Laboratory Identification of Clinically Important Aerobic Actinomycetes

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A battery of morphological, physiological, and biochemical tests, including paper chromatographic analysis of whole cell hydrolysat es, was used to study 177 cultures of aerobic actinomycetes. One hundred thirty-five of the 177 were submitted as diagnostic laboratory specimens; of these, 129 were identified as belonging to 1 of 10 genus or species groups. The tests and procedures used were found to be relatively easy to perform and interpret. On the basis of the results, a flow chart was devised to permit the step by step identification of unknown clinical isolates of these organisms.

Aerobic actinomycetes produce a variety of diseases in man and animals. Until recently, the proper identification of these organisms has been difficult because of a lack of reliable differentiating tests, and workers in clinical microbiology laboratories have therefore been forced to depend upon morphological criteria that tend to be highly variable. During the past 15 years several investigators, most notably Gordon and his associates (3, 5–13, 22), have described a number of physiological tests useful in classifying some of the aerobic actinomycetes and have more precisely characterized the clinically important species. More recently Lechevalier and her co-workers (16–20) introduced analysis of whole cell hydrolysates for the stereosomer of diaminopimelic acid (DAP) and monosaccharides as an aid in identification; they also proposed some taxonomic changes on the basis of their work. They suggested the formation of a new genus, Actinomadura, to include the organisms formerly called Nocardia madurae, N. peltierii, and N. dassonvillei.

The purpose of the present study is to evaluate some of these tests and procedures to determine their value and practicality for the identification of aerobic actinomycetes by a diagnostic microbiology laboratory. A scheme was developed which rendered the identification of these organisms relatively easy and precise and which was adaptable to the needs and capabilities of individual laboratories.

MATERIALS AND METHODS

Nomenclature. The nomenclature used in this report is that recommended by Gordon and Lechevalier and their co-workers. The study organisms will be referred to by the following generic and species names: (i) Nocardia, including N. asteroides (13), N. brasiliensis (10), and N. caviae (12); (ii) Actinomadura (19, 20), including A. madurae, A. pelletierii, and A. dassonvillei (all previously classified in the genus Nocardia); (iii) Streptomyces, only including S. somaliensis, S. griseus, S. albus, S. fradiae, and a heterogeneous group of saprophytic Streptomyces sp. (7); (iv) Micromonospora sp. (20); (v) "farcinica group"; and (vi) "rhodochrous group." Farcinica group refers only to those organisms which produce the disease bovine farcy of Northern Africa. The recent discovery that they contain mycobacterial fatty acids (18) makes the genus uncertain. The species name farcinica has in the past been associated with cultures which are now thought to be N. asteroides (13). Rhodochrous group refers to the group of organisms described by Gordon (4, 9, 11); a suitable genus has not been agreed upon.

Cultures. A total of 177 cultures were studied. Of these, 42 were reference cultures obtained from stock culture collections; the sources are indicated in Table 1. The cultures received as N. rubra and N. corallina were considered to be in the rhodochrous group in accordance with Gordon (9). The other 135 cultures were obtained from the diagnostic laboratory (Mycology Section, Center for Disease Control) and were treated as "unKnowns." Although they were received over a 20-year period, most were submitted between 1970 and 1972. No attempt was made to study all the isolates received by the laboratory during this 2-year period, but most isolates of unusual species and a cross section of the more common species were examined. All of the 135 were from cases of human or animal disease.

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**Table 1. Sources of reference cultures**

| Culture                  | Source                                                                 |
|--------------------------|------------------------------------------------------------------------|
| *Actinomadura dassonvillei* | 90—Ruth Gordon, Rutgers University (1250); ATCC 22219                  |
|                          | 115—Ruth Gordon (509)                                                 |
|                          | 116—Ruth Gordon (714)                                                 |
| *A. madurae*             | 111—Ruth Gordon (1091)                                                |
|                          | 135—ATCC 25292A                                                       |
| *A. pelletieri*          | 86—Norman F. Conant, Duke University (989)                             |
| *Nocardia asteroides*    | 152—ATCC 3318                                                         |
|                          | 154—ATCC 19247                                                        |
| *N. brasiilensis*        | 15—A. Gonzalez-Ochoa, Instituto de Salubridad y Enfermedades Tropicales, Mexico City (4015) |
|                          | 17—A. Gonzalez-Ochoa (4031)                                           |
|                          | 18—A. Gonzalez-Ochoa (4057)                                           |
|                          | 19—A. Gonzalez-Ochoa (4051)                                           |
|                          | 20—A. Gonzalez-Ochoa (4076)                                           |
|                          | 21—A. Gonzalez-Ochoa (4125)                                           |
|                          | 26—A. Gonzalez-Ochoa (4153)                                           |
| *N. caucae*              | 6—Ruth Gordon (622)                                                   |
|                          | 8—Ruth Gordon (736A)                                                  |
|                          | 12—Ruth Gordon (736B)                                                 |
|                          | 115—Ruth Gordon (616)                                                 |
| *Streptomyces albus*     | 144—Ruth Gordon (1205)                                                |
| *S. fradiae*             | 137—Ruth Gordon (3554)                                                |
|                          | 140—Ruth Gordon (3555)                                                |
| *S. griseus*             | 80—Ruth Gordon (676)                                                  |
|                          | 81—Ruth Gordon (3320)                                                 |
|                          | 121—Ruth Gordon (3325)                                                |
|                          | 123—Ruth Gordon (3321)                                                |
| *S. somaliensis*         | 83—Ruth Gordon (719)                                                  |
|                          | 119—Ruth Gordon (1298)                                                |
|                          | 120—Ruth Gordon (1278)                                                |
| “Farcinica group”        | 28—Ruth Gordon (1223); F. Mariat, Inst. Pasteur, Paris (FA-4)         |
|                          | 29—Ruth Gordon (1226); F. Mariat (NF 5)                               |
|                          | 30—Ruth Gordon (1242)                                                 |
|                          | 32—Ruth Gordon (1359); F. Mariat (378 IP)                             |
|                          | 33—Ruth Gordon (1360); F. Mariat (396 IP)                             |
| “Rhodochrous group”      | 84—J. D. Schneidau, Tulane University (12008) - received as *N. rubra* |
|                          | 85—J. D. Schneidau (12009) — received as *N. rubra*                  |
|                          | 117—J. D. Schneidau (12001) — received as *N. rubra*                 |
|                          | 119—J. D. Schneidau (12004) — received as *N. rubra*                 |
|                          | 138—J. D. Schneidau (13003) — received as *N. corallina*              |
|                          | 139—J. D. Schneidau (13002) — received as *N. corallina*              |
|                          | 169—Ruth Gordon (7899) — rhodochrous                                   |
|                          | 170—ATCC 15908 - *Mycobacterium rhodochrous*                          |

