Structure and Host Recognition of Serotype 13 Glycopeptidolipid from *Mycobacterium intracellulare*^{▽†}

Takashi Naka,^{1,2‡} Noboru Nakata,^{3‡} Shinji Maeda,^{4‡} Reina Yamamoto,^{1,5} Matsumi Doe,⁶
Seiko Mizuno,^{1,5} Mamiko Niki,¹ Kazuo Kobayashi,⁷ Hisashi Ogura,^{1,8}
Masahiko Makino,³ and Nagatoshi Fujiwara^{1*}

Departments of Bacteriology¹ and Virology,⁸ Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan; MBR Co. Ltd., Osaka 560-8552, Japan²; Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo 189-0002, Japan³; Molecular Epidemiology Division, Mycobacterium Reference Center, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo 204-8533, Japan⁴; Department of Development Nourishment, Faculty of Human Development, Soai University, Osaka 559-0003, Japan⁵; Department of Chemistry, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan⁶; and Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan⁷

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The *Mycobacterium avium*-*M. intracellulare* complex (MAIC) is divided into 28 serotypes by a species-specific glycopeptidolipid (GPL). Previously, we clarified the structures of serotype 7 GPL and two methyltransferase genes (*orfA* and *orfB*) in serotype 12 GPL. This study elucidated the chemical structure, biosynthesis gene, and host innate immune response of serotype 13 GPL. The oligosaccharide (OSE) structure of serotype 13 GPL was determined to be 4-2'-hydroxypropanoyl-amido-4,6-dideoxy- β -hexose-(1 $\rightarrow$ 3)-4-*O*-methyl- α -L-rhamnose-(1 $\rightarrow$ 3)- α -L-rhamnose-(1 $\rightarrow$ 3)- α -L-rhamnose-(1 $\rightarrow$ 2)- α -L-6-deoxy-talose by using chromatography, mass spectrometry, and nuclear magnetic resonance (NMR) analyses. The structure of the serotype 13 GPL was different from those of serotype 7 and 12 GPLs only in *O*-methylations. We found a relationship between the structure and biosynthesis gene cluster. *M. intracellulare* serotypes 12 and 13 have a 1.95-kb *orfA-orfB* gene responsible for 3-*O*-methylation at the terminal hexose, *orfB*, and 4-*O*-methylation at the rhamnose next to the terminal hexose, *orfA*. The serotype 13 *orfB* had a nonfunctional one-base missense mutation that modifies serotype 12 GPL to serotype 13 GPL. Moreover, the native serotype 13 GPL was multiactylated and recognized via Toll-like receptor 2. The findings presented here imply that serotypes 7, 12, and 13 are phylogenetically related and confirm that acetylation of the GPL is necessary for host recognition. This study will promote better understanding of the structure-function relationships of GPLs and may open a new avenue for the prevention of MAIC infections.

The increase of drug-resistant mycobacteria and the number of immunocompromised hosts including the HIV epidemic are important problems. The *Mycobacterium avium*-*M. intracellulare* complex (MAIC) is distributed ubiquitously in the environment and is the most common isolate of nontuberculous mycobacteria, which are now one of the most important environmental pathogen-disseminated infectious agents in both immunocompromised and immunocompetent hosts (26, 31, 39).

The most characteristic feature of mycobacteria is richness in lipids. These hydrophobic cell wall components contribute to the surface properties and are considered to play important roles in their pathogenesis through the host immune responses (8, 17). MAIC expresses a glycopeptidolipid (GPL) as one of the representative lipid components. Structurally, the GPL is composed of two parts, a common tetrapeptido-amino alcohol core and a serotype-specific oligosaccharide (OSE) elongated

from 6-deoxy-talose (6-d-Tal). D-Phenylalanine-D-*allo*-threonine-D-alanine-L-alaninol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol), which is modified with an amido-linked 3-hydroxy or 3-methoxy C₂₆-C₃₄ fatty acid at the *N* terminus of D-Phe, and D-*allo*-Thr and terminal L-alaninol are further linked to a 6-d-Tal and 3,4-di-*O*-methyl rhamnose (3,4-di-*O*-Me-Rha), respectively. This portion is called the serotype-nonspecific GPL (apolar GPL). Serotype-specific GPLs (polar GPLs) are produced by extending individual OSE residues from the 6-d-Tal. MAIC species are divided into 28 serotypes by serological reaction and distinctive patterns of polar GPLs on thin-layer chromatography (TLC) (7, 38). The GPL is considered to play crucial roles in the physiology of the bacteria and the host responses to MAIC infection. Several biological and immunological functions of GPLs have been reported (9, 34), but the roles of GPLs are not fully elucidated. Recently, several genes involved in GPL biosynthesis have been characterized (10, 29). To better understand the biological functions and significance of GPLs, we need to clarify the structure and biosynthetic pathways of GPLs.

