Seroreactive species-specific lipooligosaccharides of *Mycobacterium mucogenicum* sp. nov. (formerly *Mycobacterium chelonae*-like organisms): identification and chemical characterization

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Strains of the new species *Mycobacterium mucogenicum* exhibit physiological and biochemical features very similar to those of the other species of the *Mycobacterium fortuitum* complex. To define taxonomic criteria for easy identification of *M. mucogenicum*, the glycolipid patterns of the reference strains and of 32 environmental and clinical isolates were examined by TLC. It was concluded that all *M. mucogenicum* strains of smooth colony morphology contained species-specific alkali-labile glycoconjugates. Three different patterns were observed among the strains of the smooth colony type. Fractionation followed by conventional chemical analyses of the purified glycolipids showed the specific glycolipids to be lipooligosaccharides (LOS). The three LOS showed a similar fatty acid composition consisting of straight chain (dodeca-, tetradeca-, hexadecanoyl and hexadecenoyl) and methyl-branched (2,4-dimethyleicosanoyl and 2,4-dimethyleicosenoyl) fatty acyl substituents. The most commonly encountered LOS (present in 76% of the smooth strains) contained a tetraacylated pentasaccharide composed of four moles of glucose and one mole of a 2,4-di-O-methylhexose. A LOS composed of arabinose, glucose and mannose was present in 20% of the smooth strains, whereas the newly proposed type strain of *M. mucogenicum* (ATCC 49650) was the only strain that contained a LOS composed of glucose and galactose. Serological studies clearly differentiated most of the strains of *M. mucogenicum* from those of the other members of the *M. fortuitum* complex, and demonstrated the existence of serovars within the former species. Altogether, these data confirm the validity of the new species but show ATCC 49651 to be the most representative strain.

Keywords: *Mycobacterium mucogenicum*, lipooligosaccharides, inhibition ELISA, seroreactivity

INTRODUCTION

The rapidly growing non-pigmented mycobacteria are a large and complex group of environmental species,

Abbreviations: GPL, glycopeptidolipid; LOS, lipooligosaccharide; MCLO, *Mycobacterium chelonae*-like organisms; PIM, phosphatidylinositolmannoside.

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some of which cause human disease. The most important pathogens among these are the members of the *Mycobacterium fortuitum* complex. Six taxonomic groups are recognized in this complex. These are *M. fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium chelonae*, *Mycobacterium abscessus* and two unnamed groups, one of which is the *M. fortuitum* third biovariant complex, and the other the *Mycobacterium chelonae*-like organisms (MCLO). They are recognized as a cause of numerous nosocomial infections, including

surgical wound infection following cardiac surgery and augmentation mammoplasty, and catheter-related infections. Pulmonary and disseminated cutaneous diseases are also caused by these mycobacteria (Wallace, 1994; Wallace *et al.*, 1983). MCLO have been implicated in nosocomial outbreaks of peritonitis which occurred in 1976 and 1978 in Washington State, involving the use of hospital-based automated chronic peritoneal dialysis machines (Band *et al.*, 1982). MCLO were later identified as a cause of nosocomial infections associated with haemodialysis in Louisiana (Bolan *et al.*, 1985) and California (Lowry *et al.*, 1990). In all cases, MCLO were recovered from tap water used in the dialysis process. MCLO are a group of mycobacteria common in potable water, more specifically tap water. A collection of 87 sporadic clinical isolates of these organisms has been recently characterized (Wallace *et al.*, 1993). The majority of these isolates (62%) were respiratory isolates, and two strains were isolated from patients with AIDS. Among 33 non-respiratory isolates, 20 were of clinical significance.

Based on their morphological and biochemical characteristics, MCLO were included in the *M. fortuitum* complex (Lévy-Frébault *et al.*, 1983; Silcox *et al.*, 1981; Wallace *et al.*, 1993). More recently, however, the name *Mycobacterium mucogenicum* sp. nov. was proposed for this group (Springer *et al.*, 1995) on the basis of their rRNA gene sequences. This proposal is in agreement with the mycolic acid pattern of MCLO, which has been shown to be unique within the *M. fortuitum* complex (Lévy-Frébault *et al.*, 1983; Muñoz *et al.*, 1997; Wallace *et al.*, 1993), but further studies are still needed to clarify the taxonomic status of *M. mucogenicum*.

