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Rapid Method for the Detection and Identification of Mycolic Acids in Aerobic Actinomycetes and Related Bacteria[1]

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A rapid method for the identification of lipids characteristic of the genera *Corynebacterium, Mycobacterium, Nocardia,* and the "rhodochrous group" has been developed. Modifications of previously described methods make this procedure suitable for use in the clinical laboratory. Thin-layer chromatography is used to demonstrate the presence of the lipid characteristic of *Nocardia* spp. (type A) in some corynebacteria, nocardias, and members of the "rhodochrous group." Precipitation in ether and ethanol is used to demonstrate the presence of mycobacterial mycolic acids. Since this procedure can be carried out in less than 2 days and the lipids are extracted from the same batch of cells grown for diaminopimelic acid and whole-cell sugar analyses, it can readily be added to the battery of tests performed in reference laboratories that deal with aerobic actinomycetes and related bacteria.

Differentiation of mycobacteria from corynebacteria, nocardias, and the "rhodochrous group" on the basis of biochemical and morphological characteristics is often difficult and sometimes impossible to accomplish (4, 13). All four groups include species with type IV cell wall (*meso*-diaminopimelic acid, with major amounts of arabinose and galactose) (2, 14). Each group, however, has characteristic lipids, viz.: corynebacteria, corynemycolic acids; mycobacteria, mycolic acids; and nocardias and the "rhodochrous group", nocardomycolic acids (1). All three varieties of lipids are α-branched and β-hydroxylated; they differ in carbon skeleton size. The carbon skeletons of the mycolic acids are composed of 80 to 90 carbon atoms, whereas those of the nocardomycolic acids have 40 to 60 carbon atoms, and the corynemycolic acids have about 30 carbon atoms. A lipid thought to be specific for *Nocardia* sp. (lipid characteristic of *Nocardia* spp., type A [LCN-A]) (10) was detected by Mordarska (8). Since then, it has been found in a few isolates of *Corynebacterium* (9) and in over 200 isolates of *Nocardia* spp. and the "rhodochrous group." It has never been found in species of *Mycobacterium, Actinomadura,* or *Oerskovia* (12).

The methods presently used to identify the different lipid types are time-consuming and may require sophisticated and expensive equipment (6, 7). We have developed a simple technique for isolating and identifying the taxonomically important fatty acids based on the methods of Kanetsuna and Bartoli (5) and Mordarska et al. (11). Our method demonstrates the presence of either mycobacterial mycolic acids or LCN-A. The procedure may be completed in less than 2 days. It is appropriate for use in diagnostic laboratories on microorganisms known to have type IV cell walls.

MATERIALS AND METHODS

Organisms. The microorganisms included in this study all had type IV cell walls and are listed in Table 1. All organisms were grown at 28°C in Trypticase soy broth (BBL) on a rotary shaker operated at 90 strokes/min. After 3 to 7 days, the cells were killed by exposure to 1% formalin for 24 h and harvested by centrifugation. They were then washed twice in distilled water, covered with absolute alcohol, and incubated at 37°C overnight. The cells were then dried at 45°C and were ground with a mortar and pestle to facilitate weighing.

Saponification. According to the method of Moss et al. (12), 1 ml of 5% NaOH in 50% aqueous ethanol ($NaOH\text{-}CH_3OH$) was added to 50 mg of dried cells in a tube (13 by 100 mm) capped tightly with a Teflon-lined cap. The cells were heated at 100°C for 15 min. The saponificate was cooled and acidified with 1.5 ml of 1 N HCl.

Extraction. Three milliliters of diethyl ether was added to the saponificate. The mixture was then agitated and centrifuged for 3 to 5 min at 1,000 to 1,500 rpm. After the supernatant was separated, the

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TABLE 1. *Microorganisms tested, weights of their lipid precipitates in ether-ethanol (1:2, vol/vol), and results of TLC*

