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Trehalose-Containing Lipooligosaccharides of *Mycobacterium gordonae*: Presence of a Mono-*O*-methyltetra-*O*-acyltrehalose "Core" and Branching in the Oligosaccharide Backbone[†]

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ABSTRACT: Past evidence has indicated that *Mycobacterium gordonae*, as isolated from soil and as an occasional opportunistic pathogen, exists as a serocomplex. We now demonstrate that the basis of seroreactivity and diversity is a novel series of alkali-labile, trehalose-containing lipooligosaccharides (LOS). The structures from two strains were established by per-*O*-methylation, partial acid hydrolysis, infrared and high-field NMR spectroscopy, electron-impact MS, and fast atom bombardment/mass spectrometry of the native lipooligosaccharides and hydrolysis products. The structure of the major lipooligosaccharide, LOS-I, of *M. gordonae* strain 989 was defined as 2-*O*-CH_3-4-*O*-CH_3CO-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-CH_3-α-L-Rha*p*-(1→3)-[β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-2,3,4,6-tetra-*O*-acyl-α-D-Glc*p*, which was further glycosylated at C-3 of the terminal 2-*O*-CH_3-4-*O*-CH_3CO-α-L-Fuc*p* by an incompletely defined *N*-acyl derivative of 4-amino-4,6-dideoxy-2,3-di-*O*-CH_3-Gal*p*. The structure of the major lipooligosaccharide, LOS-I, of a second strain of *M. gordonae* (strain 990) was defined as α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-[β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-2,3,4,6-tetra-*O*-acyl-α-D-Glc*p*. The other minor LOSs from both strains were also defined. Both families of LOSs from the two strains contain a novel mono-6′-*O*-CH_3-2,3,4,6-tetra-*O*-acyltrehalose unit, representing the first example of such a unit among the LOSs isolated to date from mycobacteria. Also, the more polar antigenic products, LOS-I, -II′, -II″, and -III from *M. gordonae* 989 and LOS-I, -II, and -II′ from *M. gordonae* 990, are characterized by branching of the oligosaccharide backbone, the first instance of sugar branching in these products. In the case of LOS-I and -III from *M. gordonae* 989, the branch consists of a terminal (*t*)-β-D-Xyl*p* unit, whereas in LOS-II′ and -II″, they are (*t*)-3-*O*-CH_3-β-D-Xyl*p* and (*t*)-α-D-Ara*f*, respectively. Similarly, the more polar antigenic glycolipids of *M. gordonae* 990 are characterized by branching in the oligosaccharide backbone, a single terminal β-D-Xyl*p* residue in the case of LOS-I and LOS-II′, and an α-D-Ara*f* unit in LOS-II″. In some members of the LOS family, the unique branching of the oligosaccharide backbone was absent. The LOSs reacted strongly with antiserum raised against the homologous strain and only weakly against that to the heterologous strain, demonstrating that *M. gordonae*, like the *Mycobacterium avium* complex, is a serocomplex, but based on the trehalose-containing LOSs rather than the glycopeptidolipids.

During the last few years, it has become evident that diseases due to mycobacteria (mycobacterioses) other than tuberculosis occur more frequently than was earlier assumed, especially in patients with acquired immunodeficiency syndrome. Precedent demonstrates that most of these nontuberculosis mycobacterial species are endowed with large quantities of a variety of glycolipids, which contain novel oligosaccharides, often with novel nonreducing-end sugars (McNeil et al., 1989). These haptenic oligosaccharides are of sufficient antigenicity as to evoke corresponding specific antibodies which allow serodiagnosis of some individual mycobacterioses, such as leprosy (Gaylord & Brennan, 1987). One class of such glycolipids, the glycopeptidolipids (GPL),[1] notably from members of the *Mycobacterium avium* complex, have been examined in detail, and the structures of the oligosaccharide haptens from most of the predominant serovars of the *M. avium* serocomplex have been fully elucidated (Chatterjee et al., 1987; McNeil et al., 1987b). A second group of surface antigens, the so-called alkali-labile, lipooligosaccharides (LOS) of mycobacteria (Hunter et al., 1983), exhibit an unusual principle in carbohydrate chemistry: the putative reducing

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[1] Abbreviations: LOS, lipooligosaccharide; OSE, oligosaccharide; GPL, glycopeptidolipids; PGL, phenolic glycolipids; Rha*p*, rhamnopyranose; Xyl*p*, xylopyranose; Glc*p*, glucopyranose; Ara*f*, arabinofuranose; DeoxyHex, deoxyhexose; Hex, hexose; MeDeoxyHex, *O*-methylated deoxyhexose; MeHex, *O*-methylated hexose; Pent, pentose; PBS, phosphate-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; *m*-NBA, *meta*-nitrobenzyl alcohol; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay; GC/MS, gas chromatography/mass spectrometry; FAB/MS, fast atom bombardment/mass spectrometry; NMR, nuclear magnetic resonance; amu, atomic mass units; *J*, coupling constant; 2D ^{1}H COSY, two-dimensional chemical shift correlated spectroscopy.

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end is occupied by the nonreducing α,α'-trehalose unit. The known trehalose-containing glycolipids described to date are the pyruvylated glycolipids of *Mycobacterium smegmatis* (Saadat & Ballou, 1983) and the more glycosylated antigenic variety found in *Mycobacterium kansasii* (Hunter et al., 1983, 1985), *Mycobacterium szulgai* (Hunter et al., 1988), *Mycobacterium malmoense* (McNeil et al., 1987a), and *Mycobacterium tuberculosis* (Canetti) (Daffe et al., 1991). The other class of glycolipids unique to mycobacteria are the phenolic glycolipids (PGL). These are glycosides of phenolphthiocerol dimycocerosate, in which the glycosyl units are reflective of an individual species. The most notable of these is the *O*-methylglucose-, *O*-methylrhamnose-containing triglycoside of the phenolic glycolipid of *Mycobacterium leprae*, which has been used widely in the serodiagnosis of leprosy (McNeil et al., 1989).

Mycobacterium gordonae (formerly *Mycobacterium aquae*), which is sometimes known as the "tap water bacillus", is a slow-growing scotochromogenic acid-fast bacillus (Runyon group II) with characteristic cultural and biochemical properties. *M. gordonae* is frequently isolated in diagnostic laboratories but is almost always considered nonpathogenic. It is widely distributed in the environment and, as a result of its presence in water sources, has often been involved in nosocomial pseudoinfections and pseudoepidemics. More recently, however, sporadic cases of *M. gordonae* infection have occurred, primarily in patients with prosthetic devices (Lohr et al., 1978), compromised immunity (Chan et al., 1984), chronic pulmonary disease (Kumar & Varkey, 1980), or a history of trauma (Barber et al., 1991), to the extent that *M. gordonae* can now be considered as a potential opportunistic pathogen in patients with advanced HIV-1 infection (Barber et al., 1991; Weinberger et al., 1992).

Goslee et al. (1976) recognized that *M. gordonae* could be grouped in a fashion similar to the *M. avium* serocomplex and suggested the presence of at least seven distinct serotypes based on seroagglutination tests (Schaefer, 1965, 1967). In a later study, Brennan et al. (1982) demonstrated that the dominant, surface antigens of the seven serotypes of *M. gordonae* were alkali-labile glycolipids and, accordingly, were not similar to the antigenic glycolipids of *M. avium*. Since *M. gordonae* is now regarded as an opportunistic respiratory tract pathogen in patients with HIV infection, we have addressed the structures of these glycolipids in order to provide new tools for the identification and serodiagnosis of *M. gordonae*, allow its differentiation from other atypical mycobacteria, and ultimately aid in the promulgation of better chemotherapy in light of the stubborn drug resistance of isolates of *M. gordonae* (Barber et al., 1991).

EXPERIMENTAL PROCEDURES

Growth of Mycobacteria and Extraction of Lipids. Isolates of *M. gordonae* (strains no. 989 and 990 from the collection previously maintained at National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) were grown in glycerol–alanine salts medium at 37 °C for 10 weeks, and the entire suspension was autoclaved and dried at 50 °C in crystallizing dishes. The dried cells (approximately 100 g) were extracted twice with $CHCl_3$–CH_3OH (2:1; 40 mL/g of cells) at 37 °C overnight (Hunter et al., 1983). The dried organic extracts were dissolved in $CHCl_3$–CH_3OH–H_2O (4:2:1; 700 mL) (Folch et al., 1957); the contents of the lower organic phase yielded approximately 26 and 12 g of washed lipid from *M. gordonae* 989 and 990, respectively.

Purification of LOSs. Washed lipids were dissolved in $CHCl_3$ and applied to a column (3 × 40 cm) of silica gel (60–200 mesh) (Baker Chemical Co., Phillipsburgh, NJ) equilibrated in $CHCl_3$ and eluted with increasing concentrations of CH_3OH as described previously (Daffe et al., 1991). Fractions (10 mL) were collected and monitored by TLC in $CHCl_3$–CH_3OH–H_2O (65:25:4) for glycolipid content after spraying with 10% sulfuric acid in ethanol and heating at 110 °C for 5 min (Hunter et al., 1988; Daffe et al., 1991).

