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Distribution, Characterization of Mycobacterial Glycolipids and Host Responses

Nagatoshi Fujiwara

Additional information is available at the end of the chapter

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1. Introduction

Robert Koch discovered the acid-fast bacterium that is the pathogenic germ of tuberculosis (TB) in 1882 [1]. TB is a major public health problem in the world and the leading cause of death from a single infectious agent. The World Health Organization estimates that one third of the world's population is infected with *Mycobacterium tuberculosis*, and annually reports the global burden of the disease caused by TB. Over 8 million new cases and nearly 1.5 million deaths from TB occur each year, presenting a significant threat to the world health [2]. The pathogenicity of mycobacteria is related to their ability to evade being ingested by macrophages, produce latent infection, and induce delayed-type hypersensitivity lesions such as granulomas. Moreover, the global spread of multi- and extensively drug-resistant TB (MDR-TB and XDR-TB, respectively) and the number of immunocompromised hosts, including victims of the human immunodeficiency virus (HIV) epidemic, are important problems [3].

The mycobacteria include the TB-causative acid-fast bacteria that are widely pathogenic to humans: *M. tuberculosis*, *M. avium-intracellulare* complex (MAC), *M. leprae*, and *M. bovis*, the source of the only available TB vaccine, Bacillus Calmette Guérin (BCG). The acid-fast bacteria are rich in lipids, and many mycoloyl glycolipids, such as cord factor/trehalose-6,6'-dimycolate (TDM), phenolic glycolipid (PGL), sulfolipid (SL), glycopeptidolipid (GPL), phosphatidylinositol mannoside (PIM), lipomannan (LM), and lipoarabinomannan (LAM), are distributed in the cell wall [4-7]. Among them, glycolipids specific to the mycobacteria play a key role in the pathogenesis, because mycobacteria have large amounts of lipids that possess pleiotropic activities. The complex interaction between a range of mycobacterial components and the host cause the pathogenesis.

In this review, the distribution of major glycolipids in several *Mycobacteria* and structural analyses using mass spectrometry (MS) are described. MS and MS/MS are useful to analyze

the glycosyl linkage, composition, and sequence of the sugar moiety. These results make it possible to discuss the heterogeneity and biosynthesis of glycolipids, and host responses to them. We hope that this review will promote better understanding of the structure-function relationships of glycolipids and open new avenues for prevention of infectious diseases.

2. Distribution of glycolipids and phospholipids in mycobacteria

The cell envelope surrounds the cytoplasm and is important for bacterial physiology and protection of microorganisms from their environment. Unlike other pathogenic microbes, the cell envelope of the acid-fast bacteria, including mycobacteria, is wax-like. The characteristic component is mycolic acids (MAs) which are α -branched β -hydroxy fatty acids (FAs), and have species-specific carbon-chain lengths and subclasses (α , methoxy, keto, dicarboxy, epoxy, etc.). The total lipid fraction was extracted with chloroform/methanol (3:1 and 2:1 v/v) and developed by two-dimensional thin-layer chromatography (TLC). Many glycolipids and phospholipids were detected, as shown in Fig. 1. Some glycolipids, such as cord factor/TDM and trehalose 6-monomycolate (TMM), exist ubiquitously in mycobacteria, and other glycolipids, including SLs and penta-acyl trehalose, exist only in virulent strains [8]. In general, the mycobacterial species have heterogeneous compositions and concentrations of glycolipids. According to current models of the mycobacterial cell envelope, which are ultimately based on an idea of Minnikin [9], arabinogalactan-attached MAs are believed to form one highly-arranged leaflet of a second membrane-like structure adjacent to the cell-wall skeleton (CWS). Complemented by other lipids forming the outer leaflet, this structure represents a very hydrophobic barrier that is responsible for the resistance to certain drugs [10]. The free glycoconjugates of MA, in particular TMM and cord factor/TDM, appear to be more difficult to localize, and occasionally they are described or depicted as distributed on the cell surface or buried more deeply in the cell envelope. A proposed structure of the mycobacterial cell envelope is shown in Fig. 2 [4, 11, 12].

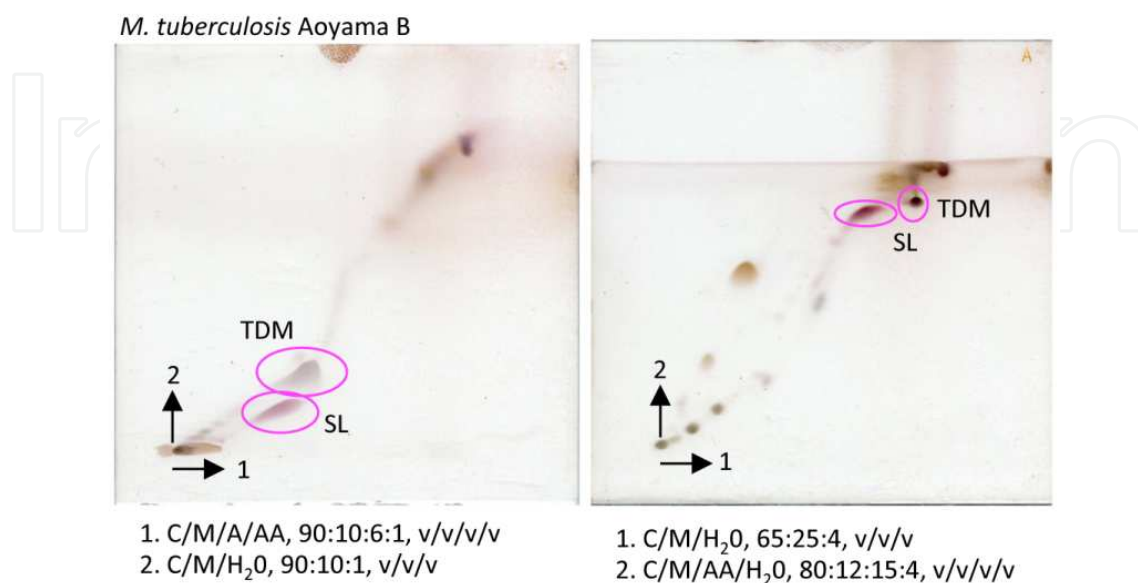


Figure 1. TLC of total lipids derived from *Mycobacterium tuberculosis* Aoyama B strain.

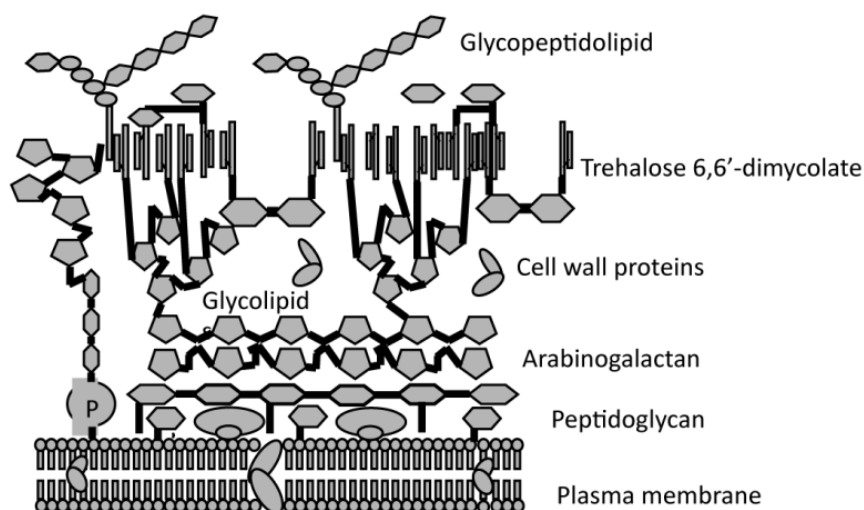


Figure 2. Schematic representation of mycobacterial cell envelope.

3. Host responses to mycobacterial glycolipids

Mycobacteria are intracellular pathogens. Mycobacterial infection has dual consequences for the host: development of inflammatory lesions and clearance of the pathogen. Genetic regulation and expression of cell-mediated immunity and delayed-type hypersensitivity play critical roles in the outcome. Cell-mediated immunity participates in host defense, whereas delayed-type hypersensitivity is involved in the development of granulomatous inflammation. After invasion to the host cell, the mycobacteria multiply inside macrophages. The mechanism of pathogenesis is focused on evading the host's killing system and induction of delayed-type hypersensitivity [13]. Certain cell wall glycolipids, such as cord factor/TDM, SL, and LAM [5-7], are involved in the host-pathogen interaction, and induce the primary host immune responses.

Peptides and proteins with a wide range of antigenic moieties are recognized by the host immune system using major histocompatibility complex (MHC) class I or II. The recognition of lipids is important for host defense against mycobacterial infection as well as other antigenic responses. Several bacterial lipid antigens are recognized by specific T cells. MA derived from mycobacteria is a lipid antigen that stimulates CD1b-restricted T cells [14, 15]. Other immunogenic mycobacterial glycolipids, glucose-6-monomycolate (GMM), PIM, and LAM, are available for loading onto CD1 molecules and recognized by specific T cells [16, 17].

