**Mycobacterium poriferae** sp. nov., a Scotochromogenic, Rapidly Growing Species Isolated from a Marine Sponge

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Five strains of a rapidly growing, orange, scotochromogenic *Mycobacterium* species were isolated from a marine sponge. Although they displayed similarities to *M. aurum* and *M. parafortuitum*, they proved to belong to a proposed new species, *Mycobacterium poriferae* sp. nov. (ATCC 35087). We distinguished the type strain of *M. poriferae* from that of *M. aurum* by its pattern of carbon sources used, acid production from carbon sources, and amidases, by its ability to tolerate 5% sodium chloride, its failure to use benzamide as sole nitrogen source, and its use of 1-serine as sole carbon and nitrogen source. We distinguished the type strain of *M. poriferae* from that of *M. parafortuitum* by its pattern of carbon sources used, acid production from carbon sources, and amidases, by its ability to tolerate 5% sodium chloride, its failure to grow at 42°C, its strong pigmentation, its failure to reduce nitrate, its failure to tolerate 0.025% hydroxylamine hydrochloride, its ability to use 1-serine as dual carbon and nitrogen source, and its failure to use acetamide as dual carbon and nitrogen source. The pattern of mycolic acid derivatives produced by acid methanolysis of whole organisms was that of the group typified by *M. avium* and which contains α-mycolates, ketomycolytates, and wax ester derivatives.

The demosponge *Halichondria bowerbanki* contains the bicyclic aryl carotenoid isorenieratene (lo), a pigment found from as a major carotenoid in certain *Mycobacterium* species and in the brown members of the Chlorobiaceae. Since a previous study showed that the carotenoid content in explants as a result of studies designed to isolate and identify pigmented mycobacteria associated with *H. bowerbanki*.

**MATERIALS AND METHODS**

Organisms. Living specimens of the marine sponge *H. bowerbanki*, known commonly as the “crumb-of-bread sponge,” were obtained from Gulf Specimen Company (Panacea, Fla.). They were used for the preparation of cell suspensions within 24 h of their arrival.

Primary isolation. A cell suspension was prepared from cut sponge pieces by mixing 1 volume of sponge pieces cut to approximately 3 by 3 mm and enough sterile seawater to make 3 volumes. The mixture was reduced to a suspension of cells and cell fragments by shearing with a Waring blender operated at low speed for 1 min. All equipment was sterile, and asptic techniques were used. This suspension then was inoculated by loop slants of Middlebrook 7-H-11 medium (1). Slants were incubated either in air or 5% CO₂ in air at 20 to 30°C for 4 to 12 weeks. Colonies of orange acid-fast organisms were purified by subculture on Tsukamura modified Sauton medium containing 5% (wt/vol) NaCl or medium supplemented with 1.0% (wt/vol) mannitol, 1.0% (wt/vol) sucrose, or 0.1 M monoethylamine.

Maintenance of strains. Pure stock cultures of *Mycobacterium poriferae* were maintained under refrigeration after 1 week of growth at 28°C in a 5% CO₂ atmosphere on tryptic soy agar (TSA) with 5% (vol/vol) glycerol added. Routine subculturing from original stocks stored at 4°C was carried out at least every 6 to 8 weeks. Freeze-dried type strains of *Mycobacterium aurum* (ATCC 23366) and *Mycobacterium parafortuitum* (ATCC 19686) were obtained from the American Type Culture Collection, Rockville, Md., and maintained on Löwenstein-Jensen medium, Ogawa egg medium, and Sauton agar for use in comparison tests with *M. poriferae* (see Table 2). Slants were incubated in air at 37°C for 2 to 4 weeks and then subcultured or stored in a refrigerator for future subculturing.