Immunological Procedures. Polyclonal rabbit antisera were obtained by injecting a 1 mL suspension of heat-killed, lyophilized whole cells of *M. gordonae* strains 989 and 990 in PBS at an optical density of 0.5 read at a wavelength of 525 nm. Rabbits were immunized every 2 weeks and serum was tested by ELISA, starting 1 week after the second injection. When the serum titer reached 1:500 against the whole-cell immunogen, rabbits were bled and the serum was collected. Polyclonal rabbit antisera were tested against pure LOS preparations by coating 96-well polystyrene plates (Dynatech, Inc., Chantilly, VA) with serially diluted LOS concentrations from 20 to 1.25 μg/mL in 50 μL of ethanol, which were then dried. Whole cells were coated at 50 μg/mL in pH 9.6 carbonate–bicarbonate buffer in a humidified incubator at 37 °C, overnight. Plates were blocked at room temperature for 5 min with PBST (0.05% Tween 80 in PBS) and then incubated with polyclonal rabbit antisera serially diluted in PBST for 1 h at 37 °C. After washing three times with PBS, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G serum was added for 0.5 h at 37 °C; the plates were then washed four times with PBS before addition of substrate [3,3′,5,5′-tetramethylbenzidine (TMB)]. The reaction was finally stopped with 2 M H_2SO_4, and absorption was read at 450 nm.

Degradation of LOSs. Deacylation of LOSs and Isolation of OSEs. Pure LOS was deacylated as described previously (Hunter et al., 1983, 1988). The products were partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ phase was backwashed twice with H_2O. The combined aqueous phases, which contain the neutral OSE, were purified by gel filtration on a column (1 × 175 cm) of Bio-Gel P-2 (200–400 mesh; Bio-Rad Laboratories, Richmond, CA) in H_2O, and the purity of the resulting water-soluble OSE was established by TLC (Hunter et al., 1988). The resulting organic phases served as a source of fatty acids; these were converted to their methyl esters with 3 M HCl in CH_3OH (0.5 mL) at 80 °C overnight followed by TRI-SIL (Pierce Chemical Co., Rockford, IL) at 80 °C for 20 min and subjected to GC/MS (Besra et al., 1992).

Location of Acyl Residues on LOSs. In order to establish the location of acyl functions on the oligosaccharide backbone, the native LOS was subjected to the neutral alkylating conditions of Prehm (1980) and purified on a C_{18} Sep-Pak cartridge (Waters Associates, Inc., Milford, MA) as described (Waeghe et al., 1983; York et al., 1986). The naturally acylated, *O*-methylated LOS was alkylated using the Hakomori methodology (Hakomori, 1964; Stellner et al., 1973) and purified on a C_{18} Sep-Pak cartridge (Waters Associates) as described previously (Waeghe et al., 1983; York et al., 1986).

Glycosyl Composition of LOSs by Alditol Acetates. Purified LOS was hydrolyzed in 2 M TFA (250 μL) at 120 °C for 3 h (McNeil et al., 1987c). Glycosyl residues were reduced with NaB^2H_4 and the resultant alditols *O*-acetylated (McNeil et al., 1987c). The alditol acetates obtained were examined by GC and GC/MS.

Derivatizations of Oligosaccharides. The OSEs arising from the corresponding LOSs were per-*O*-trideuteriomethylated using the Hakomori method (Hakomori, 1964) and purified on a C_{18} Sep-Pak cartridge (Waters Associates) (Waeghe et al., 1983; York et al., 1986). Alternatively, O-methyl and O-trideuteriomethylated derivatives of the OSE samples were prepared using the sodium hydroxide methylation procedure (Ciucani & Kerek, 1984) as described by Dell (1990). In order to establish glycosyl linkage patterns, pure per-O-CH_3-, per-O-C^2H_3-OSE was hydrolyzed with 2 M TFA at 120 °C for 2 h. The hydrolysates were dried, reduced with NaB^2H_4, and O-acetylated with acetic anhydride at 120 °C for 1 h.

In some cases, the per-O-CH_3-, per-O-C^2H_3-OSE was partially hydrolyzed with 2 M TFA at 90 °C for 1 h to generate smaller, partially per-O-CH_3, per-O-C^2H_3 oligosaccharide fragments. The hydrolysates were dried, reduced with NaB^2H_4, and alkylated with $C_2{}^2H_5I$ (Hakomori, 1964), and the resultant products were recovered by chromatography on a C_{18} Sep-Pak cartridge as described previously (Waeghe et al., 1983; York et al., 1986).

Periodate Cleavage of OSE-I. OSE-I (5 mg) was dissolved in a solution of $NaIO_4$ (2 mL, 0.1 M in 50 mM sodium acetate buffer, pH 5) and stirred in the dark for 2 days at room temperature, followed by addition of ethylene glycol (20 μL) with stirring continued for 3 h. $NaBH_4$ (100 mg) was added and the reaction mixture left overnight. The reaction mixture was neutralized with acetic acid (pH 5.0), dialyzed, and freeze-dried.

Absolute Configuration of Glycosyl Residues. To establish the absolute configuration of sugars, the per-O-CH_3-, per-O-C^2H_3 OSE was demethylated (Hough & Theobald, 1963) and treated with 75 μL of 1 M (S)-(+)-2-butanolic hydrochloride or (R)-(–)-2 butanolic hydrochloride, followed by 50 μL of TRI-SIL (Pierce Chemical Co.) (Gerwig et al., 1978). The resultant products were examined by GC/MS and compared to authentic standards.

Other Analytical and Chromatographic Procedures. Intact glycolipids, their per-O-alkylated or deacylated derivatives, and fragments were hydrolyzed under a variety of conditions [2 M TFA at 90 or 120 °C for 1–3 h; 1 M HCl in CH_3OH at 80 °C for 0.5–3 h; anhydrous HF (1 mL) at 0 °C for 2 h] in order to arrive at optimal conditions for the generation of monosaccharides. Base-catalyzed per-O-acetylation was performed with either anhydrous pyridine–Ac_2O (1:1 v/v) at 80 °C or 1-methylimidazole–Ac_2O (1:5 v/v) at room temperature (deuterioacetic anhydride was used for per-O-deuterioacetylated derivatives). Acid-catalyzed per-O-acetylation was effected with trifluoroacetic anhydride–acetic acid (2:1 v/v) at room temperature (deuterioacetic acid was used for per-O-deuterioacetylated derivatives). For time course experiments, samples were dried in reactive vials and were dissolved in a small volume (10–20 μL) of the acetylating reagent and incubated at the desired temperature. At suitable time points, aliquots (1 μL) were withdrawn and loaded directly onto the probe for FAB/MS analyses. For full per-O-acetylation, the reagents were dried under nitrogen and samples were redissolved in methanol for aliquoting into the FAB matrix. m-Nitrobenzyl alcohol (m-NBA) was the matrix of choice for the per-O-acetylated derivatives of the OSE samples, but monothioglycerol was also used for comparison.

Time course methanolyses of native OSE-I samples were performed as described by Dell (1990) and Dell et al. (1990) for per-O-methylated glycans. Samples were aliquoted from the reaction mixture for FAB/MS analyses.

Intact glycolipids were analyzed by TLC on silica gel aluminum sheets (Merck, Darmstadt, Germany) in $CHCl_3$–CH_3OH–H_2O (65:25:4 and 60:30:6). For analysis of oligosaccharides or per-O-alkylated oligosaccharides, 1-butanol–pyridine–H_2O (6:4:3 or 10:3:3) was employed (Hunter et al., 1988).

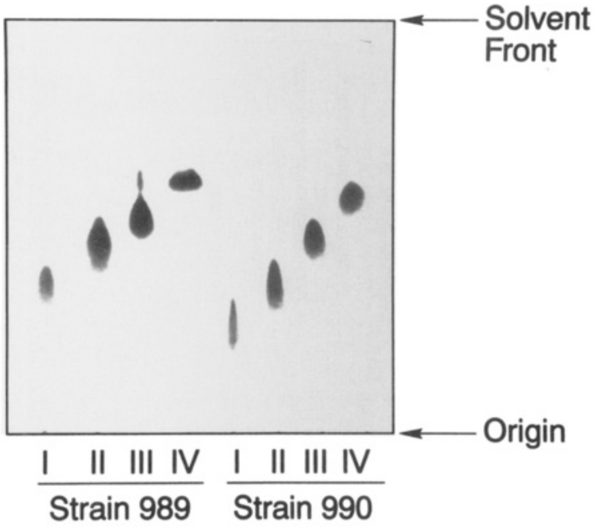

FIGURE 1: Thin-layer chromatogram of purified lipooligosaccharides (LOS-I–IV) isolated from *M. gordonae* strains 989 and 990. Glycolipids were located with 10% H_2SO_4 in ethanol followed by heating at 110 °C. Solvent: $CHCl_3$–CH_3OH–H_2O (65:25:4).