4. Mycoloyl glycolipids

Acid-fast bacteria produce several mycoloyl glycolipids that are composed of sugar and MA. The carbon-chain lengths and subclasses of MAs are species-specific. *Nocardia*, *Rhodococcus*, and *Diezia* species have short carbon-chain length MAs, and the subclass is mainly the α type. MAs of *Mycobacterium* species have long carbon-chains and are of various subclasses (α , methoxy, keto, dicarboxy, and epoxy types). In general, the average number of carbons in MAs increases from *Dietzia*, *Rhodococcus*, *Nocardia*, and *Gordona* to *Mycobacteria*. The MAs

are extracted with *n*-hexane or chloroform after alkaline-hydrolysis of heat-killed bacteria, and the subclasses are detected by using TLC developed with methyl ester derivatives (Fig. 3). The molecular species of the MA subclasses are determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using an Ultraflex II (Bruker Daltonics, Billerica, MA, USA) with 10 mg/ml 2,5-dihydroxybenzoic acid (DHB) in chloroform-methanol (1:1, v/v) as a matrix, and analyzed in Reflectron mode with an accelerating voltage operating in positive mode at 20 kV (Fig. 4). The MAs with short carbon-chains can be analyzed by using gas-chromatography mass spectrometry GC/MS. The trimethylsilyl derivatives of MA methyl esters are volatile, and the GC/MS spectra show specific fragment patterns that are assigned to detailed structures (Fig. 5), although the MAs with long carbon chains are difficult to vaporize and detect by GC/MS [18-20]. In addition, MAs are analyzed by using high performance liquid chromatography (HPLC) [21]. The resolution of HPLC is poor compared to that of GC/MS. GMM, fructose-6-monomycolate (FMM), and mannose-6-monomycolate (MMM) are produced by acid-fast bacteria cultured with their respective carbon source: glucose, fructose, and mannose. The structures are composed of MAs attached to glucose, fructose, and mannose, respectively, at the C6 position [20, 22]. Recently, it was reported that *M. tuberculosis* produces GMM by transferring MA to glucose from cord factor/TDM, and thus evades the host immune system [23]. The molecular weights of mycoloyl glycolipids are measured by MALDI-TOF MS, and this information makes it possible to determine the combination of MA subclasses [24]. Cord factor/TDM is probably the most prominent and best-studied MA-containing compound of mycobacteria, and shows pleiotropic activities, including the development of granulomatous inflammation, anti-tumor immune response, and adjuvant effects based on the induction of proinflammatory and type 1 helper T cell (Th1)-related cytokines from host cells [25-27]. We demonstrated that administration of cord factor/TDM could induce delayed-type hypersensitivity-like lesions and foreign-body granulomas in euthymic and athymic mice, regardless of preimmunization with mycobacteria and cord factor/TDM. In fact, preimmunized mice challenged with cord factor/TDM developed more severe lesions than unimmunized mice. At the active lesion, CC-chemokines attracting monocytes, proinflammatory cytokines, and immunoregulatory cytokines were found. The inflammatory and cytokine responses were augmented in immunized mice challenged with cord factor/TDM. As a result, cord factor/TDM can induce both foreign body-type (nonimmune) and hypersensitivity-type (immune) granulomas by acting as a nonspecific irritant and T-cell-dependent antigen, and both nonimmune and immune mechanisms participate in granulomatous inflammation induced by mycobacterial infection [13, 28]. Moreover, rhodococcal cord factor/TDM induced milder granulomatous lesions than a mycobacterial one, implying that the proinflammatory responses induced by cord factor/TDM are in proportion to the carbon-chain length and subclasses of MAs in addition to carbon species [20, 29]. Recently, it was clarified that macrophage-inducible C-type lectin (mincle) is an essential receptor for cord factor/TDM [30]. Mincle is a receptor for sugar, and our previous result showed that the proinflammatory responses are MA-dependent. I expect the discovery of other unknown receptors for cord factor/TDM in addition to mincle.

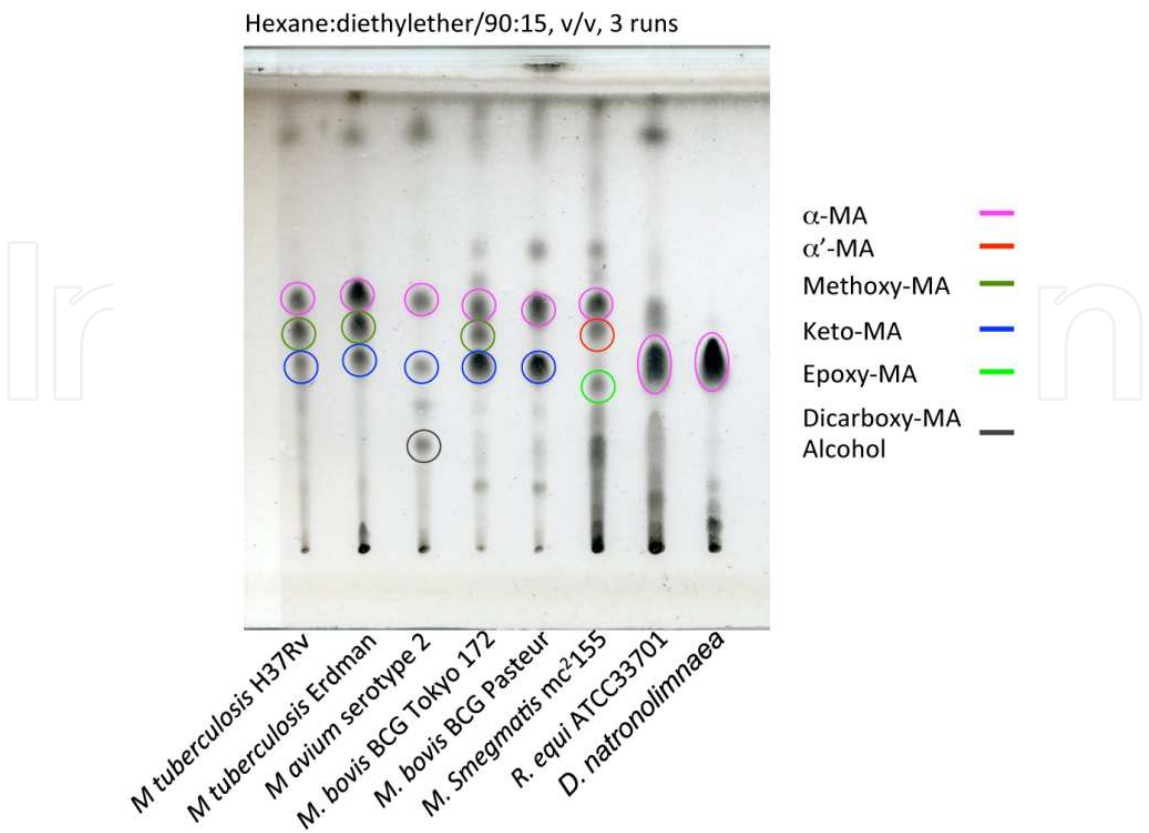


Figure 3. TLC of mycolic acid methyl esters derived from *Mycobacterium*, *Rhodococcus*, and *Dietzia* species.

