimodal cluster; therefore, the data were further analyzed by finding the deviation of the S value of each strain from the average internal similarity (Si) of all strains which made up the *M. kansasii* group and from the Si of *M. marinum* strains. The frequency distribution of the deviation of the various strains is plotted in Fig. 2. The Si for *M. kansasii* was 90.9%, and the Si for *M. marinum* was 84%. The *M. kansasii* strains exhibited a Gaussian distribution, whereas all other photochromogenic mycobacteria were heterogeneously distributed. Identical results were obtained by comparing the S value of each strain to the Si value of *M. kansasii* or to the Si value of all other photochromogenic mycobacteria as a whole. These results indicated that, in contrast to data obtained for *M. kansasii*, the data referring to *M. marinum* should be further analyzed.

| Strain no. | Source | Observations |
|------------|--------|--------------|
| 16, 21, 22, 29, 34 | American Type Culture Collection | ATCC 927, 9823, 9819, 11564, 15100 Denver, Colo. |
| 16-7; 17-20 | Werner B. Schaefer, Ernest H. Runyon | Sender's no. 2693, 2692, 2514, 2337, 2314, 2312, 689, 2735, 2734, 2733, 2732, 2693 |
| 23 | Kevin Anderson | Salt Lake City, Utah |
| 38-59 | John H. Seabury | Adelaide, South Australia |
| 1 | Womack Army Hospital, Fort Bragg, Calif. | New Orleans, La. |
| 2 | U.S. Naval Hospital, San Diego, Calif. | No. 63 isolated from sputum |
| 3 | California Dept. of Public Health | Isolated from sputum |
| 3 | U.S. Naval Hospital, Pensacola, Fla. | |
| 3 | Florida State Board of Health | |
| 25 | Washington State Dept. of Health | |
| 26 | Virginia State Dept. of Health | |
| 28, 37 | Maryland State Dept. of Health | |
| 30, 35 | 1st U.S. Med. Lab., Maryland | |
| 31 | Louisiana U.S. Public Health Service Hospital | |
| 33 | Pennsylvania Dept. of Health | |
| 36 | Delaware State Board of Health | |
| 60-62 | Alabama Dept. of Public Health | |
| 24 | 3rd U.S. Army Hospital, Georgia | |
### Table 2. Properties exhibited by Mycobacterium marinum and M. kansasii in 53 tests

| Character | M. marinum (63 strains) | M. kansasii (139 strains) |
|-----------|-------------------------|--------------------------|
| Niacin    | - (22)*                 | - (2)                    |
| Nitroreductase, 1+ or > | (3) + (99)            |
| Nitroreductase, 3+ or > | 0 + (91)              |
| Catalase  | 40 mm or < of bubbles... | 0.5% Tween 5%             |
|           | 50 mm or > of bubbles... | Pigment              |
|           | After heating at 68 C... | Growth on marinum)   |
|           | 5 days                   | 40%                     |
|           | 10 days                  | 2-week                 |
|           | 1 week                   |                         |
|           | 5 weeks                  |                         |
| Arylesulfatase | 2-week reaction, ± or > | 2-week reaction, 3+ or > |
| Pigment increase after | + (97) + (99)       |
| 1 hr of light exposure | + (90) + (96)       |
| 2-weeks of light exposure | - (48) + (100)     |
| Temp of growth | 22 C + (96) - (15)  |
|            | 37 C + (100) + (100) |
|            | 40 C - (0) + (95)    |
| Growth on | Amithiazone (10 µg/ml) | + (81) - (5) |
|           | TCH (400 µg/ml)         | + (95) + (98) |
|           | TTC (10 µg/ml)          | - (18) - (38) |
| Growth in presence of | INH (0.2 µg/ml)       | + (97) + (85) |
|           | INH (1.0 µg/ml)         | + (94) - (27) |
|           | INH (5 µg/ml)           | + (71) + (57) |
|           | SM (2 µg/ml)            | + (66) + (92) |
|           | SM (10 µg/ml)           | - (3) + (50) |
|           | PAS (2 µg/ml)           | + (52) + (95) |
| Tellurite reduced | 3 days - (0) - (0) |
|           | 6 days + (21) - (4)    |
|           | 10 days + (68) - (15) |
| 5% NaCl tolerance | - (3) - (0)       |