**General tests.** All cultures were streaked on Brain Heart Infusion agar plates to determine purity before use. If bacterial contamination was noted or if two colony types were seen, individual colonies were picked and transferred to agar in tubes; the resulting culture was then tested. Test media were inoculated with a wire loop from 2- to 3-week-old cultures grown on Sabouraud dextrose agar, or with a Pasteur pipette from 3- to 14-day-old cultures grown in Trypticase soy broth in flasks on a rotary shaker. Except for temperature studies, all tests were carried out at room temperature; however, if isolates did not grow well at room temperature, an incubation temperature of 35 C was used. Most tests were performed at least twice for each culture.

**Morphological tests.** Gross morphology was observed after 2 to 3 weeks of growth on Sabouraud dextrose agar in cotton-plugged tubes. Microscopic morphology was examined by using slide cultures or direct examination of microcolonies on agar plates. Bennett agar (12) was used initially, but it was later found that tap water agar (20 g of crude agar in 1,000 ml of tap water) was better for the study of sporulation for cultures which were able to grow on this medium. Slide cultures were retained and examined for 2 to 4 weeks, and agar plates for 4 to 8 weeks. Acid-fast stains were made by the modified Kinyoun cold-staining procedure in which 1% sulfuric acid is used as the decolorizing agent (2).

**Physiological tests.** With minor modifications, these tests were performed by the methods of Gordon and associates (5, 6).

**Casein decomposition.** Ten grams of skim milk powder in 100 ml of distilled water and 2 g of agar in 100 ml of distilled water were autoclaved separately, mixed, and poured into four-compartment petri dishes (25 ml in a 15- by 100-mm dish). A large inoculum of a culture grown on Sabouraud dextrose agar was streaked across one of the quarter sections, and the plate was examined at 7 and 14 days for clearing of the casein underneath and around the growth.

**Tyrosine, xanthine, hypoxanthine decomposition.** Either 0.5 g of tyrosine or hypoxanthine or 0.4 g of xanthine was mixed with 10 ml of distilled water and autoclaved. This suspension was mixed with 100 ml of sterile nutrient agar (pH 7.0; peptone, 5 g; beef extract, 3 g; agar, 15 g; distilled water, 1,000 ml).

The mixture was allowed to cool to 50 C and then was poured into four compartment dishes as described above. Great care was taken to keep the crystals evenly distributed in the agar. The plates were inoculated as above and examined weekly for the clearing of granules underneath and around the inoculum; the tyrosine and hypoxanthine were observed for 4 weeks and the xanthine for 3 weeks.

**Hippurate decomposition.** Tubes with 5 ml of test broth (tryptone, 10 g; beef extract, 3 g; yeast extract, 1 g; glucose, 1 g; Na₂HPO₄, 5 g; sodium hippurate, 10 g; distilled water, 1,000 ml) were inoculated with cultures grown on solid media and incubated for 6 weeks. One milliliter of supernatant fluid was mixed with 1.5 ml of 50% (vol/vol) sulfuric acid, and the mixture was examined 4 h later. A positive result was
indicated by the appearance of benzoic acid crystals in varying amounts.

**Urea decomposition.** Tubes with sterile urea broth (BBL dehydrated) were inoculated with several drops of a broth culture suspension. The tubes were read at weekly intervals for 4 weeks and observed for the development of an alkaline reaction. An uninoculated tube was incubated with the tests for color comparison.

**Esculin decomposition.** Tubes with sterile esculin broth (esculin, 1 g; ferric citrate, 0.5 g; peptone, 10 g; NaCl, 5 g; distilled water, 1,000 ml) and control tubes containing the same base broth without esculin were inoculated in the same manner as for the urea test. The tubes were observed weekly for 4 weeks for the blackening of the broth.

**Lysozyme resistance.** Ninety-five milliliters of sterile glycerol broth (peptone, 5 g; glycerol, 70 ml; distilled water, 1000 ml) was mixed with 5 ml of lysozyme solution (100 mg of lysozyme [Difco] in 100 ml of 0.01 N hydrochloric acid sterilized by Seitz filtration), and the mixture was dispensed in test tubes. These tubes and controls containing glycerol broth without lysozyme were inoculated with one drop of culture suspension with a Pasteur pipette. Readings were made weekly for 4 weeks. The result was considered positive if good growth was noted in both tubes, negative if growth was good in the control tube but poor or absent in lysozyme, and equivocal if growth was poor in both tubes.

**Acid from carbohydrates.** The following base medium was used: (NH₄)₂HPO₄, 1 g; KCl, 0.02 g; MgSO₄·7H₂O, 0.2 g; agar, 15 g; distilled water, 1,000 ml. Fifteen milliliters of a 0.04% solution of bromocresol purple was added to each liter of base medium, and the pH was adjusted to 7.0. Then 0.5 ml of a 10% solution of each carbohydrate sterilized by Seitz filtration was added to tubes containing 5 ml of base medium. The tubes were inoculated with several drops of a broth culture suspension with a Pasteur pipette and incubated for 4 weeks. An acid reaction, indicated by the appearance of a yellow color, was considered positive. The following carbohydrates were used: arabinose, fructose, galactose, inositol, lactose, mannitol, mannose, rhamnose, sorbitol, trehalose, and xylose.