Microorganism	Source	Dry wt of precipitate (mg/50 mg of dry organisms)	LCN-A[a] present in TLC
Corynebacterium hoagii	ATCC 7005	0.7	+
C. pseudodiphthericum	ATCC 10700	0.4	+
C. renale	ATCC 19412	0.4	+
C. xerosis	ATCC 373	0.4	+
Mycobacterium fortuitum	CDC, Mycobacteriology Branch[b] (51)	4.1	−
M. fortuitum	CDC, Mycobacteriology Branch[b] (56)	5.5	−
M. fortuitum	CDC, Mycobacteriology Branch[b] (57)	6.3	−
M. fortuitum	CDC, Mycobacteriology Branch[b] (58)	6.3	−
M. fortuitum	CDC, Mycobacteriology Branch[b] (59)	5.9	−
M. phlei	CDC, Mycobacteriology Branch[b] (41)	8.1	−
M. phlei	CDC, Mycobacteriology Branch[b] (42)	8.0	−
M. phlei	CDC, Mycobacteriology Branch[b] (43)	4.0	−
M. phlei	CDC, Mycobacteriology Branch[b] (44)	4.7	−
M. smegmatis	CDC, Mycobacteriology Branch[b] (46)	6.5	−
M. smegmatis	CDC, Mycobacteriology Branch[b] (47)	8.3	−
M. smegmatis	CDC, Mycobacteriology Branch[b] (48)	5.1	−
M. smegmatis	CDC, Mycobacteriology Branch[b] (49)	5.1	−
Rhodochrous group	ATCC 271	0.9	+
Rhodochrous group	ATCC 999	1.4	+
Rhodochrous group	ATCC 11048	1.3	+
Rhodochrous group	ATCC 14343	0.4	+
Rhodochrous group	NCTC 8154	0.7	+
Nocardia asteroides	ATCC 19247	0.5	+
N. asteroides	CDC, Mycology Division (N1)	0.4	+
N. asteroides	CDC, Mycology Division (N2)	1.6	+
N. asteroides	CDC, Mycology Division (N9)	1.1	+
N. asteroides	CDC, Mycology Division (N14)	0.8	+
N. asteroides	CDC, Mycology Division (N87)	1.8	+
N. asteroides	CDC, Mycology Division (N114)	0.3	+
N. asteroides	CDC, Mycology Division (N287)	1.7	+
N. brasiliensis	ATCC 19019	1.5	+
N. brasiliensis	CDC, Mycology Division (N7)	0.7	+
N. brasiliensis	CDC, Mycology Division (N20)	0.5	+
N. brasiliensis	CDC, Mycology Division (N21)	0.6	+
N. caviae	Rutgers University (616)[c]	1.0	+
N. caviae	Rutgers University (736-A)	1.5	+
N. caviae	Rutgers University (736-B)	1.3	+
N. caviae	CDC, Mycology Division (N6)	2.3	+
N. caviae	CDC, Mycology Division (N8)	1.2	+
N. caviae	CDC, Mycology Division (N112)	1.1	+
N. caviae	CDC, Mycology Division (N113)	2.1	+
N. caviae	CDC, Mycology Division (N201)	1.5	+
N. caviae	CDC, Mycology Division (N243)	1.8	+
N. caviae	CDC, Mycology Division (N245)	1.5	+

[a] LCN-A, Lipid characteristic of *Nocardia* sp., type A.
[b] Courtesy of Hugo David.
[c] Courtesy of Ruth Gordon.

extraction procedure was repeated, and the supernatants were combined in a tube that had previously been accurately weighed. The total supernatant was evaporated to dryness in a 45°C oven. The residue was washed off the wall of the tube with 1 ml of ether, and the mixture was evaporated to dryness again.

The residue, representing the lipids, was dissolved in 0.1 ml of diethyl ether, and 0.2 ml of ethanol was added. The mixture was refrigerated at 4°C for 2 h to precipitate the mycolic acids (LCN-A, if present, remained in the supernatant). After the mixture was centrifuged at 1,000 to 1,500 rpm for 3 to 5 min, the supernatant was separated and saved. Any residue in the tared tube was dried and weighed. The precipitate formed after the addition of ethanol was considered to consist primarily of mycolic acids. The supernatant, which was to be tested for the

presence of LCN-A, was evaporated to dryness.