### Table 2.—Continued

| Character | M. marinum (63 strains) | M. kansasii (139 strains) |
|-----------|-------------------------|--------------------------|
| Tolerance to oleate | 0.025% + (71) - (38) |
|            | 0.05% + (55) - (9)    |
|            | 0.1% - (31) - (2)     |
| Growth on 0.5% nicotinamide | + (94) + (80) |
| Growth on dye agar | MacConkey agar - (0) - (0) |
|            | Malachite green - (41) - (17) |
|            | Methyl violet - (27) + (93) |
|            | Pyronin B + (67) + (100) |
|            | Eosin Y + (92) + (99)  |
|            | Biebrich scarlet + (94) + (99) |
| Nitrite inhibition | 12.5 mm - (2) - (37) |
|            | 25 mm - (22) - (6)    |
| Growth on corn meal agar | - (8) - (5)       |
| Colony morphology on oleic acid-albumin-agar | Smooth S, T, D, or I |
|            | variety + (83) - (4)  |
|            | Rough R type - (38) - (12) |
|            | Smooth K variety - (12) + (85) |
| Pigmented in dark | - (0) - (1)       |
| 50% acid-fast by Z-N | + (98) + (100) |
| Cellular morphology on smear | Short rods to coccoid + (69) - (40) |
|            | Medium to long rods + (69) + (68) |
| Growth rate at 35°C | At 7 days or less + (67) - (0) |
|            | Greater than 7 days - (33) + (100) |

*a* Figures in parentheses represent percentage of positive results in the indicated tests. When 50% or more of the strains gave a positive result in the indicated test, the character was considered positive. Abbreviations: TCH, thiophen-2-carboxylic acid hydrazide; TTC, triphenyltetrazolium chloride; INH, isoniazid; SM, streptomycin; PAS, p-aminosalicylic acid; Z-N, Ziehl-Neelsen.

*b* Indicates per cent positive of strains tested.

### Numerical taxonomy: cluster analysis (M. marinum).

The S values between each pair of the organisms identified as M. marinum were arranged in decreasing order, as described by Lessel and Holt (21). and a differentially shaded similarity matrix was prepared (Fig. 3). Of the 63 strains, 43 were included in a large cluster which appeared to include three small ones. Surprisingly, M. balnei ATCC 11564 and M. marinum ATCC 927 did not seem to be related to the organisms included in the above clusters.

### Colony morphology.

The growth of mature colonies of the aquatic photochromogenic mycobacteria on Lowenstein-Jensen egg medium was

either rough and dry, smooth and hemispherical, or slightly wrinkled with a central elevation. Mixtures of these colony types were also observed. Colony types observed on 7H10 agar were very similar to the smooth Kw forms reported for M. kansasii (39). Usually, they had a granular or wrinkled surface and with transmitted light they exhibited a diffuse dark central spot. The rough R and smooth D-forms were also frequently found. Macroscopically, when grown in the dark, the colonies were buff, but they tended to have more pigmentation than M. kansasii strains.

All M. marinum strains studied gave only
restricted rough R growth in corn meal agar. On oleic acid-albumin-agar, the colonies were either smooth D, smooth S, a delicate-granular smooth S, rough R, or a mixture of these. The colony form more frequently observed by Navalkar et al. (26) was the delicate granular smooth S. The typical smooth K colony described previously in M. kansasii (20) was not observed in the M. marinum group of strains.

Growth and the effect of temperature. The effect of the incubation temperature on the growth rate of photochromogenic mycobacteria is shown in Fig. 4. The temperature at which the growth rate of the recently isolated strain was maximal (optimal temperature for growth) was identical to that of a strain maintained in the laboratory for a long time. The effect of temperature on the growth of one strain of M. kansasii is also depicted. These observations indicated that the optimal temperature for growth did not change with continued subculturing in the laboratory. Thus, M. marinum could be differentiated from M. kansasii by finding the ratio of the density of growth at 33 °C to the density of growth at 37 °C after a suitable incubation time (8 to 10 days). Because M. marinum exhibited a higher growth rate at 33 °C than at 37 °C, the temperature ratio as defined above should be higher than 1.0, and the opposite should be applicable to M. kansasii.

The results of the temperature-ratio test applied to 55 strains of aquatic photochromogens and 10 strains of M. kansasii are depicted in Table 3. The results of the experiments agreed with the expectations: the majority of the aquatic photochromogenic mycobacteria exhibited a temperature ratio over 1.0, whereas all M. kansasii strains exhibited a temperature ratio lower than 1.0.

Drug susceptibility pattern. As indicated in Table 4, the photochromogenic aquatic strains under investigation exhibited various degrees of resistance to isoniazid, streptomycin, and p-aminosalicylic acid. All were sensitive to kanamycin, and the majority of the strains were sensitive to all of the other drugs tested.

Differential diagnosis. All of the 202 strains of mycobacteria studied shared the common property of photochromogenicity. They could be divided into two large groups on the basis of the results of three tests: temperature ratio, nitrate reductase, and resistance to amithiazole. One group, corresponding to the species M. kansasii, was fairly homogeneous as determined by numerical taxonomy methods; all strains of M. kansasii exhibited a temperature ratio lower than 1.0, reduced nitrate to nitrates, and were sensitive to amithiazole. The other group, composed of 63 strains, was observed to be heterogeneous but shared the following common properties: a high temperature-ratio test (>1.0), inability to reduce nitrates to nitrates, and resistance to amithiazole. The key tests that can be useful in the differential diagnosis of M. kansasii and M. marinum are indicated in Table 5.