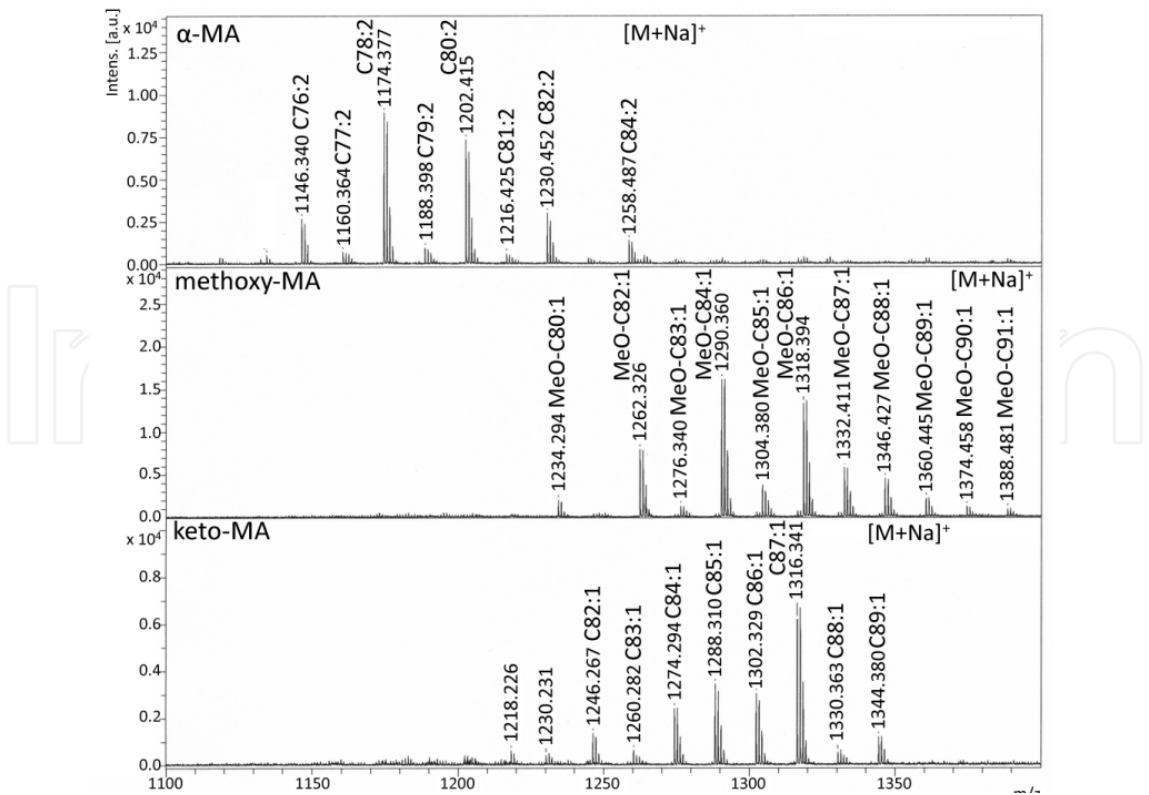


Figure 4. MALDI-TOF MS spectra of mycolic acid methyl ester subclasses from *M. tuberculosis* H37Rv.

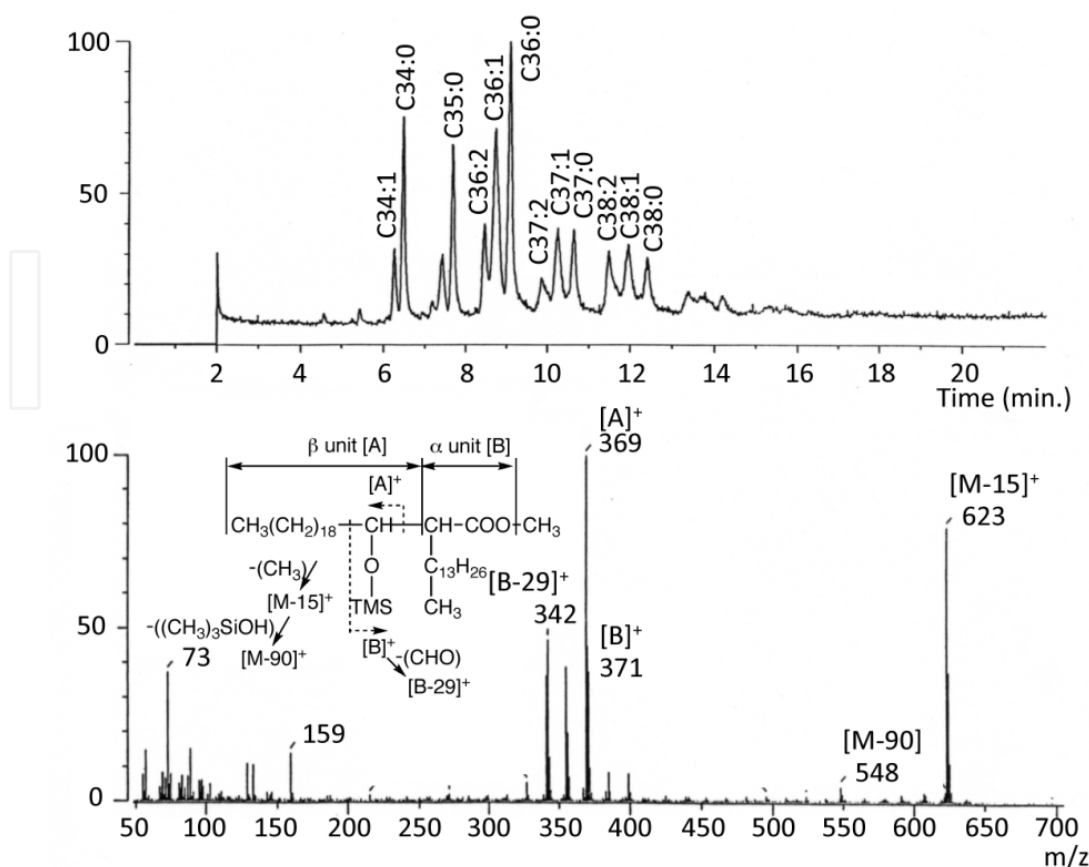


Figure 5. Total ion chromatogram and mass spectrum of mycolic acid trimethylsilyl derivatives from *R. equi*.

5. Glycopeptidolipid (GPL)

GPLs are produced by MAC, *M. scrofulaceum*, *M. chelonae*, *M. fortuitum*, and *M. smegmatis*. Structurally, a 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 FA at the N-terminal of D-Phe; 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 [31]. This portion is common to all serotypes, and is called the serotype-nonspecific GPL (apolar GPL), which exhibits antigenicity [11]. Serotype-specific GPLs (polar GPLs) are further glycosylated with a variable haptenic OSE at 6-d-Tal. We determined the structures of the serotype 7, 13, and 16 GPLs and identified the gene clusters completing the OSE biosynthesis [32-34]. In addition, two methyltransferase genes of serotype 7- and 12-specific GPL biosynthesis were characterized [35]. The standard technique to classify MAC strains has employed serologic typing based on the OSE residue of the GPL. Recently, the biosynthetic pathway of various haptenic OSEs has been explored, and the genes encoding the pathways have been identified and characterized [36-38]. At present, 31 distinct serotype-specific polar

GPLs have been identified biochemically, and the complete structures of 17 GPLs are defined [12, 33]. The GPL present on the cell wall is considered to affect colony morphology. The MAC colony phenotype spontaneously changed from a smooth to a rough type, and this was due to a mutation lacking GPLs [39, 40]. The polar GPLs produced by MAC species are of particular interest because they are considered to be correlated with the physiology of the bacteria and the host responses to MAC infection, for example, colony morphology, sliding motility, biofilm formation, immune modulation, and virulence [40-43].

We have demonstrated the applicability of serodiagnosis of MAC pulmonary diseases using the GPL and GPL core antigens, and have also shown that the levels of GPL and GPL core antibodies reflect disease activity [44-46]. The GPLs are produced by MAC species but are absent in *M. tuberculosis*, making it possible to distinguish MAC from tuberculous mycobacteria [44, 47]. An anti-GPL antibody is produced in the sera of patients and the level reflects the extent of disease, which is useful in diagnosis and treatment [48, 49]. GPLs are one of the immunologically active molecules characteristic of MAC, and serotype-specific GPLs participate in the pathogenesis and immunomodulation in the host [50, 51]. It has been reported that the GPL core plays a role in suppression of mitogen-induced blastogenic response in spleen cells [52]. In addition, the immunomodulating activity of GPL on macrophage functions is serotype-dependent [53]. The serotype 4 GPL promotes phagocytosis and inhibits phagosome-lysosome (P-L) fusion, whereas the GPLs of serotypes 9 and 16 exhibit no effect on phagocytosis and P-L fusion. The serotype 8 GPL shows concomitant stimulation of both phagocytosis and P-L fusion. Because the GPL core, but not OSE, is common in all serotypes, the OSE of GPL may be involved in the mechanism of inhibition of P-L fusion, which is mediated through mannose receptors of macrophages [54]. The serotype 4 GPL inhibits the lymphoproliferative response to mitogens [51]. Thus, host responses to GPLs vary with the MAC serotype. It is reported that the uptake by and growth in macrophages of a MAC mutant with a gene in the GPL synthesis pathway inactivated by a transposon insertion were decreased [55]. The pathogenicity of GPL may comprise both a common peptide core and an OSE elongated from 6-d-Tal. The GPL is a pleiotropic molecule and participates in the pathogenesis of MAC disease. Elucidation of the structure-activity relationship of GPL is required for a better understanding of the pathogenesis.

6. Structural analysis of GPL

The polar GPL is species-specific in the portion of the OSE. MS is very useful to analyze the structure of OSE sequences and linkage positions in addition to total molecular weight. In this review, I describe detailed methods and results of structural analyses of some GPLs. The procedure for structural analysis of OSE is summarized in Fig. 6.

