Simplified Approach to Identification of Aerobic Actinomycetes by Thin-Layer Chromatography

JOSEPH L. STANECK AND GLENN D. ROBERTS

Section of Clinical Microbiology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55901

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A system has been developed for the identification of aerobic actinomycetes in the clinical laboratory based on analysis of whole cells for diaminopimelic acid and carbohydrates and on the ability of the organism to decompose casein, tyrosine, and xanthine media. The whole-cell analyses were performed by a simple thin-layer chromatographic procedure that is described. Eighteen reference cultures were correctly identified and, subsequently, 35 isolates from clinical material were grouped by using this system. The method is well suited for use in routine clinical laboratories.

Infections due to the aerobic actinomycetes, particularly of the genus Nocardia, are being reported with greater frequency in situations that favor the opportunistic invasion and multiplication of microorganisms. Nocardiosis has been associated with malignancies (13, 16), pulmonary alveolar proteinosis (5), immunosuppressive therapy (1, 2, 14), and steroid treatment (15), and it will be a problem in patients compromised by either disease or therapy. Bacteriology, mycobacteriology, and mycology laboratories should be capable of providing rapid detection, accurate identification, and, if necessary, susceptibility data on organisms such as species of Nocardia, Streptomyces, and Actinomadura.

A recent article by Goodfellow (7) suggested that chemotaxonomic markers should be important in the classification of microorganisms. The chemical composition of cell walls has been accepted by Lechevalier and Lechevalier (12) as a criterion for the classification of aerobic actinomycetes. Becker et al. (3) introduced a system of paper chromatography able to separate the taxonomically important stereoisomers of diaminopimelic acid (DAP). Lechevalier (11) described a system to identify carbohydrates for diagnostic purposes. Chromatographic analysis of cell walls was recently used by Berd (4) in an extensive study of the classification of aerobic actinomycetes.

Our paper presents an adaptation of the paper chromatographic system to thin-layer chromatography (TLC). The use of TLC simplifies the techniques, materials, and time necessary for analysis and encourages the adoption of chromatographic systems for diagnostic purposes by the routine clinical laboratory.

MATERIALS AND METHODS

Cultures. Reference cultures (Table 1) were obtained from Center for Disease Control laboratory surveys and from Ruth Gordon (Rutgers University). Unknown cultures were obtained from clinical specimens submitted to our laboratory between December 1972 and December 1973 and from frozen stock cultures of strains originally isolated from clinical material. Working stock cultures were maintained on brain heart infusion (BHI; Difco) agar slants. Incubation of all cultures was at 30 C. Primary isolations from clinical specimens were made on either BHI agar, Lowenstein-Jensen medium (Difco), or sheep blood agar plates, depending on the nature of the specimen. The modified cold acid-fast staining method of Kinyoun (6), with 1% sulfuric acid substituted for acid-alcohol in the decolorizing step, was used to determine acid fastness.

Decomposition media. The medium for determining casein decomposition was made by dissolving 10 g of dehydrated skim milk in 100 ml of distilled water and autoclaving. A separate 100-ml solution of 2% agar in distilled water was also autoclaved. After cooling to 45 C, these two solutions were mixed and poured into plates.

The tyrosine or xanthine medium consisted of 23 g of nutrient agar, 5 g of tyrosine (or 4 g of xanthine), and 1 liter of distilled water. The ingredients were mixed, adjusted to pH 7.0, autoclaved, and poured into plates; the plates were gently swirled while the medium cooled to obtain a smooth suspension of the amino acid.

A heavy inoculum of the test organism was streaked onto a section of each type of plate and incubated. The plates were observed for 14 days for areas of...
clearing around the bacterial growth, which would indicate decomposition of the substrate.

**Identification procedure.** The following routine consists of variations of the work of Gordon and Mihm (9, 10), Lechevalier and Lechevalier (11, 12), Becker et al. (3), and Berd (4).

Any slowly growing aerobic colony consisting of small, branched cells that often, but not necessarily, were partially acid fast was suspected of belonging to the aerobic actinomyces group. Strains not well isolated on primary media were restreaked on BHI agar plates to obtain several isolated colonies that were picked and suspended in 2 ml of BHI broth to form a heavy inoculating suspension. Decomposition media were streaked with inoculum, incubated, and observed for 14 days.

