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Identification of trehalose dimycolate (cord factor) in *Mycobacterium leprae*

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Abstract Glycolipids of *Mycobacterium leprae* obtained from armadillo tissue nodules infected with the bacteria were analyzed. Mass spectrometric analysis of the glycolipids indicated the presence of trehalose 6,6′-dimycolate (TDM) together with trehalose 6-monomycolate (TMM) and phenolic glycolipid-I (PGL-I). The analysis showed that *M. leprae*-derived TDM and TMM possessed both α- and keto-mycolates centering at C78 in the former and at C81 or 83 in the latter subclasses, respectively. For the first time, MALDI-TOF mass analyses showed the presence of TDM in *M. leprae*.

Keywords: Trehalose 6,6′-dimycolate; Trehalose 6-monomycolate; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Thin-layer chromatography; *Mycobacterium leprae*

1. Introduction

Mycolic acids and mycolyl glycolipids are unique and ubiquitous components of mycobacterial cell envelopes. Among such components, trehalose 6,6′-dimycolate (TDM) was first isolated as cord factor from highly virulent *Mycobacterium tuberculosis* showing cord-like growth on the surface culture in liquid media [1–3]. TDM of *M. tuberculosis* was recognized as one of the virulence factors capable of inhibiting fusion of phagosome with lysosome in infected macrophage [4]. However, on the other hand, the TDM was considered to be associated with host defence against the mycobacterial infection since it induced immune responses such as type 1 T cell activation and the formation of granuloma in the mycobacteria-infected lesion [5–7]. Wang et al. have reported that a high proportion of patients infected with *Mycobacterium leprae* possess IgG antibody against TDM of unknown origin as well as trehalose 6-monomycolate (TMM), a biosynthetic precursor of TDM [8]. These observations suggest the possible existence of both TDM and TMM in *M. leprae*. Previously, TDM has been isolated from almost all species of culturable mycobacteria [9,10], and also TMM was isolated from *M. leprae*, however, the search for TDM in *M. leprae* has been unsuccessful [11]. The possible reason for not being able to identify TDM, may be due to (1) inadequate supply of *M. leprae*, (2) negligible amount of the product, (3) technically inefficient to identify TDM. Recent development of newer techniques such as MALDI-TOF mass spectrometry has enabled us to identify even several pg amounts of products. Therefore, the present study was designed as an attempt to directly detect TDM in *M. leprae*, by use of newer technologies. In the process, higher amounts of *M. leprae* phenolic glycolipid-I (PGL-I) was obtained which was analyzed by MALDI-TOF mass spectrometry.

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Abbreviations: TDM, trehalose 6,6′-dimycolate; TMM, trehalose 6-monomycolate; *M. leprae*, *Mycobacterium leprae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *M. bovis*, *Mycobacterium bovis*; TLC, thin-layer chromatography; MALDI-TOF mass, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

2. Materials and methods

2.1. Sources for extraction of glycolipids

M. tuberculosis Aoyama B (ATCC 31726) and *Mycobacterium bovis* BCG Connaught (ATCC 35745) were grown at 37 °C on Sauton's medium for four weeks as surface pellicles until early stationary phase. Cultivated mycobacterial strains were used for extraction of glycolipids. Because *M. leprae* cannot be cultivated in any artificial media, armadillo tissue nodules infected with *M. leprae* (Thai 53 strain) were used for the extraction of glycolipids.

2.2. Extraction of glycolipids and mycolic acid methyl esters

Glycolipids were extracted according to the methods described previously [12]. In brief, bacterial culture or tissues infected with *M. leprae* [13] were autoclaved at 121 °C for 15 min and collected by centrifugation. Lipids were extracted from homogenized tissue with 20 volumes of chloroform/methanol (2:1, v/v) three times with vigorous grinding. The two phases were separated in a funnel, the lower organic phase was collected, and the solvent was evaporated from the organic phase. The total lipids were separated by solvent fractionation and tetrahydrofuran-soluble fraction was further separated by thin-layer chromatography (TLC) on silica gel plates (Uniplate; Analtech Inc. Newark, DE) with the solvent system of chloroform/methanol/water (90:10:1, by vol.) or chloroform/methanol/acetone/acetic acid (90:10:6:1, by vol.). Glycolipid spots were visualized with a 9 M H_2SO_4 spray followed by charring at 200 °C for 10 min or with iodine vapor for preparative purposes.