Chromatography. The dried LCN-A residue was taken up in 0.1 ml of diethyl ether. Ten microliters was spotted onto a silica gel G precoated thin-layer chromatography (TLC) plate (20 by 20 cm; 0.5 mm thick; Brinkman Co.). Ascending TLC was performed for 1 h with petroleum ether-diethyl ether-glacial acetic acid (90:10:1, vol/vol) used as the solvent system. The spots were applied 1 cm apart with a capillary pipette in a line 1.3 cm above the bottom of an inactivated TLC plate. The plates were placed in a glass container (22 by 22 by 10 cm) lined with filter paper into which 100 ml of the solvent had been added 1 h previously. The plates were leaned against the sides of the container, although the edges of the silica gel were not allowed to contact the solvent-saturated filter paper. The container was sealed with a well-greased lid. After development, the chromatograms were air dried, and the spots became visible about 5 min after the plates were exposed to iodine vapors. The method of Mordarska et al. (11) can also be followed; in this procedure, the plates are dried and then redeveloped in pure methanol before the spots are developed in iodine vapor. The methanol moves most of the nondiagnostic lipid spots with the solvent front, but leaves the LCN-A in place. Since development of the plates with pure methanol failed to move the diagnostic lipid, it was considered to be the LCN-A described by Mordarska.

RESULTS AND DISCUSSION

The chromatograms of the corynebacteria, nocardias, and "rhodochrous group" all exhibited a characteristic pattern. The LCN-A spot was readily discernible: it was the most intensely colored of the spots, with a low R_f (Fig. 1). In our system, the R_f of the LCN-A spot from nocardias and the "rhodochrous group" ranged from 0.08 to 0.1, whereas that from the corynebacteria was slightly lower. The LCN-A spot is sometimes shaped like a mushroom cap. We found that all of the *Corynebacterium* spp., "rhodochrous group," and *Nocardia* spp. isolates surveyed had an LCN-A spot in their chromatograms (Table 1). None of the chromatograms obtained from *Mycobacterium* spp. isolates had such a spot.

The weights of the residues in the tared tubes were arrayed in two discrete clusters (Table 1). The residue weights of the 13 *Mycobacterium* spp. cultures ranged from 4.0 to 8.3 mg per 50 mg of dry organisms. The residue weights of the 31 *Corynebacterium* spp., "rhodochrous group," and *Nocardia* spp. cultures ranged from 0.3 to 2.3 mg per 50 mg of dry organisms. Precipitated mycolic acids constitute the residues of the mycobacteria (5). Kanetsuna and Bartoli (5) also observed that the residue weights of the nocardias (which were significantly lower than those of the mycobacteria) are not the result of precipitated mycolic acids. For the statistical analysis, the residue weight values of the mycobacteria and those of the corynebacteria, nocardias, and "rhodochrous group" were considered as two separate sets of data. The means ($\bar{N} = 1.08$, $\bar{M} = 5.99$; N signifies the corynebacteria, nocardias and "rhodochrous group" and M signifies the mycobacteria) and the standard deviations ($s_N = 0.56$, $s_M = 1.45$) were computed for the two sets of data. The point 2.45 is midway between $\bar{N}$ and $\bar{M}$ on the standardized scale; that is, $\bar{N} + 2.44\ s_N = M - 2.44\ s_M = 2.45$. The value of 2.5 mg of precipitate per 50 mg of dry organisms was taken as the division point between the two groups.

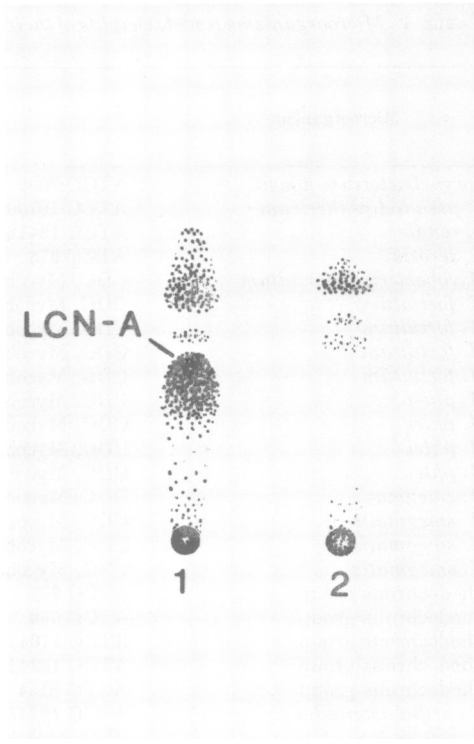

FIG. 1. *Thin-layer chromatograms of representative isolates. (1) Nocardia asteroides (ATCC 19247); (2) Mycobacterium fortuitum (51).*