The chemical structures of only 16 GPLs have been defined (9). Recently, we determined the structures of the serotype 7 and 16 GPLs and identified the gene clusters completing the OSE biosynthesis (13, 14). In addition, two methyltransferase genes of serotype 7- and 12-specific GPL biosynthesis were

* Corresponding author. Mailing address: Department of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. Phone: 81 6 6645 3746. Fax: 81 6 6645 3747. E-mail: fujiwara@med.osaka-cu.ac.jp.

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‡ These authors contributed equally to this work.

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characterized (30). In this process, we found that the structure of the serotype 13 GPL is close to that of the serotype 7 and 12 GPLs. In epidemiological serotyping, Tsang et al. (37) showed that clinical isolates of serotypes 7, 12, and 13 were found in around 10% of non-AIDS patients. However, it was difficult to distinguish serotypes 7, 12, and 13 by only serological and chromatographic techniques because of their structural similarity. The phylogeny of some MAIC strains based on GPL biosynthesis genes has been reported (23). In this study, the complete structure of the serotype 13 GPL was determined, and the genetic relationship between the serotype 7, 12, and 13 GPL biosynthesis was clarified. Moreover, the host innate immune recognition of antigenic serotype 13 GPL and the importance of structural modification were shown. We discuss the phylogeny of MAIC strains on the basis of these GPL biosynthesis genes and the relationship between GPL structure and immunogenicity.

MATERIALS AND METHODS

Bacterial strains and preparation of GPL. *M. intracellulare* serotype 13 (ATCC 35769, ATCC 25122), serotype 7 (ATCC 35847), and serotype 12 (ATCC 35762) strains were purchased from the American Type Culture Collection (Manassas, VA). The GPL preparation was performed as described previously (14, 18). Each strain of *M. intracellulare* was grown on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI) with 0.5% glycerol and 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) at 37°C for 2 to 3 weeks. The heat-killed bacteria were sonicated, and crude lipids were extracted with chloroform-methanol (2:1 [vol/vol]). The crude lipids were hydrolyzed with 0.2 N sodium hydroxide in methanol at 37°C for 2 h, followed by neutralization with 6 N hydrochloric acid. Alkaline-stable lipids were partitioned by a two-layer system with chloroform-methanol (2:1 [vol/vol]) and water. The organic phase was evaporated and precipitated with acetone to remove any acetone-insoluble components. The supernatant was washed (chloroform-methanol, 95:5 [vol/vol]) and eluted (chloroform-methanol, 1:1 [vol/vol]) with a Sep-Pak silica cartridge (Waters Corporation, Milford, MA) for partial purification. The GPL was completely purified by preparative TLC of Silicagel G (Uniplate; 20 by 20 cm, 250 μ m; Analtech, Inc., Newark, DE). The TLC plate was developed with chloroform-methanol-water (65:25:4 and 60:16:2 [vol/vol/vol]), until a single spot was obtained. The TLC plate was sprayed with 20% sulfuric acid in ethanol and was charred at 180°C for 3 min. The GPL was detected as a brownish-yellow spot. To recover the GPL, the TLC plate was exposed to iodine vapor, and the GPL spot was marked. The silica gels of the GPL spot were scraped off, and the GPL was eluted with chloroform-methanol (2:1 [vol/vol]). The native GPL was purified by the same method as the alkaline-stable GPL, omitting the hydrolysis with 0.2 N sodium hydroxide.

Preparation of OSE moiety. β -Elimination of the GPL was performed with alkaline borohydride, and the OSE elongated from D-*allo*-Thr was released (14, 18). The GPL was stirred in a solution of equal volumes of ethanol and 10 mg/ml sodium borodeuteride in 0.5 N sodium hydroxide at 60°C for 16 h. The reaction mixture was decationized with Dowex 50W X8 beads (Dow Chemical Company, Midland, MI) and evaporated under nitrogen to remove boric acid. After partition into two layers of chloroform-methanol (2:1 [vol/vol]) and water, the upper aqueous phase was recovered and evaporated, and the OSE was purified as an oligoglycosyl alditol.

MALDI-TOF MS and MALDI-TOF MS/MS. The molecular species of the intact GPL was determined by the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) with an Ultraflex II (Bruker Daltonics, Billerica, MA). One microgram of the GPL-dissolved chloroform-methanol (2:1 [vol/vol]) was applied to the target plate, and 1 μ l of 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform-methanol (1:1 [vol/vol]) was added as a matrix. The intact GPL was analyzed in the Reflectron mode with an accelerating voltage operating in positive mode at 20 kV (4). Then, the fragment pattern of the OSE was analyzed with the MALDI-TOF MS/MS mode. The OSE and 10 mg/ml 2,5-dihydroxybenzoic acid was dissolved in ethanol-water (3:7 [vol/vol]) and applied to the target plate according to the method for intact GPL.