To determine the subclass composition of the mycolic acids in each mycobacterial TMM and TDM, mycolic acid methyl esters were prepared by alkaline hydrolysis of glycolipids. The glycolipids were hydrolyzed with 1.25 M NaOH in 90% methanol at 70 °C for 1 h and the resultant mycolic acids were then extracted with *n*-hexane after acidification with HCl, followed by methylation with benzene/methanol/H_2SO_4 (10:20:1, by vol.) [14]. Mycolic acid methyl esters from each

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glycolipid were fully separated into subclasses by TLC with the solvent system of benzene in a draft chamber under reduced pressure.

2.3. Mass spectrometry analysis

Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF mass) was carried out on a Voyager DE-STR (Applied Biosystems, Tokyo, Japan) with pulsed UV light (337 nm) from an N_2 laser, essentially according to the method reported previously [14]. TDM and TMM were analyzed in the reflectron mode by the instrument operated at 20 kV in the positive ion mode. The 2,5-dihydroxybenzoic acid (2,5-DHB) matrix was used at a concentration of 10 mg/ml in chloroform/methanol (2:1 v/v). Typically, 5 μl of TDM or TMM samples (5 μg) in chloroform/methanol (2:1 v/v) solution and 5 μl of the matrix solution were mixed, and 1.5 μl of the mixture was applied on a sample plate. An external mass spectrum calibration was performed using calibration mixture 2 of the Sequazyme Peptide Mass Standards kit (Applied Biosystems), including known peptide standards in a mass range from 1290 to 5700 Da. The molecular mass of mycobacterial TMM, TDM, and PGL-I was determined based on the quasimolecular mass ions $[M+Na]^+$ by the reflectron mode. In general, nominal number of atomic mass is used for calculation of number of molecular mass. However, there is a slight difference between nominal mass number and accurate mass number read from spectrometry [15]. For instance, when both of the two molecules of mycocerosates ($R_1$and R_2) in PGL-I are C32, the nominal mass number $[M+Na]^+$ of PGL-I ($C_{124}H_{232}O_{19}$) is $[(C \times 124) + (H \times 232) + (O \times 19) + (Na \times 1)] = 2047$ (2024 + 23), but the accurate number is 2050.15 $[(12.0107 \times 124) + (1.00794 \times 232) + (15.9994 \times 19) + 22.9898]$. The nominal mass numbers are given in the text.

3. Results

3.1. TLC analysis of mycolic acids methyl ester

To determine the subclass composition of the mycolic acids in each mycobacterial TMM and/or TDM, mycolic acid methyl esters were analyzed by TLC. The TLC analysis indicated that fatty acid methyl esters had two spots corresponding to either α- and keto-mycolic acid (Fig. 1A). The same two spots pattern was observed for *M. bovis* BCG Connaught (BCG-C) and *M. leprae* while three spots were detected for *M. tuberculosis* corresponding to α-, methoxy-, and keto-mycolic acid [16,17]. The bottom spot in ML lane could be cholesterol from armadillo's tissues (data not shown).

3.2. MALDI-TOF-MS analysis of TMM

We separated the final solvent extracts of *M. leprae* into four fractions (M1–M4). Fig. 1B shows a thin-layer chromatogram of the TMM and TDM from *M. tuberculosis* and the solvent fractionated glycolipids from *M. leprae*. *M. leprae* exhibited bands, which were faint, but significantly reddish glycolipid-like, migrating close to bands of TDM or TMM of *M. tuberculosis*. The major bands of M2 and M4 migrated close to TDM and TMM positions of *M. tuberculosis*, respectively. Therefore, we tried to carefully analyze the bands which may correspond to TDM and TMM by mass spectrometry.