**Growth at 10 C.** Sabouraud dextrose agar slants were inoculated from broth cultures and immediately placed in a refrigerated water bath set at 10 C. Control tubes were inoculated and placed at room temperature.

**Whole cell hydrolysate analyses.** The cultures were grown in Trypticase soy broth in flasks on a rotary shaker for 3 days to 2 weeks and killed with 1% (wt/vol) Formalin. They were harvested by centrifugation, washed once in distilled water and then in 95% alcohol, and thoroughly dried in small petri dishes in a 45 C oven. To facilitate weighing, some of the dried preparations had to be ground with a mortar and pestle before hydrolysis.

**DAP analysis.** The method of Becker et al. (1) was used for a DAP analysis. Ten milligrams of the dried cells was placed in a 5-ml ampoule to which was added 1 ml of 6 N hydrochloric acid. The ampoule was sealed and put in an oven at 100 C for 18 h. After cooling, the hydrolysate was filtered with 4.25-cm filter paper attached to a 20-ml beaker with a paper clip. The material on the paper was washed with three or four drops of distilled water. The liquid hydrolysate was evaporated to dryness on a steam bath. To make sure all hydrochloric acid was removed, 1 ml of water was twice added and evaporated. The hydrolysate was reconstituted with 0.3 ml of distilled water, and 10 mliter was spotted on Whatman no. 1 chromatography paper. Ten microliters of 0.01 M dl-DAP (Sigma Chemical Co., St. Louis, Mo.), which contained both the meso- and L isomers, was spotted as a standard. Descending chromatography was performed by using methanol-water-10 N hydrochloric acid-pyridine (80:17.5:2.5:10 by volume) for 16 to 18 h. After the paper was dried, it was dipped in 0.2% acetic acid ninhydrin and heated in an oven at 100 C for 2 min to reveal the spots. DAP spots were olive green which faded to yellow; the L isomer moved several centimeters ahead of the meso-isomer. Other amino acid spots were puple; this color rapidly faded and these spots moved ahead of the DAP spots.

**Monosaccharide analysis.** This was performed according to the method of Lechevalier and Lechevalier (16) with minor modifications. One hundred milligrams of dried cells was placed in an ampoule with 2 ml of 1.0 N sulfuric acid. After it was sealed, the ampoule was put in boiling water for 2 h. Its contents were then transferred to a centrifuge tube, and saturated barium hydroxide was added dropwise until the pH was 5.0 to 5.5; this was determined with short-range pH paper and then checked with a pH meter. The mixture was centrifuged, and the supernatant was filtered into 50-ml beakers to which had been added 2 ml of chloroform as an anti-contami- nant. The material was evaporated to dryness in a 45 C oven overnight and reconstituted with 0.3 ml of distilled water. Thirty microliters of the hydrolysate was spotted on Whatman no. 1 paper. Ten microliters of a mixture of sugars (arabinose, galactose, glucose, mannose, rhamnose, ribose, and xylose), each in a 1% concentration, was spotted as a reference standard. A hydrolysate of a known culture of A. madurae or A. pelletieri was spotted as a reference standard for madurose. (Madurose was originally so named because it was found in A. madurae; it has recently been identified as 3-O-methyl-D-galactose [17].) Descending chromatography was performed with the organic (top) phase of a mixture of n-butanol-water-pyridine-toluene (5:3:3:4 by volume) as the solvent. After being developed for 48 to 54 h, the paper was dried and the spots were revealed by spraying with acid aniline phthalate (3.25 g of phthalic acid dissolved in 100 ml of water-saturated butanol plus 2 ml of aniline) and heating in an oven at 110 C for 3 min. Hexoses (galactose, glucose, mannose, and rhamnose) produced brown spots, and pentoses (arabinose, ribose, and xylose) yielded reddish spots. The spots moved from the origin in the following sequence: galactose, glucose, mannose, arabinose, xylose, ribose, and rhamnose (usually off the sheet). Madurose moved about the same distance as
xylose but was easily identifiable because it produced a brown spot.

RESULTS

Of the 135 isolates received from the Center for Disease Control diagnostic laboratory, 129 were identified on the basis of the results of the battery of tests performed with the 42 stock cultures as reference strains. The identities are shown in Table 2. Six cultures could not be definitely identified. Two of the cultures thought to represent *Micromonospora* sp. were subsequently sent to Mary Lechevalier at Rutgers University and confirmed by her as being correctly identified; these two then served as reference strains for this group.

*N. asteroides* was the most common species identified; next in frequency, in descending order, were *N. brasiliensis*, *Streptomyces* sp., and *A. madurae*. All of the *Streptomyces* sp. other than *S. somaliensis* resembled the reference strains of *S. griseus* and were distinctly different from the strains of *S. fradiae*. However, positive species identification was not attempted because characterization of the members of this genus is still incomplete. Six isolates were from countries other than the United States: three isolates of *A. pelletieri* (two from India and one from Australia), and one each of *A. dassonvilliei* (India), *A. madurae* (India), and *S. somaliensis* (Mexico).

Table 3 shows the results of the morphological, physiological, and biochemical tests for 12 genus or species groups, including all 171 identifiable cultures (42 reference and 129 diagnostic cultures). In this table the category *Streptomyces* sp. includes the reference strains of *S. griseus* and *S. albus* and all the diagnostic isolates that resembled *S. griseus*; *S. fradiae* and *S. somaliensis* are listed separately because there were some major differences between these species and the rest of the *Streptomyces*. In Table 4 the same results are presented for each group, excluding *S. fradiae*, with either "positive," "negative," or "variable" indicated for each test evaluated. A test was considered positive (+) for a group if 95% or more of the isolates gave positive results, and was considered negative (−) if 5% or less gave positive results; all other tests were considered variable (V).

