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Identification of Atypical Mycobacteria by Thin-Layer Chromatography of Their Surface Antigens

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The knowledge that the surface (Schaefer) antigens of certain smooth-colony atypical mycobacteria are multiglycosylated C-mycosidic peptidoglycolipids was used to devise a sensitive thin-layer chromatographic (TLC) procedure for the identification of *Mycobacterium avium/M. intracellulare/M. scrofulaceum* serotypes. TLC maps of the type-specific peptidoglycolipids from 17 of the 31 serotypes are presented. The primary use of the technique is to corroborate results obtained by seroagglutination. Without the aid of seroagglutination, the TLC procedure almost invariably requires the availability of reference strains or the specific peptidoglycolipids derived therefrom.

Advances in classifying mycobacteria other than *Mycobacterium tuberculosis* have been accompanied by increasing recognition of the widespread distribution of these organisms in nature. It is now well established that some of these, especially the group III non-photochromogens, can cause a variety of human syndromes which may mimic infection caused by *M. tuberculosis* (11, 17). However, not all group III mycobacteria are highly virulent, and hence proper identification of infective organisms is of paramount importance. The late Werner Schaefer (12, 13) developed a seroagglutination procedure for the identification and classification of atypical mycobacteria based on their ability to form smooth colonies and to elaborate specific surface antigens (characteristics not shared by the rough colony-forming *M. tuberculosis*). When seroagglutination was applied to strains in Runyon's four groups (11), several serological subgroupings were recognized, among them one encompassing many non-photochromogens and scotochromogens and composed of 31 distinct but related serotypes: the *Mycobacterium avium/M. intracellulare/M. scrofulaceum* (MAIS) complex (5, 10, 16).

Marks, Jenkins, and colleagues (6, 8, 9), in a search for other easily identifiable features characteristic of atypical mycobacteria, recognized an array of closely related polar and apolar lipids shared by smooth-colony strains in the MAIS complex. Initially they distinguished between six serotypes of organisms classified as *M. intracellulare* or *M. avium* on the basis of the thin-layer chromatographic (TLC) profile which these characteristic lipids assumed (8). Later, Jenkins et al. (6) differentiated between *M. scrofulaceum* serotypes 41, 42, and 43 in like fashion. Sehrt et al. (14) essentially confirmed the observations of Marks and Jenkins and satisfactorily differentiated between *M. avium* serotypes 1, 2, and 3 and 13 serotypes of *M. intracellulare*. None of these workers attempted to identify the characteristic lipids, except to conclude that they were glycolipids based on their reaction to orcinol-H_2SO_4.

In ongoing research (P. J. Brennan and M. B. Goren, submitted for publication), preliminary results of which have been reported (2; P. J. Brennan, O. Brokl, M. B. Goren, and W. B. Schaefer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K102, p. 203), we have determined that both the polar and apolar Marks-Jenkins specific lipids are closely related to the known mycoside C-peptidoglycolipids (see references 1 and 4 for reviews on C-mycosides). In addition, the Schaefer antigens are also structured on a C-mycoside core, are much more glycosylated than those hitherto recognized, and, surprisingly, correspond to the more polar entities among the Marks-Jenkins specific lipids. This relevation at once raised the possibility of identifying the specific antigens by chemical means, thereby offering a procedure corroborative to seroagglutination. Herein we describe simple procedures for the manipulation of the whole-lipid extract from certain atypical mycobacteria leading to TLC profiles of the serologically ac-

tive variety, which, because of their uniqueness, allows independent recognition of most individual serotypes.

MATERIALS AND METHODS

Strains of mycobacteria. Known serotypes were obtained from the stock of atypical mycobacterial cultures acquired by W. B. Schaefer and maintained at National Jewish Hospital (for the numbering system in vogue, see references 5 and 16). Those used were: *M. avium* serotype 2 and the following *M. intracellulare* serotypes: 7 (VII), 8 (Davis), 9 (Watson), 10 (IIIa), 12 (Howell), 14 (Boone), 15 (Dent), 16 (Yandle), 18 (Altmann), 19 (Darden), 23, 24, 25, 41 (*M. scrofulaceum*), 42 (Lunning), and 43 (Gause). The identity of each of these strains was confirmed by the Schaefer seroagglutination procedure (12, 13). The origins of the unknown serotypes sent to us for identification are described in Fig. 2.

