Antigenic Relationships of the *Mycobacterium fortuitum*-*Mycobacterium chelonae* Complex

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We used a combination of chemical analysis, seroagglutination, and an enzyme-linked immunosorbent assay to examine the glycolipids of the rapidly growing mycobacteria in order to define the basis of the antigenicity of these organisms. This dual chemical and serological approach established that the typing antigens of *Mycobacterium chelonae* subsp. *chelonae* belong to the polar C-mycoside glycopeptidolipid class, the major component of which contains a trisaccharide composed of 3,4-di-*O*-methylrhamnose, rhamnose, and 6-deoxytalose. The glycolipid patterns of *M. chelonae* subsp. *abscessus* and *M. chelonae* subsp. *chelonae* were identical. The same approach showed that there is no relationship between *Mycobacterium fortuitum* biovar *fortuitum* and *Mycobacterium fortuitum* biovar *peregrinum*. The specific lipids of *M. fortuitum* biovar *peregrinum* belong to the alkali-stable C-mycoside glycopeptidolipid class and are different from those of *M. chelonae* subsp. *chelonae*. *M. fortuitum* biovar *fortuitum* isolates produced three patterns of alkali-labile lipids with features of lipooligosaccharide antigens. Moreover, strains and antisera of *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* used in combination produced no cross-reactions. Thus, chemical analysis of the type-specific antigens is a powerful adjunct to serology for studying the antigenic relationships among rapidly growing mycobacteria, and our results support the reinstitution of "*Mycobacterium peregrinum*" as a species distinct from *M. fortuitum*.

Chemical analysis and serology (either agglutination or an enzyme-linked immunosorbent assay [ELISA]) of the specific lipid antigens of many slowly growing nontuberculous mycobacteria have proved to be powerful tools for examining the fundamental differences among species of *Mycobacterium*. This dual approach has been used with members of the *Mycobacterium avium*-*Mycobacterium intracellulare*-*Mycobacterium scrofulaceum* (MAIS) complex (5, 6, 8), *Mycobacterium simiae* serovars I and II (unpublished data), *Mycobacterium kansasii*, *Mycobacterium szulgai*, and other species (P. J. Brennan and S. W. Hunter, Fed. Proc. 41:1173, 1982; S. W. Hunter, R. C. Murphy, K. Clay, M. B. Goren, and P. J. Brennan, J. Biol. Chem., in press). Accordingly, the characteristic antigens of nontuberculous mycobacteria have been found to belong to the following two classes: the C-mycoside glycopeptidolipids (6) and the trehalose-containing lipooligosaccharides (Brennan and Hunter, Fed. Proc. 41:1173, 1982; Hunter et al., in press). These antigens can be readily differentiated by thin-layer chromatography (TLC) after treatment of lipid extracts with alkali; the lipooligosaccharides are alkali labile and destroyed, whereas the glycopeptidolipids retain their basic structure and lipophilicity due to amide-linked acyl residues and simply lose acetyl substituents in the process (7, 9).

The realization that rapidly growing nontuberculous mycobacteria are opportunistic invaders of postoperative wounds and pulmonary lesions (3, 11, 23) has led to renewed interest in the use of the inherent antigenicity of these organisms in identification and in the charting of epidemiological trends. *Mycobacterium chelonae* corr. has been clearly differentiated from *Mycobacterium fortuitum* (13), and a number of parameters suggest that *M. chelonae* is the legitimate species name of mycobacteria that have been called, among other names, "*Mycobacterium abscessus*" and "*Mycobacterium borstelense*" (19). However, instead

of one simple taxon, some experts have argued for the designation of two subspecies, *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* (14). Likewise, *M. fortuitum* has been differentiated into two biovars, *M. fortuitum* biovar *peregrinum* and *M. fortuitum* biovar *fortuitum* (15). In the present study we supplemented serology with the fundamental chemistry of the specific lipid antigens to understand better the basis of the antigenicity and surface structures of these molecules. In essence, the opportunistic members of Runyon group IV (17) were examined in light of new information on the immunochemistry of the mycobacteria.

MATERIALS AND METHODS

Mycobacteria. The standard strains used were obtained from the serotyping culture collection and from the Trudeau Mycobacterial Culture Collection, both of which are housed at the National Jewish Hospital and Research Center/National Asthma Center under the auspices of National Institutes of Health, the United States-Japan Co-operative Medical Sciences (Tuberculosis) Program. A total of 33 strains were obtained from patient specimens supplied by R. J. Wallace, Department of Microbiology, University of Texas Health Center, Tyler, and H. M. Sommers, Department of Pathology, Northwestern Memorial Hospital, Chicago, Ill. These included 9 strains from sputa, 12 strains from wounds, 2 strains from bone abscesses, 3 strains from breast abscesses, 2 strains from corneal ulcers, 1 strain from abdominal wall tissue, 1 strain from lung tissue, 1 strain from pleural fluid, and 2 strains from unknown sources. All of the strains were routinely grown in 5 ml of 7H11 broth (9) and incubated for 5 days at 37°C. Samples (100 µl) were spread onto five 7H11 plates which were incubated at 37°C for 1 week. The growth from three plates was harvested into 10 ml of phenol-phosphate-buffered saline (PPBS) by scraping the plates with a bent capillary pipette and transferring the resulting suspension into a sterile screw-capped tube (20 by 125 mm). The