Gross morphology was extremely variable and could be radically altered by change in media or temperature and growth in screw-capped test tubes. However, when the cultures were grown on Sabouraud dextrose agar in cotton-plugged tubes at room temperature (22–25 C), a few generalizations could be made. *A. madurae* and *A. pelletieri* were glabrous and varied from colorless to pink to bright red. *A. dassonvilliei* closely resembled many of the *Streptomyces* sp., with an abundance of grossly visible aerial mycelia. The rhodochrous group cultures were soft to mucoid, often colored yellow, orange, or salmon. The farcinica group cultures were of two morphological types. One type was whitish with a warty texture and extremely slow growing, whereas the other was creamy white, soft, and rapid growing. The four isolates of *Micromonospora* sp. were glabrous and orange, but as they aged a black color developed on the surfaces. The appearance of the *Nocardia* sp. and *Streptomyces* sp. did not permit generalization because of their great variability in texture, color, and presence of grossly visible aerial mycelia.

All of the isolates, except for some of those in the rhodochrous group, had a microscopic morphology consisting of well-formed filaments with true branching (Fig. 1). The rhodochrous group microscopically consisted either of just coccobacillary forms or of rudimentary filaments (Fig. 2). Microscopic aerial mycelia were abundant in most of the cultures of the three *Nocardia* sp., the *Streptomyces* sp., and *A. dassonvilliei* (Fig. 1). A short, sparse aerial mycelium was sometimes seen in the cultures of *A. madurae*, *A. pelletieri*, and the farcinica group and was rarely noted in the rhodochrous group. *Micromonospora* sp. produced no aerial mycelium. Spores borne on the aerial hyphae in medium and long chains were usually seen in the cultures of the *Streptomyces* sp. and *A. dassonvilliei* (Fig. 1). *Micromonospora* sp. showed the characteristic spores borne singly along the vegetative hyphae (Fig. 3). Sporulation was uncommon in the *Nocardia* sp. and *A. madurae*, and it was never seen with *A. pel-

### Table 2. Identity of cultures received from the diagnostic laboratory

| Culture                  | Isolates* |
|--------------------------|-----------|
| *Nocardia asteroides*    | 71        |
| *N. brasiliensis*        | 15        |
| *Streptomyces* sp.       | 14        |
| *Actinomadura madurae*   | 10        |
| *Nocardia caviae*        | 4         |
| "Rhodochrous group"     | 4         |
| *Micromonospora* sp.     | 4         |
| *Actinomadura dassonvilliei* | 3    |
| *A. pelletieri*          | 3         |
| *Streptomyces somaliensis* | 1     |
| "Farcinica group"       | None      |
| Unidentified             | 6         |

*Total, 135.*
| Determination                        | Actinomadura despensi | A. madurae | A. petieri | Micrococcus sp. | Micrococcus aerogenes | N. brasiliensis | S. fradiae | S. luteus | S. marcescens | Pediococcus group | Biochemical group |
|-------------------------------------|-----------------------|------------|-----------|----------------|-----------------------|----------------|------------|-----------|----------------|-------------------|------------------|
| No. of isolates                     | 6                     | 12         | 4         | 4              | 71                    | 22             | 8          | 19        | 2              | 4                 | 5                | 12             |
| Decomposition of                    |                       |            |           |                |                       |                |            |           |                |                   |                  |                |
| Casein                              | 69                    | 12         | 4         | 4              | 1                     | 22             | 0          | 19        | 2              | 4                 | 0                | 0              |
| Hypoxanthine                        | 6                     | 4          | 2         | 0              | 0                     | 15             | 8          | 19        | 0              | 0                 | 0                | 0              |
| Tyrosine                            | 6                     | 9          | 3         | 3              | 0                     | 22             | 0          | 19        | 2              | 4                 | 0                | 9              |
| Xanthine                            | 6                     | 0          | 0         | 0              | 0                     | 0              | 8          | 19        | 0              | 0                 | 0                | 0              |
| Esculin                              | 4                     | 12         | 2         | 4              | 69                    | 22             | 8          | 18        | 0              | 0                 | 5                | 8              |
| Urea                                | 2                     | 0          | 0         | 0              | 0                     | 50             | 21         | 8         | 10             | 0                 | 0                | 8              |
| Hippurate                           | 6                     | X          | X         | X              | X                     | 7              | 0          | X         | X              | X                 | 0                | 67             |
| Lysozyme resistance                 | 6                     | 0          | 0         | 0              | 0                     | 71             | 22         | 8         | 0              | 0                 | 0                | X              |
| Acid from                           | 4                     | 12         | 0         | 0              | 0                     | 1              | 0         | 0         | 13             | 2                 | 0                | 0              |
| Arabinose                           | 67                    | 100        | 0         | 0              | 0                     | 1              | 0         | 0         | 68             | 100               | 0                | 0              |
| Fructose                            | 10                    | 0          | 4         | 4              | 46                    | 22             | 8          | X         | 1              | 0                 | 2                | 11             |
| Galactose                           | 3                     | 0          | 0         | 0              | 0                     | 3              | 21         | 0         | X              | 1                 | 0                | 2              |
| Glucose                             | 6                     | 12         | 4         | 3              | 65                    | 22             | 8          | 19        | 2              | 3                 | 5                | 12             |
| Inositol                            | 0                     | 3          | 0         | 0              | 0                     | 1              | 21         | 8         | 6              | 0                 | 0                | 1              |
| Lactose                             | 0                     | 25         | 0         | 0              | 0                     | 1              | 96         | 100       | 32             | 0                 | 20               | 33             |
| Mannitol                            | 5                     | 12         | 0         | 0              | 0                     | 1              | 21         | 7         | 18             | 0                 | 0                | 2              |
| Mannose                             | 4                     | X          | X         | X              | X                     | 4              | X          | 2         | X              | X                 | 2                | 9              |
| Rhamnose                            | 4                     | 12         | 0         | 1              | 26                    | 0              | 0         | 9         | 0              | 0                 | 0                | 1              |
| Sorbitol                            | X                     | X          | X         | X              | 0                     | X              | 0         | X         | X              | X                 | 1                | 9              |

*Percent in italics, calculated on the basis of the number of isolates tested.