GC of alditol acetates and of partially O-alkylated, partially O-acetylated alditols was conducted on a DB-23 capillary column as described (Bozic et al., 1988). GC/MS analyses were performed on a Hewlett-Packard 5890 A gas chromatograph connected to a Hewlett-Packard 5970 mass selector detector using a 12-m HP-1 Hewlett-Packard column (Besra et al., 1992).

FAB/MS was performed on a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan FAB gun operated at 10 kV. The matrices used were monothioglycerol for most analyses and m-NBA specifically for per-O-acetylated derivatives of the OSE samples. Spectra were recorded on oscillographic chart paper and manually counted.

NMR was performed on a Bruker ACE-300. LOSs were dissolved in C^2H_3Cl–$C^2H_3O^2H$ (2:1; 0.5 mL) before and after exchanging protons in C^2H_3Cl–$C^2H_3O^2H$; OSEs were dissolved in ^2H_2O (0.5 mL). Acetone was added to the samples, and its signal was assigned to δ 2.225 for ^1H and δ 31.4 for ^{13}C NMR, which corresponds to δ 0 for tetramethylsilane. 2D COSY was conducted by using the Bruker double quantum filter phase-sensitive COSY software (SI 1 = 1024, SI 2 = 2048, SW 1 = 1373, SW 2 = 2747). IR spectra were recorded on a Nicolet MX-1 FTIR spectrometer (Nicolet Instrument Co., Madison, WI) and were measured as a thin film on NaCl.

RESULTS

Isolation and Antigenicity of LOSs from M. gordonae Strains 989 and 990. When the crude lipid extracts from *M. gordonae* strains 989 and 990 were applied to a silica gel column that was irrigated with $CHCl_3$ followed by increasing concentrations of CH_3OH in $CHCl_3$, four carbohydrate-containing lipids were resolved from each isolate: LOS-I (670 mg), LOS-II (197 mg), LOS-III (59 mg), and LOS-IV (24 mg) from *M. gordonae* 989; and LOS-I (90 mg), LOS-II (36 mg), LOS-III (14 mg), and LOS-IV (20 mg) from *M. gordonae* 990 (Figure 1). The antigenicity of LOS-I–IV from the two strains was tested against rabbit antiserum raised to the homologous and heterologous strains (Figure 2). The

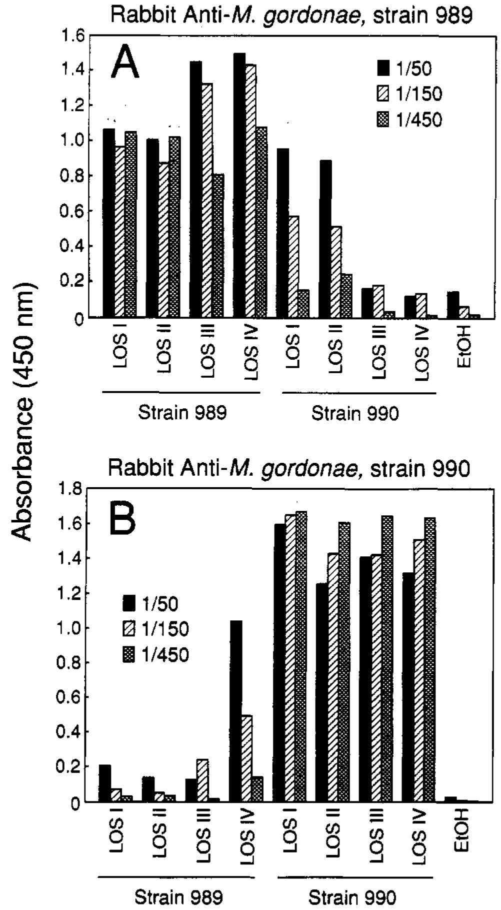

FIGURE 2: ELISA reactivity of polyclonal antibodies to various purified LOS (*M. gordonae* strains 989 and 990). Each well was coated with LOS (1.25–20 μg/mL) in 50 μL of ethanol and dried; whole cells were coated at 50 μg/mL. Plates were then blocked and incubated with polyclonal rabbit antisera serially diluted in PBST for 1 h at 37 °C. The reaction was finally stopped with 2 M H_2SO_4 and absorption read at 450 nm.

antigens were highly species-specific in that they showed only weak cross-reactivity against the antiserum to the heterologous strain.

Upon treatment of pure LOS-I–IV from both strains of *M. gordonae* with 0.2 N NaOH and subsequent TLC of the proceeds, all semblances of the original glycolipids had disappeared. Therefore, they conformed to the specification of the alkali-labile LOS class (McNeil et al., 1989; Hunter et al., 1983; Brennan, 1988). The water-soluble OSEs produced by deacylation were purified by gel filtration on a Bio-Gel P-2 column to yield the corresponding oligosaccharides, OSE-I (180 mg), OSE-II (62 mg), OSE-III (18 mg), and OSE-IV (8 mg) from strain 989 and OSE-I (34 mg), OSE-II (12 mg), OSE-III (5 mg), and OSE-IV (3 mg) from strain 990.

Glycosyl Composition and Anomeric Configurations of All LOSs. Hydrolysis of the LOSs with 2 M TFA and capillary GC and GC/MS of the alditol acetates established the identities and relative amounts of glycosyl residues (Tables I and II). It was clear from this analysis that LOS-II (strain 989) and LOS-II (strain 990) were not pure, as nonstoichmetric amounts of Ara and 3-*O*-CH_3-Xyl, and Ara and Xyl, respectively, were present. GC/MS of the $(CH_3)_3Si$-(*R*)-(−)-2-butyl glycosides and $(CH_3)_3Si$-(*S*)-(+)-2-butyl glycosides conducted on the fully demethylated LOS as described (McNeil et al., 1987b; Gerwig et al., 1978) from strains 989 and 990 demonstrated that the Rha*p* and Fuc*p* were in the L absolute configuration, whereas Glc*p*, Xyl*p*, and Ara*f* were in the D absolute configuration (results not shown). In addition, it was immediately suspected, on the basis of precedent (Hunter et al., 1988; Daffe et al., 1991) and relative TLC mobility, that LOS-I and -II (strain 989) may contain glycosyl residues additional to those observed by GC/MS. Also, evidence of IR absorption bands at 1541 and 1637 cm^{-1} in the spectrum of the more polar antigenic LOS-I (strain 989) pointed to an acylated amino sugar (Daffe et al., 1991). High-resolution ^{13}C NMR and ^{1}H NMR of OSE-I–IV (strain 989) showed the existence of a CH–NH carbon signal (Bundle et al., 1973; Steinblatt et al., 1982) at 51.08 ppm in OSE-I and -II. Moreover, ^{13}C NMR indicated the presence of ten anomeric carbons in OSE-I (LOS-I; strain 989), which differed from the original sugar analysis of nine residues. Since LOS-I and LOS-III (strain 990) were identical in sugar analysis, it was suspected that LOS-III is a structural analog of LOS-I, differing only in the absence of the acylated amino sugar. Indeed, a comparison of both ^{1}H NMR spectra revealed H-1 (δ 5.39, $J_{1,2}$ = 3.25 Hz) and H-6 (δ 1.15, $J_{5,6}$ = 6.8 Hz) of the amino sugar. On the basis of the NMR data, heteronuclear $^{13}C/^{1}H$ 2D COSY, and data from the literature (Bradbury et al., 1984; Gorin et al., 1975; Hunter et al., 1983, 1988; Lemieux et al., 1966; Pozsgay et al., 1981), it was possible to assign the following resonances for H-1 and C-1 of OSE-I (strain 989): β-D-Xyl*p*- (^{1}H, δ 4.40, $J_{1,2}$ = 7.2 Hz; ^{13}C, 105.0 ppm), 2 × β-D-Glc*p*- (^{1}H, δ 4.64 and 4.71, both $J_{1,2}$ = 7.4 Hz; ^{13}C, 104.8 and 104.4 ppm), 2-*O*-CH_3-α-L-Rha*p*- (^{1}H, δ 5.11, $J_{1,2}$ = <1.5 Hz; ^{13}C, 100.2 ppm), 2 × α-D-Glc*p*- (^{1}H, δ 5.16, $J_{1,2}$ = 3.6 Hz; ^{13}C, 93.8 and 94.5 ppm), α-L-Rha*p*- (^{1}H, δ 5.21, $J_{1,2}$ = <1.5 Hz; ^{13}C, 99.7 ppm), α-L-Rha*p*- (^{1}H, δ 5.24, $J_{1,2}$ = <1.5 Hz; ^{13}C, 101.9 ppm), amino sugar (^{1}H, δ 5.39, $J_{1,2}$ = 3.7 Hz; ^{13}C, 98.6 ppm), 2-*O*-CH_3-α-L-Fuc*p*- (^{1}H, δ 5.58, $J_{1,2}$ = 3.7 Hz; ^{13}C, 97.4 ppm). The chemical shift and coupling constants (<1.5 Hz) of the Rha*p* residues were indicative of an α-configuration, and the assignment of the δ 4.40 signal ($J_{1,2}$ = 7.2 Hz) to β-D-Xyl*p* was based on the large coupling constant and similarity of the chemical shift value to that reported (Lemieux et al., 1966). The anomeric configurations of glycosyl residues of LOS-II–IV from strain 989 and LOS-I–IV from strain 990 were assigned in a similar fashion from the ^{1}H and ^{13}C NMR data.