Characterization of isolates. Prior to their use in the tests listed below, all strains were examined for purity microscopically by both the Ziehl-Neelsen acid-fast and the Gram staining methods. The characterization for species identification was conducted after the method of Tsukamura (20), which is an Adansonian numerical classification approach. In all growth tests, the strains of *M. poriferae* were incubated at 28°C in air; at the appropriate reading times, tests were scored as positive or negative according to the outcome of at least two of the three replicate test cultures. When a test was negative at the first reading, it was continued for 2 more weeks and read again. Test results were used to determine percent similarity (S%) by the formula S% = (NS/NT) × 100, where NS is the number of characteristics with similar outcomes and NT is the total number of characteristics tested. With the use of a 90% phonon line, those strains showing S% greater than or equal to 90% were considered to be the same species and those differences found were considered interspecies variation. In the case of S% < 90%, the strains were considered different species exhibiting interspecies variation (22).

Acid fastness. In addition to the test of acid fastness by the Ziehl-Neelsen method, acid fastness was confirmed with the Kinyoun method and the Blair fluorochrome technique.

Selected cell lipids. A slightly modified Hecht and Causey method (2) was used to establish the presence of mycobacterial mycolic acids and the absence of LCN-A (lipid characteristic of *Nocardia* spp.). Modifications were the use of...
methanol rather than ethanol for esterification, measurement of the dry weight of extracted cells rather than unextracted, and evaporation in a rotary evaporator rather than an oven.

Further characterization of mycolic acids was done by the method of Minnikin et al. (7) with the methanolsysis carried out at 50°C for 16 h. Thin-layer chromatography was performed on Silica Gel G (Merck) activated for 20 min at 100°C and run in a solvent system of light petroleum (boiling point, 30 to 60°C)-acetone (97:3) in the first direction three times and toluene-acetone (97:3) once in the second direction.

Growth rate. Categorization for rapid or slow growth was made on Löwenstein-Jensen medium and Tsukamura modified Sauton medium (17). The Tsukamura technique (20) for matching the inoculations was used. The cultures were grown at 28 to 30°C in air. Rapid growth was defined as abundant growth on Löwenstein-Jensen medium in 4 or 7 days on Sauton medium. Tsukamura's definition of abundant growth was used throughout these studies; that is, the presence of macroscopically evident growth as opposed to maximal or heavy growth. A trace of growth was considered negative.

Temperature effect on growth. Cultures were incubated at 20, 25, 30, 35, 37, 40, and 45°C on Löwenstein-Jensen medium. A definite bacterial carpet after 1 week was considered positive; a trace of growth was considered negative.

Survival at 60°C. Löwenstein-Jensen slants were inoculated by loop and placed for 4 h in a 60°C water bath. The cultures were next incubated for 7 days at 28°C in air. Survivors showed abundant growth at 7 days.

Cell morphology. A smear of 1-week-old growth was made on a slide and heat fixed lightly. The slide was scored and broken to about 10 mm square, attached with colloidal silver foil. The bacteria were scored as scotochromogenic if pigment reaction to light was scored as negative.

Enzymatic activities. Arylsulfatase activity at 3 days, nitrate reductase after 2 h, and 68°C and semiquantitative catalase activities were tested as described by Vestal (28).

Effect of pH on growth. TSA with 5% (vol/vol) glycerol added was sterilized, and then the pH was adjusted aseptically with 10% potassium hydroxide or 10% (vol/vol) hydrochloric acid to produce media with pHs of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. A positive test result was the presence of abundant growth after 2 or 4 weeks of incubation.

Iron uptake. Iron uptake was tested as described by Vestal (28).

Tolerance to 5% sodium chloride. Cultures on Löwenstein-Jensen medium with and without 5% (wt/vol) sodium chloride were incubated at 28°C for 2 weeks. Growth on both media was scored as tolerance.

Tolerance to selected organic chemicals. Löwenstein-Jensen medium containing either 0.05 or 0.10% (wt/vol) sodium salicylate was inoculated and incubated at 28°C for 3 weeks. Abundant growth was scored as a positive test for salicylate tolerance. Tolerance to picric acid (18), sodium p-aminosalicylate (PAS) (14, 22), hydroxylamine hydrochloride (19, 22), and thiophen-2-carboxylic acid hydrazide (TCH) (22) was tested by standard methods.

Degradation of selected organic chemicals. The isolates were screened for degradation of sodium salicylate (17) and PAS (15, 16).

Tween hydrolysis. Tween hydrolysis was tested as by Wayne et al. (29) and Kilburn et al. (4).