GC/MS of carbohydrates. To determine the glycosyl composition and linkage position, gas chromatography/mass spectrometry (GC/MS) of partially methylated alditol acetate derivatives was performed. Perdeuteromethylation was con-

FIG. 1. TLC patterns and MALDI-TOF MS spectra of serotype 7, 12, and 13 GPLs. (A and B) The alkaline-stable lipids derived from *M. intracellulare* serotype 13 ATCC 35769 and ATCC 25122 (A) and the purified serotype 7, 12, and 13 GPLs (B) were developed on TLC plates with solvent systems of chloroform-methanol-water (60:16:2 and 65:25:4 [vol/vol/vol]). (C) The MALDI-TOF MS spectra of serotype 7, 12, and 13 GPLs were acquired using 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform-methanol (1:1 [vol/vol]) as a matrix, and the molecular ions were detected as [M+Na]⁺ in positive mode. Intens., intensity; a.u., arbitrary units.

ducted by the modified procedure of Hakomori (14, 15). The OSE was dissolved with a mixture of dimethyl sulfoxide and sodium hydroxide, followed by the addition of deuteromethyl iodide. After stirring at room temperature for 15 min, the reaction mixture was separated by a two-layer system of water and chloroform. The chloroform-containing perdeuteromethylated OSE layer was collected, washed with water two times, and evaporated completely. Partially deuteromethylated alditol acetate derivatives were prepared from perdeuteromethylated OSE by hydrolysis with 2 N trifluoroacetic acid at 120°C for 2 h, reduction with 10 mg/ml sodium borodeuteride at 25°C for 2 h, and acetylation with acetic anhydride at 100°C for 1 h (14, 19). GC/MS was performed using a benchtop ion trap mass spectrometer (GCMS-QP2010 Plus; Shimadzu Corp., Kyoto, Japan) equipped with a fused capillary column (SP-2380 and Equity-1; 30 m, 0.25-mm inner diameter [ID]; Supelco, Bellefonte, PA). Helium was used

FIG. 2. MALDI-TOF MS/MS spectra of serotype 13, 7, and 12 OSEs (A, B, and C, respectively). The fragment ions by each glycosyl cleavage were detected, and the assigned fragment patterns are illustrated. Arrowheads indicate the characteristic mass numbers of the serotype 13, 7, and 12 OSEs. The matrix was 10 mg/ml 2,5-dihydroxybenzoic acid in ethanol-water (3:7 [vol/vol]), and it was performed in the MS/MS mode. Intens., intensity; a.u., arbitrary units.

as the carrier gas, and the flow rate was 1 ml/min. The temperature program for alditol acetate derivatives was started at 60°C, increased 40°C/min to 220°C, and held for 15 min, followed by an increase of 10°C/min to 260°C and holding for 10 min. The molecular separator and ion source energies were 70 eV, and the accelerating voltage was 8 kV.

NMR of GPL. The OSE was dissolved in deuterium oxide. To define the anomeric configurations of each glycosyl residue, ¹H and ¹³C nuclear magnetic resonance (NMR) was employed. Both homonuclear correlation spectrometry (COSY) and ¹H-detected [¹H, ¹³C] heteronuclear multiple-quantum correlation (HMQC) were recorded with a Bruker AVANCE-600 (Bruker BioSpin Corp. Billerica, MA), as described previously (14, 18). Ten microliters of acetone was added to the sample, and its chemical shift values, 2.04 ppm (proton) and 29.8 ppm (carbon), were used as internal controls.

Sequencing of *orfA-orfB* region of *M. intracellulare* serotype 13. PCR was used to amplify the *orfA-orfB* region (30) of *M. intracellulare* serotype 13 (ATCC 35769 and ATCC 25122), using primers *orfA*-F (5'-GCGGATCCAGTGTGCAGACG AGCGGAAC-3'), *orfA*-R (5'-GCGAATTCTTATCGAGAAAAATAAAAG-3'), *orfB*-F (5'-GCGGATCCACTGCTAGACT CCGCCACCAT-3'), and *orfB*-R (5'-GCGAATTCCTACACCTTCACGGCGAGTC-3'). The amplified fragment was sequenced using a BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA), and a sequence analyzer (ABI3130xl; Applied Biosystems).

Transformation of *M. intracellulare* serotype 13 strain with serotype 12 *orfB*. The *orfB* fragments from serotype 12 (sero12-*orfB*) and serotype 13 (sero13-*orfB*) strains were amplified and cloned into pVV16, an expression plasmid vector for mycobacteria, downstream of the *hsp60* promoter. *M. intracellulare* serotype 13 ATCC 35769 was transformed with pVV16-sero12-*orfB* and pVV16-sero13-*orfB* by electroporation, and hygromycin- and kanamycin-resistant colonies were isolated. Alkaline-stable lipids were prepared from heat-killed bacteria, and productive GPLs were identified by TLC, MALDI-TOF MS, MALDI-TOF MS/MS, and GC/MS.