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suspension was allowed to stand for approximately 3 weeks at room temperature before it was used in the seroagglutination procedure. The growth on the remaining two plates was harvested in sterile physiological saline and used as a source of lipid for TLC analysis and ELISA. Larger quantities of *M. chelonae* were grown in a Micro-Ferm fermentor (model MF-205; New Brunswick Scientific Co.) in Sauton medium (pH 6.8) modified by adding casein hydrolysate (22). The bacteria were harvested by siphoning and then autoclaved and lyophilized.

TLC for strain identification. The procedure developed previously for extracting lipids from the MAIS complex (9) was used without alteration for the rapidly growing mycobacteria. The lipids were dried and dissolved in CHCl_3 - CH_3OH (2:1), and a portion was applied directly to TLC plates, which were developed in CHCl_3 - CH_3OH - H_2O (60:12:1). All plates were sprayed with orcinol- H_2SO_4 and photographed (9). Where indicated, an alkali treatment was applied as described previously (9).

ELISA. The ELISA procedure which we used is a modification of a procedure described recently (12). Intact or alkali-stable lipids of *M. chelonae* subsp. *chelonae*, *M. chelonae* subsp. *abscessus*, and *M. fortuitum* biovar *peregrinum* were prepared as described above for TLC, and were suspended in ethanol with sonication. Samples (50 μl) were added to each well of a 96-well polystyrene microtiter plate, allowed to dry overnight, and washed in a phosphate-buffered saline-0.1% Tween 80 solution (PBS-Tween) for 10 min. Rabbit antiserum (50 μl) was added, and after a 30-min incubation at room temperature, the serum was aspirated and the wells were washed four times with PBS-Tween. Finally, 50 μl of 1:1,000-diluted goat anti-rabbit immunoglobulin G peroxidase-linked conjugate (Sigma Chemical Co.) in PBS-Tween was added to each well, and the preparations were incubated for 45 min. After additional washes, 50 μl of *O*-phenylenediamine- H_2O_2 substrate in citrate phosphate buffer (12) was added, and the plates were incubated in the dark for 30 min. The reaction was stopped by adding 2.5 N H_2SO_4 , and absorption was read at 490 nm with a Micro-ELISA Reader (Dynatech Laboratories, Inc.).

Seroagglutination and absorption. The procedure used was modified from the method of Schaefer (18). A 3-week-old suspension of mycobacteria in PPBS was adjusted to an optical density at 525 nm of 0.3 with a Coleman Junior spectrophotometer. A 500- μl amount of this suspension and 500 μl of rabbit antisera prepared as described previously (18) against *M. fortuitum* biovar *fortuitum* strains Smith-6196 and Teichmiller, *M. fortuitum* biovar *peregrinum* strains New Mexico-680 and 12660, *M. chelonae* subsp. *chelonae* strains TMC 1544 and Ryder, and *M. chelonae* subsp. *abscessus* strain TMC 1543 were placed in tubes (12 by 75 mm). A control tube containing 500 μl of PPBS was also used with the organism suspensions. Tubes were shaken and incubated at 37°C. Using indirect light, we viewed agglutination after 4 and 24 h. The reactions were recorded as 4+ (complete agglutination) to 0 (no reaction). An identification was assigned when a 4+ reaction occurred with one pair of antisera and no reaction was recorded with the other antisera. (The working titers of the rabbit antisera were determined to be the highest dilutions of the sera that gave complete agglutination of a homologous strain.)

Sera were absorbed with nonhomologous strains as follows. A heavy suspension of the test organism was prepared by centrifugation for 10 min at 1,000 relative centrifugal force and suspended in an equal volume of PPBS. A sample (500 μl) was mixed with an equal volume of 1:10-diluted

antiserum and incubated overnight at 4°C. The absorbed antiserum was removed after centrifugation at 1,000 relative centrifugal force for 15 min, serially diluted, and titrated against homologous and heterologous strains. Those antisera that reduced the nonhomologous serum titer fourfold or less were considered to have low antibody-absorbing capacities and to be antigenically different from the corresponding type. The strains with high antibody-absorbing capacities were considered antigenically similar to the corresponding type.