Sole carbon and/or nitrogen sources. Organic acids were tested as sole carbon sources by the method of Tsukamura (23). Other nutrients also were tested by the method of Tsukamura (22).

Acid formation from carbon sources. The formation of acid from selected carbon sources was assayed by the method of Tsukamura (22).

RESULTS AND DISCUSSION

Seven acid-fast strains were isolated from the sponge cell preparations; of these, six survived and were characterized. Of the six, we concluded that five belong to the genus Mycobacterium and represent a heretofore undescribed species that is an orange, marine, scotochromogenic rapid grower. All five strains contained mycolic acids that precipitated from ether-ethanol, lacked LCN-A, and gave chromatographic patterns of their whole cell methanolsylates that matched established mycobacterial species. The results of 116 other tests of characterization on the five mycobacterial isolates are presented in Table 1. The type strain, no. 47, ranged in similarity to the other four strains from 94.0 to 97.4%. Overall the five strains' similarities to each other ranged from 92.3 to 97.4%.

The hypothetical mean organism (HMO) for the five strains was established by the method of Tsukamura and Mizuno (24). The HMO is positive for characters that are positive with frequencies of 60, 80, and 100% and negative for characters with positive frequencies of 40, 20 and 0% except for nicotinamidase activity, which is positive in the HMO. The type strain is the same as the HMO except that it is positive for 1,3-butylen glycol as carbon source. Strain no. 3 showed 96.6% similarity with the HMO; no. 6, 98.3%; no. 47, 99.1%; no. 49, 94.9%; and no. 80, 94.9%. This level of homogeneity is well above the 90% phenon line used routinely for species establishment as proposed by Tsukamura and others.

The type strain was chosen by a numerical approach. Since no. 47 had the highest average similarity among strains (96.4%), the highest similarity to the hypothetical mean organism (99.1%), and the highest value among the lowest similarities to the other strains (94.6% to no. 49 and no. 80), it was designated the type strain. It was deposited with the American Type Culture Collection as M. poriferae (ATCC 35087). The specific epithet which recognizes the phylum
Porifera was chosen in keeping with the custom of indicating the source of isolates in the binomial of many members of this genus.

Comparison of the results of the characterization tests with known species supports the conclusion that the sponges harbored a new Mycobacterium species. While the properties of *M. poriferae* placed it in the *M. parafortuitum* complex (1), it was too dissimilar to each member of the complex to represent any of them. *M. poriferae* is most similar to *M. aurum* and *M. parafortuitum* with 82.3% similarity to each based on a comparison of 87 characters (24). We performed a battery of tests to verify that the type strain of *M. poriferae* could be distinguished experimentally from the type strains of these two most closely related species. We chose those tests for which the HMO character of *M. poriferae* was different from *M. aurum* or *M. parafortuitum* (24) plus the assays for nicotinamidase activity since only 40% of the *M. poriferae* strains are positive despite the HMO's being positive. We also added a test for tolerance to 5% sodium chloride since *M. poriferae* was isolated from a marine environment. At least 9 differences were required between the type strains for less than 90% similarity. Since the tests we excluded from our battery represented reported similarities between the type strains, any variation from the literature results would only have increased the dissimilarity between the type strains. Our tests gave 12 differences from *M. aurum* and 16 differences from *M. parafortuitum* as shown in Table 2. Therefore, the *M. aurum* type strain was no more than 86.4% similar to the type strain of *M. poriferae*, and the *M. parafortuitum* type strain was no more than 81.8% similar.

We distinguished *M. poriferae* from *M. aurum* by its use of benzoate and malonate as sole carbon sources, its failure to use L-arabinose, galactose, and myo-inositol as sole carbon sources, its failure to produce acid from myo-inositol, and its inability to use L-arabinose, L-serine, acetamide, benzamidase, and its ability to use 1,3-butylene glycol, L-rhamnose, acid from carbon sources (L-arabinose, galactose, lactose, L-rhamnose, sorbitol), sole nitrogen sources (benzamide, nitrite), dual carbon and nitrogen sources (nicotinamide, acetamide, benzamide).