*Not tested.
| Determination                  | Actinomadura | A. madura | A. pellicera | Micromonospora | Nocardiopsis astroides | N. brasiliensis | N. caviae | Streptomyces | S. fedae | S. somaliensis | Furcincus group | Rhodochrous group |
|-------------------------------|--------------|-----------|--------------|----------------|------------------------|----------------|-----------|--------------|----------|----------------|------------------|------------------|
| Trehalose                     | 1            | 11        | 4            | 2              | 19                     | 22             | 7         | 18           | 0        | 0              | 1                | 7                |
|                               | 17           | 92        | 100          | 50             | 29                     | 100            | 88        | 100          | 50       | 0              | 20               | 58               |
| Xylose                        | 5            | 12        | 0            | 4              | 0                      | 0              | 0         | 18           | 2        | 0              | 1                | 1                |
|                               | 83           | 100       | 0            | 100            | 0                      | 0              | 0         | 95           | 100      | 0              | 0                | 8                |
| Growth at 10C                 | 3            | X         | X            | 4              | X                      | X              | X         | 8            | X        | 0              | 12               |                  |
|                               | 50           |           |              |                |                        |                |           |              |          | 0              | 100              |                  |
| Acid fast                     | 0            | 0         | 0            | 1              | 68                     | 21             | 5         | 2            | 2        | 0              | 5                | 9                |
|                               | 0            | 0         | 0            | 25             | 96                     | 96             | 63        | 11           | 100      | 0              | 100              | 75               |
| Morphology                    | Filaments    | 6         | 12           | 4              | 4                      | 66*            | 20*       | 8            | 11*      | 1*             | 2*               | 5*               |
|                               |              | 100       | 100          | 100            | 100                    | 100            | 100       | 100          | 100      | 100            | 100              | 17               |
| Aerial mycelium               | 6            | 10        | 2            | 0              | 63*                    | 20*            | 8         | 11*          | 1*       | 2*             | 2*               | 2*               |
|                               | 100          | 83        | 50           | 0              | 96                     | 100            | 100       | 100          | 100      | 40             | 25               |                  |
| Spores                       | 4            | 2         | 0            | 4              | 8*                     | 0              | 1         | 10*          | 0        | 1*             | 0                | 0                |
|                               | 67           | 17        | 0            | 100            | 12                     | 13             | 91        | 0            | 50       | 0              | 0                |                  |
| DAP                           | Meso         | 6         | 12           | 4              | 4                      | 66*            | 18*       | 8            | 0        | 0              | 0                | 3*               |
|                               |              | 100       | 100          | 100            | 100                    | 100            | 100       | 100          | 100      | 0              | 100              | 100              |
| L                             | 0            | 0         | 0            | 0              | 0                      | 0              | 19        | 2            | 4        | 0              | 0                |                  |
|                               | 0            | 0         | 0            | 0              | 0                      | 0              | 0         | 100          | 100      | 100            | 0                | 0                |
| Sugars in hydrolysate         | Arabinose    | 1*        | 0            | 0              | 4                      | 66*            | 18*       | 8            | 0        | 0              | 3*               | 8*               |
|                               |              | 17         | 0            | 0              | 0                      | 100            | 100       | 100          | 0        | 0              | 100              | 100              |
| Galactose                     | 3*           | 12        | 4            | 2              | 66*                    | 18*            | 8         | 8            | 0        | 0              | 3*               | 8*               |
|                               |              | 50         | 100          | 100            | 50                     | 100            | 100       | 100          | 50       | 0              | 100              | 100              |
| Madurose                      | 0            | 12        | 4            | 0              | 0                      | 0              | 0         | 0            | 0        | 0              | 0                | 0                |
|                               | 0            | 100        | 100          | 0              | 0                      | 0              | 0         | 0            | 0        | 0              | 0                | 0                |
| Xylose                        | 0            | 0         | 0            | 4              | 0                      | 0              | 0         | 0            | 0        | 0              | 0                | 0                |
|                               | 0            | 0         | 0            | 100            | 0                      | 0              | 0         | 0            | 0        | 0              | 0                | 0                |
| Glucose                       | 6            | 12        | 4            | 4              | 66*                    | 18*            | 8         | 16*          | 2        | 1              | 3*               | 8*               |
|                               | 100          | 100       | 100          | 100            | 100                    | 100            | 100       | 100          | 25       | 100            | 100              |                  |
| Mannose                       | 0            | 12        | 4            | 4              | 66*                    | 18*            | 8         | 11*          | 2        | 1              | 3*               | 8*               |
|                               | 0            | 100        | 100          | 100            | 100                    | 100            | 100       | 100          | 58       | 100            | 100              | 100              |
| Ribose                        | 6            | 12        | 4            | 4              | 66*                    | 18*            | 8         | 16*          | 2        | 2              | 3*               | 8*               |
|                               | 100          | 100       | 100          | 100            | 100                    | 100            | 100       | 100          | 50       | 100            | 100              |                  |

* Asterisk, some isolates were not evaluated for in situ microscopic morphology, growth at 10 C, or composition of whole cell hydrolysate.