Isolation of the glycopeptidolipid antigen of *M. chelonae*. Lyophilized cells (31 g) were extracted with 1.22 liter of CHCl_3 - CH_3OH (2:1) with stirring at room temperature for 18 h; this was followed by further extraction with 0.5 volume of extractant at 50°C. The dried lipid was dissolved in 300 ml of CHCl_3 - CH_3OH - H_2O (8:4:3) and centrifuged, and the separated phases were washed with separate upper and lower phases. The combined lower phases were dried, and dissolved in 30 ml of CHCl_3 - CH_3OH (2:1), and this was followed by the addition of an equal volume of 0.2 M NaOH in CH_3OH (9). The mixture was maintained at 37°C for 20 min, neutralized with glacial acetic acid (750 μl), dried on a rotary evaporator, and partitioned between 50 ml of CH_2Cl_2 - CH_3OH (2:1) and 10 ml of water.

The dried organic phase containing the washed alkali-stable glycopeptidolipids was applied to a column of silicic acid-Celite (2:1) which was irrigated with CHCl_3 (500 ml), followed by 300 ml each of 2, 3.5, and 5% CH_3OH in CHCl_3 . The CH_3OH -containing eluates were collected in fractions of about 50 ml each, dried, washed, and examined by TLC.

Isolation of r-Ose_n from the polar glycopeptidolipid of *M. chelonae*. To isolate reduced oligosaccharide (r-Ose_n), partially purified polar glycopeptidolipid from a silicic acid column was subjected to alkaline borohydride reductive cleavage (β -elimination) as follows. To 148 mg of the lipid were added 5 ml of ethanol and 1 ml of water (8). The suspension was heated at 60°C and briefly sonicated to aid dissolution; 1 N NaOH (4 ml) was added, followed by 454 mg of NaBH_4 and a small crystal of ^3H -labeled NaBH_4 . The mixture, which was in a tightly sealed tube, was allowed to react at 60°C for 21 h. Then it was neutralized with 2 M CH_3COOH , dried on the rotary evaporator, and partitioned between equal volumes of CHCl_3 - CH_3OH (2:1) and water. The dried aqueous phase was applied to a column (102 by 2 cm) of Sephadex G-15, which was eluted with water. Fractions (2 ml) were analyzed for carbohydrate (8) and counted for radioactivity. Incidental to this operation, the organic phase from the β -elimination reaction was chromatographed on thin-layer plates in CHCl_3 - CH_3OH -2% CH_3COONa (45:3:0.3) and compared with a similar product derived from the glycopeptidolipid antigen of *M. intracellulare* serovar 9 (6, 8).

Other analytical procedures. Most of the chemical procedures used have been described previously (6, 8). Cellulose TLC plates (0.1 mm) were obtained from E. Merck, and silica gel TLC plates (Redi-Plates) were obtained from Fisher Scientific Co. Reducing sugars were located on cellulose thin-layer plates with aniline-oxalate (6). Paper chromatography was conducted as described previously (8) by using an AgNO_3 -NaOH dip reagent to locate the oligosaccharides. The oligosaccharides were hydrolyzed with 2 M CF_3COOH in steam for 3 h. Alditol acetates were prepared as described previously (8). Gas-liquid chromatography was conducted with a Hewlett-Packard model 5800A gas chromatograph attached to a Hewlett-Packard model 3380A integrator. Further details are provided in the legend to Fig. 4.

TABLE 1. Comparison of strains identified as *M. chelonae* by traditional biochemical means, seroagglutination, and TLC

Strain ^a	Identification based on biochemical reactions	Antiserum reactions				Identification based on serology	Identification based on TLC of lipid antigens
		<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i>		
Sullivan	<i>M. chelonae</i> subsp. <i>chelonae</i>	—	++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
TMC 1542	<i>M. chelonae</i> subsp. <i>chelonae</i>	—	+	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
TMC 1544	<i>M. chelonae</i> subsp. <i>chelonae</i>	—	—	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
3-29	<i>M. chelonae</i>	—	—	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
4-28	<i>M. fortuitum</i>	—	++++	++++	++++	<i>M. fortuitum</i> biovar <i>peregrinum</i> , <i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
6-11	<i>M. chelonae</i>	—	++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
1-8	<i>M. chelonae</i>	—	++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
3-7	<i>M. chelonae</i>	—	++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
3-16	<i>M. chelonae</i>	—	+	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
3-22	<i>M. chelonae</i>	—	+++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
6-40	<i>M. chelonae</i>	—	+++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
8-1	<i>M. fortuitum</i>	—	+	++++	+++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
8-12	<i>M. fortuitum</i>	—	++	++++	+++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
8-13	<i>M. fortuitum</i>	—	++	++++	+++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
8-21	<i>M. fortuitum</i>	—	+	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>
TMC 1543	<i>M. chelonae</i> subsp. <i>abscessus</i>	—	+++	++++	++++	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i> , <i>M. chelonae</i> subsp. <i>abscessus</i>

^a All isolates except strains Sullivan, TMC 1542, TMC 1544, and TMC 1543 were obtained from R. J. Wallace and H. M. Sommers.