Earlier studies have demonstrated that most mycobacterial species contain large quantities of a variety of complex lipids, including species-specific glycoconjugates, which possess a potential value in taxonomy, epidemiology and serodiagnosis, and may also play a role in the pathogenicity of these organisms (for a review see Brennan *et al.*, 1990). In recent years examination of the glycolipid content of members of the *M. fortuitum* complex has greatly contributed to the knowledge of this closely related group of organisms, both in terms of taxonomic relatedness and of structures of compounds elaborated by the members of the group. For instance, a class of antigenic mycobacterial glycolipids belonging to the family of C-mycoside glycopeptidolipids (GPLs) has been identified in *M. abscessus*, *M. chelonae*, *M. peregrinum* and some strains of the *M. fortuitum* third biovariant complex (Lanéelle *et al.*, 1996; López Marín *et al.*, 1991, 1994; Tsang *et al.*, 1984). Chemical analysis, seroagglutination and ELISA have established that the GPLs of *M. chelonae* and *M. abscessus* have identical structures and differ from those of *M. peregrinum* and the *M. fortuitum* third biovariant (Lanéelle *et al.*, 1996; López Marín *et al.*, 1991, 1994; Tsang *et al.*, 1984). In contrast, a lipooligosaccharide (LOS) has been characterized in some strains of the third biovariant complex of *M. fortuitum* (Lanéelle *et al.*, 1996), whereas

two different specific antigenic acylated trehaloses, a diacyl- and a triacyltrehalose, have been described in *M. fortuitum* (Ariza *et al.*, 1994; Gautier *et al.*, 1992; Sempere *et al.*, 1993). Thus, the analysis of the glycolipid content of members of the *M. fortuitum* complex validated the DNA-DNA homology studies which had recently established that *M. fortuitum* and *M. peregrinum* belonged to two different species (Kusunoki & Ezaki, 1992; Lévy-Frébault *et al.*, 1986). Previously they had been considered as biovarieties of the same species (*M. fortuitum* bv. *fortuitum* and *M. fortuitum* bv. *peregrinum*, respectively). The glycolipid analysis also confirmed the heterogeneity of the strains grouped in the so-called *M. fortuitum* third biovariant complex, which have been shown to differ one from the other by biochemical tests, genetic studies and sensitivity to antimicrobial drugs (Kirschner *et al.*, 1992; Wallace *et al.*, 1991). Consequently, we investigated the glycolipid content of three reference strains and 32 clinical and environmental isolates of *M. mucogenicum*, the most recent species to be included in the *M. fortuitum* complex, in order to: (i) evaluate the homogeneity of the lipid pattern of strains belonging to this species; (ii) evaluate the antigenicity of the crude lipids of *M. mucogenicum* and define the chemical basis of the seroreactivities; (iii) define the taxonomic relatedness of the species to the other members of the *M. fortuitum* complex on the basis of the class of species-specific glycolipids synthesized by *M. mucogenicum* and of the serological cross-reactions.

METHODS

Bacterial strains, media and growth conditions. This study was performed with three reference strains of *M. mucogenicum* (ATCC 49649, ATCC 49650 and ATCC 49651) and 32 strains isolated from clinical and environmental sources (Table 1). These isolates were identified as *M. mucogenicum* by standard cultural, biochemical and physiological procedures as described previously (Muñoz *et al.*, 1997). Their mycolic acid patterns, and fatty acid and alcohol profiles were determined by TLC and GC, respectively. In addition, the characterization of the hypervariable region within the 16S rRNA (Muñoz *et al.*, 1997) was performed in selected isolates.

All strains were grown on plates of Sauton's agar (Sauton, 1912) incubated at 33 °C. Isolated colonies of 2-week-old cultures were examined for colony characteristics with a stereomicroscope and an external light source. For the isolation of glycolipids, cells from 2-week-old cultures of either pure rough or smooth colonies of *M. mucogenicum* were harvested from plates by scraping. For comparison, the type strains of *M. chelonae* (NCTC 946), *M. fortuitum* (ATCC 6841), *M. peregrinum* (ATCC 14467) and a reference strain of the *M. fortuitum* third biovariant complex (ATCC 49403) were grown on Sauton's medium as surface pellicles in 250 ml glass flasks at 33 °C for 2 weeks, and the cells were harvested as previously described (Lanéelle *et al.*, 1996; López Marín *et al.*, 1994).

Extraction and purification of glycolipids. Glycolipids were extracted from harvested bacteria (approximately 2 g wet wt cells) as described by Daffé *et al.* (1991). The glycolipid content of lipid extracts thus obtained was examined by TLC

on silica-gel-coated plates (G-60, 0.25 mm thickness, Merck) developed with chloroform/methanol (90:10, v/v) or with chloroform/methanol/water (30:8:1, 65:25:4 or 60:35:8, by vol.). The glycolipids were visualized by spraying plates with 0.2% (w/v) anthrone in concentrated H_2SO_4 , followed by heating at 120 °C. Molybdenum blue spray reagent (Sigma) was used for revealing phosphorus-containing substances. The lipid extracts were dissolved in chloroform and applied to a column of silica gel (70–230 mesh, Merck) equilibrated in chloroform. The column was first eluted with chloroform, and then with increasing concentrations of methanol in chloroform (5, 10, 20, 30, 50 and 100%, v/v). The different eluates were concentrated and examined by TLC using the eluting solvents mentioned above. The chromatographic fractions eluted with chloroform/methanol (1:1, v/v) and 100% methanol contained the most polar glycolipids, consisting of phospholipids and some anthrone-reactive substances. These two fractions were pooled and applied on a DEAE-cellulose column (Merck) to purify the unidentified glycoconjugates. The DEAE-cellulose column was eluted with chloroform and with chloroform containing increasing concentrations of methanol, and the purification process was monitored by TLC.