The remaining suspension was added to a 250-ml flask containing 100 ml of BHI broth and incubated with constant shaking at 30°C until the broth became turbid. These cells were killed with formalin (final concentration, 1%) for 24 h at room temperature and collected by centrifugation. The cells were washed once in distilled water and once in 95% ethanol and then dried by suitable means (vacuum desiccation, forced air, or overnight drying at 45 C). The dried cells were analyzed for carbohydrates and DAP as described below.

The identification is made as shown in Table 2.

**Hydrolysis and chromatography.** The procedure of Becker et al. (3) was followed for the hydrolysis of whole cells preceding DAP analysis. Approximately 3 mg (dry weight) of cells was placed into a small ampoule with 1 ml of 6 N hydrochloric acid. The sealed ampoule was kept at 100°C in an oven for 18 h. After cooling, the hydrolysate was filtered through Whatman no. 1 paper. The filtrate was evaporated to dryness in a boiling water bath, redissolved in 1 ml of distilled water, and taken to dryness again. This residue was dissolved in 0.3 ml of distilled water, and 2 μl of it was applied at the base line of the TLC sheet (Chromagram-Eastman Kodak no. 6064 cellulose without fluorescent indicator). Ascending TLC was performed with the solvent system methanol-distilled water-6 N HCl-pyridine (80:26:4:10, vol/vol) for approximately 3.5 h. After the chromatogram was air dried, spots were visualized by spraying with 0.2% ninhydrin in acetone and heating at 100°C for 3 min. As a DAP standard, 1 μl of 0.01 M dl-DAP (Sigma Chemical Co.), which contains both meso- and l-DAP isomers, was used. The DAP spots were seen as gray-green fading to yellow, with the l isomer moving ahead of the meso isomer (Fig. 1). With hydrolysates, amino acid spots appeared purple or red and migrated ahead of the DAP spot. The easy visualization, after development, of 0.5 μl of the 0.01 M standard solution of dl-DAP indicated the ability of the system to detect as little as 1 μg of a DAP isomer. Sample application, development, and identification required less than 4 h.

The carbohydrate analysis was based on the work of Lechevalier (11). Approximately 25 mg (dry weight) of cells was placed into an ampoule with 1.5 ml of 1 N sulfuric acid. The sealed ampoule was heated for 2 h in a boiling water bath. After cooling, the hydrolysate was transferred to a 15-ml conical centrifuge tube, and saturated barium hydroxide was added dropwise

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**Table 1. Reference cultures and source**

| Strain                        | No. | Source                   |
|-------------------------------|-----|--------------------------|
| *Nocardia asteroides*         | 3   | CDC laboratory survey    |
| *N. brasiliensis*             | 2   | CDC laboratory survey    |
| *N. cauive*                   | 1   | CDC laboratory survey    |
| Streptomyces species          | 1   | CDC laboratory survey    |
| Actinomadura dassonvieli      | 2   | Ruth Gordon (NCTC#434, 711) |
| *A. madurae*                  | 1   | Ruth Gordon (#1092)      |
| *Micromonospora species*      | 2   | Ruth Gordon (#3450, #3640) |
| "Rhodochrous" group           | 5   | Ruth Gordon (#1240, W#5408, #1346, #382, and #1296) |
| *S. somaliensis*              | 1   | Ruth Gordon (#1448)      |

* CDC, Center for Disease Control.

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**Table 2. Identification of aerobic actinomycetes**

| DAP isomer | Carbohydrate products* | Decomposition media | Organism                          |
|------------|------------------------|---------------------|-----------------------------------|
| L-DAP      | +                      | +                   | +                                 |
| meso-DAP   | +                      | +                   | +                                 |
| Ara + Gal  | +                      | +                   | +                                 |
| Gal or none| +                      | +                   | +                                 |
| Gal + Mad  | +                      | +                   | +                                 |
| Ara + Xyl* | +                      | +                   | +                                 |

* Ara, Arabinose; Gal, galactose; Mad, madurose; Xyl, xylose.

* Strains of the "rhodochrous" group that fail to decompose tyrosine are difficult to distinguish from *Nocardia* by these or other biochemical tests; differentiation depends on colonial and microscopic morphology (4, 8).

* With or without galactose.