- Cultivation of *Mycobacterium avium-intracellulare* complex
Middlebrook 7H11 agar, 37°C, 2-3 weeks
- Extraction of lipids fraction (Chloroform/Methanol, 2:1 v/v)
- Mild alkaline hydrolysis (0.2 N KOH)
- Purification of glycopeptidolipid (GPL)
- Structural analysis of GPL

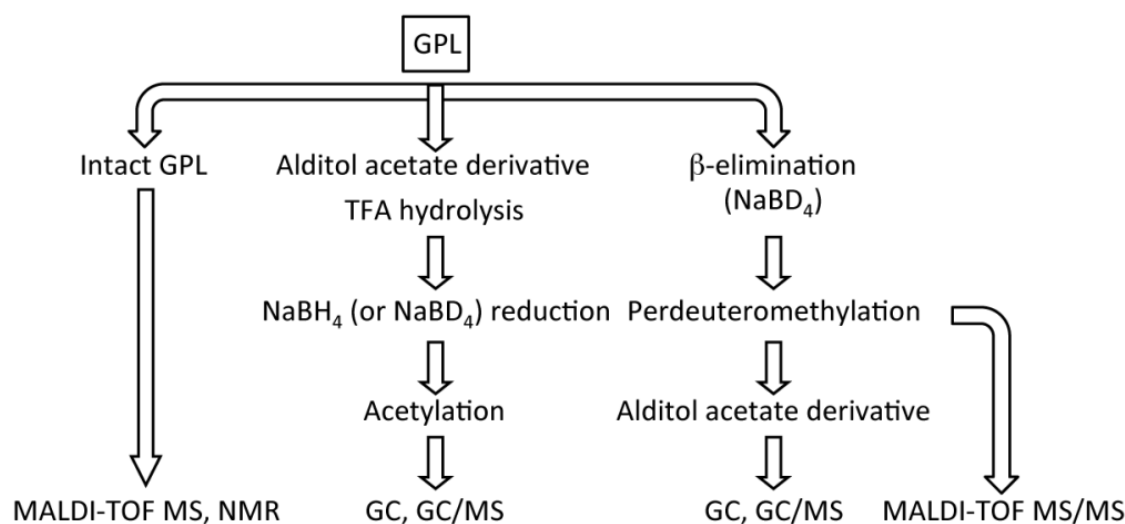


Figure 6. Procedure for structural analyses of OSE.

6.1. Preparation of GPL

MAC was grown on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI, USA) with 0.5% glycerol and 10% Middlebrook OADC enrichment (Difco) at 37°C for 2-3 weeks. Heat-killed bacteria were sonicated, and total lipids were extracted with chloroform-methanol (2:1, v/v). The total 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, v/v) and water. The organic phase was evaporated and precipitated with acetone to remove any acetone-insoluble components. The supernatant was partially purified with a Sep-Pak Silica Cartridge (Waters Corporation, Milford, MA, USA). The GPL was completely purified by preparative TLC of silicagel G (Uniplat; 20 × 20 cm, 250 μm; Analtech, Inc., Newark, DE, USA). The TLC plate was developed with chloroform-methanol-water (65:25:4 and 60:16:2, v/v), until a single spot was obtained.

6.2. Preparation of OSE moiety

β-Elimination of the GPL was performed with alkaline borohydride, and the OSE elongated from *D-allo*-Thr was released [34, 56]. 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, USA), and evaporated under nitrogen to remove boric acid. After partition into two layers of chloroform-methanol (2:1, v/v) and water, the upper aqueous phase was recovered and evaporated, and the OSE was purified as an oligoglycosyl alditol.

6.3. Molecular weight of intact GPL

The molecular species of the intact GPL was determined by MALDI-TOF MS. One μg of the GPL dissolved with chloroform-methanol (2:1, v/v) was applied to the target plate, and 1 μl of 10 mg/ml DHB in chloroform-methanol (1:1, v/v) 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 [57]. The peak ions of intact GPL were detected in sodium adduct form, $[\text{M}+\text{Na}]^+$ as the main molecular-related ion; representative spectra of some GPLs are shown in Fig. 7. The mass numbers identified the proposed structures of each GPL.

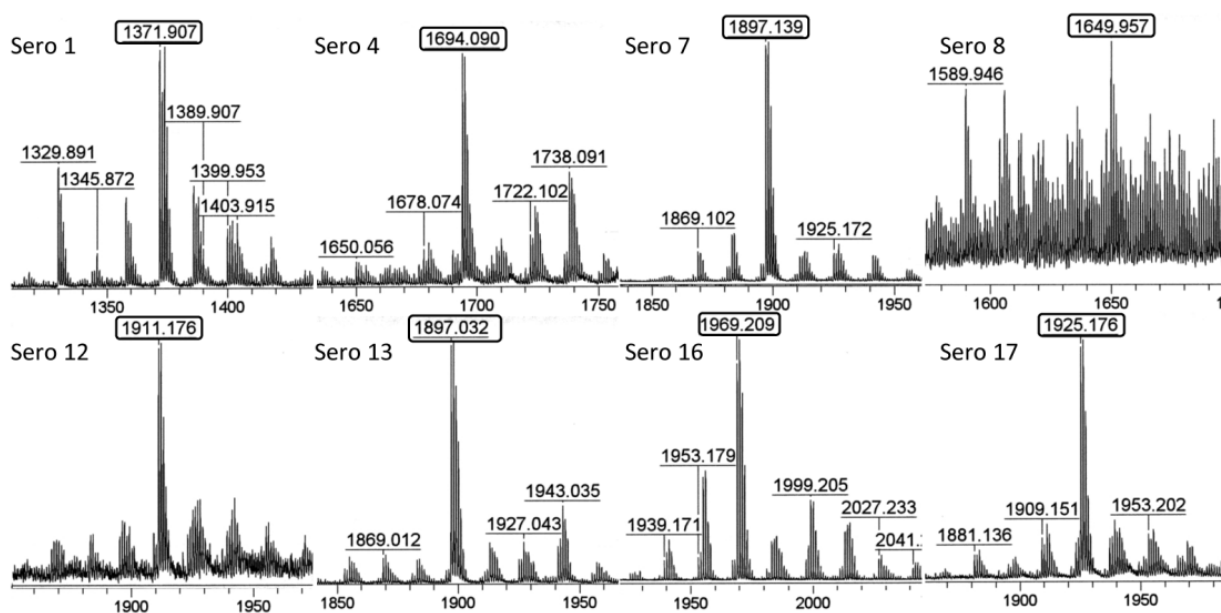


Figure 7. MALDI-TOF MS spectra of representative GPLs.

6.4. Glycosyl sequences of OSE.

The molecular weight of the OSE portion is measured by MALDI-TOF MS. The OSE and 10 mg/ml DHB were dissolved in ethanol-water (3:7, v/v) and applied to the target plate

according to the method for intact GPL. To determine the glycosyl sequence of the OSE, MALDI-TOF MS/MS analysis of the oligoglycosyl alditol from the OSE was performed. The spectrum afforded the molecular ion $[M+Na]^+$, together with the characteristic mass increments in the series of glycosyloxonium ions formed on fragmentation at each glycosyl linkage from both terminal sugars to their opposites, respectively. It was shown the representative assignments of intact serotype 13 GPL and its OSE with the proposed structure in Fig. 8.

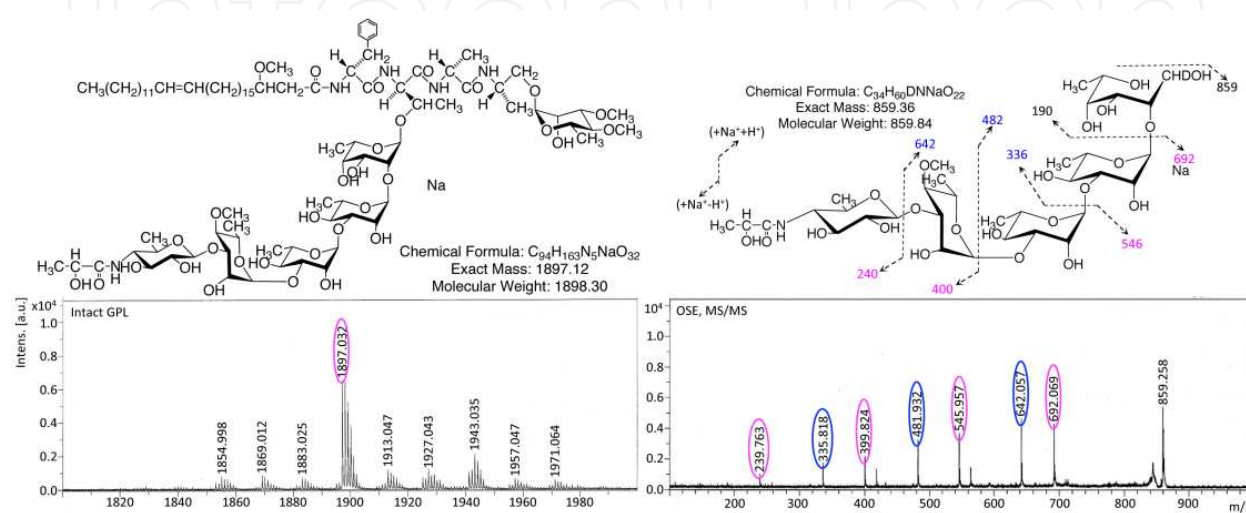


Figure 8. MALDI-TOF MS and MS/MS spectra of serotype 13 GPL. The molecular weight of the intact GPL was detected as m/z 1897 for $[M+Na]^+$ and fixed the proposed structure. The MS/MS spectra clearly show that the each ion was assigned to the fragmentation at each glycosyl linkage from both terminal sugars to their opposites, respectively.