### TABLE 1. Characteristics of five strains of *M. poriferae*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>% Strains positive</th>
</tr>
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<tbody>
<tr>
<td>Acid fastness (Ziehl-Neelsen, Kinyoun, and Blair fluorochrome); Gram stain; rapid growth (Sauton, Lowenstein-Jensen); growth at 20, 25, 30, 35, 37°C: short rods; smooth colonies; pigmentation; scotochromogenicity; urease; catalase (qualitative, semiquantitative, and 68°C); growth at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5; iron uptake; 5% NaCl tolerance; salicylate tolerance (0.05 and 0.10%); picric acid tolerance (0.10 and 0.2%; PAS tolerance (0.2%); hydroxylamine hydrochloride tolerance (0.00625 and 0.0125%); TCH tolerance; Tween hydrolysis (7 days); sole carbon sources (acetate, pyruvate, citrate, succinate, malate, benzoate, malonate, fumarate, glucose, galactose, trehalose, mannose, d-xylene, sorbitol, fructose, sucrose, ethanol, L-propanol, propylene glycol, 1-butanol, glycerol); acid from carbon sources (glucose, mannitol, sucrose, d-xylene, mannose); sole nitrogen sources (L-glutamate, L-serine, acetamide, pyrazinamide, succinamide, nitrate); dual nitrogen and carbon sources (glucosamine, L-glutamate, L-serine, monoethanolamine, trimethylenediamine)</td>
<td>100</td>
</tr>
<tr>
<td>Pyrazinamidase; sole carbon sources (mannitol, 2,3-butylene glycol)</td>
<td>80</td>
</tr>
<tr>
<td>Sole nitrogen sources (nicotinamide, urea)</td>
<td>60</td>
</tr>
<tr>
<td>Nicotinamidase; sole carbon sources (1,3-butylene glycol, inositol); acid from carbon sources (inositol, trehalose)</td>
<td>40</td>
</tr>
<tr>
<td>Sole carbon source (L-arabinose)</td>
<td>20</td>
</tr>
<tr>
<td>Growth at 40 and 45°C; survival at 60°C; arsylurate; nitrate reductase; acetamidase; allantoinase; benzamidase</td>
<td>0</td>
</tr>
<tr>
<td>Isonicotinamidase; succinamidase; growth at pH 4.5 and 9.0; salicylate degradation (0.05 and 0.10%), PAS degradation; hydroxylamine hydrochloride tolerance (0.0250% and 0.0500%); sole carbon sources (isobutanol, 1,4-butylen glycol, L-rhamnose); acid from carbon sources (L-arabinose, galactose, lactose, L-rhamnose, sorbitol); sole nitrogen sources (benzamide, nitrite), dual carbon and nitrogen sources (nicotinamide, acetamide, benzamide)</td>
<td>40</td>
</tr>
</tbody>
</table>

* Four weeks until positive reading.
sponding to ketomycolates, ω-carboxymycolates, and 2-ecicosanol homologs with a smaller amount of material possessing α-mycolate mobility present. M. poriferae is unusual in sometimes having a weak α-mycolate spot in all strains and at other times having a strong α-mycolate spot. Production of a small amount of α-mycolate is a property also found in the newly described species M. vaccae (27). Among the other species in the M. avium group are two members of the M. parafortuitum complex, M. aurum and M. neoaurum (8). Two other members of the M. parafortuitum complex, M. parafortuitum and M. vaccae, fall into a different mycolic acid group, which contains α′-mycolates in addition to the types already named (8).

On the basis of clustered matching coefficients, Tsukamura et al. (26) grouped together M. fortuitum, M. aurum, M. abcessus, M. chelonei, M. phlei, M. parafortuitum, M. chubuense, M. aichense, and M. chitae. From this group M. poriferae shares the most similarity with M. aurum and M. parafortuitum, as stated previously; however, M. poriferae and M. fortuitum share an unusual morphological similarity. Both organisms have a non-acid-fast body (11), even though overall the similarity between them is only 74.2%. In acid-fast-stained M. poriferae samples it was an ovoid blue region found at the end of the red rod with the larger diameter and occurred typically in older cultures.