Glycosyl Linkage Analysis of All LOSs. The glycolipids were per-*O*-trideuteriomethylated followed by analysis of the partially per-*O*-methylated, partially per-*O*-trideuteriomethylated, and partially per-*O*-acetylated alditol derivatives (Tables III and IV). These results revealed that OSE-III (strain 989) contained three nonreducing terminal glycosyl residues (α-D-Glc*p*, β-D-Xyl*p*, and 2-*O*-CH_3-α-L-Fuc*p*) and a 2,3-linked α-L-Rha*p* with the remaining residues being 1→3-linked (Table III). However, glycosyl linkage analysis of OSE-I (strain 989) (Table III) clearly showed that the 2-*O*-CH_3-α-L-Fuc*p* residue was not terminal but was, in fact, substituted at the 3-position by the terminal amino sugar. It was obvious from glycosyl linkage analysis of LOS-II (strain 989) that it was a mixture of two components, -II′ and -II″, where OSE-II′ contained a (*t*)-3-*O*-CH_3-β-D-Xyl*p* residue and OSE-II″ a (*t*)-α-D-Ara*f* residue. OSE-IV (strain 989) was found to be devoid of a 2,3-linked α-L-Rha*p* residue as well as (*t*)-β-D-Xyl*p*, (*t*)-3-*O*-CH_3-β-D-Xyl*p*, or t-α-D-Ara*f* residues. The strain 990 product demonstrated the presence of three non-reducing terminal glycosyl residues, α-L-Rha*p*, α-D-Glc*p*, and β-D-Xyl*p*, a 2,3-linked α-L-Rha*p*, and a 2-linked 3-*O*-CH_3-α-L-Rha*p*. All other sugar residues were 1→3-linked (Table IV). OSE-I, -II′, and -II″ (strain 990) also showed

Table I: Glycosyl Composition of LOS-I–IV from *M. gordonae* Strain 989 As Revealed by GC/MS Analysis of Alditol Acetates

name of the *O*-Ac-*O*-CH$_3$-alditol identified by GC/MS	t_R (min)[a]	name of the glycosyl residue	mol/mol of LOS			
			I	II	III	IV
1,3,4,5-tetra-*O*-Ac-2-*O*-CH$_3$-fucitol	7.26[b]	2-*O*-CH$_3$-Fuc	1.0	1.0	1.0	1.0
1,3,4,5-tetra-*O*-Ac-2-*O*-CH$_3$-rhamnitol	7.26[b]	2-*O*-CH$_3$-Rha	0.9	1.0	1.0	1.0
1,2,3,4,5-penta-*O*-Ac-rhamnitol	7.70	Rha	2.3	2.2	2.3	2.3
1,2,3,4,5-penta-*O*-Ac-arabinitol	7.72	Ara		0.5		
1,2,4,5-tetra-*O*-Ac-3-*O*-CH$_3$-Xylitol	7.34	3-*O*-CH$_3$-Xyl		0.4		
1,2,3,4,5-penta-*O*-Ac-xylitol	7.87	Xyl	1.0		0.9	
1,2,3,4,5-penta-*O*-Ac-6-*O*-CH$_3$-glucitol	8.76	6-*O*-CH$_3$-Glc	0.8	0.7	0.7	0.7
1,2,3,4,5,6-hexa-*O*-Ac-glucitol	9.68	Glc	3.2	2.9	3.1	2.7

[a] GC/MS analyses were performed as described in the Experimental Procedures. [b] Identities revealed by coelution with authentic standards by GC.

Table II: Glycosyl Composition of LOS-I–IV from *M. gordonae* Strain 990 As Revealed by GC/MS Analysis of Alditol Acetates

name of the *O*-Ac-*O*-CH$_3$-alditol identified by GC/MS	t_R (min)[a]	name of the glycosyl residue	mol/mol of LOS			
			I	II	III	IV
1,2,3,5-tetra-*O*-Ac-4-*O*-CH$_3$-rhamnitol	8.11	4-*O*-CH$_3$-Rha		1.1	0.4	0.9
1,2,4,5-tetra-*O*-Ac-3-*O*-CH$_3$-rhamnitol	8.20	3-*O*-CH$_3$-Rha	0.9	1.2	0.9	1.1
1,2,3,4,5-penta-*O*-Ac-rhamnitol	8.54	Rha	3.2	2.1	2.3	2.2
1,2,3,4,5-penta-*O*-Ac-arabinitol	8.57	Ara		–[b]		
1,2,3,4,5-penta-*O*-Ac-xylitol	8.70	Xyl	0.8	–[b]		
1,2,3,4,5-penta-*O*-Ac-6-*O*-CH$_3$-glucitol	9.69	6-*O*-CH$_3$-Glc	1.0	0.9	0.9	0.9
1,2,3,4,5,6-hexa-*O*-Ac-glucitol	10.70	Glc	3.0	3.0	3.0	3.0

[a] GC/MS analyses were performed as described in Experimental Procedures. [b] Molar ratio for Xyl and Ara could not be determined due to poor release under the hydrolysis conditions, but the ratio of Ara:Xyl was approximately 1.6:1.

Table III: Glycosyl Linkage Analysis of Per-*O*-CH$_3$-OSE-I–IV[a] from *M. gordonae* 989

name of the *O*-Ac-*O*-CH$_3$-*O*-C^{2}H$_3$ alditol identified by GC/MS	name of the glycosyl residue and linkage pattern[b]	mol/mol of per-*O*-CH$_3$-OSE				
		I	II′	II″	III	IV
1,3,5-tri-*O*-Ac-2-*O*-CH$_3$-3,4-*O*-C^{2}H$_3$-rhamnitol	→3)-2-*O*-CH$_3$-Rha*p*	1.1	1.0	1.0	1.0	1.0
1,2,3,5-tetra-*O*-Ac-4-*O*-C^{2}H$_3$-rhamnitol	→2, →3)-Rha*p*	1.3	1.3	1.3	1.1	
1,3,5-tri-*O*-Ac-2,4-di-*O*-C^{2}H$_3$-rhamnitol	→3)-Rha*p*	1.1	1.0	1.0	1.0	1.8
1,3,5-tri-*O*-Ac-2-*O*-CH$_3$-4-*O*-C^{2}H$_3$-fucitol	→3)-2-*O*-CH$_3$Fuc*p*	1.3	1.1	1.1		
1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-C^{2}H$_3$-glucitol	*t*-Glc*p*	1.0	1.0	1.0	1.0	1.0
1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-C^{2}H$_3$-glucitol	→3)-Glc*p*	1.7	2.0	2.0	1.7	2.3
1,3,5-tri-*O*-Ac-6-*O*-CH$_3$-2,4-di-*O*-C^{2}H$_3$-glucitol	→3)-6-*O*-CH$_3$-Glc*p*	0.8	1.0	1.0	0.8	0.8
1,4-di-*O*-Ac-2,3,5-tri-*O*-C^{2}H$_3$-arabinitol	*t*-Ara*f*			0.4		
1,5-di-*O*-Ac-2,3,4-tri-*O*-C^{2}H$_3$-xylitol	*t*-Xyl*p*	0.7			0.8	
1,5-di-*O*-Ac-3-*O*-CH$_3$-2,4-di-*O*-C^{2}H$_3$-xylitol	*t*-3-*O*-CH$_3$-Xyl*p*		0.4			
1,5-di-*O*-Ac-2-*O*-CH$_3$-3,4-di-*O*-C^{2}H$_3$-fucitol	*t*-2-*O*-CH$_3$-Fuc*p*				0.9	0.9

[a] OSE-I–IV were per-*O*-trideuteriomethylated with C^{2}H$_3$I (Hakomori, 1964). The resulting per-*O*-CH$_3$, per-*O*-C^{2}H$_3$ OSE (I–IV) were hydrolyzed, reduced with NaB^{2}H$_4$, per-*O*-acetylated, and examined by GC/MS. [b] *t* = terminal; the →2, →3) and →3) designations mean that the OH at these positions of the glycosyl residue were originally linked to another glycosyl unit.