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FIG. 1. Separation of DAP isomers by TLC (origin at bottom). All of the following were hydrolysates except the standard solution. 1, N. asteroides; 2, Streptomyces sp.; 3, dL-DAP (Sigma Chemical Co.); 4, Actinomadura dassonvillei; 5, S. somaliensis. The L-DAP is the faster-moving spot. Note long smear of amino acids migrating ahead of DAP in hydrolysates.

until the pH was between 5.2 and 5.5 (determined with pH paper). The precipitate was removed by centrifugation and discarded. The supernatant fluid was evaporated in a 50-ml beaker under a stream of air, and the residue was redissolved in 0.3 ml of distilled water (any insoluble material remaining at this step was removed by centrifugation); 1 μl of this hydrolysate was applied to the base line of the TLC sheet (Chromagram) as well as 1 μl of each of two standard solutions. The first contained galactose, arabinose, and xylose, each at 1% concentration. The second solution contained rhamnose, mannose, glucose, and ribose, also each at 1%.

Ascending TLC was performed with the solvent system n-butanol-distilled water-pyridine-toluene (10:6:6:1, vol/vol) for approximately 4 h. Spots were visualized by spraying the chromatogram 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 at 100 C for 4 min. Hexose spots were yellow after heating, and pentose spots were brown. The carbohydrates migrated in the following sequence from the origin (slowest to fastest component): galactose, glucose, arabinose, mannose, xylose, ribose, and rhamnose (Fig. 2). Madurose (3-O-methyl-D-galactose), if present in a hydrolysate, migrated the same distance as xylose but could be distinguished by its yellow color. In this system, 1 μg (1-μl spot of a 0.1% solution) of any standard carbohydrate could easily be visualized.

TLC. The sides of a 2-liter glass beaker were lined with filter paper, and 80 ml of the appropriate solvent was added. The beaker was then tightly covered with aluminum foil and the solvent was allowed to migrate up the filter paper for at least 1 h before use to saturate the atmosphere within the “tank.” A sheet of chromatogram, 18 cm long, was cut to a convenient width. No activation of this material was required for use. Samples were spotted with a capillary pipette 1.0 cm apart at an origin 1.3 cm from the bottom edge of the chromatogram sheet. A light stream of air facilitated drying of the spots. The prepped strip was placed into the “tank” so that the origin was in the solvent and the strip leaned against a “spacer” positioned between the upper back portion of the strip and the side of the beaker (a cylinder of tape, adhesive side out, worked very well). It was important that the edges of the strip not touch the saturated filter paper lining or the sides of the beaker. The top of the strip was replaced and the strip was developed for approximately 3.5 to 4 h. The strip was then moved, dried, sprayed with the appropriate reagent, and heated for visualization of the spots.

RESULTS

The 18 known strains of aerobic actinomycetes were coded, randomized, and treated as unknowns; 14 were easily identified according to the criteria in Table 2. Three cultures showed no decomposition, contained meso-DAP, and were found to have galactose and arabinose in their cell walls, thus resembling the N. asteroides group. However, the smooth appearance and creamy texture of the colonies and the small branching or coccoid microscopic appearance were strongly suggestive of the “rhodochrous” group, and these
strains therefore were classified as such. One strain demonstrated casein and tyrosine decomposition and contained meso-DAP but produced only galactose. This strain was suspected of being either *Actinomadura madurae* or *A. pelletieri*; in a repeat test with application of 5 μl of hydrolysate to the TLC plate, a faint madurose spot was detected, confirming the identification. In a clinical situation, further tests, such as carbohydrate fermentations as described by Berd (4), would have to be used to differentiate between *A. madurae* and *A. pelletieri*. Acid-fast staining at times was difficult to interpret and was therefore given little consideration in the identification.

Subsequently, 35 strains, collected from recent clinical isolations and from stock cultures of previous clinical isolations, were subjected to analysis by this system. All could be classified easily and included the following isolates: 23 *N. asteroides*, one *N. brasiliensis*, one *N. caviae*, eight *Streptomyces* sp., and two *A. dassonvillei*. One of the two *A. dassonvillei* isolates was sent to the Center for Disease Control and our identification was confirmed; the other has been tentatively identified as *A. dassonvillei* by Ruth Gordon.

Most of our identifications were made within approximately 8 days after initial isolation of the organism. Although decomposition media were always held for 14 days, we have found that, when the inoculum is adequate, changes rarely occur in the pattern beyond the 8th day. Thus, a tentative identification at approximately 8 days was often possible based on the decomposition pattern and supported by the chromatographic analyses.

Identification was delayed in the case of slowly growing strains. Among our reference strains, *Micromonospora* sp. and *A. madurae* required relatively long incubation periods in broth cultures to reach densities suitable for chemical analyses, and two strains of *A. pelletieri* failed to grow to sufficient densities for analyses and were therefore not included in this study.