RESULTS

Seroagglutination of *M. chelonae* subspecies. Table 1 shows the agglutination reactions of reference strains which had been classified as *M. chelonae* subsp. *chelonae* or *M. chelonae* subsp. *abscessus*, as well as clinical isolates, some of which were considered *M. fortuitum* based largely on biochemical reactions (19). There was no difference in the agglutination reactions of the *M. chelonae* subsp. *chelonae* strains and the one *M. chelonae* subsp. *abscessus* reference strain. With some of the clinical isolates (strains 8-1, 8-12,

and 8-13), slightly weaker, although probably insignificant, reactions occurred with *M. chelonae* subsp. *chelonae* antisera. Strain 4-28 gave a distinctly positive reaction with the *M. fortuitum* biovar *peregrinum* antisera, but full corroboration of this identification had to await TLC analysis. *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* strains reacted to variable extents with *M. fortuitum* biovar *peregrinum* antiserum, but there were no responses to *M. fortuitum* biovar *fortuitum* sera.

TLC of the lipid antigens of *M. chelonae* subsp. *abscessus*. Figure 1A shows the results of chromatography of the total

FIG. 1. (A) TLC of the intact lipids from reference strains and clinical isolates of *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* and one isolate of *M. fortuitum* biovar *peregrinum* (lane 5). The solvent used was $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (60:12:1), and the spray used was orcinol- H_2SO_4 . (B) TLC of the alkali-stable lipids under the same conditions. Lane 1, Strain Sullivan; lane 2, strain TMC 1542; lane 3, strain TMC 1544; lane 4, strain 3-29; lane 5, strain 4-28; lane 6, strain 6-11; lane 7, strain 1-8; lane 8, strain 3-7; lane 9, strain 3-16; lane 10, strain 3-22; lane 11, strain 6-40; lane 12, strain 8-1; lane 13, strain 8-12; lane 14, strain 8-13; lane 15, strain 8-21; lane 16, strain TMC 1543. GPLS, Glycopeptidolipids.

untreated lipids from the strains shown in Table 1. With one exception (strain 4-28 [Fig. 1A, lane 5]), the profiles of the lipids of all isolates were identical, albeit not particularly striking. Nevertheless, spots with a yellow to golden hue were evident, and, as indicated elsewhere (7, 9), such a response to the orcinol spray is highly indicative of the presence of glycopeptidolipids. These molecules are resistant to alkali, whereas the majority of the nonspecific lipids shown in Fig. 1A are susceptible. The products of mild treatment with NaOH were predominantly yellow-tinged spots and were fewer in number, presumably due to the absence of multiacetylated varieties (Fig. 1B). Again, all isolates of *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* were identical in this respect, and the isolates for which biochemical parameters suggested identification as *M. fortuitum* but serology suggested otherwise were obviously *M. chelonae*. The one exception (strain 4-28 [Fig. 1B, lane 5]), for which serology suggested identification as *M. fortuitum* biovar *peregrinum*, produced a spectrum of glycopeptidolipids that was characteristic of *M. fortuitum* biovar *peregrinum* (see below).

Structure of the polar glycopeptidolipid from *M. chelonae*. The yield of washed lipids from a typical batch of 31 g of *M. chelonae* cells grown in modified Sauton medium was 3.23 g. Such preparations were treated with 0.2 M NaOH in order to destroy the unwanted glycerides and simplify the pattern of glycopeptidolipids, thereby facilitating isolation of the species-specific polar glycopeptidolipid (2). Irrigation of silicic acid to which the alkali-stable lipids had been applied with CHCl_3 , followed by irrigation with 2% CH_3OH in CHCl_3 , removed the unwanted fatty acids. Subsequent irrigation with 3.5% CH_3OH in CHCl_3 removed the apolar glycopeptidolipids, whereas 3.5 and 5% CH_3OH elutriants provided the polar (presumed antigenic) glycopeptidolipids. About 340 mg of total glycopeptidolipids was recovered in the process, of which about 140 mg was of the polar variety. This latter mixture was subjected to reductive β -elimination in the

presence of ^3H -labeled NaBH_4 (8), and the water-soluble products were applied to a Sephadex G-25 column (Fig. 2). The mixed carbohydrate-containing fractions yielded 17 mg of ^3H -labeled r-Ose_n that was largely free of inorganic reaction products. The lipid products of the β -elimination reaction were subjected to TLC in $\text{CHCl}_3\text{-CH}_3\text{OH}$ -2% CH_3COONa (45:30:0.3) (8). The major product had a mobil-

FIG. 2. Gel filtration on a Sephadex G-15 column (110 by 1.5 cm) of the water-soluble products from reductive (NaB^3H_4) alkaline cleavage of the polar glycopeptidolipids from *M. chelonae*. About 20 mg (2.35×10^6 cpm) of rhamnose equivalents was applied, and about 17 mg (2×10^6 cpm) was recovered in the mixed carbohydrate peak.

ity which was identical to that of similarly derived products from MAIS complex serovars (6, 8). This product was isolated by preparative TLC and hydrolyzed, and gas-liquid chromatography of the sugar (as its alditol acetate) showed that it was 3,4-di-*O*-methylrhamnose (data not shown). Thus, the fatty acylpeptidyl-monoglycosyl backbone of the glycopeptidolipid from *M. chelonae* seems to be identical to that from serovars of the MAIS complex (8).