Table IV: Glycosyl Linkage Analysis of Per-*O*-CH$_3$-OSE-I–IV[a] from *M. gordonae* 990

name of the *O*-Ac-*O*-CH$_3$-*O*-C^{2}H$_3$ alditol identified by GC/MS	name of the glycosyl residue and linkage pattern[b]	mol/mol of per-*O*-CH$_3$-OSE					
		I	II′	II″	III′	III″	IV
1,5-di-*O*-Ac-2,3,4-tri-*O*-C^{2}H$_3$-rhamnitol	*t*-Rha*p*	1.1			0.4		
1,5-di-*O*-Ac-4-*O*-CH$_3$-2,3-di-*O*-C^{2}H$_3$ rhamnitol	*t*-4-*O*-CH$_3$-Rha*p*		1	1		0.5	0.8
1,2,3,5-tetra-*O*-Ac-4-*O*-C^{2}H$_3$-rhamnitol	→2, →3)Rha*p*	1.0	0.8	0.8			
1,3,5-tri-*O*-Ac-2,4-di-*O*-C^{2}H$_3$-rhamnitol	→3)Rha*p*	1.1	1.2	1.2	1.7	1.7	2.1
1,2,5-tri-*O*-Ac-3-*O*-CH$_3$-4-*O*-C^{2}H$_3$-rhamnitol	→2)-3-*O*-CH$_3$-Rha*p*	1.1	1.2	1.2	1.1	1.1	0.8
1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-C^{2}H$_3$-glucitol	*t*-Glc*p*	1.1	1.1	1.1	1.1	1.1	0.8
1,3,5-tri-*O*-Ac-2,4,6-tri-*O*-C^{2}H$_3$-glucitol	→3)Glc*p*	2.2	2.1	2.1	1.8	1.8	2.1
1,3,5-tri-*O*-Ac-6-*O*-CH$_3$-2,4-di-*O*-C^{2}H$_3$-glucitol	→3)-6-*O*-CH$_3$-Glc*p*	1.1	1.1	1.1	1.1	1.1	1.1
1,5-di-*O*-Ac-2,3,4-tri-*O*-C^{2}H$_3$-xylitol	*t*-Xyl*p*	0.7	1.2				
1,4-di-*O*-Ac-2,3,5-tri-*O*-C^{2}H$_3$-arabinitol	*t*-Ara*f*			0.4			

[a] OSE-I–IV were per-*O*-trideuteriomethylated with C^{2}H$_3$I (Hakomori, 1964). The resulting per-*O*-CH$_3$, per-*O*-C^{2}H$_3$-OSE-(I–IV) were hydrolyzed, reduced with NaB^{2}H$_4$, per-*O*-acetylated, and examined by GC/MS. [b] *t* = terminal; the →2, →3) and →3) designation means that the OH at these positions of the glycosyl residue were originally linked to another glycosyl unit.

this unique branching feature and were distinguished from each other by (*t*)-β-D-Xyl*p* and (*t*)-α-D-Ara*f* at the 2-position of the 2,3-linked α-L-Rha*p*, respectively, as well as *O*-methylation at C-4 of the terminal rhamnosyl residue. OSE-III was also found to be a two-component mixture, OSE-III′ and -III″; both were devoid of any branched sugar residues. OSE-III′ was essentially identical to OSE-I but lacked a (*t*)-β-D-Xyl*p* residue, and OSE-III″ differed from OSE-III′ in that the (*t*)-α-L-Rha*p* residue was replaced by (*t*)-4-*O*-CH$_3$-α-L-Rha*p*. OSE-III″ and OSE-IV showed the same glycosyl linkage pattern, indicating that the two are identical; however, since they were separable as their respective LOSs by TLC, they must differ in the nature or number of the acyl functions. The glycosyl linkage patterns for the products from strains

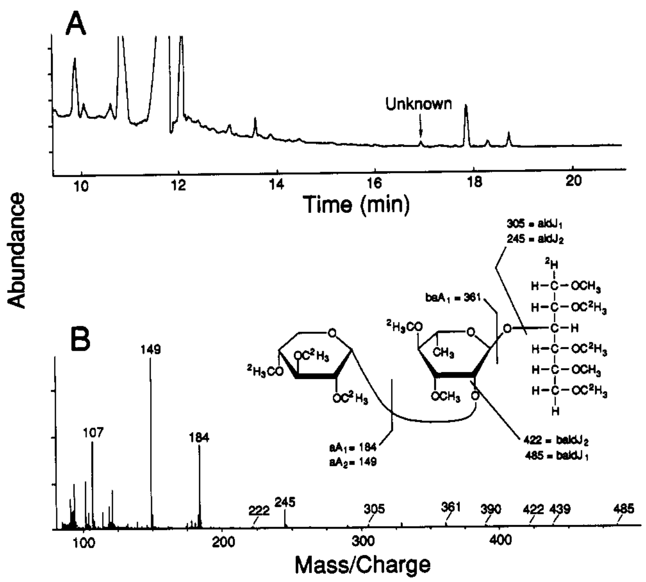

FIGURE 3: GC/MS analysis of fragmented per-*O*-C^2H_3-OSE-I. (A) Total-ion chromatogram of the mixture of per-*O*-alkylated oligosaccharides and per-*O*-alkylated alditols prepared from per-*O*-CH_3, per-*O*-C^2H_3 OSE-I. (B) Electron impact mass spectrum of compound X.

989 and 990 are summarized in Tables III and IV, which, in general, indicate that they are characterized by unique structural features in all segments of the molecules in terms of *O*-acylation, *O*-methylation, and the nonreducing ends, and particularly in terms of branching from the main oligosaccharide backbone.

Confirmation of Location of the Branched (t)-β-D-Xylp, (t)-β-D-3-O-CH₃-Xylp, and (t)-α-D-Araf Residues on Some of the LOSs. In order to establish the exact location of the (*t*)-β-D-Xyl*p* residue, which could be located at the 2- or 3-position of the α-L-Rha*p* residue, the per-*O*-CH_3-, per-*O*-C^2H_3-OSE-I (strain 989) was fragmented into smaller oligosaccharides by partial acid hydrolysis, treated with NaB^2H_4, and per-*O*-methylated as described (McNeil et al., 1982). The resulting complex mixture of partially per-*O*-methylated, partially per-*O*-trideuteriomethylated alditol fragments was subjected to GC/MS analysis (Figure 3A). The mass spectrum of the crucial oligosaccharide alditol (unknown) used in locating the (*t*)-β-D-Xyl*p* glycosyl residue is shown in Figure 3B. The presence of $aldJ_1$ ion at *m/z* 305 clearly shows that the (*t*)-β-D-Xyl*p* is located at the 2-position of the 2,3-linked α-L-Rha*p* residue. It is also expected that the (*t*)-3-*O*-CH_3-β-D-Xyl*p* (OSE-II′), (*t*)-α-D-Ara*f* (OSE-II″), and (*t*)-β-D-Xyl*p* (OSE-III) are located at the 2-position of the branched 2,3-linked α-L-Rha*p* residue from a biosynthetic standpoint. Similarly, it is expected that (*t*)-β-D-Xyl*p* (OSE-I, OSE-II′) and (*t*)-α-D-Ara*f* (OSE-II′) from strain 990 are located at the 2-position of the branched 2,3-linked α-L-Rha*p* residue. These conclusions were further substantiated by isolation of this same oligosaccharide alditol by HPLC, which was then hydrolyzed, reduced with NaB^2H_4, per-*O*-acetylated, and examined by GC/MS.

Structures of OSE-II–IV from Strain 989. Structural elucidation of OSE-II–IV (strain 989) proved relatively easy and provided the strategy for application to the other OSEs.

The FAB spectrum of OSE-III afforded a major molecular ion signal at *m/z* 1447, corresponding to $[M + Na]^+$ of $MeDeoxyHex_2DeoxyHex_2MeHex_1Hex_3Pent_1$. Several fragment ions were also observed, each of which could be assigned to one or more partial sequences consistent with the proposed composition. To afford unambiguous sequence analysis, it was thus essential to derivatize the sample in order to direct the fragmentation along a limited number of well-defined pathways, as well as differentiating the nonreducing and reducing end fragments (Dell, 1987).