Major band in M4 in Fig. 1B was analyzed using BCG-C as a reference. The mass spectra of TMM from BCG-C showed a biphasic distribution of pseudomolecular ions, $[M+Na]^+$ (Fig. 2A). In the higher mass ranges of BCG-C TMM, dominant ions were detected at *m/z* 1555, 1583, 1597, 1611 and 1625 due to $[M+Na]^+$ of C82, C84, C85, C86 and C87 keto mycolyl TMM, and the major mass ions in the lower mass ranges were detected at *m/z* 1455, 1483, 1511 and 1539 due to $[M+Na]^+$ of α-mycolyl TMM centering at C78, respectively (Fig. 2A) [18]. On the other hand, *M. leprae* derived TMM showed in lower mass ranges at *m/z* 1427, 1455, 1483, 1511 and 1539 due to $[M+Na]^+$ of α-mycolyl TMM centering at C78 same to that of BCG-C (Fig. 2B). In the higher mass ranges, dominant ions were shifted lower and the major ions were detected at *m/z* 1541, 1569 and 1597 (Fig. 2B), indicating the major keto-mycolyl TMM consisted of C81, C83 or C85 mycolate, respectively. The molecular species of TMM from *M. leprae* and that from BCG-C are summarized in Table 1. These results indicate that *M. leprae* possess trehalose 6-monomycolate, with C78 α- and C83 keto-mycolates, as the major molecular species.

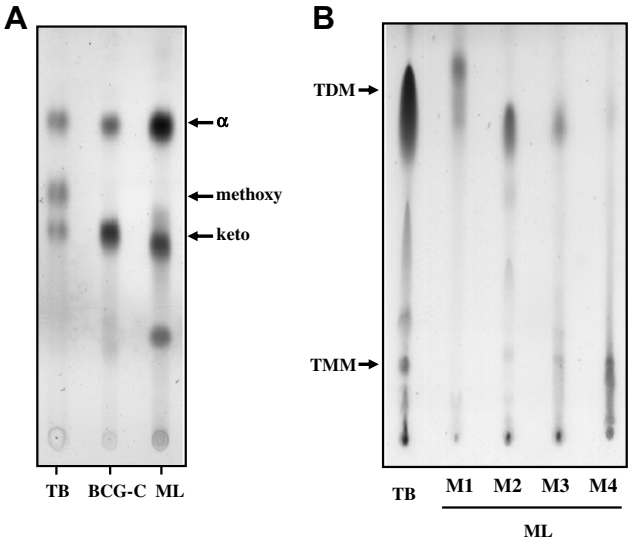

Fig. 1. Thin-layer chromatograms (TLC) of solvent extracts from tetrahydrofuran soluble fraction and mycolic acid methyl ester subclasses of *M. tuberculosis, M. bovis* BCG Connaught, and *M. leprae*. (A) TLC of mycolic acid methyl ester subclasses with the solvent system of benzene/methanol/H_2SO_4 (10:20:1, by vol.). Mycolic acid methyl ester subclasses of *M. tuberculosis* (TB); α-, methoxy- and keto-mycolic acid methyl esters are shown with the mycolic acid methyl ester mixture of glycolipids derived from armadillo tissue nodules infected with *M. leprae* (ML), and those of *M. bovis* BCG Connaught (BCG-C). (B) TLC of solvent extracts from *M. leprae* (ML) and *M. tuberculosis* (TB) with the solvent system of chloroform/methanol/acetone/acetic acid (90:10:6:1, by vol.). Trehalose monomycolate (TMM) and trehalose di-mycolate (TDM) bands of TB were identified previously and used as references in this TLC. Fractions 1–4 separated from the final extracts of *M. leprae* and designated M1–4.