Paper chromatography of ^3H -labeled r-Ose_n from the *M. chelonae* subsp. *chelonae* glycopeptidolipid showed chromatographic mobility in the region of known hexose-containing disaccharides (Fig. 3). The ^3H -labeled r-Ose_n was hydrolyzed, chromatographed on cellulose thin layers, and stained with aniline-oxalate for reducing sugars. Only two such sugars were located, and these corresponded to rhamnose and 3,4-di-*O*-methylrhamnose. However, gas-liquid chromatography of the alditol acetates from ^3H -labeled r-Ose₃ showed that the following three components were present (Fig. 4): the acetates of 6-deoxytalitol, rhamnitol, and 3,4-di-*O*-methylrhamnitol. Obviously, as in the case of other r-Ose_n (5), the nonreducing 6-deoxytalitol occurred in the ^3H -labeled r-Ose_n from *M. chelonae*. Thus, the r-Ose_n is a 6-deoxyhexose-containing trisaccharide (r-Ose₃) which has chromatographic properties like those of hexose-containing disaccharides. In the glycopeptidolipid antigens from MAIS complex serovars, rhamnose always occupies the second position and is linked to 6-deoxytalitol through an α -1 $\rightarrow$ 2 linkage, whereas *O*-methyl-6-deoxyhexoses occupy ultimate or penultimate positions (5). Hence, through a process of direct analysis, comparison with the products from the MAIS complex, and deduction, we propose the following incomplete and tentative structure for the r-Ose₃ from *M. chelonae*: 3,4-di-*O*-methylrhamnose-(1 $\rightarrow$?)—rhamnose-(α -1 $\rightarrow$ 2)—6-deoxytalitol.

FIG. 3. Descending paper chromatography on Whatman no. 1 paper of ^3H -labeled r-Ose_n from *M. chelonae* subsp. *chelonae* along with raffinose, maltose, and trehalose. Oligosaccharides were located with AgNO_3 -NaOH. The solvent used was 1-butanol-pyridine-water (6:4:3).

FIG. 4. Gas-liquid chromatography of the alditol acetates derived from pure ^3H -labeled r-Ose_n of *M. chelonae* subsp. *chelonae*. About 35 μg of the r-Ose_n was hydrolyzed with 2 M CF_3COOH in a steam bath for 3 h, and the products were reduced and acetylated. The following operating conditions were used: 3% SP-2340 on 100-120 Supelcoport; 175°C; 60 ml of N_2 per min. Me, Methyl; RHA, rhamnose; 6-dTAL, 6-deoxytalose.

ELISA and the glycopeptidolipid antigens of rapidly growing mycobacteria. The application of the ELISA to glycopeptidolipids per se, including those of the MAIS complex, *M. simiae* serovars I and II, *Mycobacterium mageritense*, *M. chelonae* subsp. *chelonae*, *M. chelonae* subsp. *abscessus*, and *M. fortuitum* biovar *peregrinum*, will be reported separately (C. V. Knisely, V. L. Barr, A. Y. Tsang, J. K. McClatchy, and P. J. Brennan, manuscript in preparation). Briefly, whole lipid extracts from *M. chelonae* subsp. *chelonae*, *M. chelonae* subsp. *abscessus*, and *M. fortuitum* biovar *peregrinum* strains reacted readily in the ELISA with serum to the homologous strain. Alkali treatment of the extracts did not diminish the activity, thereby verifying that the alkali-stable glycopeptidolipids were the active substances. The reactions of *M. chelonae* subsp. *chelonae* lipid with anti-*M. chelonae* subsp. *abscessus* serum were only about one-third the reactions of the homologous pair. Likewise, when the reverse reaction was conducted, there was not full equivalence. However, less than full correspondence even among different strains belonging to the one MAIS complex serovar is common with the ELISA (D. L. Yanagihara, unpublished data).

Seroagglutination of *M. fortuitum* biovars. Table 2 shows the agglutination reactions of reference strains and a selection of clinical isolates of *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum*. Strains of *M. fortuitum* biovar *fortuitum* which produced unequivocal seroagglutination and TLC results did not cross-react with *M. fortuitum* biovar *peregrinum*, *M. chelonae* subsp. *abscessus*, and *M. chelonae* subsp. *chelonae* antisera. Thus, strain 5-34 proved to be *M. fortuitum* biovar *fortuitum* and not *M. chelonae*. On the other hand, strain 7-56 proved to be *M. fortuitum* biovar *peregrinum*. The same relationship was true for 18 other isolates supplied by R. J. Wallace and H. M. Sommers. Although *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* cross-reacted with *M. fortuitum* biovar *peregrinum* antisera, there was no cross-reaction with *M. fortuitum* biovar *fortuitum* antisera. Thus, our results support the hypothesis that there is a major antigenic difference between *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* and corroborate the results of Jenkins et al. (13).