Immunological procedures

Preparation of antiserum. Rabbit antisera were obtained by intradermal injection (in multiple sites) of 1 ml of a water-in-oil emulsion prepared by mixing 0.5 ml PBS (0.145 M NaCl, 0.01 M K_2HPO_4/KH_2PO_4 , pH 7.2) containing 5 mg lipid extract of either strain 51 or 54s of *M. mucogenicum* and 0.5 ml Freund's incomplete adjuvant. The emulsion was injected into 3-month-old New Zealand White rabbits. Three weeks later, the animals received a booster dose of 3 mg lipid extract in water-in-oil emulsion, as described above, and were then bled at 2-week intervals.

Direct ELISA. Lipids were suspended at 500 $\mu g\ ml^{-1}$ in hexane/ethanol (1:1, v/v). Samples (50 μl) were applied to the wells of a polystyrene microtitre plate (Nunc-Immuno Plate I) and allowed to evaporate at 37 °C overnight. After saturation and washing, 100 μl of the rabbit sera, diluted 1:1000 (v/v) in 0.4% (w/v) powdered skimmed milk in PBS, was added and the plates incubated for 2 h at 37 °C. After washing, alkaline phosphatase conjugated to whole anti-rabbit IgG (Sigma) and *p*-nitrophenyl phosphate substrate (Sigma) were used for the colour reaction. Absorbance was measured at 410 nm with a Multiskan apparatus (Flow Laboratories). Each value was measured in triplicate.

Inhibition ELISA. The inhibition of the reaction between 25 μg solid-phase lipid extract of either strain 51 or 54s of *M. mucogenicum* and specific rabbit anti-(lipids of the given strain of *M. mucogenicum*) serum diluted 1:100 (v/v) in PBS by the whole lipid extract, the purified alkali-labile glycolipid isolated from strain 51 or by D-glucose, D-mannose, D- or L-arabinose was performed as follows. The inhibitory compounds were suspended at 500, 250, 125 and 100 $\mu g\ ml^{-1}$ in either hexane/ethanol (1:1, v/v) or 0.4% (w/v) powdered skimmed milk in PBS. Then, 50 μl of these suspensions, 40 μl of 0.4% (w/v) powdered skimmed milk in PBS and 10 μl rabbit anti-(lipids of either strain 51 or 54s of *M. mucogenicum*) serum (diluted 1:100, v/v, in PBS) were incubated for 2 h at 37 °C; the mixtures were deposited in wells that had previously been coated with 25 μg whole lipid extract from the given strain and blocked by 200 μl 30% (w/v) powdered skimmed milk in PBS. The mixture was left for 2 h at 37 °C before rinsing. The colour reaction used anti-rabbit IgG (Sigma) and *p*-nitrophenyl phosphate substrate (Sigma) as

above. A control for no inhibition was performed using 10 μl serum diluted 1:100 (v/v) in PBS and 90 μl buffer; a blank control was obtained by the development of the complete ELISA reaction in wells not coated with lipids.

Analytical techniques

Alkaline deacylation. To determine the stability of the compounds towards alkali, the crude lipid extracts and the purified glycolipids were deacylated according to Brennan & Goren (1979).

Sugar characterization. Samples (1–5 mg) were hydrolysed with 2 M CF_3COOH solution at 110 °C for 4 h. After evaporation, the hydrolysates were partitioned between diethyl ether and water. The aqueous phase was recovered and divided into two portions. One portion was dried under nitrogen and trimethylsilylated (Sweeley *et al.*, 1963). The remaining portion of the aqueous phase was reduced by $NaBH_4$ and peracetylated with acetic anhydride/pyridine (1:1, v/v) overnight at room temperature. The trimethylsilylated and alditol acetate derivatives of sugars were analysed by GC.

Fatty acid composition of glycolipids. The organic phases from the acid hydrolysis and from the alkaline deacylation (as described above) were treated with diazomethane. The resulting fatty acid methyl esters were analysed by GC and GC-MS.