6.5. GC/MS of carbohydrates

To determine the glycosyl composition and linkage position, GC/MS of partially methylated alditol acetate derivatives was performed. Perdeuteromethylation was conducted by the modified procedure of Hakomori [34, 58]. The OSE was dissolved with a mixture of dimethylsulfoxide 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 twice with water, 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 [34, 59]. GC/MS was performed using a fused capillary column (SP-2380 and Equity-1; 30 m, 0.25 mm ID, Supelco, Bellefonte, PA). The linkage positions were acetylated, and no related positions were previously deuteromethylated. The linkage positions were assigned by the fragmentation patterns of partially deuteromethylated alditol acetate derivatives and retention time of the peaks. The representative fragmentation patterns of partially methylated alditol acetate derivatives are shown in Fig. 9.

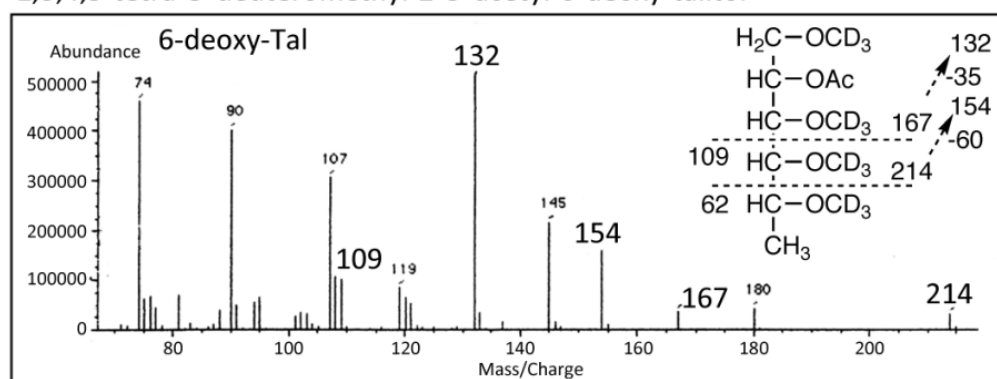
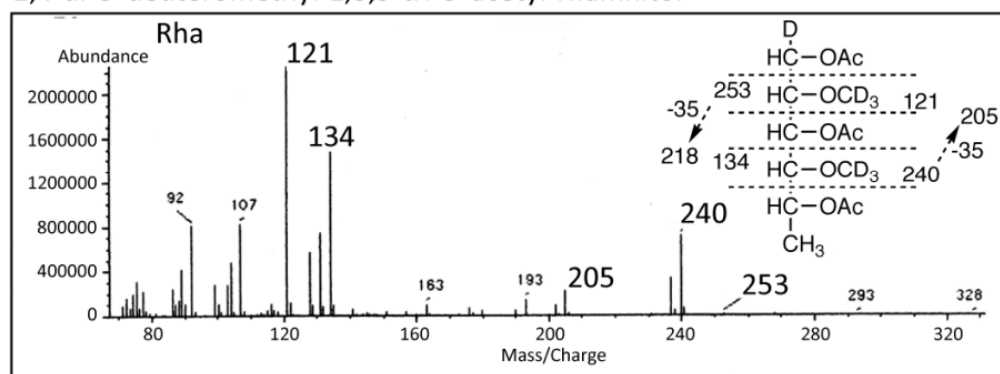
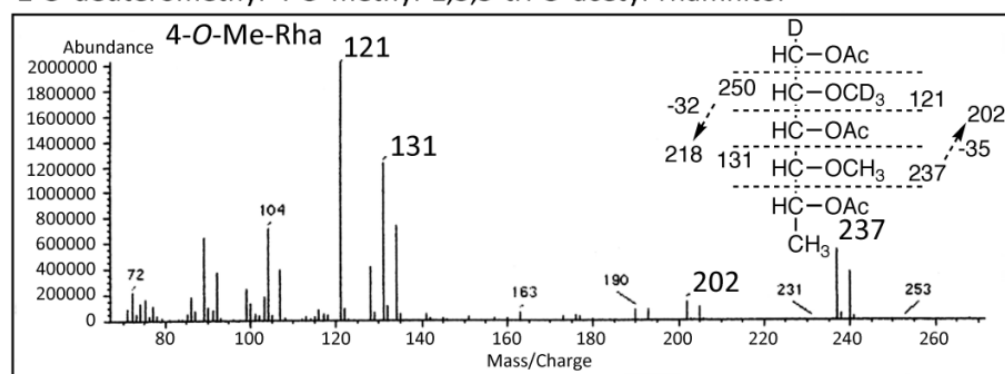
1,3,4,5-tetra-*O*-deuteromethyl-2-*O*-acetyl-6-deoxy-talitol2,4-di-*O*-deuteromethyl-1,3,5-tri-*O*-acetyl-rhamnitol2-*O*-deuteromethyl-4-*O*-methyl-1,3,5-tri-*O*-acetyl-rhamnitol

Figure 9. Representative mass spectrum of partially deuteromethylated alditol acetate derivatives.

6.6. Nuclear magnetic resonance (NMR) of GPL

The OSE was dissolved in deuterium oxide. To define the anomeric configurations of each glycosyl residue, ^1H and ^{13}C nuclear magnetic resonance (NMR) was employed. Both homonuclear correlation spectrometry (COSY) and ^1H -detected [^1H , ^{13}C] heteronuclear multiple-quantum correlation (HMQC) were performed as described previously [34, 56]. The ^1H NMR and ^1H - ^1H homonuclear COSY analyses of the OSE derived from the GPL revealed distinct anomeric protons with corresponding H1-H2 cross-peaks in the low-field region, and the chemical shift and coupling constant are indicative of α -anomers and a β -hexosyl unit, respectively.

7. Biosynthesis gene of GPL

7.1. Isolation of cosmid clones carrying *rtfA* gene and sequence analysis

First, we constructed the *M. intracellulare* cosmid library. Genomic DNA of MAC was prepared by mechanical disruption of bacterial cells, which was accomplished by homogenizing a bacterial pellet with glass beads in phosphate-buffered saline, followed by phenol-chloroform extraction, and precipitation with ethanol. Genomic DNA fragments randomly sheared to 30–50 kb fragments during the extraction process were fractionated and electroeluted from agarose gels using Takara Recochip (Takara, Kyoto, Japan). These DNA fragments were rendered blunt-ended using T4 DNA polymerase and dNTPs, followed by ligation to dephosphorylated arms of pYUB412 (*Xba*I-*Eco*RV and *Eco*RV-*Xba*I). After *in vitro* packaging using Gigapack III Gold extracts (Stratagene, La Jolla, CA, USA), recombinant cosmids were introduced into the *E. coli* STBL2 [F-*mcrA* D (*mcrBC*-*hsdRMS*-*mrr*) *endA1 recA1 lon gyrA96 thi supE44 relA1 l-* D (*lac-proAB*)]. PCR was used to isolate cosmid clones carrying the rhamnosyltransferase (*rtfA*) gene with primers *rtfA*-F (5'-TTTGGAGCGACGAGTTCATC-3') and *rtfA*-R (5'-GTGTAGTTGACCACGCCGAC-3'). *RtfA* encodes an enzyme responsible for the transfer of Rha to 6-d-Tal in the OSE [38, 60]. We isolated the cosmid clones #49 (accession no. AB274811) and #253 (accession no. AB355138), which are responsible for the biosynthesis of GPL 7 and 16, respectively. The insert of a cosmid clone was sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 310 gene analyzer (Applied Biosystems). The putative function of each open reading frame (*orf*) was identified by similarity searches between the deduced amino acid sequences and known proteins using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and FramePlot (<http://www.nih.gov/~jun/cgi-bin/frameplot.pl>), and the DNASIS computer program (Hitachi Software Engineering, Yokohama, Japan). The similarity of protein sequences of each ORF was compared to those of serotype 2, 4, 7, and 16 GPLs, and the genetic maps for GPL biosynthetic cluster are summarized in Fig. 10 [32, 36, 61]. The OSE of serotype 1 GPL produced by *M. avium* serotype 1 strain is composed of α -L-Rha-(1→2)-6-d-L-Tal, which is the core OSE of all serotypes [12]. *M. avium* serotype 1 strain (NF113) was transformed with pYUB412-cosmid clone #253 containing the serotype 16-specific gene cluster. The transformant produced a serotype 16 GPL with a different R_f value on TLC compared to serotype 1 GPL. The molecular weights of intact GPLs and the fragment patterns of their OSEs were completely equivalent to the serotype 16 GPL. Next, we identified some *orf* functions of #253. We hypothesized that *orf1*, 16, and 17 in #253 were correlated with the glycosyltransferase by the similarity of ORF sequences, in addition to *rtfA*. They were compared to the productive GPLs of serotype 1 transformants inserted with the combination of *orf1*, 16, and 17. We determined that *orf1*, 17, and 16 were responsible for the elongation from 6-d-Tal-Rha to 6-d-Tal-Rha-Rha-Rha-Rha, and that these glycosyltransferases operated in this order, as shown in Fig. 11. After the elongation of an OSE, the OSE may be modified by aminotransferase, methyltransferase, and acyltransferase, and serotype 16 GPL may be completed.