**DISCUSSION**

Compared with paper chromatography, TLC provides the advantages of simplified equipment, increased sensitivity, rapid development, and ease of handling, all of which are important in the routine clinical laboratory. The increased sensitivity permits analysis with smaller culture volumes of pathogenic organisms. Tanks for

![Fig. 2. Separation of carbohydrates from aerobic actinomycetes hydrolysates by TLC (origin at bottom). Carbohydrates are listed in the order of migration, starting at the origin (slowest to fastest moving components). 1, *N. asteroides*: galactose, arabinose, ribose; 2, *A. madurae*: galactose, madurose, ribose; 3, standards: galactose, arabinose, xylose; 4, standards: glucose, mannose, ribose, rhamnose; 5, *Micromonospora* sp.: galactose, arabinose, xylose, ribose; 6, *Streptomyces somaliensis*: ribose.](image-url)
descending chromatography and ventilated drying hoods are unnecessary. The decreased development time hastens identification and thus permits the laboratory to provide a better diagnostic service. In our experience, TLC for DAP and carbohydrate analyses has been more manageable than the paper system and, in less time with less expense, has yielded similar information as paper chromatographic methods with respect to the diagnostically important carbohydrates: galactose, arabinose, xylose, and madurose. These factors should encourage other routine clinical laboratories to consider using the determination of chemotaxonomic markers for diagnostic purposes.

The modified system for the identification of aerobic actinomycetes relies heavily on the extensive and thorough characterization of these organisms by Berd (4), Lechevalier and Lechevalier (12), and Gordon and Mihm (8-10), and we are in no way suggesting that this system should be interpreted taxonomically beyond its clinical limits; these authors all have reported strains of aerobic actinomycetes that fit loosely into the identification system that we have used. Isolates that fail to conform to our system should be subjected to further biochemical testing by the primary laboratory or by a reference laboratory. However, our modified system can be most useful in the diagnosis of infections caused by this group of organisms and provides an accurate basis for studies to determine the incidence and significance of these infections.

The commercially available flexible Chromagram cellulose plates were found to be extremely convenient and represented a considerable improvement over glass plates for TLC, particularly because they can be cut to size with scissors and are easily handled throughout all steps of the procedure. No problems were encountered in the system to identify the isomers of DAP. Occasionally, in carbohydrate analysis a sample applied too heavily produced significant streaking; however, a repeat test with a smaller application would reveal an interpretable pattern. Conversely, a faint pattern of sugars could be made more distinctive by increasing the size of sample applied. Although Lechevalier and Lechevalier (12) report the presence of glucose, mannose, and ribose in almost all cultures that they studied, we found only ribose consistently in all isolates. Glucose and mannose, both yellow-staining spots, were distinguishable, although not completely separated, from the maroon-staining arabinose spot if the three components were run in a mixture containing equal concentrations of each. However, it is conceivable that either glucose or mannose spots might be masked by the presence of a greater amount of arabinose, since these three carbohydrates migrate close to one another. The failure of this system to detect glucose or mannose could thus be explained since those hydrolysates that contain arabinose showed a predominance of arabinose in their carbohydrate patterns, both in this chromatographic system and in the report by Lechevalier (11). This fact does not detract from the clinical usefulness of the system because the diagnostically important sugars—arabinose, galactose, and xylose—when present dominate the carbohydrate patterns and are easily interpreted. This limitation should be borne in mind if this chromatographic system is used outside of the diagnostic context outlined here.

The variability of the acid-fast stain can be misleading. A positive acid-fast reaction suggests final identification of a Nocardia sp., but the lack of acid fastness does not rule out this genus. The use of a heavy uniform inoculum for the decomposition media is preferable for valid results. This system fails to differentiate A. pelletieri from A. madurae and further tests, such as carbohydrate fermentations, are required. Members of the "rhodochrous" group, some species of Mycobacterium, and Corynebacterium can present a profile similar to that of N. asteroides in this system and, in these instances, the gross and microscopic morphologies must be considered in an attempt to assess true branching (4).

In the past 9 months, the modified system of identification of aerobic actinomycetes has enabled us to decrease significantly the time of reporting results, so that the laboratory data are of active rather than historical interest to the clinician. From December 1972 to December 1973, we recovered 17 different isolates of N. asteroides. The use of chromatography enabled us to identify two strains of A. dashionvillii, one from a blood culture after 16 days of incubation and the other from an ocular exudate; although no clinical significance could be assigned to these isolations, we are encouraged that, with further efforts, more members of the aerobic actinomycetes group may be found in clinical material.

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