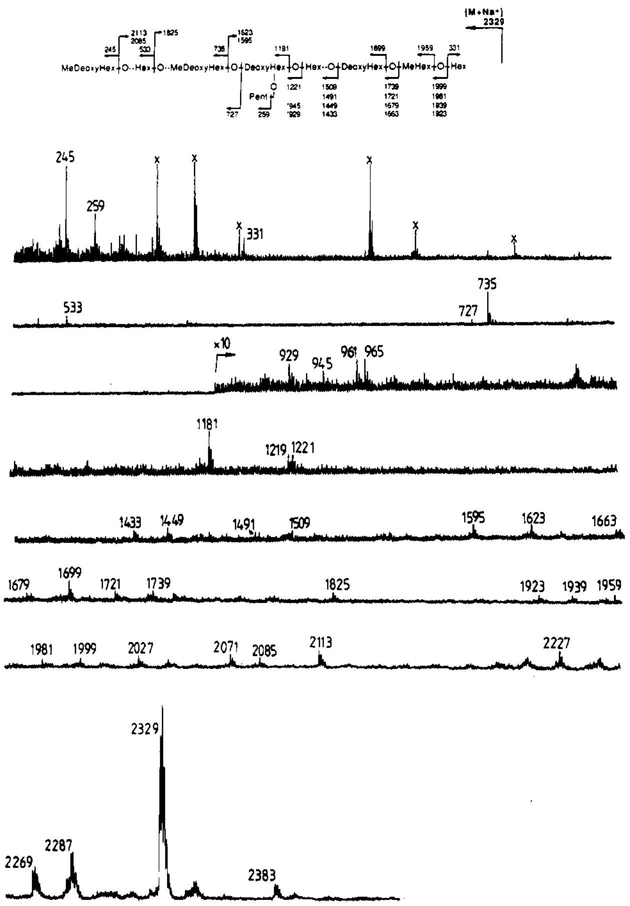

FIGURE 4: FAB spectrum of per-*O*-acetylated OSE-III (strain 989). All ions were sodiated except A-type ions. Fragment ions were assigned as illustrated. Signals at *m/z* 961 and 965 probably arose from double cleavage, corresponding to structure II of the sequence $[MeDeoxyHexHexMeDeoxyHexDeoxyHex(OH) + Na]^+$ and $[(HO)DeoxyHex(Pent)HexDeoxyHex^+]$, respectively. The signal at *m/z* 2027 resulted from ring cleavage at the terminal Hex, i.e., 28 amu higher than *m/z* 1999. Signals at *m/z* 2071 and 2287 were the underacetylated species of signals at *m/z* 2113 and 2329 minus acetic acid, respectively. Other signals were assigned as follows: *m/z* 2227 and 2269 (2287 and 2329 minus acetic acid, respectively) and 2383 (corresponds to the molecular ion at *m/z* 2329 with one trifluoroacetyl group substituting an acetyl group). Peaks marked with "x" were matrix derived. Spectra were acquired using the *m*-nitrobenzyl alcohol (*m*-NBA) matrix.

The spectrum of per-*O*-methylated OSE-III gave a quasi-molecular ion $[M + NH_4]^+$ at *m/z* 1736 and A-type fragment ions (Dell, 1987) at *m/z* 567, 901, 1105, and 1279, corresponding to $(MeDeoxyHex_2Hex_1)^+$ and $(MeDeoxyHex_2Hex_1\text{-}DeoxyHex_1Pent_1)^+$, respectively. These assignments were verified with per-*O*-trideuteriomethylated derivatives and established the partial sequence: $(MeDeoxyHex_2Hex_1)$-DeoxyHex(Pent)HexDeoxyHex$(MeHex_1Hex_1)$ (Dell, 1987).

Further sequence data were afforded by the FAB spectrum of the per-*O*-acetylated derivatives (Figure 4), acquired using the *m*-nitrobenzyl alcohol (*m*-NBA) matrix. In addition to the quasi-molecular ion $[M + Na]^+$ at *m/z* 2329 and the A-type ions at *m/z* 245, 259, 331, 533, 735, 1181, 1699, and 1959 (see Figure 4 for assignments), two other series of cleavage ions were observed. Glycosidic β-cleavage with a hydrogen transfer and/or ring cleavage afforded the sodiated reducing end fragment ion pairs of 28 amu apart, at *m/z* 2113/

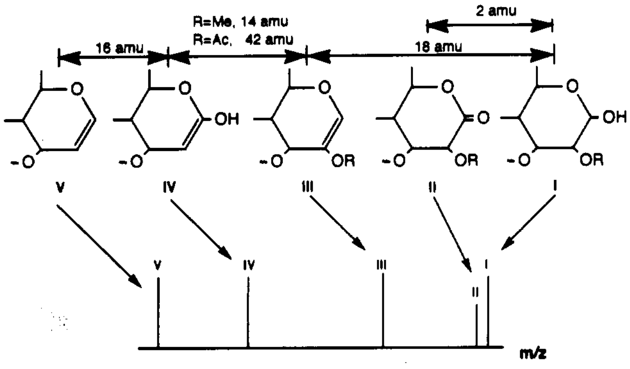

FIGURE 5: Schematic diagram illustrating the characteristic patterns of signals observed in the FAB/MS spectra of per-*O*-acetylated derivatives run in the *m*-NBA matrix.

2085, 1623/1595, and 1825 (lacking the β-cleavage ion) (Dell, 1987).

Finally, clusters of sodiated nonreducing end fragment ions resulting from several possible modes of glycosyl cleavage were also observed at distinct mass intervals, giving the characteristic pattern of signals (Figure 5) uniquely associated with FAB spectra of per-*O*-acetylated derivatives of glycans run in *m*-NBA matrices (Dell, 1990; Dell et al., 1990; Reason et al., 1991). Signals corresponding to structure I were seen (Figure 4) at *m/z* 1999, 1739, 1509, and 1221, accompanied by signals 18 amu lower, of structure III; while signals corresponding to structure V were seen at *m/z* 1923, 1663, 1433, 929, and 727 accompanied by signals 16 amu higher, of structure IV. (Structure II was relatively minor in this spectrum.) The mass difference between the I/III pair and the IV/V pair is dependent on the substituent on the C-2 of the residue at the cleavage site, i.e., 14 amu for cleavage at 2-*O*-Me-DeoxyHex, and 42 amu for cleavage at other residues (2-*O*-Ac). Elimination of the Pent residue 2-linked to the DeoxyHex yielded the signal pair at *m/z* 945/929. In summary, FAB/MS analyses of the derivatives of OSE-III unambiguously defined its sequence as MeDeoxyHex-HexMeDeoxyHexDeoxyHex(Pent)HexDeoxyHexMe-HexHex, as well as confirming the substituents on the C-2 of each residue.

The sequence of sugars in OSE-II, -II′, and -IV was not determined from first principles. However, the glycosyl composition (Table I), glycosyl linkage analysis (Table II), and comparison with OSE-I (see later) and -III infer the following conclusions. OSE-II′ and -II″ appear to have the same sequence as OSE-I except that (*t*)-3-*O*-CH_3-β-D-Xyl*p* and a (*t*)-α-D-Ara*f* replace the (*t*)-β-D-Xyl*p* residue of OSE-I for OSE-II′ and -II″, respectively. OSE-IV also appears to have the same sequence as OSE-I, except that it is devoid of a branched sugar residue and the acylated amino sugar.

Structure of OSE-I, Strain 989, and Its Novel Amino Sugar. (i) Conventional Analysis. The location of the nitrogen-carrying carbon was established by homo- and heteronuclear ($^{13}C/^{1}H$) COSY NMR. The CH–NH carbon signal at 51.08 ppm was found to correlate with a proton resonance at δ 4.62 in the heteronuclear $^{13}C/^{1}H$ COSY NMR spectrum. In the homonuclear ^{1}H COSY NMR spectrum, it was possible to assign the ring protons of the amino sugar through connectivities from H-1 (δ 5.40, $J_{1,2}$ = 3.25 Hz), H-2 (δ 3.48, $J_{2,3}$ = 10.8 Hz), H-3 (δ 3.85, $J_{3,4}$ = 4.0 Hz), H-4 (δ 4.62), H-5 (δ 4.30), and H-6 (δ 1.15, $J_{5,6}$ = 6.8 Hz), and on the basis of these coupling constants, the *galacto* configuration for the novel amino sugar was established. On summary of the NMR data, we conclude that this sugar is based on 4-amino-4,6-dideoxygalactopyranose. Confirmation of its identity was afforded by HF hydrolysis, $NaB^{2}H_4$ reduction, and per-*O*-acetylation of OSE-I. This reaction produced in low yield a compound whose mass spectrum was consistent with 4-acetamido-1,5-di-*O*-acetyl-4,6-dideoxy-2,3-di-*O*-methylgalactitol (*m/z* 98, 118, 142, 158, 162, 202). The acetyl function at N-4 was presumably aroused by the removal of the native acyl function at some point during the procedure followed by *N*-acetylation.