3.3. MALDI-TOF-MS analysis of TDM

TDM from BCG-C showed a diverse distribution of mass ions according to the combination of di α-, α- and keto-, and di keto-mycolic acid subclasses and each molecular species, that leads to a multiphasic distribution of mass ions due to the dominant combination of α–α, α–keto and keto–keto dimycolyl TDM (Fig. 2C). Given the small sample size, the identification of *M. leprae* TDM was achieved primarily on the thin-layer chromatographic behavior and MALDI-TOF mass analysis, in comparison with the analytical results from BCG-C possessing the same mycolic acid subclasses. Fig. 2D shows the positive MALDI-TOF mass spectra of *M. leprae* TDM (Fig. 1B, M2). In contrast to TDM from BCG-C, TDM from *M. leprae* showed a distinctive mass ion distribution shifted to lower mass ranges due to the major combinations of α–α dimycolyl TDM and α–keto dimycolyl TDM with a small shoulder due to keto–keto dimycolyl TDM. In *M. leprae* TDM, major

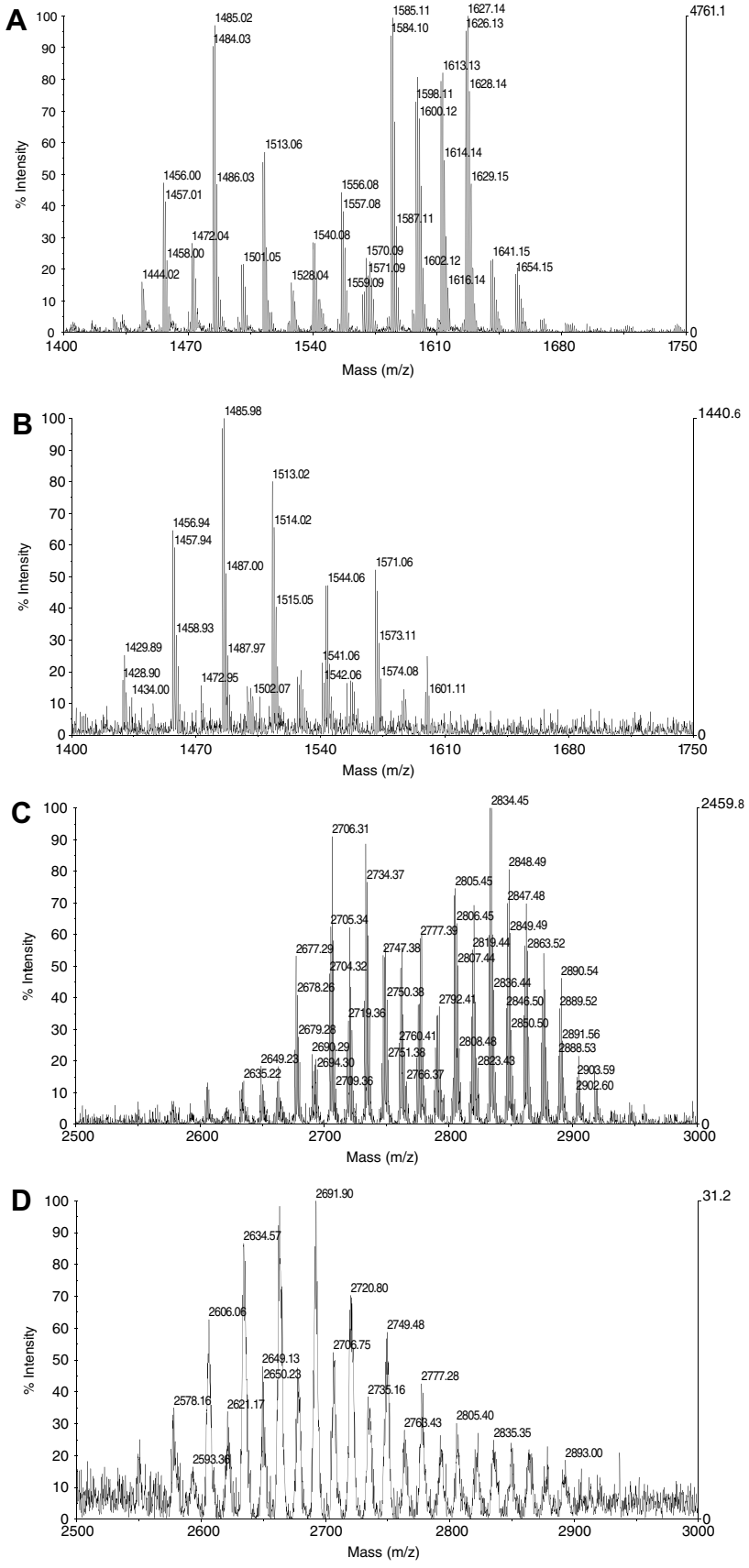

Fig. 2. MALDI-TOF-MS spectra of TMM and TDM. (A) TMM of *M. bovis* BCG Connaught, (B) TMM of *M. leprae* Thai 53, (C) TDM of *M. bovis* BCG Connaught, (D) TDM of *M. leprae* Thai 53.

Table 1
MALDI-TOF mass spectrometry data of the individual types of TMM from *M. leprae* (Thai 53) and *M. bovis* BCG (Connaught)

Species (strain)	Mycolic acid type[a]	Total carbon number of TMM mycolate																	
		74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91
M. leprae	I	1427		**1455**		**1483**	1497	**1511**	1525	1539	1553								
(Thai 53)	II			1471		1499		1527	**1541**	1555	**1569**	1583	1597						
M. bovis BCG	I			**1455**	1469	**1483**		**1511**		1539									
(Connaught)	II									**1555**	1569	**1583**	**1597**	**1611**	**1625**	1639	1653	1667	1681