Host recognition of native and alkaline-treated serotype 13 GPLs. The host recognition of GPLs was estimated by activations of HEK-blue-2 and -4 cells (InvivoGen, San Diego, CA). HEK-blue-2 and -4 cells are HEK293 cells stably transfected with multiple genes for recognition of Toll-like receptor 2 (TLR2) and TLR4 (including the coreceptors MD2 and CD14). In addition, HEK-blue-2 and -4 cells stably express an optimized alkaline phosphatase gene engineered to

be secreted (sAP) and placed under the control of a promoter inducible by several transcription factors, such as NF-κB and alkaline phosphatase-1. HEK-blue-2 and -4 cells were seeded at a concentration of 2×10^5 cells/ml in 96-well flat-bottom tissue culture plates and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂ for 3 days. The adherent HEK-blue-2 and -4 cells were stimulated by native and alkaline-treated serotype 13 GPLs. After 24 h of incubation, NF-κB activation was assayed by the levels of sAP in the supernatant. The sAP was measured in duplicate using QUANTI-Blue (InvivoGen) according to the manufacturer's instructions. As positive controls, we used lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO) for TLR4 and Pam3CSK4 (InvivoGen) for TLR2. Two independent experiments were performed.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the NCBI GenBank database under accession number AB557690.

RESULTS

Purification and molecular weight of intact GPL. The serotype 13 GPLs from *M. intracellulare* ATCC 35769 and 25122 were detected as spots on TLC plates and showed the same *R_f* value (Fig. 1A). Because serotype 13 GPL was predicted to be very close structurally to the serotype 7 and 12 GPLs, the *R_f* values were compared on TLC plates developed with two different chloroform-methanol-water solvent systems (65:25:4 and 60:16:2 [vol/vol/vol]), respectively. Interestingly, the *R_f* value of the serotype 13 GPL was lower than that of the serotype 12 GPL and almost the same as that of the serotype 7 GPL in both developing systems (Fig. 1B). The intact molecular weight of each GPL was determined. The MALDI-TOF MS spectrum of the serotype 13 GPL showed *m/z* 1,897 for [M+Na]⁺ as the main molecular ion in positive mode (Fig.

1C). This mass number is identical to that of the serotype 7 GPL ($[M+Na]^+$: 1,897) and 14 atomic mass units lower than that of the serotype 12 GPL ($[M+Na]^+$: 1,911).

Glycosyl sequence of serotype 13 OSE. To determine the glycosyl sequence of the OSE, MALDI-TOF MS/MS of the oligoglycosyl alditol from serotype 13 OSE was performed. The spectrum afforded the molecular ion $[M+Na]^+$ at m/z 859, together with the characteristic mass increments in the series of glycosyloxonium ions formed on fragmentation at m/z 240, 400, 546, and 692 from the *N*-acylated Hex to 6-d-Tal, and at m/z 190, 336, 482, and 642 from 6-d-Tal to *N*-acylated Hex (Fig. 2A). In comparison, the fragment patterns of the cleaved terminal *N*-acylated Hex of the OSEs were m/z 254 and 628 in serotype 7 and m/z 254 and 642 in serotype 12, and those next to the terminal Hex were m/z 400 and 482 in serotype 7 and m/z 414 and 482 in serotype 12 (Fig. 2B and C). Together with the intact molecular weight of each GPL (Fig. 1B), these results strongly implied that serotype 13 GPL has no *O*-methyl group in the terminal *N*-acylated Hex but does have an *O*-methyl group added to the Rha next to the terminal Hex.

Carbohydrate composition and linkage analyses. GC/MS analysis of the perdeuteromethylated alditol acetate derivative from serotype 13 OSE was performed to determine the glycosyl composition. The total ion chromatography (TIC) of the GC/MS spectrum of serotype 13 GPL derivatives was compared to those of serotype 7 and 12 GPL derivatives (Fig. 3A). Previous reports showed that the carbohydrate compositions of the serotype 7 GPL were 6-d-Tal, Rha, and 4-2'-hydroxypropanoyl-amido-3,6-dideoxy-2-*O*-Me-Hex, and those of the serotype 12 GPL were 6-d-Tal, Rha, 4-*O*-Me-Rha, and 4-2'-hydroxypropanoyl-amido-3,6-dideoxy-3-*O*-Me-Hex (5, 13). Comparison of the retention times and mass spectra of GC/MS determined that serotype 13 GPL was composed of 6-d-Tal, Rha, 4-*O*-Me-Rha, and another terminal *N*-acylated Hex. As shown in Fig. 3B, the perdeuteromethylated alditol acetate derivative of the terminal *N*-acylated Hex was assigned to 2,3-di-*O*-deuteromethyl-1,5-di-*O*-acetyl-4-2'-*O*-deuteromethylpropanoyl-deuteromethylamido-4,6-dideoxy-hexitol from the fragment pattern (m/z 62, 108, 121, 168, 209, 222, 269, and 303). These results confirmed that the *O*-methyl group was deleted from the terminal *N*-acylated Hex and added to the C-4 position at Rha next to the terminal Hex. Taken together, these results established the sequence and linkage arrangement of 4-2'-hydroxypropanoyl-amido-4,6-dideoxy-Hex-(1→3)-4-*O*-Me-Rha-(1→3)-L-Rha-(1→3)-L-Rha-(1→2)-6-d-Tal exclusively.