Growth of mycobacteria. Cultures were grown for about 3 weeks at 37°C in plastic petri dishes (90 by 15 mm) containing 20 ml of the 7H11 agar (3). Usually two to three such plates yielded more than the requisite amount of dry bacteria (see next paragraph). Bacilli were scraped from the surface and transferred to preweighed Kimax screw-capped culture tubes (13 by 100 mm) and dried to a crisp opaqueness in vacuo over P_2O_5 (at least 48 h). The dried bacilli could thus be maintained indefinitely at 4°C without harm.

Extraction of lipids. Chloroform-methanol (2:1, vol/vol; 32 μl/mg of bacteria) was added to the dry bacteria (10 to 14 mg) in culture tubes (13 by 100 mm) with Teflon-lined screw caps. The tubes were treated in a sonic bath (Varian Aerograph) for 5 s and extracted at 50°C for 18 h. Tubes were centrifuged at 1,500 × *g* for 30 min, followed by storage of the clear supernatant at 4°C in tubes tightly closed with Teflon-lined caps.

Deacetylation of lipids. NaOH (0.2 M) in methanol (100 μl) was added to an equal volume of the lipid extract in screw-capped tubes, which were then kept at 37°C for 20 min. The mixture was neutralized with 2.5 μl of glacial acetic acid and dried in the fume cupboard with a stream of nitrogen at 37°C. Chloroform-methanol (2:1, 500 μl) followed by water (100 μl) was added to the dry residue, which was Vortex mixed and centrifuged (1,500 × *g*, 30 min). The upper aqueous phase was discarded, and the lower chloroform phase was dried with a stream of nitrogen. The contents were redissolved in 100 μl of chloroform-methanol (2:1, vol/vol) before chromatography.

TLC. Thin-layer plates (20 by 20 cm or 20 by 10 cm; 0.25 to 0.30 mm thick) were prepared from Silica Gel H (Merck; supplied by SMI, Emeryville, Calif.). They were dried in a hot-air oven for 30 min at 110°C, cleaned by an acetone run, air dried, and reactivated at 110°C for 30 min before use. Samples (20 μl) of the deacylated lipid extracts were applied as small discrete spots to each of four plates (using a hair dryer to conserve the spot dimension) at a distance of 1 to 2 cm up from the plate's end. The following three developing solvents were used in 15- to 17-cm runs in paper-lined tanks: solvent I, chloroform-methanol-water (60:27:4 by volume); solvent II, chloroform-methanol-water (65:25:4 by volume); and solvent III, chloroform-methanol-water (60:16:2 by volume). A fourth solvent (solvent IV, chloroform-methanol-water, 45:5:0.5 by volume) was used specifically to examine the nonpolar C-mycosidic peptidoglycolipids.

Developed chromatograms were dried at room temperature for at least 30 min and then lightly sprayed with a solution of 0.1% (wt/vol) orcinol in 40% (vol/vol) H_2SO_4 (9). Heating for 5 to 7 min in a hot-air oven at 110°C produced the characteristic yellow-gold color described by Marks et al. (8). Clean glass plates were then clamped over the developed chromatograms, thereby stabilizing the colors for several weeks, and thus the chromatograms were amenable to periodic inspection.

RESULTS AND DISCUSSION

A key step in the present procedure involves mild saponification of the extracted lipids before TLC. Acetyl groups on the constituent sugars are thereby removed, but otherwise the C-mycosidic peptidoglycolipids are unaffected (Brennan and Goren, submitted for publication). The ensuing enhanced polarity of the characteristic lipids allows for greater resolution and, at the same time, diminishes the number of those polar peptidoglycolipids which differ only in the number of attached acetyl groups. Above all, mild saponification destroys practically all of the nonspecific more orthodox mycobacterial lipids. Otherwise, the sheer mass of these interferes with resolution of the characteristic kinds of lipids.

In our hands the ethanol-ether extractant, as used by Marks and Szulga (9), performed inconsistently in solubilizing all of the polar (serologically active) variety of characteristic lipids (for instance, those from *M. intracellulare* serotype 8 were often not at all obvious). Chloroform-methanol extractions, as described above, did not suffer from this disadvantage.