GC and GC-MS. GC and GC-MS analyses of fatty acid methyl esters and both alditol acetate and trimethylsilyl derivatives of saccharides were performed on a Hewlett-Packard 5890A chromatograph connected to a Hewlett-Packard 5989X mass selective detector using a fused-silica capillary column (12 m $\times$ 0.25 mm internal diameter) with cross-linked methyl silicone (HP-1, Hewlett-Packard) as the stationary phase. The injector and transfer line temperatures were 275 and 280 °C respectively. The carrier gas was helium with a flow rate of 1 ml min^{-1} , and the split ratio was approximately 1:50. For the analysis of fatty acid methyl esters, the oven was programmed to rise at 8 °C min^{-1} from 175 °C to 300 °C, followed by a 15 min hold at 300 °C. The temperature programme that was applied to trimethylsilyl and alditol acetate derivatives involved a 100 °C hold for 2 min, followed by a 5 °C min^{-1} rise to 280 °C. The chromatograms were integrated by using a HP3396 Series II Integrator (Hewlett-Packard). Fatty acid methyl esters and trimethylsilylated derivatives of sugars were identified by comparing their retention times with those of authentic methyl esters and alcohol standards (Supelco and Sigma). The identities of fatty acid methyl esters were also confirmed by MS.

NMR. All the NMR spectra were obtained in $CDCl_3/CD_3OD$ (2:1, v/v) at 25 °C. They were obtained with 40 ° pulses, and acquisition times of 1.56 s for the ^{13}C -NMR spectrum (spectral width 5263 Hz) and 2.73 s for the 1H -NMR spectrum (spectral width 2994 Hz) were used. The 1H -NMR spectrum was recorded on a Bruker WM 250 apparatus instrument at 250 MHz, and the ^{13}C -NMR spectrum was obtained with the same instrument operating at 62.9 MHz using a Bruker J-Mod sequence. The chemical-shift reference used was that of tetramethylsilane.

RESULTS

Morphology of the colonies of *M. mucogenicum*

One physical characteristic of *M. mucogenicum*, also shown by other mycobacteria, is an ability to exhibit various colony morphologies (rough and variable smooth formats). The three reference strains, as well as

Table 1. Origin, colony morphology and LOS pattern of *M. mucogenicum* reference strains and isolates

Strain no.	Source*	Colonial morphology	LOS pattern†
ATCC 49649	Peritoneal fluid	Smooth	I
ATCC 49650	Neck abscess	Smooth	III
ATCC 49651	Postinjection buttock abscess	Smooth	I
51	Gastric lavage	Smooth	I
54s	Urine	Smooth	II
54r	Urine	Rough	—
147	Gastric lavage	Smooth	II
1	River mouth	Smooth	I
2s	River mouth	Smooth	I
2r	River mouth	Rough	—
32s	River mouth	Smooth	I
32r	River mouth	Rough	—
33	River mouth	Smooth	II
3	Seawater	Smooth	I
4	Seawater	Smooth	II
5	Seawater	Smooth	I
11	Seawater	Smooth	I
12	Seawater	Smooth	I
13	Seawater	Smooth	I
14	Seawater	Smooth	I
15	Seawater	Smooth	I
20	Seawater	Smooth	I
34	Seawater	Smooth	I
35	Seawater	Smooth	I
45	Seawater	Rough	—
46	Seawater	Smooth	I
98	Sewage water	Rough	—
102	Sewage water	Smooth	I
106s	Sewage water	Smooth	II
106r	Sewage water	Rough	—
112	Sewage water	Rough	—
131	Sewage water	Rough	—
205	Sewage water	Rough	—
207	Sewage water	Rough	—
208	Sewage water	Smooth	I

* River mouth, at the mouth of the River Besos, one of the two rivers that flow through the suburbs of Barcelona; seawater, at different points in coastal waters about 200 m offshore; sewage water, at the admittance and discharge points of the sewage treatment plant at the town of Vilafranca del Penedès, situated about 50 km from Barcelona.

† Patterns I–III defined by the sugar composition of the LOS-containing fractions; — indicates no pattern detected.

22 out of the 32 strains that we isolated, displayed a smooth morphology (Table 1, Fig. 1a, b, c). The remaining 10 strains showed a rough morphology (Table 1, Fig. 1d). Three types of smooth colonies were observed (Fig. 1). The reference strains ATCC 49649 and ATCC 49651, and all the smooth isolates that elaborated the glycolipid pattern I (Table 1), formed circular, convex colonies with entire margins (Fig. 1a), whereas the strains which contained glycolipids of pattern II (Table 1) exhibited irregular, raised colonies

with complex margins (Fig. 1b). The newly proposed type strain ATCC 49650 was the only strain examined that formed circular, convex colonies with a slightly translucent border (Fig. 1c) and showed the glycolipid pattern III (Table 1).