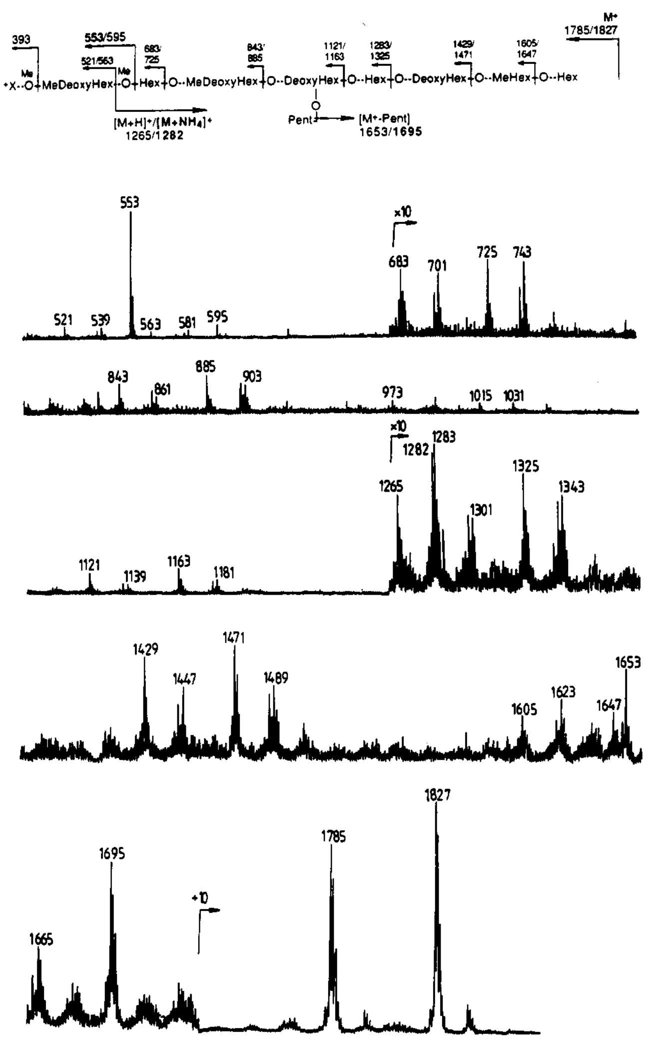

FIGURE 6: FAB/MS spectra of the methanolyzed native OSE-I (strain 989) after 1 min at room temperature.

(ii) FAB/MS Analysis. Further structural information on the acyl function and OSE-I was obtained by FAB/MS analysis. The FAB spectrum of the native sample afforded two major molecular ion clusters separated by 42 amu. Each cluster was comprised of M^+ and its sodium and potassium adducts, defining M as 1785 and 1872. Since the calculated mass for OSE-1 minus the X moiety is 1424, X^+ is thus 362 or 404 amu if the additional moiety of 42 amu, probably an acetyl group, is located on X. The FAB spectrum of the native OSE-I afforded several weak fragment ion signals, but these were of little importance in establishing an unambiguous sequence. However, when the native sample was subjected to time course methanolysis/esterification (Dell, 1990), unexpected results were obtained. After standing at room temperature for 1 min, an aliquot of the native OSE-I in CH_3OH–HCl, when loaded directly onto the probe, afforded the FAB spectrum reproduced in Figure 6. In the molecular ion region, the pair of signals 42 amu were observed predominantly

Table V: Postulated Structures of OSE-I–IV of *M. gordonae* Strain 989 As Determined by FAB/MS and by the Origins of Individual Fragments That Had Been Deduced by GC/MS

OSE-I	*N*-acyl-4-amino-4,6-dideoxy-2,3-di-*O*-CH$_3$-α-Gal*p*-(1→3)-2-*O*-CH$_3$-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-CH$_3$-α-L-Rha*p*-(1→3)-[β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH$_3$-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-II′	*N*-acyl-4-amino-4,6-dideoxy-2,3-di-*O*-CH$_3$-α-Gal*p*-(1→3)-2-*O*-CH$_3$-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-CH$_3$-α-L-Rha*p*-(1→3)-[3-*O*-CH$_3$-β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH$_3$-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-II″	*N*-acyl-4-amino-4,6-dideoxy-2,3-di-*O*-CH$_3$-α-Gal*p*-(1→3)-2-*O*-CH$_3$-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-CH$_3$-α L-Rha*p*-(1→3)-[α-D-Ara*f*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH$_3$-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-III	2-*O*-CH$_3$-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-CH$_3$-α-L-Rha*p*-(1→3)-[β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH$_3$-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-IV	2-*O*-CH$_3$-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-CH$_3$-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH$_3$-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*

as M$^+$ with very little cationization. Sequence ions were observed for glycosidic cleavage at every single residue of both molecular ion species, giving pairs of nonreducing end fragment ions of structure III at *m/z* 1605/1647, 1429/1471, 1283/1325, 1121/1163, 843/885, 683/725, and 521/563 (see Figure 6 for assignments). Each of these ions was also accompanied by signals 16 and 18 amu higher, corresponding to structures II and I, respectively. In addition, methanolyzed products were observed at *m/z* 1282 for the ammonium adduct of M having lost the nonreducing terminal X$^+$-MeDeoxyHex moiety, and the corresponding methyl glycoside of X$^+$-MeDeoxyHex was generated at *m/z* 553. Interestingly, the signal at 42 amu higher, *i.e.*, 595, was very minor, suggesting that the putative acetate is susceptible to acid hydrolysis when the X$^+$-MeDeoxyHex moiety is released from the oligosaccharide chain. Alternatively, it is conceivable that the oligosaccharide lacking the 42 amu increment was methanolyzed more rapidly than that containing the putative acetate. The postulated structures of OSE-I–IV of strain 989 are presented in Table V.

Structures of OSE-I–IV Strain 990. Each of OSE-I–IV was per-*O*-trideuteriomethylated (Hakomori, 1964), and the resulting per-*O*-methylated, per-*O*-trideuteriomethylated OSE subjected to sequential partial acid hydrolysis, NaB^{2}H$_4$ reduction, and penta-*O*-deuterioethylation as described (Bozic et al., 1988). The resulting complex mixture of partially *O*-methylated, partially *O*-trideuteriomethylated, partially-*O*-deuterioethylated oligoglycosyl alditol fragments were analyzed by GC/MS. The results of this approach are summarized in Figures 7 and 8 for OSE-I.

The native oligosaccharides and the per-*O*-methylated, per-*O*-trideuteriomethylated OSE-I were analyzed by positive-ion FAB/MS. The (M + H)$^+$ ions were observed at *m/z* 1411 (OSE-I), *m/z* 1425 (OSE-II′ and -II″), and *m/z* 1279 and 1293 (OSE-III′ and -III″). The (M + Na)$^+$ ion was observed for OSE-IV at *m/z* 1315. These results gave a clear indication of the molecular weights of the native oligosaccharide and were in accord with the glycosyl composition analysis. Positive-ion FAB/MS of the per-*O*-methylated, per-*O*-trideuteriomethylated OSE-I gave fragment ions from which the sequence of the native OSE-I was deduced. The sequence derived from FAB/MS analysis supported that obtained by the sequential partial acid hydrolysis method. The structures of OSE-I–IV (strain 990) are shown in Table VI. The ring structure of OSE-I is illustrated in Figure 9.

Identification and Location of Acyl Groups. The fatty acids of LOSs-I–IV from *M. gordonae* strains 989 and 990 were examined by GC/MS as methyl esters as described previously (Hunter et al., 1983) and were shown to consist of saturated [McLafferty base ion at *m/z* 74 (29)], unsaturated [base ion at *m/z* 57], α-methyl-branched, β-hydroxylated fatty acid methyl esters [base ion at *m/z* 189, corresponding to C$_3$–C$_4$ bond cleavage of the trimethylsilylated derivative] (results not shown). From the mass spectrum of the individual esters as fractionated by GC, the structures of the different acids were established on the basis of McLafferty ions, molecular ions, and rearrangement ions (Ryhage & Stenhagen, 1963, 1969). Cochromatography conducted as described previously (Besra et al., 1992) helped identify the fatty acids from *M. gordonae* strains 989 and 990 as follows: C$_{15}$ mycocerosate; C$_{16}$-C$_{18}$ saturated; C$_{16}$-C$_{18}$ unsaturated; and C$_{20}$ β-hydroxy-α-methyl branched [results not shown; see Besra et al. (1992)].

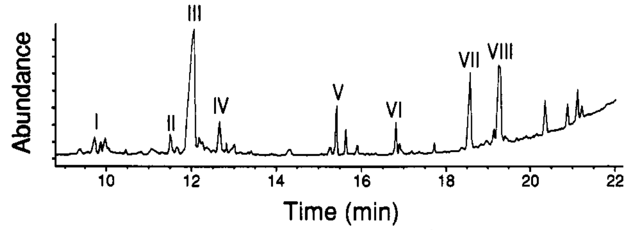

FIGURE 7: Total-ion chromatogram obtained during GC/MS of the oligoglycosyl alditol fragments I–VIII generated by sequential partial acid hydrolysis, NaB^{2}H$_4$ reduction, and penta-*O*-deuterioethylation of per-*O*-alkylated OSE-I (*M. gordonae* 990). The following information was applied to arrive at the structures of the various fragments (see Figure 8) [the nomenclature is that of Kochetkov and Chizhov (1966)] [the peak number, the GC t_R (min), and the EI/MS ions (*m/z*) and interpretation are given in succession]: peak I, 9.72 min, *m/z* 94, 163 (aA$_2$), 198 (A$_1$), 247 (aldJ$_2$); peak II, 11.50 min, *m/z* 107, 110, 196 (aA$_2$), 247 (A$_1$), 250 (aldJ$_2$); peak III, 12.05 min, *m/z* 107, 193 (aA$_2$), 196 (rA$_2$), 243, 303, 306; peak IV, 12.64 min, *m/z* 107, 110, 196 (aA$_2$), 247 (aA$_1$), 283 (aldJ$_0$), 362 (aldJ$_1$), 435 (alditol cleavage); peak V, 15.42 min, *m/z* 163 (aA$_1$), 198 (aA$_2$), 375 (bA$_1$), 266 (aldJ$_2$), 326 (aldJ$_1$), 443 (baldJ$_2$), 506 (baldJ$_1$); peak VI, 16.80 min, 149 (aA$_2$), 184 (aA$_1$), 380 (baA$_1$), 329 (baA$_2$), 283 (aldJ$_2$), 362 (aldJ$_1$), 479 (aldJ$_2$); peak VII, 18.62 min, *m/z* 107, 110, 214 (aA$_1$), 196 (rA$_2$), 231 (rA$_1$), 441 (brJ$_2$), 520 (brJ$_1$); peak VIII, 19.29 min, *m/z* 196 (aA$_2$), 247 (aA$_1$), 460 (baA$_1$), 250 (aldJ$_2$), 296 (aldJ$_0$), 463 (baldJ$_2$), 542 (baldJ$_1$).