NMR analysis of serotype 13 OSE. The 1H NMR and 1H - 1H homonuclear COSY analyses of the OSE derived from the serotype 13 GPL revealed four distinct anomeric protons with corresponding H1-H2 cross-peaks in the low-field region at δ 4.88, 4.71, 4.97 ($J_{1-2} = 1$ to 2 Hz, indicative of α -anomers), and 4.52 (a doublet, $J_{1-2} = 7.9$ Hz, indicative of a β -hexosyl unit). When further analyzed by 1H -detected [1H - ^{13}C] two-dimensional HMQC, the anomeric protons resonating at δ 4.88, 4.71, 4.97, and 4.52 had C-1s resonating at δ 102.10, 93.50, 94.00, and 103.40, respectively. The J_{CH} values for each of these protons were calculated to be 170, 170, 171, and 161 Hz by measurement of the inverse-detection nondecoupled two-dimensional HMQC (see Fig. S1 and Table S1 in the supplemental material). It was concluded that two Rha and

FIG. 3. Assignment of glycosyl composition of OSEs in serotype 13 GPL. (A) Total ion chromatogram of the alditol acetate derivatives from serotype 13 compared to those of serotype 7 and 12 GPLs. A fused SP-2380 capillary column was used as the GC column. The temperature program for alditol acetate derivatives was started at 60°C, increased to 40°C/min to 220°C, and held for 15 min, followed by an increase of 10°C/min to 260°C and holding for 10 min. (B) GC/MS spectrum of the perdeuteromethylated alditol acetate derivative from the terminal Hex in serotype 13 GPL. The pattern of prominent fragment ions is illustrated. A fused Equity-1 capillary column was used as the GC column. Ac, CH_3CO .

4-*O*-Me Rha were α -anomers and that the terminal *N*-acylated Hex was a β configuration.

Nucleotide sequence of *orfA*-*orfB* region of *M. intracellulare* serotype 13. The present study demonstrated that the difference between the chemical structures of the serotype 13 GPL and serotype 7 and 12 GPLs was whether the *O*-methyl group in the terminal *N*-acylated Hex and the next Rha were present or not. We confirmed the genetic basis of these *O*-methylations. Our previous study clarified three unique open reading frames (ORFs) for methyltransferase, named *orf2*, derived from *M. intracellulare* serotype 7, and *orfA* and *orfB*, from *M. intracellulare* serotype 12 (13, 30). *orfA* and *orfB* in *M. intracellulare* serotype 12 are responsible for 4-*O*-methylation of the Rha next to the terminal Hex and 3-*O*-methylation of the

FIG. 4. Detection of *orfA-orfB* regions and comparison of genetic maps of GPL biosynthetic cluster. (A) PCR was performed to amplify the *orfA-orfB* regions of *M. intracellulare* serotype 7, 12, and 13 strains. The primers and the amplified regions are indicated. (B) *M. intracellulare* serotype 7 ATCC 35847 and serotype 12 ATCC 35762 were sequenced in our previous work (13, 30). *M. intracellulare* serotype 13 ATCC 35769 and ATCC 25122 were sequenced in this study. The missense mutation of *orfA-orfB* regions is indicated.

terminal Hex, respectively. Therefore, we examined whether or not *M. intracellulare* serotype 13 has these ORFs. First, comparison of the *gtfB-drrC* gene cluster in *M. intracellulare* serotype 7 and 12 strains implied that *orf2* in *M. intracellulare* serotype 7 replaced *orfA-orfB* in *M. intracellulare* serotype 12. We amplified the *orfA-orfB* in the genomic DNA from *M. intracellulare* serotypes 7, 12, and 13 (Fig. 4A). Interestingly, *M. intracellulare* serotype 13 had the same-sized DNA fragment of the *orfA-orfB* region, and the nucleotide sequences were determined. The 1.95-kb *orfA-orfB* regions of the two serotype 13 strains had complete identity and showed only one nucleotide substitution from that of serotype 12: codon 105, TTC, of the *orfB* in serotype 12 was replaced by codon TCC in serotype 13 (Fig. 4B). This missense mutation induced a single amino acid substitution from Phe to Ser and implied the loss of the *orfB* activity for *O*-methylation.