* Trace amounts.
### Table 4. Results of morphological, physiological, and biochemical tests

| Determination                       | Actinomadura dassonvillei | A. madurae | A. pelletieri | Micromonaspora sp. | Nocardia asteroides | N. brasiliensis | N. caviae | Streptomycyes sp. | S. somaliensis | Farcinica group | Rhodochrous group |
|------------------------------------|---------------------------|------------|--------------|-------------------|-------------------|-----------------|-----------|-------------------|----------------|-----------------|------------------|
| **Decomposition of**               |                           |            |              |                   |                   |                 |           |                   |                 |                 |                  |
| Casein                             | +                         | +          | +            | +                 | -                 | +               | -         | +                 | -              | -               | -                |
| Hypoxanthine                       | +                         | V          | V            | -                 | -                 | V               | +         | +                 | +              | -               | V                |
| Tyrosine                           | +                         | V          | V            | +                 | +                 | -               | -         | -                 | +              | -               | V                |
| Xanthine                           | +                         | -          | -            | -                 | -                 | +               | +         | -                 | -              | -               | -                |
| Esculin                            | V                         | +          | V            | V                 | V                 | +               | V         | -                 | -              | +               | V                |
| Urea                               | V                         | X          | X            | X                 | X                 | X               | X         | V                 | X              | X               | X                |
| Hippurate                          | +                         | X          | X            | X                 | X                 | X               | X         | V                 | X              | X               | X                |
| Lysozyme resistance                | -                         | -          | -            | -                 | +                 | +               | -         | +                 | -              | X               | X                |
| Acid from                          |                           |            |              |                   |                   |                 |           |                   |                 |                 |                  |
| Arabinose                          | V                         | +          | +            | -                 | -                 | V               | -         | V                 | -              | V               | V                |
| Fructose                           | X                         | V          | -            | +                 | V                 | +               | X         | +                 | -              | V               | V                |
| Galactose                          | X                         | V          | -            | -                 | -                 | V               | -         | V                 | -              | V               | V                |
| Glucose                            | +                         | +          | +            | V                 | +                 | +               | V         | +                 | +              | +               | +                |
| Inositol                           | -                         | V          | -            | -                 | -                 | +               | V         | +                 | -              | -               | -                |
| Lactose                            | -                         | V          | -            | -                 | -                 | +               | V         | +                 | +              | +               | +                |
| Mannitol                           | V                         | +          | +            | V                 | -                 | V               | -         | V                 | V              | -               | -                |
| Mannose                            | X                         | X          | X            | X                 | V                 | V               | X         | V                 | V              | X               | X                |
| Rhamnose                           | V                         | +          | -            | V                 | V                 | -               | -         | V                 | V              | -               | -                |
| Sorbitol                           | X                         | X          | X            | X                 | X                 | -               | V         | V                 | V              | X               | V                |
| Trehalose                          | V                         | V          | +            | V                 | V                 | +               | V         | -                 | V              | V               | V                |
| Xylose                             | V                         | -          | -            | +                 | +                 | -               | -         | +                 | -              | -               | -                |
| Growth at 10°C                     | V                         | X          | X            | X                 | X                 | X               | V         | -                 | +              | +               | +                |
| Acid fast                          | -                         | -          | -            | V                 | +                 | V               | V         | +                 | -              | -               | -                |
| Morphology                         |                           |            |              |                   |                   |                 |           |                   |                 |                 |                  |
| Filaments                          | +                         | +          | +            | +                 | +                 | +               | +         | +                 | +              | V               | V                |
| Aerial mycelium                    | +                         | V          | V            | -                 | -                 | +               | V         | V                 | V              | V               | V                |
| Spores                             | V                         | V          | -            | V                 | -                 | V               | V         | V                 | V              | V               | V                |
| DAP                                |                           |            |              |                   |                   |                 |           |                   |                 |                 |                  |
| Meso                               | +                         | +          | +            | +                 | +                 | +               | +         | -                 | -              | +               | +                |
| L                                  | -                         | -          | -            | -                 | -                 | +               | -         | -                 | -              | -               | -                |
| Sugars in hydrolysate              |                           |            |              |                   |                   |                 |           |                   |                 |                 |                  |
| Arabinose                          | -                         | -          | -            | +                 | +                 | +               | +         | +                 | +              | +               | +                |
| Galactose                          | V                         | +          | V            | +                 | +                 | +               | V         | -                 | -              | -               | -                |
| Maltose                            | +                         | +          | +            | V                 | -                 | -               | -         | -                 | -              | -               | -                |
| Xylose                             | -                         | -          | -            | +                 | V                 | -               | -         | -                 | -              | -               | -                |

*Symbols: +, positive; -, negative; V, variable; X, not tested.*

Letieri, the farcinica group, or the rhodochrous group.

Most of the cultures of *N. asteroides*, *N. brasiliensis*, and the farcinica group and none of the cultures of *A. dassonvillei*, *A. madurae*, *A. pelletieri*, or *S. somaliensis* were judged to be acid fast. *N. caviae*, the rhodochrous group, *Micromonaspora* sp., and the other *Streptomycyes* sp. were variable in their reactions. Difficulties in the interpretation of the acid-
Fig. 1. Slide culture of a Streptomyces sp. on tap water agar, showing branching filaments, abundant aerial mycelium, and long chains of spores, some of which have broken off. Aerial mycelia appear darker than the vegetative, and many of them are out of the plane of focus. ×400.

Fig. 2. Slide culture of an isolate of the "rhodochrous group" on tap water agar. There are some filaments but they are short and not branching. Aerial mycelia are absent. ×400.
fast stain were frequently encountered, and a culture which was positive or negative when first tested often gave an equivocal result on a second trial.

For the most part, the physiological tests were easy to perform and yielded reproducible results. A notable exception was the test for growth at 10°C; inconsistent results were sometimes obtained on repeat trials despite the fact that all conditions were kept constant. The cultures of the farcinica group and an occasional isolate of *A. madurae* and *A. pelletieri* did not grow well in the lysozyme base broth, and in these cases the test was uninterpretable.