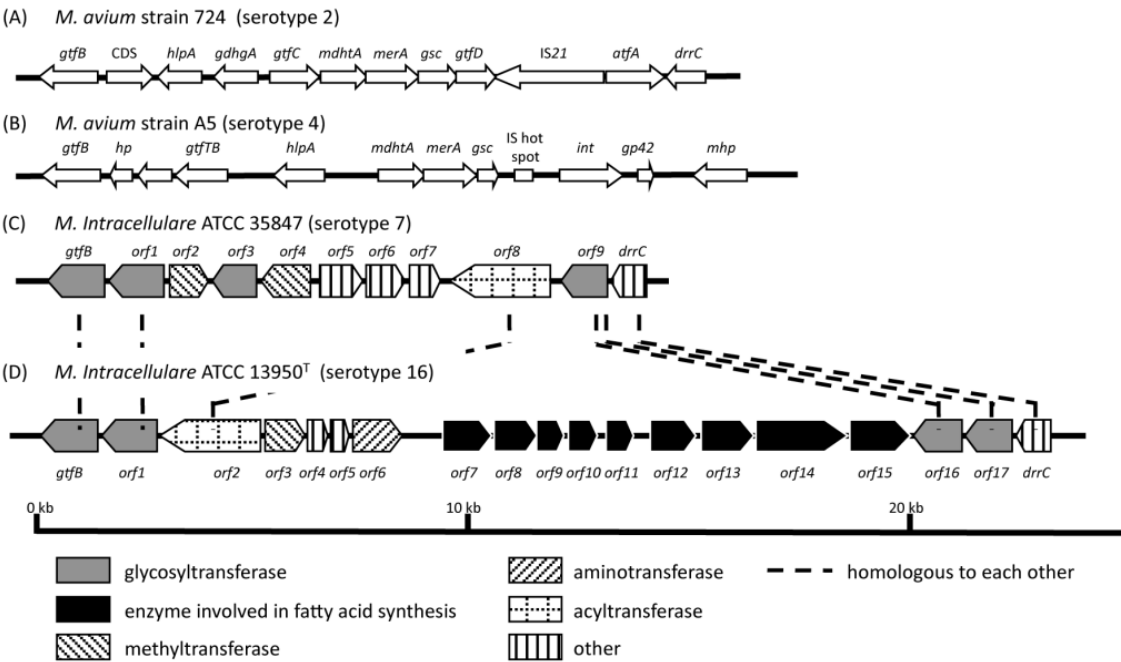


Figure 10. Comparison and overview of genetic maps of GPL biosynthetic cluster. (A), *M. avium* strain 724 (serotype 2, accession no. AF125999); (B), *M. avium* strain A5 (serotype 4, accession no. AY130970); (C), *M. intracellulare* ATCC 35847 (serotype 7, accession no. AB274811); (D), *M. intracellulare* ATCC 13950^T (serotype 16, accession no. AB355138).

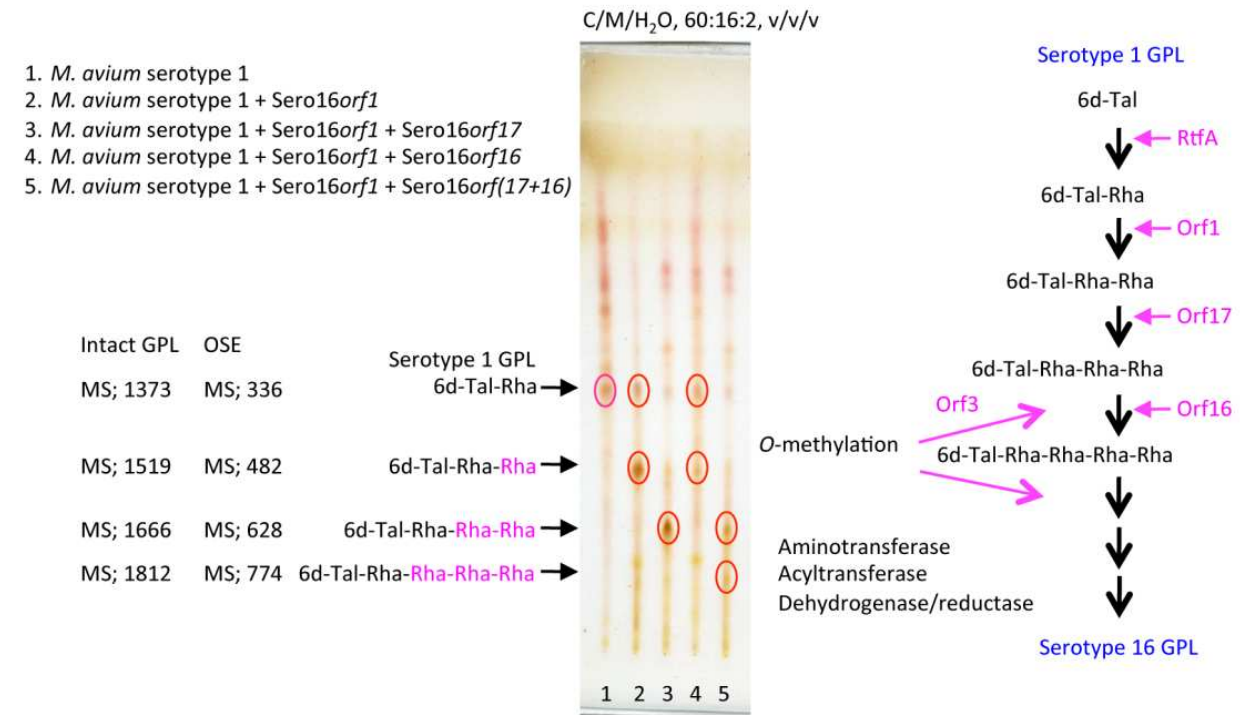


Figure 11. Proposed elongation of OSE in serotype 16 GPL.

7.2. Native conformation of GPL and host response

The native GPL was purified without alkaline treatment. The native GPLs were detected on TLC as several spots that expanded broadly and had different R_f values from that of the alkaline-treated GPL (Fig. 12). Alkaline treatment converged these spots into one spot. It was reported that the native GPLs were modified by several *O*-acetylations in the OSE portion and the alkaline treatment removed the acetylated groups [23, 33]. We are now analyzing in detail the positions and numbers of *O*-acetylations in the OSE by using MALDI-TOF MS/MS.

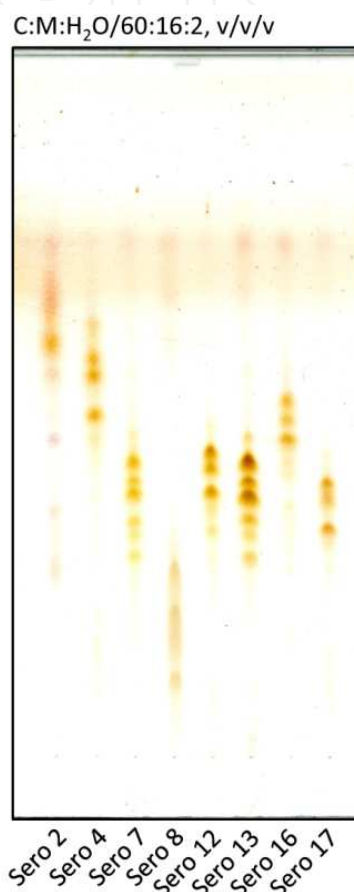


Figure 12. TLC of some native GPL fractions (non-alkali-treated).