Expression of sero12-*orfB* and sero13-*orfB* in *M. intracellulare* serotype 13. To test the functional activity of *orfB* in *M. intracellulare* serotypes 12 and 13, the sero12-*orfB* and sero13-*orfB* genes were introduced into the *M. intracellulare* serotype 13 strain. The 0.84-kb sero12-*orfB* and sero13-*orfB* were amplified and cloned into a pVV16 vector, and *M. intracellulare* serotype 13 ATCC 35769 was transformed with the resulting plasmids and the pVV16 vector. The alkaline-stable lipids derived from the transformants were developed on TLC plates, and the productive GPLs were compared to the spots of serotype 7, 12, and 13 GPLs (Fig. 5A). Both R_f values of the GPLs produced in the transformants with the pVV16 vector and sero13-*orfB* were identical to that of the serotype 13 GPL. However, the R_f value of the GPL produced in the transformant with sero12-*orfB* was the same as that of serotype 12 GPL. By MALDI-TOF MS, the main molecular weights of the

GPLs produced in the transformants with sero12-*orfB*, sero13-*orfB*, and the pVV16 vector were detected as m/z 1,911, 1,897, and 1,897, respectively, for $[M+Na]^+$ (data not shown). The fragment ions of the related glycosyl cleavage in the OSEs were analyzed by using MALDI-TOF MS/MS, and the glycosyl compositions were determined. The fragment ions of the OSEs in the pVV16 vector and sero13-*orfB* showed the same pattern as serotype 13 GPL, indicating that overexpression of sero13-*orfB* in the serotype 13 strain was not affected (Fig. 2A and 5B). The fragment ions of the OSE in sero12-*orfB*, i.e., m/z 254 and 414, were different from those of the OSE in sero13-*orfB*, i.e., m/z 240 and 400, respectively (Fig. 5B). The GC/MS spectrum of the perdeuteromethylated alditol acetate derivative of the terminal *N*-acylated Hex from sero12-*orfB* was assigned to 2-*O*-deuteromethyl-1,5-di-*O*-acetyl-4-2'-*O*-deuteromethyl-propanoyl-deuteromethylamido-4,6-dideoxy-3-*O*-methyl-hexitol from the fragment pattern (m/z 62, 105, 121, 165, 206, 222, 266, and 300) (Fig. 5C), which was identical to that of the serotype 12 GPL. These results demonstrated that the serotype 13 transformant with sero12-*orfB* but not sero13-*orfB* had an added *O*-methyl group at the C-3 position in the terminal Hex and that the productive GPL was completely changed from serotype 13 to serotype 12. In addition, we confirmed that the plasmid-deleted C-terminal 40-base region of sero12-*orfB* was completely functional and that sero12-*orfB* worked in the serotype 7 transformant. Taken together, these results indicated that sero13-*orfB* was inactivated by the missense mutation at codon 105 and that the serotype 13 GPL lacked *O*-methylation at the C-3 position of the terminal Hex.

Native conformation of serotype 13 GPL and host response. The native serotype 13 GPL was purified without alkaline treatment. The native serotype 13 GPLs were detected on TLC

FIG. 5. The productive GPLs in transformants of *M. intracellulare* serotype 13 with sero12-*orfB* or sero13-*orfB*. (A) TLC patterns of the alkaline-stable lipids derived from *M. intracellulare* serotypes 7, 12, and 13 and serotype 13 transformants (ATCC 35769) with the pVV16-vector, sero12-*orfB*, and sero13-*orfB* from left to right, developing with a solvent system of chloroform-methanol-water (60:16:2 [vol/vol/vol]). (B) MALDI-TOF MS/MS spectra of OSEs derived from the productive GPLs in transformants of *M. intracellulare* serotype 13 with sero12-*orfB*, sero13-*orfB*, and the pVV16 vector. The replaced mass numbers are indicated by arrowheads. (C) GC/MS spectrum of the perdeuteromethylated alditol acetate derivative of the terminal *N*-acylated Hex from serotype 13 transformant with sero12-*orfB*. The MALDI TOF MS/MS and GC/MS conditions are described in the legends for Fig. 2 and 3. Ac, CH₃CO.

plates as three major spots that expanded broadly and had R_f values different from that of the alkaline-treated serotype 13 GPL. These spots were converged into one spot by alkaline treatment (Fig. 6A). It was reported that some positions of OSE in GPLs are acetylated in nature (27). The molecular weights of these three spots were checked by MALDI-TOF MS. The mass numbers of m/z 1,983, 2,025, and 2,067 for $[M+Na]^+$ caused the 2- to 4-unit increases of m/z 42 (addition of acetylations) and the modification to saturated alkyl group, compared to m/z 1,897 of the alkaline-treated serotype 13 GPL, implying that native GPLs were modified by several *O*-acetylations in the OSE portion and that alkaline treatment removed the acetylated groups (Fig. 6B). In addition, several peaks at intervals of 14 atomic mass units were caused by an alkyl group, indicating that the fatty acids of the core portion were variable and that the molecular species were heterogeneous.