[a]I; α-dicyclopropanoic or dienoic, II; keto-monocyclopropanoic or monoenoic. Major homologues are shown in bold.

mass ions due to molecular species possessing C76, C78, C80 α–α dimycolic acids were detected at *m/z* 2573, 2601 and 2629, and those possessing α–keto dimycolic acids at *m/z* 2659, 2687 and 2715, and those possessing keto–keto dimycolic acids at *m/z* 2731, 2745, 2773 and 2801, respectively. The deduced molecular species of TDM from *M. leprae* and that from BCG-C are summarized in Table 2.

3.4. MALDI-TOF-MS analysis of PGL-I

The MALDI-TOF mass spectra of M2 in Fig. 1B showed the existence of TDM as described above, however, the major cluster ions in mass spectra of M2 were observed in lower mass ranges from around *m/z* 1990 to 2100. So, we analyzed the spectra in more detail. The result indicates that the cluster ions are derived from phenolic glycolipid-I (PGL-I) (Fig. 3A). The nominal mass number of the PGL-I, which is assumed to have two molecules of C32 mycocerosates, was *m/z* 2047 (Fig. 3A and B). Thus, the deduced combination of mycocerosic acids with different carbon numbers in PGL-I are shown in Fig. 3A and the general structure of PGL-I is shown in Fig. 3B.

4. Discussion

In the present study, we have directly detected TDM and TMM from armadillo tissues infected with *M. leprae*. The presence of TMM in *M. leprae* possessing C74-82 α-mycolic acids has been reported previously [8,18], however, the existence of keto-mycolate in *M. leprae* TMM was not clear. We identified keto-mycolate clearly in *M. leprae* TDM and TMM, and the chain length of keto-mycolate in *M. leprae* was found to be shorter than those from slow-growing culturable mycobacteria such as *M. tuberculosis* and *M. bovis* BCG [19].