Another major divergence from the Marks-Jenkins procedure is in the chromatographic solvents used. Not only did we have the benefit of the detailed work of Marks and Jenkins, but we had discerned the nature of the specific lipids, and our solvents are therefore tailored to their peptidoglycolipid nature. The apolar solvent (solvent IV) was adapted from the pioneering work on mycoside C-peptidoglycolipid structures (7, 15); the polar solvents arose from our own work on the novel multiglycosylated, serologically active peptidoglycolipids.

Three maps are presented in Fig. 1 demonstrating resolution of the characteristic polar, serologically active, deacetylated peptidoglycolipids from a wide range of member serotypes of the MAIS complex. (To date we have estab-

lished that the polar, intact [fully acetylated] peptidoglycolipids from serotypes 8, 9, 16, and 25 are serologically active. Those from the remaining serotypes in Fig. 1 have not yet been examined, but are presumed to be active. We do not yet know whether deacetylation, which is requisite for our TLC procedure, destroys serological activity.) A marked feature of these polar characteristic lipids is a vivid, long-lasting yellow-gold coloration with orcinol, in contrast to the apolar variety, which later assume a pink texture. This probably reflects the larger quantities of 6-deoxyhexoses in the polar varieties. (Of the several different kinds of sugars tested, only 6-deoxyhexoses [e.g., rhamnose, fucose] produced this yellow-gold response to the orcinol spray. 6-Deoxyhexoses are the characteristic sugars of the C-mycosidic peptidoglycolipids [see reference 4].) Some of the distinguishing features of the maps for the individual serotypes shown in Fig. 1 are summarized as follows. The characteristic peptidoglycolipid of serotype 2 (*M. avium* 2) migrated much farther than those from any of the other serotypes; it could be discerned only in solvents III and IV. Serotypes 9, 10, and 25 were distinguished by the slowest-running peptidoglycolipids (Fig. 1B). (The vast difference in the mobilities of the peptidoglycolipids within and among serotypes probably reflects in large part the size of the oligosaccharides which are attached to the C-mycosidic core. The more mobile peptidoglycolipids contain small oligosaccharides [e.g., tri-], whereas the slower-running type carry a larger oligosaccharide.) Several serotypes were characterized by the presence of peptidoglycolipids of intermediate mobility, for example, serotypes 8, 18, 19, and 42 in solvent II (Fig. 1C). Solvent III (Fig. 1A) retained the slowest peptidoglycolipids close to the origin and had, for the most part, the advantage of demarcating those serotypes having peptidoglycolipids with relatively rapid mobility (e.g., 12, 14, 23, 24, 41, and 43) from one another and from other serotypes. This information is tabulated in Table 1. With some serotypes (e.g., 7 and 16) it was difficult to decide whether their peptidoglycolipids belong to the "medium mobile" or "fast-moving" groups. In these cases, the number of lipid spots and their mobilities relative to those from other serotypes facilitated identification. Also, the serotype 24/43 pair, among others, was difficult to distinguish by TLC (Fig. 1A). Fortunately, co-chromatography of their peptidoglycolipids showed two spots, and this ploy may be used to identify unknown cultures (see below). Slight modifications in the composition of solvent III and greater familiarity with the number of lipids and their relative intensities should help to resolve obvious difficulties in distinguishing between serotypes in the fast-moving group. In general, it is important that several solvents be used, and we would like to emphasize that the three listed here are not inviolable; prevailing conditions (e.g., humidity) may mitigate slight alterations in the ratios of chloroform to methanol to water. In time we hope to produce similar chromatographic patterns for the serologically active peptidoglycolipids from the remaining serotypes of the MAIS complex.

One reason for developing the TLC procedure was to corroborate results gathered from seroagglutination assays, and in this respect the method is reliable, simple (in our hands), and indeed most satisfying. A challenging test of the greater worth of the procedure is to determine whether it is capable of blind identification of serotypes and how useful it might be if seroagglutination were not directly available.