To clarify the host recognitions of serotype 13 GPL via TLRs, we stimulated HEK-blue-2 and -4 cells with native and alkaline-treated serotype 13 GPLs. The native serotype 13 GPL significantly activated HEK-blue-2 cells in a dose-dependent manner, but HEK-blue-4 cells did not respond. The alkaline-treated serotype 13 GPL without *O*-acetylation did not activate either HEK-blue-2 or -4 cells. Reacetylated alkaline-treated serotype 13 GPLs with *O*-acetyl groups substituted for all hydroxy groups of OSE activated HEK-blue-2 cells, although the level of activation was less than that of the native form (Fig. 6C). Moreover, we confirmed that only the native

serotype 13 GPL stimulated mouse bone marrow-derived macrophages via TLR2 by using C57BL/6 and TLR2 knockout mice (see Fig. S2 in the supplemental material).

DISCUSSION

The structural heterogeneity of the GPLs in MAIC species is reflected in their morphology, virulence, and pathogenicity (2, 3, 24) and may be meaningful in phylogenetic classification. Actually, epidemiological studies show that the isolates of MAIC serotypes from patients are heterogeneous and important for assessing the prognosis of pulmonary MAIC disease (25, 37). Chatterjee and Khoo (9) proposed grouping the three types of GPLs by OSE structure, and the group 2 GPLs included the serotype 12, 17, and 19 strains. The serotype 7 and 16 GPLs determined in our previous studies also belong to the group 2 GPLs (13, 14). The group 2 GPLs have in common 6-d-Tal-Rha-Rha and serotype-individual sugars elongated from the second Rha. In addition, except for the serotype 19 GPL, group 2 GPLs carry an unusual substituent, *N*-acylated amido sugar, as the terminal Hex. Aspinall et al. (1) mentioned that the terminal sugar residue of serotype 12 GPL is a derivative of viosamine (4-amino-4,6-dideoxyglucose). The structural difference between serotype 7 and 12 GPLs in group 2 was due to the functions of three methyltransferase genes, *orf2*, *orfA*, and *orfB* (13, 30). In this study, we found that the serotype 13 GPL was structurally very close to those of serotypes 7 and 12, and we determined the novel structure of

FIG. 6. TLC patterns, MALDI-TOF MS spectra, and TLR recognition of the native and alkaline-treated serotype 13 GPL. (A) The TLC plate was developed with a solvent system of chloroform-methanol-water (60:16:2 [vol/vol/vol]). Three major spots of native GPL are indicated by the numbers from top to bottom. (B) The major spots were purified and their molecular ions were measured by MALDI-TOF MS. The condition is described in the legend for Fig. 1. (C) HEK-blue-2 and -4 cells (2×10^5 cells/ml) were stimulated with native, alkaline-treated, and re-acetylated serotype 13 GPLs. After 24 h of incubation, NF- κ B activation was assessed by measuring the levels of secreted alkaline phosphatase (sAP) in the supernatant by using QUANTI-Blue. The data are means $\pm$ standard deviations (SD) for two experiments done in duplicate.

the serotype 13 GPL to be 4-2'-hydroxypropanoyl-amido-4,6-dideoxy- β -hexose-(1 $\rightarrow$ 3)-4-*O*-methyl- α -L-rhamnose-(1 $\rightarrow$ 3)- α -L-rhamnose-(1 $\rightarrow$ 3)- α -L-rhamnose-(1 $\rightarrow$ 2)- α -L-6-deoxy-talose. This result clarified that the serotype 13 GPL is structurally different from the serotype 7 and 12 GPLs in the *O*-methylation of the terminal *N*-acylated Hex and Rha next to the terminal Hex. Serotype 13 GPL lacked the *O*-methyl group in the terminal *N*-acylated Hex, although serotype 7 and 12 GPLs had one at the C-2 and C-3 positions, respectively. The composition and position of the *N*-acyl group at the terminal Hex were completely identical in these three GPLs. At the Rha next to the terminal Hex, serotype 12 and 13 GPLs have an *O*-methyl group at the C-4 position, and this modification is present in all group 2 GPLs except for the serotype 7 GPL, suggesting that this methyl group may play a role in MAIC physiology and virulence. These results also implied that *M.*

intracellulare serotypes 7, 12, and 13 are very close phylogenetically.

We investigated the relationship between the structure and biosynthetic pathway and tried to verify the phylogenetic classification of serotypes 7, 12, and 13 by genetic analysis of GPL biosynthesis. We previously reported the nucleotide sequences of the *gtfB-drrC* region, which completely determine each serotype-specific GPL in serotypes 7 and 12 (13, 30), and found the sequence of the serotype 13 gene cluster (unpublished data). The genetic organizations of the *gtfB-drrC* regions in serotype 7, 12, and 13 gene clusters closely resemble each other. Seven common ORFs are conserved in *gtfB-drrC* clusters, suggesting that these three serotypes diverged from a common ancestor. The *orfA-orfB* region in serotypes 12 and 13 replaced *orf2* in serotype 7. Only one nucleotide substitution was found in the 1.95-kb segment in *orfA-orfB* of serotypes 12