The importance of Toll-like receptor (TLR)-mediated responses has been studied in tuberculous infections. Means et al. reported that *M. tuberculosis* activated both TLR2 and TLR4, whereas heat-killed *M. tuberculosis* and MAC activated only TLR2 [62]. It was observed that MyD88- and TLR2-deficient mice have increased susceptibility to MAC infection compared to TLR4-deficient and wild-type mice [63]. These lines of evidence suggest that TLRs are related to host recognition of the MAC components containing GPLs and affect MAC infections. To clarify the host recognitions of GPLs via TLRs, we stimulated HEK-blue-2, and -4 cells (InvivoGen, San Diego, CA, USA) with native and alkaline-treated GPLs. HEK-blue-2 and -4 cells are HEK293 cells stably transfected with multiple genes for recognition of TLR2 and TLR4 (including the co-receptors MD2 and CD14). The native GPL

significantly activated HEK-blue-2 cells in a dose-dependent manner, but HEK-blue-4 cells did not respond (Fig. 13). The alkaline-treated GPL without *O*-acetylation did not activate either HEK-blue-2 or -4 cells. Re-acetylated alkaline-treated GPLs with *O*-acetyl groups substituted for all hydroxy groups of the OSE activated HEK-blue-2 cells, although the level of activation was lower than that of the native form. Moreover, we confirmed that only the native GPL stimulated mouse bone marrow-derived macrophages via TLR2 by using C57BL/6 and TLR2 knockout mice. Brennan and Goren first proposed that de-acetylated GPLs as alkaline-stable lipids, made it possible to classify serotyping [12, 31]. Schorey and colleagues clarified that serotype 1 and 2 GPLs can function as TLR2 agonists and promote macrophage activation in a TLR2 and MyD88-dependent pathway [64, 65]. They reported that the acetylated and methylated groups of GPLs were necessary for GPL-TLR2 interaction as a molecular requirement. Taken together with our results, native GPLs are a TLR2 agonist, and it may be important for GPL-TLR2 interaction to balance the hydrophobicity and hydrophilicity of the GPL molecules.

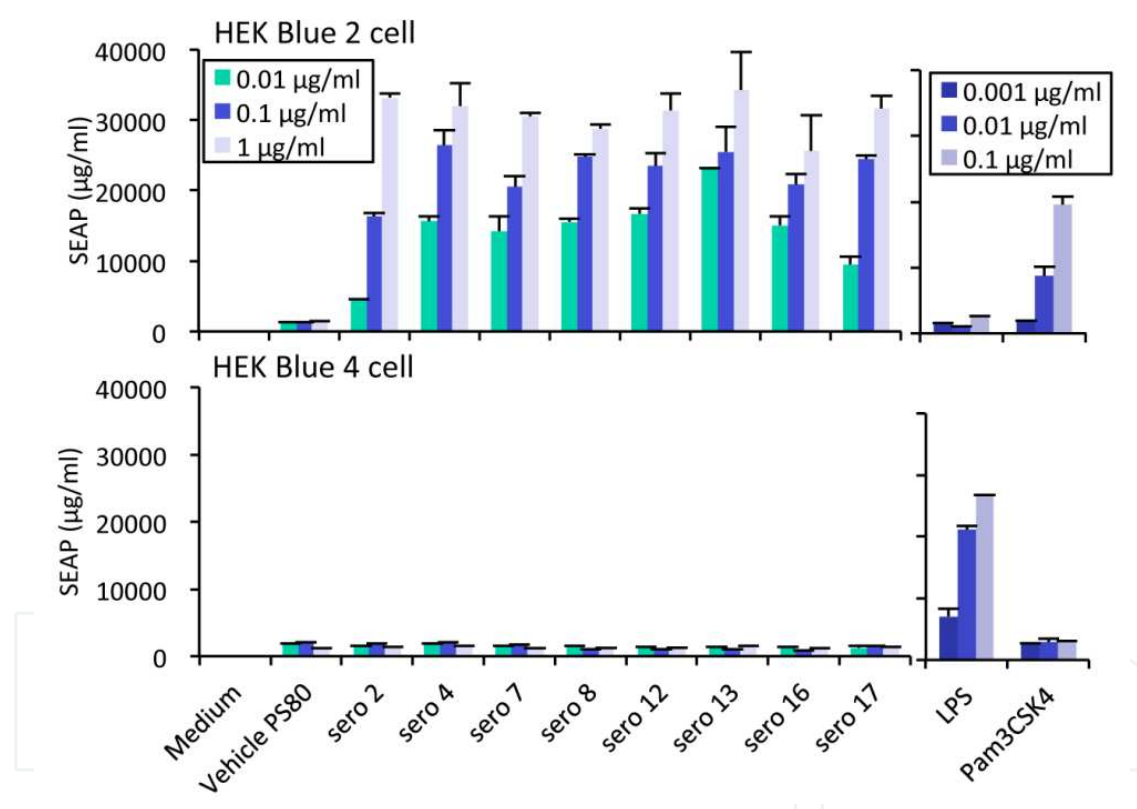


Figure 13. Native GPLs activate cells through TLR2.

8. Sulfolipid (SL)

Mycobacterial SLs are classified into three types by the number of attached short acyl chains. SL-1 is the predominant type, and SL-2 and -3 are intermediate types. The structure of SL-1 was identified as 2-palmitoyl(stearoyl)-3-phthioceranyl-6,6'-bis-hydroxyphthioceranyl-trehalose-2'-sulfate [66, 67]. The presence of SLs in mycobacteria is strain-specific. Several studies have demonstrated a significant relationship between SL-1

and virulence, such as the amount of SL-1 biosynthesis in virulent strains, and the inhibition of P-L fusion in macrophages by SL-1. SL-1 modulates superoxide release and secretion of interleukin (IL)-1 β and tumor necrosis factor (TNF)- α by blocking activation of human macrophages and neutrophils [68, 69]. In contrast to these studies, it has been reported that *pks2* disruption and SL-1 deficiency do not significantly affect the replication, persistence, and pathogenicity of *M. tuberculosis* in mice, guinea pigs, or cultured macrophages [70, 71]. The pathogenicity of SL-1 is controversial. SL-1 does not induce granulomatous inflammation, but rather inhibits it and the release of TNF- α induced by cord factor/TDM [72]. SL-1 could contribute to virulence at an early stage of mycobacterial infection by counteracting the immunopotentiating effect of cord factor/TDM. On the other hand, it is reported that SL-3, 2-palmitoyl(stearoyl)-3-hydroxyphthioceranol-trehalose-2'-sulfate, is mainly recognized by CD1b-restricted T cells as a lipid antigen. The other tetraacylated and triacylated SLs (SL-1 and -2) were unable to stimulate diacylated SL (SL-3)-specific T cell clones, which implies that immunogenic SL-3 are not generated inside the antigen-presented cells [73].

SLs of *M. tuberculosis* have been implicated in the virulence of this organism by inhibiting the P-L fusion in macrophages, thus probably promoting the intracellular survival of *M. tuberculosis*, but the pathogenic role remains controversial. The gene, *pks2*, responsible for SL synthesis was identified and disrupted [74]. The *pks2* mutant defective in an early step of SL biosynthesis had no obvious growth defect in infected mice. By contrast, growth of a strain lacking MmpL8, a transporter of SL in *M. tuberculosis*, was highly attenuated in a mouse model of tuberculosis [70]. Although initial replication rates and containment levels of the MmpL8 mutant were identical, compared with the wild type, a significant attenuation of the mutant strain in time-to-death was observed. Early in infection, differential expression of cytokines and cytokine receptors revealed that the mutant strain less efficiently suppresses key indicators of a Th1-type immune response, suggesting an immunomodulatory role for SLs in the pathogenesis of tuberculosis [75].

Recently, Kummer et al. demonstrate that PapA2 and PapA1 are responsible for the sequential acylation of SL1 biosynthesis. Disruption of *papA2* and *papA1* in *M. tuberculosis* confirmed their essential role in SL-1 biosynthesis and their order of action. BALB/c mice infected by aerosol with wild-type, Δ *papA2*, and Δ *papA1* mutants showed no significant difference in the ability of the bacteria to grow or persist through the time to death of the mice. The loss of SL-1 did not appear to affect bacterial replication or trafficking. They suggested that the functions of SL-1 are specific to human infection [76].

9. Phenolic glycolipid (PGL)

The glycosylated phenolphthiocerol dimycocerosates (PDIM), so-called PGLs, are produced by a limited group of mycobacterial species, and most of them are pathogenic in humans [77]. PGL is distributed in *M. leprae* as a unique antigen, and inhibits the lymphoproliferative responses and suppresses monocyte oxidative responses [78-80]. It has also shown that disruption of PGL synthesis results in loss of the virulent phenotype

without significantly affecting the bacterial load during disease in experimental models using mice and rabbits, and loss of PGL was found to correlate with an increase in the release of the pro-inflammatory cytokines *in vitro* [81, 82]. A PGL purified from *M. leprae* has been used as antigen in an enzyme-linked immunosorbent assay. Antibodies directed against the lipid were detected in sera of leprosy patients but not in sera from uninfected controls or patients infected with other mycobacteria, including *M. tuberculosis*. The antibody response distinguished between the *M. leprae* lipid and the structurally related PGL from *M. kansasii*. Similar to serodiagnosis of MAC disease using GPL, this assay based on PGL antigen has considerable potential as a specific serodiagnostic test for infection with *M. leprae* [83, 84].