**Description of the type strain of Mycobacterium poriferae sp. nov. (no. 47 = ATCC 35087T).** Mycobacterium poriferae (po.rif.' er.ae, L. gen., of the Porifera, the phylum of sponges). This species, isolated from cell suspensions of a marine sponge, consists of rapidly growing, strongly acid-fast, gram-positive rods which frequently appear coccoid. No branching or Y-shaped cells occur, but cells may become enlarged on one end and display an ovoid, non-acid-fast body. Cells are 0.7 to 2.5 μm wide and 1.1 to 4.9 μm long. Nondividing cells in cultures are typically 2.2 to 2.3 μm wide by 2.7 to 3.1 μm long. Optimal growth occurs at 28 to 30°C on various media including Middlebrook 7-H-11 and tryptic soy agar with 5% (vol/vol) glycerol. A pH range from 5.0 through 8.5 is tolerated. Growth on Middlebrook 7-H-11 agar after 4 days produces smooth, moist, shiny, dome-shaped colonies with a nearly translucent apron which disappears on continued growth. M. poriferae is a strongly orange scotochromogen. High-molecular-weight mycolic acids precipitate with ether-ethanol in the range of 4.1 to 4.6 mg/50 mg of cells, LCN-A is absent, and two-dimensional thin-layer chromatography gives a mycolic acid pattern of the M. avium type, which has α-mycolates, ketomycolates, and wax ester derivatives, although some strains may have α′-mycolates present also.

M. poriferae produces catalase but is negative for arylsulfatase (3 days) and nitrate reductase. Urease, pyrazinamidase, and nicotinamidase are present, while acetamidase, allantoinase, benzamidase, isonicotinamidase, and succinamidase are absent. Sodium chloride (5%), salicylate, picric acid, PAS, and TCH are tolerated; neither salicylate nor PAS is degraded. Hydroxyamine is tolerated to 0.0125%. Iron uptake and Tween hydrolysis (7 days) are positive. Carbon sources utilized include acetate, pyruvate, citrate, succinate, malate, benzoate, malonate, fumarate, glucose, galactose, trehalose, mannnose, D-xylose, mannitol, sorbitol, fructose, sucrose, ethanol, 1-propanol, propylene glycol, 1-butanol, 2,3-butyylene glycol, and glyceral but not 1,3-butyylene glycol, 1,4-butyylene glycol, isobutanol, inositol, L-arabinose, or L-rhamnose. Acid is produced from glucose, mannitol, sucrose, D-xylose, and mannose but not from trehalose, inositol, L-arabinose, galactose, lactose, L-rham-

**TABLE 2. Differentiation of M. poriferae from M. aurum ATCC 23366T and M. parafortuitum ATCC 19686T**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>M. poriferae</th>
<th>M. aurum</th>
<th>M. parafortuitum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>H</td>
<td>%P</td>
</tr>
<tr>
<td>Growth at 42°C</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Acetamidase</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Allantoicinase</td>
<td>+</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td>Acetoin</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Malonate</td>
<td>+</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>1,3-Butylene glycol</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>2,3-Butylene glycol</td>
<td>+</td>
<td>+</td>
<td>80</td>
</tr>
<tr>
<td>Inositol</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Benzoate</td>
<td>+</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Succrose</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Acid from inositol</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Acid from arabinose</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

*Abbreviations: T, result of our comparison of type strains; H, HMO from Tsukamura and Mizuno (24); %P, percentage of positive strains, using data from Saio et al. (12) on the left of the column and from Tsukamura et al. (25) on the right.

* Previous tests indicate 3% NaCl; our panel used 5% NaCl.
FIG. 1. Two-dimensional thin-layer chromatogram of a whole-organism methanolysate of *M. portiera* (ATCC 35087). A triple development with petroleum ether (boiling point, 30 to 60°C)—acetone (97:3, vol/vol) in the first direction was followed by a single development with petroleum ether (boiling point, 30 to 60°C)–water. Abbreviations: A, α-mycolate; C, ketomycolate; D, ω-carboxymycolate methyl ester; E, 2-eicosanol and homologs; F, non-hydroxylated fatty acid methyl esters; ?, unknown component.

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LITERATURE CITED