FIG. 7. Scheme of the relationship between GPL biosynthesis ORFs encoded the methyltransferases and their structures.

and 13, and *orfB* in serotype 13 was inactivated. In general, it is unusual for an ORF inactivated by a missense mutation to remain in the genome because it is a burden for the bacterium to transcribe and translate an inactivated ORF. Thus, the *M. intracellulare* serotype 13 strain must have diverged from an *M. intracellulare* serotype 12 organism recently. Serotype 13 GPL also has 4-*O*-Me-Rha. *orfA* is responsible for this methylation. In previous studies, we demonstrated that the *orfB* activity had incapacitated the *orf2* activity, which synthesizes an *O*-methyl group at the C-2 position of the terminal Hex of *M. intracellulare* serotype 7. We also showed that the *orf2* activity was independent of *orfA* activity in *M. intracellulare* serotype 12 (30). The relation of methyltransferases, *orfA*, *orfB*, and *orf2* is summarized in Fig. 7.

GPLs are correlated with colony morphology, sliding motility, biofilm formation, immune modulation, and virulence (2, 3, 16, 34). GPLs have several significant features. They are produced in MAIC species and absent from *Mycobacterium tuberculosis*, making it possible to distinguish MAIC from tuberculous mycobacteria (11, 20). An anti-GPL antibody is produced in the sera of patients and reflects the disease, which is useful in diagnosis and treatment (21, 22, 25). Moreover, it was reported that ethambutol-susceptible and -resistant MAIC strains of serotype 1 had different GPL profiles. The susceptible strain expressed only the polar serotype 1 GPL, and the resistant strain expressed several apolar GPLs. The efficacy of antibiotics may be affected by the GPL profile through differences in cell wall permeability (19). On the other hand, the importance of TLR-mediated responses has been studied in tuberculous infections. Means et al. (28) reported that *M. tuberculosis* activated both TLR2 and TLR4, whereas heat-killed *M. tuberculosis* and *M. avium* activated only TLR2. It was observed that MyD88- and TLR2-deficient mice have increased susceptibility to *M. avium* infection compared to TLR4-deficient and wild-type mice (12). These lines of evidence suggest that TLRs are related to host recognition of the MAIC components containing GPLs and affect MAIC infections. Brennan and Goren (6) first proposed that GPLs were alkaline-stable lipids and made it possible to classify serospecificity by the unique, variable deacetylated OSE sequences (9). We did not detect any biological activity of these alkaline-

treated GPLs on splenocytes and bone marrow macrophages of mice in *in vitro* stimulation. Recently, Schorey and colleagues (35, 36) clarified that serotype 1 and 2 GPLs can function as TLR2 agonists and promote macrophage activation in a TLR2- and MyD88-dependent pathway. They reported that the acetylated and methylated groups of GPLs were necessary for GPL-TLR2 interaction as a molecular requirement. In this study, we purified both native and alkaline-treated serotype 13 GPLs and clarified the acetylation patterns of serotype 13 GPL. It was confirmed that the native acetylated form of serotype 13 GPL was recognized via TLR2 and that the deacetylated form by alkaline treatment was not recognized. The serotype 13 GPL has one *O*-methyl group next to the terminal *N*-acylated Hex that was stable regardless of alkaline treatment. Taken together, an acetyl rather than a methyl group was necessary for host immune response via TLR2. The completely acetylated derivative of alkaline-treated serotype 13 GPL partially recovered the HEK-blue-2 activation, compared to the native form containing 2 to 4 acetylated groups. It may be important for GPL-TLR2 interaction to balance the hydrophobicity and hydrophilicity of the molecule. Recht and Kolter (32) reported that the acetylation of GPL affects sliding motility and biofilm formation by deleting the *atf1* gene, which is responsible for acetylation on the 6-d-Tal of GPL core in *Mycobacterium smegmatis*. Rhoades et al. (33) reported that the *Mycobacterium abscessus* GPLs were related to smooth and rough colony morphology and that the GPLs in the outermost portion of the cell wall masked underlying phosphatidyl-*myo*-inositol mannosides involved in stimulating the innate immune response via TLR2. In contrast, our results suggest that the species-specific acetylated GPL is effective in host recognition as a TLR2 agonist independent of phosphatidyl-*myo*-inositol mannosides and that it plays important roles directly in host innate immune responses. Regulating the acetylation of GPL may control the MAIC pathogenicity by, for example, developing the inhibitor of ATF1.

The present study demonstrated the chemical structure and biosynthesis gene cluster of the serotype 13 GPL of *M. intracellulare* and host innate immune response via TLR2. Serotype 13 GPL should be included in group 2 GPLs, and the phylogenetic relationship of serotype 7, 12, and 13 strains was par-

tially clarified by the GPL. We propose that the lipid components in the cell envelope are important for MAIC infection and that the structure modification must be taken into account. These findings shed light on the better understanding of the structure-function relationships of GPLs and may open a new avenue for the prevention of MAIC infections.

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