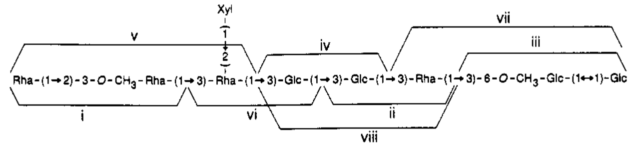

FIGURE 8: Sequence of sugars in OSE-I (*M. gordonae* strain 990), as derived from analysis of the individual fragments described in Figure 7.

In order to determine the location of these acyl residues on the oligosaccharide backbones, the pure LOS-I from *M. gordonae* strains 989 and 990 were *O*-methylated with methyl trifluoromethanesulfonate in trimethyl phosphate (Prehm, 1980), *O*-ethylated with iodoethane using the Hakomori method (1964), hydrolyzed, reduced, and *O*-acetylated and

Table VI: Postulated Structures of OSE-I–IV of *M. gordonae* Strain 990 As Determined by FAB/MS and by the Origins of Individual Fragments That Had Been Deduced by GC/MS

OSE-I	α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-[β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-II′	4-*O*-CH_3-α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-[β-D-Xyl*p*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-II″	4-*O*-CH_3-α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-[α-D-Ara*f*-(1→2)-]-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-III′	α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(⟷)-α-D-Glc*p*
OSE-III″	4-*O*-CH_3-α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*
OSE-IV	4-*O*-CH_3-α-L-Rha*p*-(1→2)-3-*O*-CH_3-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-CH_3-α-D-Glc*p*-(1⟷1)-α-D-Glc*p*

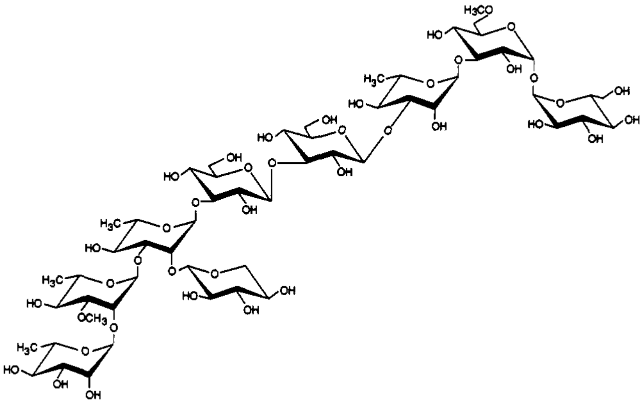

FIGURE 9: Structure of OSE-I from *M. gordonae* strain 990.

the resulting alditol acetates examined by GC/MS. It was clear from the results (not shown) that the (*t*)-α-D-Glc*p* unit of *M. gordonae* strains 989 and 990 must be fully substituted by *O*-acyl residues, i.e., four acyl residues must be esterified at the 2-, 3-, 4-, and 6-*O*-hydroxyl positions of the (*t*)-α-D-Glc*p*. Thus, LOS-I from both sources contains a unique glycosidically linked mono-6′-*O*-CH_3-tetra-*O*-acyltrehalose unit. The precise location of individual acyl functions was not established. It was also concluded that the 3-linked 2-*O*-CH_3-α-L-Fuc*p* residue of *M. gordonae* 989 was substituted at the 4-position by an acyl function which was earlier shown to be an acetate.

DISCUSSION

It is clear from the present paper that the glycolipids isolated from *M. gordonae* strains 989 and 990 belong to the so-called alkali-labile, lipooligosaccharide family and possess unique structural features at the "reducing acylated end", at the "nonreducing end", and also within the internal segment with evidence of branching. Because the structures are complex and there is some heterogeneity in some fractions, some of the data are ambiguous although generally consistent with the proposed structures. There is clear evidence of novel branching within the oligosaccharide backbone, thereby providing additional epitopes with the potential for greater specific antigenicity. This feature provides the most characteristic property of this group of glycolipids. *M. gordonae* strain 989 was characterized by branching in LOS-I and -III with a (*t*)-β-D-Xyl*p* residue, whereas in LOS-II′ and -II″, the branching sugar residue was represented by (*t*)-3-*O*-CH_3-β-D-Xyl*p* and (*t*)-α-D-Ara*f*, respectively. Similarly, the branching residues in LOS-I and -II′ were found to be (*t*)-β-D-Xyl*p*, whereas in LOS-II″, the residue was represented by (*t*)-α-D-Ara*f* from *M. gordonae* strain 990. A third structural feature which characterizes this intriguing set of glycolipids is the presence of a novel acylated amino sugar, which was found only in *M. gordonae* strain 989. Extensive analyses by high-resolution NMR and FAB/MS coupled with degradative reactions allowed one to deduce the structure of the amino sugar as being a 4-amino-4,6-dideoxy-2,3-di-*O*-CH_3-α-galactopyranosyl residue. The absolute configuration of the amino sugar is uncertain. The presence of a (*t*)-α-D-Ara*f* unit in LOS-II″ was unexpected in that Ara*f* has become a hallmark of the arabinogalactan and lipoarabinomannan of the cell walls of mycobacteria and other actinomycetes (McNeil et al., 1987c; Daffe et al., 1993), giving rise to the classification of a chemotype IV cell wall. However, arabinofuranose, as it appears at the nonreducing termini of arabinogalactan or arabinomannan, is invariably in the β-configuration (i.e., β-D-Ara*f*), whereas this newly discovered, branched D-Ara*f* unit on LOS-II″ of *M. gordonae* strain 989 is in the α-configuration, suggesting different biosynthetic origins. All of the other sugars in this family of LOS antigens have been encountered previously but in different types of glycolipids on different species, with the possible exception of the novel *N*-acylamino sugar (Daffe et al., 1991; Hunter et al., 1988).

Clearly, the LOSs of *M. gordonae* are highly antigenic, supporting the evidence that this class of glycolipid is the dominant surface antigen of the respective organism (Hunter et al., 1983, 1985, 1988). However, much more surprising was the virtual lack of cross-reactivity between the products from both strains. This evidence indicates that for the first time a serocomplex within *Mycobacterium* can be based on other than the glycopeptidolipids (Brennan, 1988). In the case of the glycopeptidolipids of the *M. avium* complex, and the LOSs of *M. kansasii* and *M. szulgai*, serological dominance is resident in the terminal/nonreducing sugar arrangements of the most polar products. Accordingly, it would seem that, in the case of the *M. gordonae* strains, serological response is largely determined by the linear chain end group (i.e., the *N*-acyl-4-amino-4,6-dideoxy-2,3-di-*O*-CH_3-α-Gal*p* and 2-*O*-CH_3-α-L-Fuc*p* residues in the case of strain 989 and the α-L-Rha*p*, 4-*O*-CH_3-α-L-Rha*p* units in strain 990) rather than the branched end groups which are common.

Recently, sporadic cases of *M. gordonae* infection have been reported involving skin and soft tissues, trauma, and underlying immunosuppression (Barber et al., 1991; Weinberger et al., 1992) which are contrary to the earlier belief that *M. gordonae* does not cause disease but is a benign commensal (Wolinsky et al., 1979). In the case of individuals with advanced immunodeficiency and who are susceptible to a wide variety of microorganisms of low pathogenicity, infections due to *M. gordonae* cause pulmonary disease indistinguishable from that caused by *M. tuberculosis*. Such patients are often started on antituberculosis therapy and yet *M. gordonae* is resistant to isoniazid, pyrazinamide, and, in some cases, ethambutol and cycloserine (Barber et al., 1991). Due to this confusion, the diagnosis of atypical mycobacteria is important. It is likely that *M. gordonae* will be identified with increasing frequency as an opportunistic pathogen in patients with AIDS, and the fact that these opportunistic pathogens can now be unequivocally identified should allow a correct course of therapy to be administered.

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