Previously, no detectable TDM was identified by the analysis of the lipids obtained from *M. leprae* infected armadillo [9,20]. However, in our hands, we observed a meager but significant spot on TLC from *M. leprae* extract which migrated to the position of TDM of *M. tuberculosis* (Aoyama B strain). When this spot was analyzed by mass spectrometry in reference to TDM from *M. bovis* BCG Connaught (BCG-C), TDM having α–α dimycolates were observed in the lower mass ranges than *m/z* 2673 as seen in TDM from BCG-C (Fig. 2C). Therefore, both *M. leprae* and *M. bovis*

Table 2
Most probable combination of mycolic acids constructing TDM from *M. leprae* (Thai 53) and *M. bovis* BCG (Connaught)

Mass no. of TDM (*m/z*)	*M. leprae* (Thai 53)	*M. bovis* BCG (Connaught)
2573	α76:α78	
2601	α76:α80, α78:α78	
2617	α74:k82, α76:k80, α78:k78, α80:k76	
2629	α78:α80	
2645	α74:k84, α76:k82, α78:k80, α80:k78, α82:k76	
2659	α76:k83, α78:k81	
2673	α76:k84, α78:k82, α79:k81, α80:k80, α82:k78	α76:k84, α78:k82
2687	α78:k83, α80:k81	
2701	α78:k84, α79:k83, α80:k82, α81:k81, α82:k80	α76:k86, α78:k84, α80:k82
2715	α80:k83	α76:k87, α78:k85
2729		α78:k86, α80:k84
2731	k80:k83, k81:k82	
2743		α78:k87, α80:k85
2745	k81:k83	k82:k82
2757		α80:k86
2773	k83:k83	k82:k84
2787		k82:k85
2801	k83:k85, k84:k84	k82:k86, k84:k84
2815		k82:k87, k84:k85
2829		k84:k86, k85:k85
2843		k84:k87, k85:k86
2857		k85:k87, k86:k86
2871		k86:k87
2885		k87:k87

α,α-dicyclopropanoic or dienoic; k, keto-monocyclopropanoic or monoenoic mycolic acid. Molecular ions of TDM with intensities ⩾ 30% of the highest intensity observed are listed.