Extracts from 7 untyped MAIS cultures (identified as such by "biochemical" analyses [18]) were chromatographed in solvents I, II, and III. Comparison of the patterns with the maps shown in Fig. 1A, -B, and -C provided indications which tallied in most cases with the final identification of the cultures. However, we were unable or reluctant to positively identify any of the cultures on this basis. Yet if serotypes 9, 10, 25, or 42 were among the unknowns, identification would probably be possible simply by direct comparison with Fig. 1B and -C. The chromatographic mobilities of the peptidoglycolipids from the unknown cultures were next measured and compared with those in Table 1. All seven belonged to the most populous group, those with fast-moving peptidoglycolipids. With this information in hand, extracts from the unknown cultures were again chromatographed in solvent III

TABLE 1. *Classification of the chromatographic mobilities of the serologically active peptidoglycolipids (shown in Fig. 1) from MAIS serotypes*

Serotype	TLC mobility of slowest of characteristic peptidoglycolipids[a]	Category
9, 10, 25	R_f < 0.7 in solvent I (see Fig. 1B)	Slow moving
8, 18, 19, 42	R_f < 0.7 in solvent II (see Fig. 1C)	Medium mobile
12, 14, 15, 23, 24, 41, 43	R_f > 0.7 in solvents I and II and <0.5 in solvent III (see Fig. 1A)	Fast moving

[a] TLC mobility will vary. Hence we routinely include on all chromatograms a lipid extract that will serve as a focal point (e.g., that from serotype 42) and adjust R_f values accordingly.

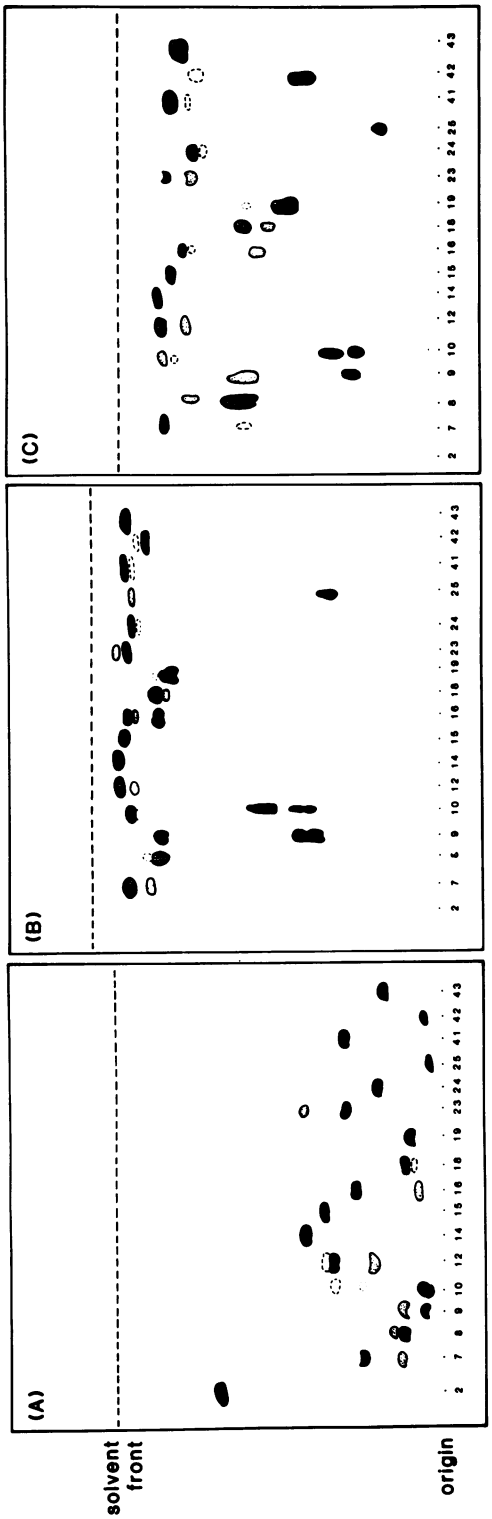

FIG. 1. *TLC of the deacetylated polar C-mycosidic peptidoglycolipids of MAIS serotypes. Full details of lipid extraction and chromatography are presented in the text. (A) Chloroform-methanol-water (60:16:2 by volume; solvent III); (B) chloroform-methanol-water (60:27:4 by volume; solvent I); (C) chloroform-methanol-water (65:25:4 by volume; solvent II). The C-mycosidic peptidoglycolipids were located with the orcinol spray, and the amount of stippling indicates the relative intensities of the spots. The apolar C-mycosidic peptidoglycolipids, which are channeled to the top of the plates, are not depicted.*

alongside those from the most appropriate serotypes (Fig. 2). Cultures designated A to G were the unknowns. Three of these (A, B, and D) corresponded to serotype 43 (Gause) and were distinguishable from serotype 24 by co-chromatography of extracts. Three others (C, E, and G) were serotype 14 (Boone), and one (F) did not correspond to any of the available serotypes.