We determined the structure of the PGL derived from BCG (Tokyo 172 strain). The PGL produced by BCG is a so-called mycoside B (PGL-BCG), and its sugar moiety is different from that of the PGL produced by *M. tuberculosis* (PGL-tb). The PGL-BCG has only a 2-O-Me-Rha branch elongated from the phenol moiety, although PGL-tb has it elongated to three sugar residues [85]. The composition of the PDIM in PGL is similar in both species. The MALDI-TOF MS spectrum of PGL-BCG showed m/z 1531 and other mass units at 14 Da intervals for $[M+Na]^+$ as molecule-related ions in positive mode (Fig. 14). In addition, the MS/MS spectrum showed fragment ion peaks m/z 1371 based on the elimination of methyl-deoxysugar; m/z 1135, 1093, based on the elimination of the C26:0, C29:0 FAs; m/z 696 based on the elimination of both C26:0 and C29:0 FAs; and m/z 535 for the phenol phthiocerol that eliminated methyl-deoxysugar and C26:0, C29:0 FAs (Fig. 14).

Reed et al. demonstrated that PGL-tb inhibits the innate immune response. Loss of PGL-tb was responsible for an increase in the release of TNF- α and IL-6 and IL-12 *in vitro*, and the PGL-tb-deficient mutant showed a phenotype with low virulence/pathogenicity [81]. The composition of the PDIM in PGL-BCG is similar to that in PGL-tb. Although purified PGL molecules by themselves had no effect on the activation of macrophages *in vitro*, we found that PGL suppressed the activation of murine bone marrow-derived macrophages elicited by total lipids. It is considered that the PGL may have a competitive inhibitory effect or mask the active site of other TLR2 agonistic lipid components, and decrease their activity.

10. Phosphatidylinositol mannoside (PIM), lipomannan (LM), and lipoarabinomannan (LAM)

PIMs and their multiglycosylated counterparts, LMs, and LAMs, are complex lipoglycans that are found ubiquitously in the envelopes of all mycobacterial species. Their structures originate from a phosphatidyl-*myo*-inositol (MPI) anchor, which is mannosylated to generate LM and further arabinosylated to give LAM [86]. The non-reducing termini of the arabinosyl side chains can be substituted by capping motifs, and LAMs are classified into three families. LAMs from slow growing mycobacteria bearing mannose caps, *i.e.* mono- or ($\alpha 1 \rightarrow 2$)-di- or trimannoside units, are designated as ManLAMs. In contrast, LAMs from fast growing mycobacteria capped by phospho-*myo*-inositol units and not capped at all, are termed PILAM and AraLAM, respectively [87]. LAMs and LMs exhibit a broad spectrum of

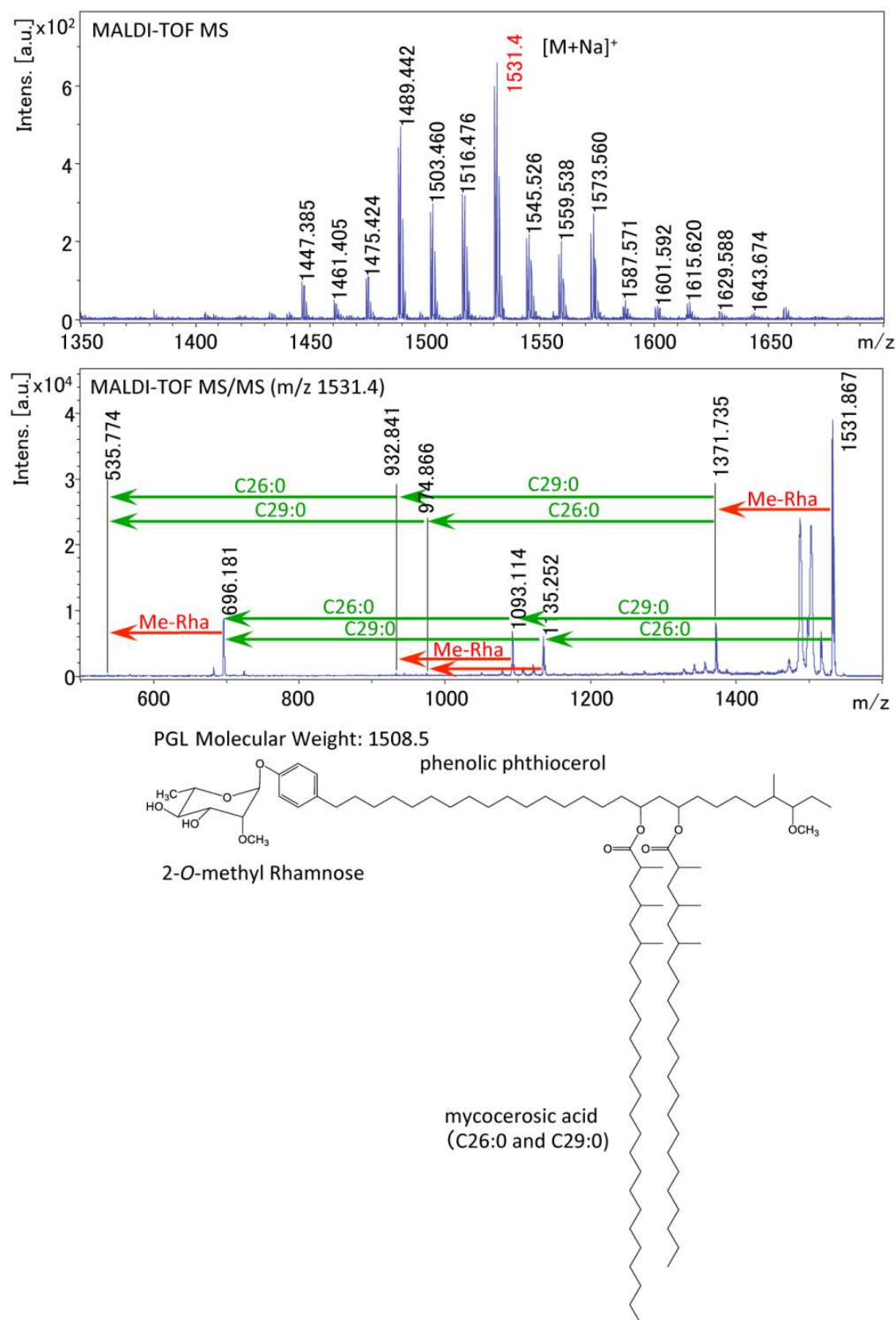


Figure 14. MALDI-TOF MS and MS/MS spectra of PGL derived from BCG Tokyo 172 strain, and proposed structure.

immunomodulatory activities, including the ability to modulate the production of macrophage-derived Th1 pro-inflammatory cytokines, most commonly TNF- α and IL-12. The ManLAM from *M. tuberculosis* is involved in the inhibition of phagosome maturation, apoptosis, and IFN- γ signaling in macrophages and IL-12 secretion of dendritic cells. ManLAMs contribute an immunosuppressive effect to the persistence of slow-growing mycobacteria in humans. In contrast, PILAMs are able to induce the release of a variety of proinflammatory cytokines. Recently it was reported that LMs from both pathogenic and nonpathogenic mycobacterial species, independent of their origin, are potent stimulators of TNF- α , IL-8, and IL-12, and activate macrophages via TLR2 [88-90]. The ManLAM/LM balance might be important to host immune responses against mycobacteria.

PIMs are composed of a phosphatidylinositol and mannose moieties. PIMs have 4–6 mannoses, the third and fourth of which are acylated in some cases. PIM₂ containing two mannoses, a main component of mycobacterial cell walls, is highly immunogenic. Several biological functions have been recently attributed to PIMs. PIM₂ was shown to recruit natural killer T cells, which play a role in the local granulomatous response [91, 92]. PIMs activate cells via TLR-2 [93]. PIM₆ as well as the ManLAMs from *M. leprae* and *M. tuberculosis* are presented by antigen-presenting cells in the context of CD1b [17]. The phosphatidylinositol moiety plays a central role in the process of binding PIMs and ManLAMs to CD1b proteins.

11. Conclusion

Mycobacterial glycolipids are pleiotropic molecules and play key roles in both microbes and hosts by acting as structural components of cell walls and immunologically active substances. A better understanding of mycobacterial glycolipids will help us develop novel diagnostics, therapeutics, and prophylactics for mycobacterial diseases.

Author details

Nagatoshi Fujiwara

Department of Bacteriology, Osaka City University Graduate School of Medicine, Japan

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