Among the strains isolated from human clinical specimens, the pure cultures (51 and 147) exhibited a smooth morphology; the remaining two strains (54s, 54r) were isolated from an originally mixed culture in which the

Fig. 1. Smooth colonies of strain ATCC 49649 and related isolates (a), isolate 54s (b) and strain ATCC 49650 (c), and rough colonies of *M. mucogenicum* (d).

strain with smooth morphology (54s) was predominant. The isolation of mixed cultures in which the smooth strain predominated over the rough strain also occurred in two aquatic isolates, i.e. the pairs 2s/2r and 32s/32r. Interestingly, this predominance of the strains with smooth morphology over the strains with rough morphology did not occur in the strains isolated from sewage water, a highly contaminated and perhaps more hostile environment. Of the nine strains isolated from sewage water, six displayed a rough morphology and three exhibited a smooth morphology. In the only mixed culture obtained from sewage water (strains 106s and 106r), the strain with a rough morphology predominated over the strain with a smooth one. The predominance of a given morphology in mixed cultures according to the origin of isolations (water-based environment, clinical specimens or sewage water) suggest that, in the case of *M. mucogenicum*, external factors may influence the production of smooth or rough colonies.

Identification of LOS in smooth strains of *M. mucogenicum*

Comparative TLC analyses of the lipid extracts of the various strains showed, as expected, the presence of ubiquitous mycobacterial lipids in all the strains examined. These included triacylglycerol, mycolate-containing glycolipids and phospholipids. In addition, one or more major spots which correspond to polar lipids, exhibiting chromatographic mobilities similar to those

of phosphatidylinositomannosides (PIMs) were detected from all the smooth strains of *M. mucogenicum* (Fig. 2); all the rough strains were devoid of these spots. The additional spots readily reacted with anthrone, a reagent that specifically reveals the presence of carbohydrates, but not with molybdenum blue, a specific reagent for phosphorylated substances (Fig. 2). Furthermore, the additional spots disappeared after treatment of lipids from smooth strains of *M. mucogenicum* with 0.2 M NaOH in methanol (a reaction that cleaves ester links). Thus, these preliminary data demonstrated that the substances present in smooth strains, and absent in the rough strains of *M. mucogenicum* (Fig. 2), conformed to the specification of acylated oligosaccharides, more commonly known as LOS (McNeil *et al.*, 1989).

Three different LOS patterns were clearly observed by TLC (Fig. 2, Table 1). Pattern I was shared by most (76%) of the smooth strains examined, including the two reference strains ATCC 49649 and ATCC 49651 (Fig. 2, lanes 1, 3 and 4–7). All these strains formed circular, convex colonies with entire margins (Fig. 1a). Pattern II (Fig. 2, lane 8) was observed in the five strains (20% of the smooth strains) which formed irregular, raised colonies with complex margins (Fig. 1b), whereas the third pattern (Fig. 2, lane 2) was exclusively found in the type strain (ATCC 49650), which formed circular and convex colonies with a slightly translucent border (Fig. 1c). These patterns were found to have been invariant during the last 2 years; no change has ever

Fig. 2. TLC (solvent, chloroform/methanol/water, 60:35:8, by vol.; colour reagent, anthrone) of the lipid extracts of the reference strains ATCC 49649 (lane 1), ATCC 49650 (lane 2), ATCC 49651 (lane 3) of *M. mucogenicum* and of smooth strains 2s, 32s, 51, 102 and 54s (lanes 4–8, respectively) and rough isolates 54r, 2r and 32r (lanes 9–11, respectively). Arrows indicate phosphorus-free alkali-labile glycolipids (LOS). The compounds exhibiting chromatographic mobilities similar to that of LOS correspond to PIMs.

been detected in the LOS pattern of the various strains studied.

Purification and chemical composition of the major LOS of *M. mucogenicum*

The lipid extracts of representative strains of LOS pattern I (strain 51 and ATCC 49649), pattern II (strain 54s) and pattern III (strain ATCC 49650), as well as those of some rough strains (2r and 32r) were fractionated by adsorption chromatography on silica gel columns to yield enriched LOS fractions. The neutral polar glycolipids (LOS) were separated from the PIMs by anion-exchange chromatography on DEAE-cellulose columns. Application of this procedure yielded pure LOS from strains exhibiting LOS patterns I and II. In the case of strain ATCC 49650 (LOS pattern III), however, LOS were eluted with PIMs; application of various chromatographic methods failed to give pure LOS, suggesting the occurrence of acidic substituents.

Analysis of the fatty acid methyl esters that result from the alkaline deacylation of LOS by GC and GC-MS showed a similar profile for the three compounds (Fig. 3). The fatty acyl substituents were identified on the basis of their retention times on GC, the m/z values of their molecular ion peaks, $[M]^+$, and of the fragment ion peaks, $[M \text{ minus } C_2-C_3-C_4]^+$, on GC-MS, and the

occurrence of ion peaks at m/z 74 (or 88) and 87 (or 101) (Ryhage & Stenhagen, 1960). The major fatty acyl component of the LOS was octadecenoyl ($C_{18:1}$). Significant amounts of lower homologues (C_{16} , C_{14} and C_{12}) were also present. In addition, methyl-branched fatty acyl substituents were identified. They consisted of 2-methyloctadecanoyl, 2,4-dimethyleicosenoyl and 2,4-dimethyleicosanoyl. The relative amounts of the different fatty acyl substituents varied slightly according to the origin of the LOS and, presumably, the growth phase of the examined strains.