The above data lead to the following classification rule. When the experimental conditions are the same as the ones described in this paper and when the microorganism in question belongs to either the genus *Corynebacterium*, the genus *Mycobacterium*, and genus *Nocardia*, or the "rhodochrous group," then: (i) The unknown isolate is a *Corynebacterium* sp., *Nocardia* sp., or member of the "rhodochrous group" if an LCN-A spot is visible on its thin-layer chromatogram. (ii) The unknown isolate is a *Mycobacterium* sp. if it has a lipid precipitate

weight of at least 2.5 mg per 50 mg of dry organisms. This rule is based on demonstration of the presence of characteristic lipids. The converse, based on the absence of those lipids, is also reasonable (if no LCN-A spot is found, the isolate is a *Mycobacterium* sp.; if less than 2.5 mg of precipitate per 50 mg of dry organisms is present, then the isolate is a *Corynebacterium* sp., *Nocardia* sp., or a member of the "rhodochrous group").

Aside from being rapid and simple, the described procedure is made more convenient because it is possible to use cells grown for routine diaminopimelic acid and whole-cell sugar analyses. As such, it is suited for addition to the diagnostic laboratory repertoire.

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LITERATURE CITED

1. **Asselineau, J.** 1966. The bacterial lipids. Holden-Day, Inc., San Francisco.
2. **Becker, B., M. P. Lechevalier, and H. A. Lechevalier.** 1965. Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13:236-243.
3. **Etémadi, A. H.** 1967. The use of pyrolysis gas chromatography and mass spectroscopy in the study of structures of mycolic acids. J. Gas Chromatogr. 5:447-456.
4. **Gordon, R. E.** 1966. Some strains in search of a genus—*Corynebacterium*, *Mycobacterium*, *Nocardia* or what? J. Gen. Microbiol. 43:329-343.
5. **Kanetsuna, F., and A. Bartoli.** 1972. A simple method to differentiate *Mycobacterium* from *Nocardia*. J. Gen. Microbiol. 70:209-212.
6. **Lanéele, M. A., J. Asselineau, and G. Castelnuovo.** 1965. Etudes sur les mycobactéries et nocardiae. IV. Composition des lipides de *Mycobacterium rhodochrous, M. pellegrino sp.*, et de quelques souches de nocardiae. Ann. Inst. Pasteur Paris 108:69-82.
7. **Lechevalier, M. P., A. C. Horan, and H. A. Lechevalier.** 1971. Lipid composition in the classification of nocardiae and mycobacteria. J. Bacteriol. 105:313-318.
8. **Mordarska, H.** 1968. A trial using lipids for the classification of actinomycetes. Arch. Immunol. Ther. Exp. 16:45-50.
9. **Mordarska, H., and M. Mordarski.** 1970. Cell lipids of Nocardia, p. 47-53. *In* H. Prauser (ed.), The Actinomycetales. Gustav Fischer, Jena.
10. **Mordarska, H., and A. Rethy.** 1970. Preliminary studies on the chemical character of the lipid fraction in *Nocardia*. Arch. Immunol. Ther. Exp. 18:455-459.
11. **Mordarska, H., M. Mordarski, and M. Goodfellow.** 1972. Chemotaxonomic characters and classification of some nocardioform bacteria. J. Gen. Microbiol. 71:77-86.
12. **Moss, C. W., M. A. Lambert, and W. H. Merwin.** 1974. Comparison of rapid methods for the analysis of bacterial fatty acids. Appl. Microbiol. 28:80-85.
13. **Tsukamura, M.** 1970. Relationship between *Mycobacterium* and *Nocardia*. Jpn. J. Microbiol. 14:187-195.
14. **Yamaguchi, T.** 1965. Comparison of the cell wall composition of morphologically distinct actinomycetes. J. Bacteriol. 89:444-453.