TABLE 2. Comparison of strains identified as *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* by traditional biochemical means, seroagglutination, and TLC

Strain	Identification based on biochemical reactions	Antiserum reactions				Identification based on serology	Identification based on TLC of lipid antigens
		<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. chelonae</i> subsp. <i>chelonae</i>		
5-34	<i>M. chelonae</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
7-82	<i>M. fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
5-8	<i>M. fortuitum</i>	++++	++++	++++	++++	Spontaneous agglutination	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-32	<i>M. fortuitum</i> biovar <i>fortuitum</i>	—	—	—	—	Untypable	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-33	<i>M. fortuitum</i> biovar <i>fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-34	<i>M. fortuitum</i> biovar <i>fortuitum</i>	+	—	—	—	Untypable	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-36	<i>M. fortuitum</i> biovar <i>fortuitum</i>	—	—	—	—	Untypable	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-37	<i>M. fortuitum</i> biovar <i>fortuitum</i>	—	—	—	—	Untypable	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-38	<i>M. fortuitum</i> group 3 ^a	—	—	—	—	Untypable	<i>M. fortuitum</i> biovar <i>fortuitum</i>
10-35	<i>M. fortuitum</i> biovar <i>peregrinum</i>	—	++++	++++	+++	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
11-19	<i>M. fortuitum</i> biovar <i>fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-20	<i>M. fortuitum</i> biovar <i>fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-21	<i>M. fortuitum</i> biovar <i>fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-22	<i>M. fortuitum</i> biovar <i>fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-23	<i>M. fortuitum</i> biovar <i>fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-24	<i>M. fortuitum</i> biovar <i>fortuitum</i>	+++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-25	<i>M. fortuitum</i>	++++	++++	++++	++++	Spontaneous agglutination	<i>M. fortuitum</i> biovar <i>fortuitum</i>
11-26	<i>M. fortuitum</i> group 3 ^a	—	++++	++	++	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
TMC 1529	<i>M. fortuitum</i>	++++	—	—	—	<i>M. fortuitum</i> biovar <i>fortuitum</i>	<i>M. fortuitum</i> biovar <i>fortuitum</i>
Starr-Glidden	<i>M. fortuitum</i> biovar <i>peregrinum</i>	—	++++	++	+++	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
TMC 1545	<i>M. fortuitum</i> biovar <i>peregrinum</i>	—	++++	+++	+++	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
Broussard	<i>M. fortuitum</i> biovar <i>peregrinum</i>	—	++++	++++	+++	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
TMC 1547	<i>M. fortuitum</i> biovar <i>peregrinum</i>	++	++++	+++	+++	Spontaneous agglutination	<i>M. fortuitum</i> biovar <i>peregrinum</i>
1-73	Rapid grower	—	+++	—	—	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>
7-56	<i>M. fortuitum</i>	—	++++	++	++	<i>M. fortuitum</i> biovar <i>peregrinum</i>	<i>M. fortuitum</i> biovar <i>peregrinum</i>

^a The unnamed group 3 biovar of *M. fortuitum* is differentiated from *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* by its ability to produce acid from inositol, mannitol, and sorbitol (4).

Chromatography of the lipids of the *M. fortuitum* biovars. TLC of the lipids from five isolates of *M. fortuitum* biovar *peregrinum* before (Fig. 5A) and after (Fig. 5B) alkali treatment revealed that the relevant and identifying spots again had the golden yellow to golden brown hue typical of the C-mycoside glycopeptidolipids. Again, we observed the characteristic accentuation and simplification of these components upon alkali treatment. Figure 6 shows the identifying lipids of a few isolates of *M. chelonae* subsp. *chelonae*

and *M. fortuitum* biovar *peregrinum* after exposure to alkali in order to enhance the comparison. A less polar solvent was used to illustrate the various polarities of the glycolipids.

Figure 7 shows the results of TLC of the intact lipids from a selection of other *M. fortuitum* isolates, mostly biovar *fortuitum*. A number of patterns were observed. Pattern 1 occurs in lanes 2 through 4, 6 through 8, 11 through 14, 17, and 19 and was characterized by a dark purple spot. Pattern 2 was more nondescript (lanes 5, 15, and 16); this pattern

FIG. 5. TLC in $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (65:25:4) of the total lipids from five strains of *M. fortuitum* biovar *peregrinum* (see Table 2) (Starr-Glidden, TMC 1545, Broussard, TMC 1547, Rapid Grower [= 1-73]) and *M. fortuitum* 7-56. (A) Before alkaline treatment. (B) After alkaline treatment. GPLS, Glycopeptidolipids.