Acid hydrolysis of the different LOS followed by the analysis of the sugar derivatives by GC demonstrated that the difference in the chromatographic mobility of the various LOS (Fig. 2) corresponded to a difference in the sugar composition (Table 2), rather than to the number of fatty acyls substituting the LOS. Glucose was the major sugar constituent of the three glycolipids, as commonly found in mycobacterial LOS. The additional sugar that typified the LOS present in most of the smooth strains of *M. mucogenicum*, i.e. LOS pattern I, corresponded to a 2,4-di-O-methylhexose (fragment ion peaks at m/z 43, 117, 189, 201, 233 and 305). Mannose and arabinose, in addition to glucose, were the sugar constituents of LOS pattern II (Table 2). Acid hydrolysis of the enriched LOS fraction from the type strain ATCC 49650 released glucose and galactose. In addition,

Fig. 3. GC profiles of methyl esters resulting from the deacylation of the LOS purified from isolate 51 of *M. mucogenicum*. Peaks A, B and C correspond to 2-methyloctadecanoate, 2,4-dimethyleicosenoate and 2,4-dimethyleicosanoate, respectively.

Table 2. Glycosyl composition of the different LOS patterns found in *M. mucogenicum*

+ and – indicate the presence and absence of the sugar, respectively.

	Pattern I	Pattern II	Pattern III
Glucose	+	+	+
Mannose	–	+	+
Arabinose	–	+	–
Galactose	–	–	+
2,4-Di-O-methylhexose	+	–	–
Glycerol	–	–	+
Inositol	–	–	+

glycerol, mannose and inositol, presumably derived from the contaminant PIMs (see above), were found (Table 2). Interestingly, the polyol constituents of PIMs, i.e. mannose, glycerol and inositol, were the only hydrosoluble products detected by the GC analysis of the acid hydrolysis products of the polar lipid fractions from the rough strains examined.

NMR analyses of the commonest LOS

Because LOS pattern I was encountered in the majority of the smooth strains of *M. mucogenicum* examined, further structural analyses were conducted on the purified LOS by ^1H - and ^{13}C -NMR. The ^1H -NMR spectrum (Fig. 4a) was informative, notably in the region of anomeric and other deshielded protons

(4.5–5.5 p.p.m.). Two doublets ($J_{1,2} = 3.5$ Hz) were observed at 5.23 and 5.32 p.p.m. and assigned to the resonances of H-1 of α -hexopyranosyl residues possessing a *gluco* configuration (Daffé *et al.*, 1988). The triplet centred at 5.07 p.p.m. may be attributed to either a deshielded H-3 or H-4 resonance of a glucopyranosyl unit that had an esterified hydroxyl group located on C-3 or C-4. Similarly, a doublet of doublets was observed at 4.85 p.p.m., assignable to the resonance of H-2 of an α -glucopyranosyl residue that had an esterified hydroxyl group located on C-2 (Daffé *et al.*, 1988, 1991; Gautier *et al.*, 1992; Lanéelle *et al.*, 1996). Analysis of the ^1H -NMR spectrum of the LOS confirmed the presence of double bonds (resonance of the ethylenic protons at 5.40 p.p.m.), long-chain fatty acyl residues (a massive signal corresponding to the resonance of methylenic protons centred at 1.30 p.p.m.), methyl-branched protons (doublet at 1.20 p.p.m.) and other features (see Fig. 4a) that were in agreement with the data obtained by GC and GC-MS of fatty acid methyl esters (see above). In addition, two sharp singlets were observed at 3.60 and 3.70 p.p.m. and assigned to the resonances of the methyl protons of the two methoxyl groups of the 2,4-di-O-methylhexosyl residue of the LOS. Analysis of the ^{13}C -NMR spectrum (Fig. 4b) confirmed the data obtained from the interpretation of the ^1H -NMR spectrum and demonstrated the occurrence of five glycosyl units and four acyl substituents in the LOS. Two signals, assignable only to the resonances of the two anomeric carbons of α,α' -trehalose (Bradbury & Jenkins, 1984), were observed at 92.60 and 94.60 p.p.m. (Fig. 4b, inset). The three other anomeric signals at 102.17, 104.40 and 104.9 p.p.m. were tentatively attributed to the di-O-methylhexopyranosyl residue and to two β -glucopyranosyl units (Bradbury & Jenkins, 1984). The existence of four deshielded signals (at >170 p.p.m.), assignable to the resonances of carbonyl, indicated the presence of four acyl substituents in the glycolipid. Thus, the LOS pattern I corresponded to a tetraacylated pentasaccharide whose complete structure is under study.