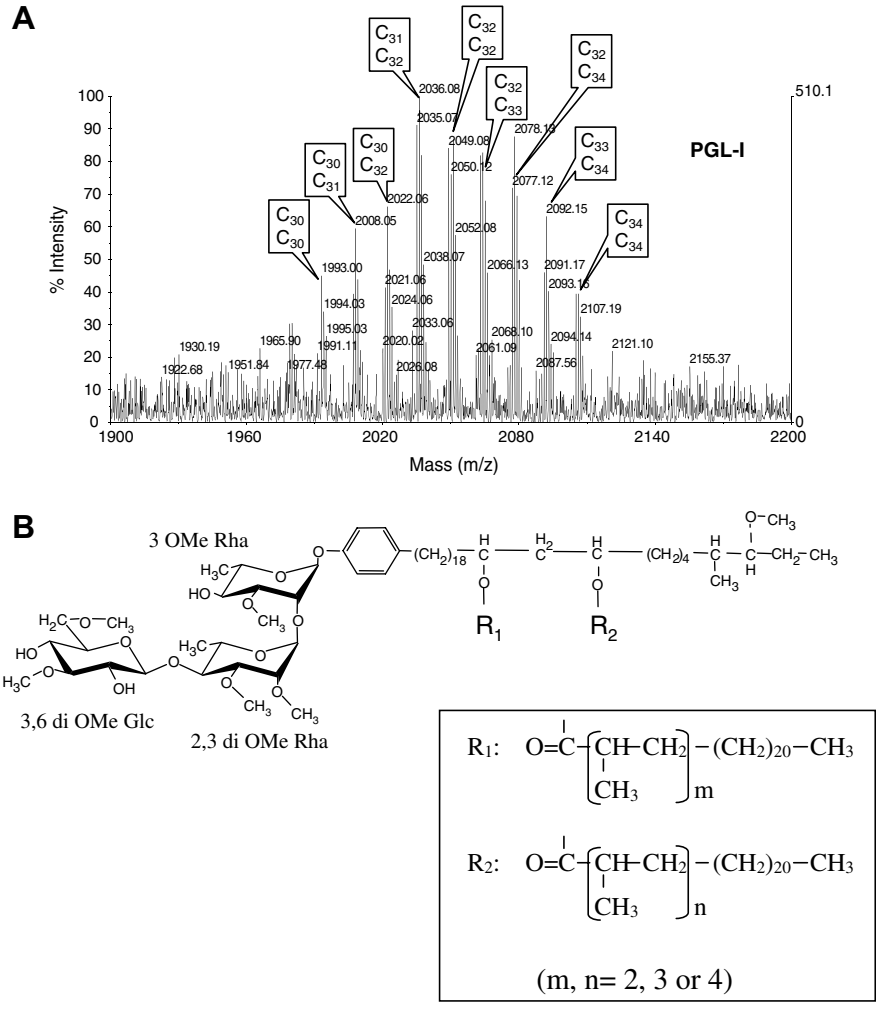

Fig. 3. MALDI-TOF mass spectra of *M. leprae* PGL-I and the deduced chemical structure. (A) The mass spectra of the major cluster ions ranging from around *m/z* 1997 (C30:C30) to 2103 (C34:C34) due to PGL-I separated by solvent fractionation, followed by TLC. C30–C34 shows carbon numbers of two sets of mycocerosate in PGL-I. (B) The chemical structure of *M. leprae* PGL-I. R indicates mycocerosate.

BCG-C can synthesize α-mycolic acids with similar sizes (C74 to C82 centering at C78), however, the subclass composition of keto-mycolate differed. *M. bovis* BCG-C synthesizes multi sub-classes of ketomycolic acids such as *cis*-monoenoic, *cis*-monocyclopropanoic, *cis*-monoenoic *cis*-monocyclopropanoic, *trans*-monoenoic and *trans*-monocyclopropanoic mycolates [21], while *M. leprae* synthesize only *cis*-monoenoic ketomycolate centering at C81 or C83 (Tables 1 and 2) [22]. Recently, the immunological role of mycolic acid has become a point of focus. The *trans*-, but not *cis*-cyclopropanation of mycolic acids in TDM induce massive inflammation in the lesion infected with *M. tuberculosis* and, in this respects, the *trans*-cyclopropane structure may be contributing to virulence [23,24]. Furthermore, recently, it was reported that the Δ*kasB* (a β-keto acyl-ACP synthase gene) mutant strain of *M. tuberculosis* synthesized shorter chain mycolate than the wild ones, and resulted in the loss of keto-mycolic acid *trans*-cyclopropanation. The *kasB* deletion induced the ability of the mutant strain to persist in infected mice for up to 600 days without causing disease [25]. Thus, the lack of *trans*-cyclopropanoic keto-mycolic acid in *M. leprae* may contribute to the low virulency in contrary to substantial quantities found in most of the slow growing pathogenic mycobacteria.

In the search for TDM, we also identified a major cluster ions in M2 fraction. Detailed analyses showed that the ions were derived from phenolic glycolipid (PGL-I), which is a major species specific glycolipid of *M. leprae*. Previously, combined GLC-mass spectroscopic analysis of the trisaccharide of PGL-I had been achieved, but TOF-mass spectrometric data have not been reported [26]. We analyzed the M1 fraction by MALDI TOF mass spectrometry and detected some clusters. Mass spectra of major clusters look like monoglycosyl phthiocerol diester and diglycosyl phthiocerol diester (data not shown), which differ from PGL-I in lacking the terminal sugars [27]. The co-existence of both PGL-I and TDM in armadillo tissue nodules infected with *M. leprae* indicates that these lipids were originated actually from *M. leprae*. Thus, this paper identifies TDM structure of *M. leprae* for the first time and shows that *M. leprae* TDM contains only α-mycolic acids and shorter chain *cis*-monoenoic keto-mycolic acids, exclusively.

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