Despite the success of this limited exercise, we realize that facile and independent identification of cultures by this means can be difficult for other laboratories. Extracts from several known serotypes are almost obligatory for even tentative identification, and the suspect serotype has to be available when a positive answer is being sought. However, just as we now supply typed cultures and specific antisera to bona fide investigators, in time the specific lipids may also become available.

In our experience, the apolar C-mycosidic peptidoglycolipids, whether examined in the natural state as in the Marks-Jenkins procedure or deacetylated as shown in Fig. 3, are generally useless for delineating MAIS serotypes. Those from most of the serotypes examined (only a representative few are depicted in Fig. 3) contain six chromatographically identical apolar C-mycosidic peptidoglycolipids. Hence we rely almost totally on the profiles of the polar lipids, achieved with the use of solvents I through III, to aid identification. It is not surprising that the polar C-mycosidic peptidoglycolipids are so uniquely characteristic of individual serotypes, since, as noted above, they are the basis of the seroagglutination procedure, whereas all other cellular fractions, including the apolar C-mycosides, are devoid of serological activity (Brennan and Goren, submitted for publication).

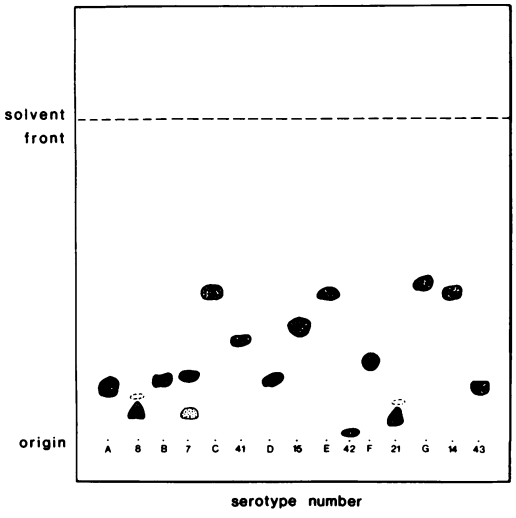

FIG. 2. *Final step in the identification of a batch of untyped cultures by the TLC procedure. See text for manipulations preceding this chromatogram. A through G represent untyped cultures. These were from a batch sent to us for identification by R. F. O'Connor, Institute of Clinical Pathology and Medical Research, Lidcombe, New South Wales, Australia. Solvent III (chloroform-methanol-water, 60:16:2) was used. See Fig. 1 and text for additional details.*

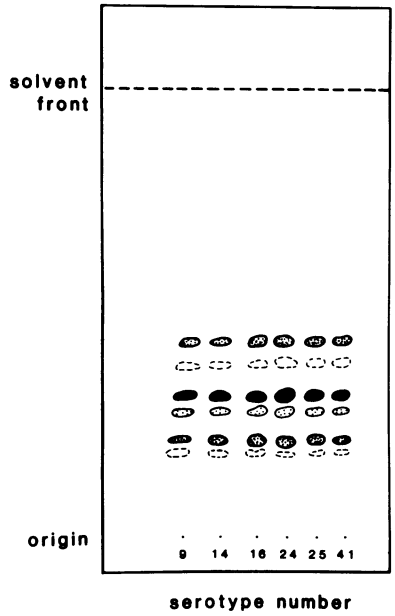

FIG. 3. *TLC of the deacetylated apolar C-mycosidic peptidoglycolipids of MAIS serotypes in chloroform-methanol-water (45:5:0.5 by volume; solvent IV). Other conditions are described in Fig. 1 and the text. The polar C-mycosidic peptidoglycolipids which are congregated at the origin are not depicted.*

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