Seroreactivity of the lipid extracts

Rabbit sera were raised against the whole lipid extracts from the representative strains of LOS patterns I and II (isolates 51 and 54s, respectively) to evaluate the antigenicity of the two LOS. In both cases the highest seroreactivities were obtained with sera produced 7 weeks after immunization. As shown in Fig. 5, the lipids from a given strain reacted readily in ELISA with the homologous serum. In contrast, the sera raised against the lipid extracts of isolates 51 and 54s reacted only weakly with the heterologous lipids (Fig. 5), demonstrating the specificity of the seroreactions and confirming the existence of at least two serovars among the strains of *M. mucogenicum*. Furthermore, the sera directed against the lipids from strains 51 and 54s, which represented 96% of the smooth strains of *M. mucogenicum*, did not recognize the lipid extracts from other members of the *M. fortuitum* complex tested, which

Fig. 4. NMR spectra of the LOS purified from isolate 51 of *M. mucogenicum*. (a) ^1H -NMR spectrum; (b) portion of the J -modulated ^{13}C -NMR spectrum. Both spectra were recorded in $\text{CDCl}_3/\text{CD}_3\text{OD}$ (2:1, v/v).

included the type strains of *M. fortuitum*, *M. peregrinum* and *M. chelonae*, and a representative strain of the third biovariant complex of *M. fortuitum*.

The seroreactivity of the lipids from isolates 51 and 54s with the homologous serum was abolished by deacylation, showing that the alkali-labile substances represented the main antigenic compounds. In addition, the lipids from the different rough strains, whose composition differed from those of strains 51 and 54s only by the absence of LOS, did not react with any of the sera. This demonstrates that the ubiquitous lipids (PIMs and

mycoloyltrehaloses) did not significantly contribute to the observed seroreactivity of lipids from smooth strains, and suggests that LOS were the antigenic substances. To test this hypothesis, the contribution of the purified LOS from strain 51 to the seroreactivity was determined.

Seroreactivity of the purified LOS of strain 51

As expected from the seroreactivity of the various lipid extracts, the purified LOS of isolate 51 reacted strongly with the serum raised against the whole lipids from

Fig. 5. Reactivity in ELISA of native lipid extracts of reference strains and isolates of *M. mucogenicum* and representative strains of the other species of the *M. fortuitum* complex, of deacylated lipids of isolates 51 and 54s of *M. mucogenicum*, and of purified LOS from strain 51 against a rabbit anti-(whole lipid antigens of strain 51) serum (black columns) and a rabbit anti-(whole lipid antigens of strain 54s) serum (white columns). Results represent the arithmetic mean for three determinations and 25 µg product was used per well. The sera were diluted in PBS (1:1000).

Fig. 6. Inhibition of the reaction between (a) a rabbit anti-(whole lipid antigens of strain 51 of *M. mucogenicum*) serum and 25 µg coated whole lipid extract of strain 51 by the LOS purified from the same strain and (b) a rabbit anti-(whole lipid antigens of strain 54s of *M. mucogenicum*) serum and 25 µg coated whole lipid extract of strain 54s by L-arabinose (■), D-arabinose (◆), D-glucose (▲) and D-mannose (●). One hundred per cent activity corresponded to an A₄₁₀ value of 1.77.

strain 51 (Fig. 5). It was clearly demonstrated that this glycolipid was the main antigen of the lipid mixture by inhibition experiments in which up to 90% of the

seroreactivity of the crude lipid from strain 51 with the homologous serum was inhibited by the LOS purified from the same strain (Fig. 6a).

Chemical basis of the seroreactivity of the lipid extracts of isolate 54s

The antigenicity of glycolipids is widely accepted as due to the carbohydrate moiety of the molecules. In mycobacteria, the presence of unusual sugar residues confers a structural diversity to the oligosaccharides that substitute a common lipid or lipopeptide core, which in turn governs the exquisite specificity of the seroreactivity of the surface-exposed glycolipids (see Brennan *et al.*, 1990). Consequently, the L- and D-isomers of arabinose, D-mannose and D-glucose, which composed the LOS of isolate 54s, were used to inhibit the reactivity of the lipid extract of strain 54s with the serum directed against the lipid extract from the same strain (Fig. 6b). D-Arabinose inhibited up to 70% of the seroreactivity, whereas the L-isomer achieved only a 20% inhibition; as expected, neither glucose, which is the ubiquitous sugar constituent of all the mycobacterial LOS characterized so far (see Brennan *et al.*, 1990), nor mannose, inhibit the reaction. These results demonstrated that the D-arabinosyl residue of the LOS is involved in the antigenicity of the molecule.