was identical to pattern 1, except that the major purple spot was missing. Pattern 3 was characterized by a very distinct dark spot (lanes 1 and 9). A total of 29 isolates with a seroagglutination response indicative of *M. fortuitum* biovar *fortuitum* were examined; pattern 1 was observed in 15, pattern 2 was observed in 12, and pattern 3 was observed in only 2. Seemingly, only pattern 1 corresponds to the pattern described previously (13). Incidentally, the pattern in lanes 10 and 18, with yellow to gold colors, was the pattern of the intact lipids from *M. fortuitum* biovar *peregrinum*. Shared characteristics of patterns 1 through 3 were the absence of any yellowish gold coloration and complete obliteration of the patterns with alkali. Figure 8, lanes 1 and 2, show pattern 1 before and after alkali treatment, and lanes 3 and 4 show pattern 3 before and after alkali treatment.

Absorption studies. Rabbit antibodies to *M. chelonae* subsp. *chelonae* were completely absorbed by strains of *M. chelonae* subsp. *abscessus*. Conversely, antibodies to *M. chelonae* subsp. *abscessus* were absorbed by strains of *M. chelonae* subsp. *chelonae*, and neither was absorbed by strains of *M. fortuitum* biovar *peregrinum*. Moreover, there was no change when *M. fortuitum* biovar *fortuitum* was used for absorption of *M. fortuitum* biovar *peregrinum* antisera (Table 3). We conclude on the basis of seroagglutination and antibody absorption that *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* are antigenically synonymous, whereas *M. fortuitum* biovar *peregrinum* may share some antigens, but is not homologous.

DISCUSSION

In this study we confirmed and extended the data of Jenkins et al. (13). Using TLC and the Schaefer seroagglutination protocol at a time that predated realization that the typing antigens and the specific lipids are synonymous, Jenkins et al. (13) observed that nonabsorbed *M. chelonae* subsp. *abscessus* antisera showed considerable cross-reac-

tion with *M. fortuitum* biovar *peregrinum* but little cross-reaction with *M. fortuitum* biovar *fortuitum* and that after absorption by *M. fortuitum* biovar *peregrinum*, the antisera still agglutinated *M. chelonae* subsp. *abscessus*. Similar absorptions were conducted in this work with *M. chelonae* subsp. *chelonae* with similar results, and our data indicated that the two *M. chelonae* subspecies show considerable homology, if not synonymy. Moreover, using the TLC approach, Jenkins et al. (13) observed that strains of *M. chelonae* subsp. *abscessus* contained a major lipid which yielded "a deep orange spot" in response to the orcinol spray. Somewhat similar descriptions had been given for the characteristic lipids of the MAIS complex (9), and we now realize that these are C-mycoside glycopeptidolipids with a precise oligosaccharide determinant and are the actual typing antigens of the MAIS complex. At that time we believed that the glycopeptidolipid antigens were restricted to the MAIS complex. However, we have since determined that in addition to being present in *M. chelonae* and *M. fortuitum* biovar *peregrinum*, these antigens are also present in *M. simiae* serovars I and II and in *M. malmoeense* (V. L. Barr, D. L. Yanagihara, A. Y. Tsang, J. K. McClatchy, and P. J. Brennan, unpublished data). The distinctive feature of the glycopeptidolipid antigen of *M. chelonae* is a 3,4-di-O-methylrharnnose substituent on the oligosaccharide moiety. Previously, we found this sugar linked only to the alaninol of the peptide entity (6, 8); in *M. chelonae* it apparently occurs at both the peptide and oligosaccharide termini. Also, the oligosaccharide, being a trisaccharide, is smaller than the tetrasaccharides found in the MAIS complex products; otherwise, the glycopeptidolipid molecules appear to be identical.

On the basis of a number of biochemical tests, an international consortium identified two subspecies of *M. chelonae*, *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* (14). In the present study we show that there is

FIG. 6. TLC in a less polar solvent, $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (60:12:1), of the lipids from three of the authenticated isolates of *M. chelonae* subsp. *chelonae* (A) and *M. fortuitum* biovar *peregrinum* (B) after alkali treatment.

FIG. 7. TLC in $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (60:16:2) of the intact lipids from strains of *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum*. Lane 1, strain 5-34; lane 2, strain 7-82; lane 3, strain 5-8; lane 4, strain 10-32; lane 5, strain 10-33; lane 6, strain 10-34; lane 7, strain 10-36; lane 8, strain 10-37; lane 9, strain 10-38; lane 10, strain 10-35; lane 11, strain 11-19; lane 12, strain 11-20; lane 13, strain 11-21; lane 14, strain 11-22; lane 15, strain 11-23; lane 16, strain 11-24; lane 17, strain 11-25; lane 18, strain 11-26; lane 19, strain TMC 1529.

essentially no difference between the two subspecies in what amounts to fundamental outer surface composition and antigenicity (2). Deoxyribonucleic acid-deoxyribonucleic acid homology studies performed by Baess (1) showed that the level of homology between *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* is 99%. Baess supported the suggestion that these two strains belong to the same species. The electrophoretic patterns of the cytoplasmic proteins (10) and the antibiotic susceptibility characteristics (21) also support synonymy between the two subspecies.