Whole cell analyses for DAP and monosaccharides presented little difficulty in technique or interpretation. There was never any doubt as to whether a hydrolysate contained the meso- or L isomer of DAP. As reported by Lechevalier and Lechevalier (16), sugar hydrolysates of most of the cultures contained glucose, mannose, and ribose. Arabinose, galactose, madurose, and xylose were the diagnostic sugars. Madurose was identified only in the *A. madurae* and *A. pelletieri* isolates; although it was identified in all of them, it occasionally produced only a very faint spot. A few isolates of *Streptomyces* sp. and *A. dassonvillei* yielded small amounts of galactose, and one isolate of the latter contained a faint trace of arabinose, but this was not considered diagnostically significant.

Of the six cultures which could not be definitely identified with the tests and procedures outlined above, one was subsequently identified by Ruth Gordon as a rare, and as yet unnamed, species of *Nocardia*. Two others closely resembled *A. dassonvillei* except that the whole cell hydrolysates contained arabinose and galactose. Of the remaining three, one resembled the cultures in the "rhodochrous group" and two were probably not true actinomycetes.

**DISCUSSION**

Of the 135 cultures received from the diagnostic laboratory, 129 (more than 95%) were positively identified as belonging to one of the major genus or species groups by using a battery of morphological, physiological, and biochemical tests. The selection of isolates to be studied from among all the cultures submitted to the diagnostic laboratory was not random but was deliberately weighted to include most of the unusual or atypical isolates. If
selection had been random, the percentage of unidentifiable isolates would probably have been even lower.

The test results obtained for the various genus and species groups were similar to those reported by Gordon in her series of publications (4–13). The only major discrepancies were the inability of the present study to differentiate \textit{A. dassonvillei} from \textit{S. griseus} by growth at 10 C and the variable results obtained here for the decomposition of hypoxanthine by \textit{A. madurae}, \textit{A. pelletieri}, and \textit{N. brasiliensis}.

On the basis of the data derived from the examination of the 171 identifiable cultures of aerobic actinomycetes (129 diagnostic plus 42 reference strains) a flow chart was prepared for the step by step identification of unknown clinical isolates (Fig. 4). With this chart it should be possible to correctly identify most of the cultures recovered or received by clinical laboratories. It must be noted, however, that by its very nature a flow chart is an oversimplified collection of data. Not every isolate of \textit{N. brasiliensis} (or \textit{A. madurae}, for example) will exactly correspond to the results in the chart. At times it may be necessary to refer to Table 3 to complete the identification of an atypical isolate by using some of the other tests.

A careful evaluation of the microscopic morphology of a culture must be performed in conjunction with the use of this flow chart. It is essential to establish that a particular isolate, especially if it is in the casein (–), xanthine (–), and tyrosine (–) group, is, in fact, an aerobic actinomycete and not a \textit{Mycobacterium} sp., a \textit{Corynebacterium} sp., a member of the rhodochrous group, or another bacterium that can give the appearance of being filamentous. Most \textit{Nocardia} sp. develop well-formed, branched filaments with aerial mycelium which is sometimes visible grossly. \textit{Mycobacterium} sp. and \textit{Corynebacterium} sp. generally have none of these features (9). With most isolates this morphological differentiation is obvious, but sometimes it is difficult if not impossible (Fig. 5). So far there is no reliable battery of physiological tests to make the differentiation. Analysis of the whole cell hydrolysates is generally not helpful, because the mycobacteria and some of the corynebacteria give results identical to those obtained with the nocardia (19). Study of cell wall lipids seems promising but is not yet practical for routine use (18).

The rhodochrous group, an entity described and discussed by Gordon (4, 9, 11), presents special problems. These organisms have a variable microscopic morphology intermediate between nocardia and mycobacteria, but generally they resemble the mycobacteria more closely. Their gross appearance—soft texture with orange, yellow, or pink color—may be helpful in identification when it is typical. Unfortunately, this group is not well characterized by physiological tests, although many of the isolates are positive for tyrosine decomposition, production of acid from sorbitol, and growth at 10 C. Whole cell hydrolysate analysis yields the same results with this group as with the nocardia and mycobacteria. The definite identification of a culture as a member of the rhodochrous group is thus often very difficult and must sometimes be established mainly by excluding \textit{Nocardia} sp., \textit{Mycobacterium} sp., and other more well-defined groups.

The farcinica group is not listed in the flow chart. Although this group cannot be differentiated from \textit{N. astroides} by the physiological or biochemical tests discussed here, the slow growing type is distinctive in its gross appearance and growth rate, and the rapid-growing type would be most likely confused with one of the rapid growing mycobacteria. Indeed, there is biochemical evidence that the farcinica group is in fact a species of mycobacteria (18). These organisms are encountered only in Northern Africa as far as is known and should not be a problem for most clinical laboratories.

The identification of some of the rarer aerobic actinomycetes, such as \textit{Micromonospora} sp., \textit{Microbispora} sp., and \textit{Micropolyspora} sp., still depends to a great extent upon morphology because these organisms have not been extensively studied with physiological tests. Whole cell sugar analysis is also useful in recognizing these genera.

Since few clinical microbiology laboratories have the personnel to set up and routinely perform the large battery of tests and biochemical procedures necessary for the use of the flow chart (Fig. 4), a simplified version of the diagnostic scheme was devised (Fig. 6). This system can be used if a culture fulfills both of the morphological criteria outlined in this flow chart, i.e., if it is a \textit{Nocardia} sp., \textit{Actinomadura} sp. or \textit{Streptomyces} sp. It involves only three physiological tests and a properly performed acid-fast stain, and it should make possible the identification of most of the common species encountered in the United States. The addition of whole cell analysis for the stereoisomer of DAP would greatly increase its accuracy. Obviously, cultures which cannot be speciated by using this chart and those for which the results

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(9) S. Gordon, J. Bacteriol. 67, 713 (1954).