DISCUSSION

The present study was undertaken to identify and chemically characterize the species-specific glycolipids of *M. mucogenicum*, the most recent species to be included in the *M. fortuitum* complex. Examination of the glycolipid content of three reference strains and 32 clinical and environmental isolates belonging to *M. mucogenicum* demonstrated that all the smooth strains of this species elaborate antigenic species-specific alkali-labile and phosphorus-free neutral glycolipids, i.e. LOS. This observation is similar to that noted by Belisle &

Brennan (1989) in the case of *Mycobacterium kansasii*. It has to be noted, however, that no correlation between the presence of LOS and the colony morphology of the strains has been observed in either *M. tuberculosis* (Lemassu *et al.*, 1992) or the third biovariant complex of *M. fortuitum* (Lanéelle *et al.*, 1996). The acylated triglucoside, the simplest form of LOS described so far, characterized in some strains of this latter heterogeneous group of organisms (Lanéelle *et al.*, 1996), is structurally similar to the one found in some strains of *M. fortuitum* (Besra *et al.*, 1992). These strains probably belong to the third biovariant complex, since only acylated trehaloses have been found in the type strain and in well-characterized strains of *M. fortuitum* (Ariza *et al.*, 1994; Gautier *et al.*, 1992; Sempere *et al.*, 1993). Apart from these two exceptions, the presence of LOS has not been reported in the other species of the *M. fortuitum* complex, i.e. *M. chelonae*, *M. abscessus* and *M. peregrinum*, which elaborate antigenic GPLs as major glycolipids (López Marín *et al.*, 1991, 1994; Tsang *et al.*, 1984). For this reason, the presence of a characteristic LOS pattern in all the smooth strains of *M. mucogenicum* investigated in the present study clearly differentiates *M. mucogenicum* from the other species that compose the *M. fortuitum* complex, and supports the recent proposal which considers *M. mucogenicum* to be a new species. Furthermore, the sera raised against the lipid extracts of the smooth strains of *M. mucogenicum* did not react with the lipid antigens of the representative strains of the other members of the *M. fortuitum* complex; this suggests that serological studies may help in the identification of strains belonging to the new species.

In the smooth strains of *M. mucogenicum* examined, three different LOS patterns were observed. The LOS present in 76% of the smooth strains examined was chemically characterized as a tetraacylated pentasaccharide; the LOS typifying the remaining strains of *M. mucogenicum* differ from the former LOS, and from the previously described LOS, by their sugar composition and their seroreactivity. It is worthy of note that the variability in the LOS patterns in mycobacterial strains belonging to the same species had previously been observed in *Mycobacterium gordonae*. Goslee *et al.* (1976) recognized seven distinct serotypes of *M. gordonae*, the dominant surface antigens of which have subsequently been characterized by Brennan *et al.* (1982) as LOS. The chemical explanation of such variability has recently been given by Besra *et al.* (1993) through the structural elucidation of LOS from two strains of *M. gordonae*. The authors showed that each strain contained a set of four major LOS, whose structures differed according to the strain examined. The purified LOS reacted strongly with the antiserum raised against the homologous strain and only weakly with the heterologous strain, demonstrating that *M. gordonae* is a serocomplex based on the structures of the LOS. Thus, like GPLs that typify the 31 serovars of the *M. avium* complex (see Brennan *et al.*, 1990), LOS may be responsible for the occurrence of serovars in a given

mycobacterial species. In the case of *M. mucogenicum*, the present study suggests the existence of at least three serovars which differ one from the other by the structure and seroreactivity of their LOS. In this connection, several studies, notably in the *M. avium* complex, have demonstrated the usefulness of determining these serovars in epidemiological studies (Falkinham, 1994; Kiehn *et al.*, 1985; McClatchy, 1981; Shaefer, 1979; Yakrus & Good, 1990). There is no doubt that epidemiological markers remain an important tool in the fight against pathogenic bacteria and fundamentally against those that give rise to epidemics. This is the case in *M. mucogenicum* which has caused several nosocomial epidemics and has been recovered in several occasions from tap water in the affected hospitals (Band *et al.*, 1982; Bolan *et al.*, 1985; Lowry *et al.*, 1990). Interestingly, strains 54s and 147, which contain the same arabinose-containing LOS, were isolated from two patients who occupied the same ward in a hospital block for 2 weeks. Although neither of the two isolations were clinically significant, the two isolates possibly belong to the same clone that was contaminating the tap water of the hospital block at that time.

Finally, based on partial sequencing of the 16S rRNA gene of the three reference strains of *M. mucogenicum* used in the present study, Springer *et al.* (1995) recently proposed the biochemically typical strain ATCC 49650 as the type strain of the new species rather than the previously recommended (Wallace *et al.*, 1993) but atypical strain ATCC 49649. In our hands, however, the newly proposed type strain is atypical in terms of LOS content. This strain is the only one that elaborated galactose-containing LOS among the 32 isolates we examined. In conclusion, we recommend that strain ATCC 49651 be considered the type strain of *M. mucogenicum*, firstly because ATCC 49651 is a biochemically typical strain of *M. mucogenicum*, secondly because it contains the most often encountered LOS characterized in the present study, and thirdly because it has a 16S rRNA gene sequence identical to that of the newly proposed type strain ATCC 49650 (Springer *et al.*, 1995).

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