The fundamental differences between *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* were revealed by simple chemical manipulations of the specific lipids. Those of *M. fortuitum* biovar *peregrinum* belong to the alkali-stable C-mycoside glycopeptidolipid class and are the serotyping antigens. On the other hand, *M. fortuitum* biovar *fortuitum* is devoid of glycopeptidolipids and is characterized by a population of distinct alkali-labile lipids. Jenkins et al. (13) also noted differences between the lipids of these two biovariants. They reported that the identifying lipid of *M. fortuitum* biovar *peregrinum* produced a "bright orange" response to the orcinol reagent, whereas extracts of *M. fortuitum* biovar *fortuitum* produced a pair of "double purple spots." In this study we found that strains which conform to the *M. fortuitum* designation by a number of parameters, including agglutination, exhibit a variety of lipid profiles, of which purple spots is just one; however, alkaline lability applies to all. Recently, we found that alkaline lability may indicate the presence of a class of novel anti-

FIG. 8. TLC in $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (64:10:1) of lipids from *M. fortuitum* isolates representing patterns 1 and 3. Lane 1, Pattern 1; lane 2, pattern 1 after alkali treatment; lane 3, pattern 3; lane 4, pattern 3 after alkali treatment.

TABLE 3. Agglutination reactions of sera prepared with strains of *M. fortuitum* biovar *peregrinum*, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus* before and after absorption

Strain used to prepare antiserum	Strain used to absorb antiserum	Homologous reaction before absorption	Homologous reaction after reaction
<i>M. fortuitum</i> biovar <i>fortuitum</i> 6196 Smith	<i>M. fortuitum</i> biovar <i>peregrinum</i> New Mexico 680	0	ND ^a
<i>M. fortuitum</i> biovar <i>peregrinum</i> New Mexico 680	<i>M. fortuitum</i> biovar <i>fortuitum</i> 6196 Smith	0	ND
<i>M. fortuitum</i> biovar <i>peregrinum</i> New Mexico 680	<i>M. chelonae</i> subsp. <i>abscessus</i> TMC 1543	4+	0
<i>M. fortuitum</i> biovar <i>peregrinum</i> New Mexico 680	<i>M. chelonae</i> subsp. <i>chelonae</i> TMC 1544	4+	0
<i>M. chelonae</i> subsp. <i>chelonae</i> TMC 1544	<i>M. fortuitum</i> biovar <i>fortuitum</i> 6196 Smith	0	ND
<i>M. chelonae</i> subsp. <i>chelonae</i> TMC 1544	<i>M. chelonae</i> subsp. <i>abscessus</i> TMC 1543	4+	0
<i>M. chelonae</i> subsp. <i>abscessus</i> TMC 1543	<i>M. chelonae</i> subsp. <i>chelonae</i> TMC 1544	4+	0
<i>M. chelonae</i> subsp. <i>abscessus</i> TMC 1543	<i>M. fortuitum</i> biovar <i>peregrinum</i> New Mexico 680	1+	0

^a ND, Not done, because there was no cross-reaction.

gens, the trehalose-containing lipooligosaccharides. The oligosaccharide substituent in the antigens from *M. kansasii* contain a trehalose moiety, xylose, 3-*O*-methylrhamnose, fucose, and a novel *N*-acyl amino sugar; in addition, the entire oligosaccharide is esterified by 2,4-dimethyltetradecanoyl groups (Hunter et al., in press). Analogous but species-specific lipooligosaccharide antigens characterize *M. szulgai*, *Mycobacterium marinum*, members of the *Mycobacterium gordonae* complex, and presumably other nontuberculous mycobacteria (P. J. Brennan, S. W. Hunter, V. L. Barr, and D. M. Wynn, unpublished data). However, despite alkaline lability, we are not yet prepared to say that the characteristic lipids of *M. fortuitum* belong to the lipooligosaccharide class, because, unlike other workers, we have been unsuccessful in associating antigenicity with the lipids by using gel diffusion and ELISA technology.

The international consortium (14) concluded that *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* should be considered a "homologous assemblage." However, Baess (1) showed that on the basis of deoxyribonucleic acid-deoxyribonucleic acid homology, *M. fortuitum* biovar *fortuitum* is more related to *M. fortuitum* biovar *peregrinum* than to other species of mycobacteria; the levels of homology range from 47 to 57%, and Baess concluded that *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* should be considered separate species. Our data support the proposal of Baess that *M. fortuitum* biovar *fortuitum* and *M. fortuitum* biovar *peregrinum* should be differentiated, and we support the revival of "*Mycobacterium peregrinum*" as a species distinct from *M. fortuitum*.

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