(11) S. Gordon, J. Bacteriol. 68, 166 (1954).

(12) S. Gordon, J. Bacteriol. 69, 106 (1956).

(13) S. Gordon, J. Bacteriol. 70, 134 (1955).

(18) S. Gordon, J. Bacteriol. 71, 266 (1955).
**Fig. 4a.** Detailed scheme for identification of aerobic actinomycetes.
Fig. 4b. Detailed scheme for identification of aerobic actinomycetes. Isolates which are casein negative. Footnote 1, several of these have been studies by Ruth Gordon (13).
Fig. 4c. Detailed scheme for identification of aerobic actinomycetes. Isolates which are casein positive and xanthine negative. Footnote 1, formerly called Nocardia turbata (19).
are equivocal or uninterpretable must be referred to a reference laboratory for more detailed evaluation.

A battery of diagnostic tests has no value unless the individual tests are properly carried out. The importance of using positive and negative controls for all tests cannot be overemphasized. Test results can be influenced by a variety of factors, including temperature, constituents of the base medium, pH, time of incubation, and size of inoculum: these factors must be kept constant to insure reliability. False-negative results can be produced by failure of the inoculum to grow well on the test medium. A variety of confusing results can be caused by apparent mold or bacterial contamination or by two actinomycetes mixed in one culture; whenever a "problem" culture is encountered, it should be streaked on an agar plate to investigate these two possibilities. All of the tests used in this study should give clearly positive or negative results, so any equivocal results must be repeated.

There are some specific precautions which apply to particular tests. The acid-fast stain, long considered of primary importance in the identification of nocardia, is, in the opinion of this author, one of the most difficult tests to standardize and interpret. It should only be performed with positive and negative controls and should be used for diagnostic purposes only in conjunction with other tests. Because gross morphology is variable, its evaluation is of limited value and is useful mainly as a means of making a tentative identification before more definitive tests are completed. The tests for decomposition of casein, xanthine, hypoxanthine, and tyrosine necessitate a large inoculum, because areas of positivity may be visible only in areas surrounding large fragments of the growth. Since agar thickness can affect the length of time needed for the test to appear positive, this should be kept constant. Casein tests which become positive only after 14 days should be considered equivocal because rare isolates of N. asteroides give this result. For testing resistance to lysozyme, use of a very small inoculum makes evaluation of the amount of growth easier.

The techniques for paper chromatographic analysis of whole cell hydrolysates for the stereoisomer of DAP and for monosaccharides presented no special problems when performed carefully. Once this technique had been learned by laboratory personnel, the analyses required less time to perform and were easier to interpret than the conventional physiological tests. Most large hospital laboratories have the

Fig. 5. Slide culture of an isolate of Mycobacterium fortuitum on tap water agar. This morphology resembles that of a Nocardia sp., but the branches are very short and no aerial mycelia are present. ×400.
ISOLATES WITH:
1) Well-formed, branching mycelium with or without aerial mycelium
2) No spores or spores formed in chains on aerial mycelium

Casein -
Xanthine +
Urea +
N. caviae

Casein -
Xanthine -
Urea + or -
N. asteroides

Casein +
Xanthine -

Urea + and acid fast +
N. brasiliensis

Urea -
and/or acid fast -

Uncertain — probably may be:
A. madurae
Streptomyces sp.
S. somaliensis
A. pelletieri
other

Casein +
Xanthine +
Urea + or -

L - DAP
Streptomyces
sp.

meso - DAP

Uncertain may be:
A. dassonvillei

Fig. 6. Simplified scheme for the identification of aerobic actinomycetes.
capacity to carry out these analyses, and it is urged that more laboratories adopt them for routine use.

This study was not intended to determine the frequency of isolation of the various aerobic actinomycetes in the laboratory or to evaluate their clinical significance. However, several observations could be made about these points. As would be expected, more than half of the isolates were *N. asteroides*, mostly from patients with suspected pulmonary or disseminated disease. *N. brasiliensis* was commonly found and was usually associated with superficial abscesses rather than true mycetomas. *A. madurae*, which has been considered important mainly as an agent of mycetoma in the United States (14), was isolated most frequently from sputa; the clinical significance of these isolations is not known. *Streptomyces* sp., all resembling *S. griseus*, were isolated from various kinds of specimens and were probably all saprophytes. Because selection of the cultures to be studied was not random, the actual frequency of the *Streptomyces* sp. is probably greater than represented here. The clinical significance of the few isolates of *N. caviae* and *A. dassonvillei* is unknown. Several of these were from sputum and blood cultures, but there was no evidence that they were pathogens in these cases. The few isolates of *A. pelletieri*, *S. somalensis*, and the farcinica group were of foreign origin.

The rhodochrous group of organisms may be more common in the diagnostic laboratory than these data indicated. Many isolates are submitted to mycobacteriology or general bacteriology laboratories instead of mycology, but the recent observation that these organisms are more closely related to nocardia may change this situation (18). There are no documented reports of human or animal disease caused by members of this group.

Finally, the isolation of four *Micromonospora* sp. is noteworthy. Before the analysis of whole cell hydrolysates was used as a diagnostic tool, many of these may have been discarded as unidentified saprophytes. Their frequency in clinical specimens thus may be much greater than was heretofore presumed. Indeed, several more suspected isolates have been found since the completion of this study. It will be interesting to see whether continued use of chromatographic analysis and careful examination of microscopic morphology result in the more frequent identification of other actinomycetes rarely encountered in the clinical laboratory, e.g., *Micropolyspora* sp., *Thermoactinomyces* sp., and *Microbispora* sp. Although these organisms are not known to invade tissue, some of them have been implicated as causes of allergic pulmonary disease (15, 21). Further studies are needed on the prevalence of these organisms in humans and on their clinical significance.

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