These few tests were selected because they could be performed in a public health or clinical...
FIG. 3. Differentially shaded similarity matrix of 63 *Mycobacterium marinux* strains. The figures correspond to the strain number indicated in Table 1. Key: ■, 90 to 100% S; □, 80 to 89% S; △, 70 to 79% S; □, below 70% S.

microbiology laboratory. None of these tests alone can be used to identify one of these species. Some points which may be useful in interpreting results from these tests are as follows. (i) Some strains of *M. marinux* may exhibit weak nitrate reductase activity, i.e., ±, 1+, 2+, which can be tolerated if the other tests fit the pattern for *M. marinux*. (ii) Occasional strains of *M. kansasii* may exhibit weak nitrate reductase (2+). (iii) The majority of *M. marinux* strains give at least ± activity in the 3-day arylsulfatase test. None of the *M. kansasii* possesses this characteristic. (iv) None of the *M. kansasii* strains has 3+ or greater 2-week arylsulfatase activity. In fact, most strains have little activity. (v) If subcultures of *M. kansasii* are heavily inoculated, growth can occur within 7 days. (vi) Some strains of *M. marinux* have a growth rate greater than 7 days on subculture at 35 to 37 C.

DISCUSSION

According to the investigations described in this report, about 78% of the photochromogenic mycobacteria isolated from chronic skin granuloma in humans appeared to belong to a tight cluster. The remaining strains exhibited various degrees of similarity to those included in the main cluster (cluster I, in Fig. 3). Surprisingly, the two strains of *M. marinux* included in the analysis for comparative purposes (*M. balnei*, Linell and Norden strain X, ATCC 11564, and *M. marinux* Arson, ATCC 927) exhibited a low overall similarity to the majority of strains isolated from human cases. Furthermore, three subclusters could be recognized within the main group. In addition, strains 57 and 59 were isolated from the same patient; strain 57 was a subculture of the initial isolate, and strain 59 was a single-colony isolate from the initial culture. Strain 57 was highly similar to the strains in cluster I, but strain 59 appeared to belong to a different species.

The above observations are difficult to interpret unless one assumes that selection of bacterial variants occurred at some time during the life cycle of the bacterial populations. This hypothesis seems to be supported by the observations about
strains 57 and 59. Since these photochromogenic mycobacteria appear to be accidental pathogens and the environments where infection occurred were of a different nature (fresh water fish tanks, swimming pools, a marine environment, and environments in which the relationship to injury or ecology is not clear), ecological factors may determine which mutant type will predominate at a given location. Yet some of the characters in these organisms appeared to be genetically stable because they were shared by all of the strains investigated.

Primary isolates of most strains of *M. marinum* require incubation at a temperature of 30 to 33 C (2, 3, 5, 6, 22), but subcultures will grow at 37 C. Thus, the temperature relationships that are characteristic of the species appear to lose their taxonomical value. However, the optimal temperature of growth characteristic of the species was shown not to shift from its norm (about 33 C). Therefore, the optimal temperature of growth also appeared to be a stable character. Indeed, an investigation of the effect of the temperature upon the growth of a laboratory strain and a recently isolated strain of *M. marinum* indicated that the temperature at which the growth rate was maximal (optimal temperature of growth) was the same for both. The results of this study suggested that the ratio of the cell density at 33 C to the cell density at 37 C could be, therefore, a valid and useful taxonomic character. This notion was confirmed by applying it to various strains of *M. marinum* and *M. kansasii*.

The results of the cluster analysis of *M. marinum* were interpreted by assuming that the various clusters would represent biotypes within a single species. These biotypes would be the consequence of selective pressures within the ecological niche in which the organism happened to be at the time.
human infection occurred. The alternative interpretation that each cluster represents a different species is also justified. However, the identity of M. marinum and M. balnei was definitely established before (5, 6, 26, 32), and all strains but one (ATCC 9823) exhibited 80% or more similarity to one or more of the strains included in the main cluster. Therefore, the evidence now available does not seem to justify the proposition that the collection of strains studied comprise more than one species. We conclude that, with the probable exclusion of the ATCC 9823 strain, all mycobacteria studied belong to the species M. marinum.

Some of the characters common to all M. marinum strains studied appeared to be useful in distinguishing M. marinum from M. kansasii, a species which was very homogeneous within the criteria adopted. The useful tests were the nitrate reductase which was positive for M. kansasii and negative for M. marinum, the arylsulfatase test which at 2 weeks was strongly positive for M. marinum and negative or weakly positive for M. kansasii, the amithiazone test which showed the M. kansasii strains to be sensitive and the M. marinum strains to be resistant, and the kanamycin test which showed the M. kansasii strains to be resistant and the M. marinum strains to be sensitive. The comparison of the optimal temperature of growth (assayed as a temperature-ratio test) and the rate of growth in solid medium was also found to be of differential value.

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