

References

- 1 WHO study group. Chemotherapy of leprosy for control programs. *World Health Organ Tech Rep Ser*, 1982; **675**.
- 2 WHO. Global leprosy situation. *Wkly Epidemiol Rec*, 2006; **81**: 309–316 (<http://www.who.int/wer>).
- 3 Ebenezer GJ, Norman G, Joseph GA *et al*. Drug resistant *Mycobacterium leprae* – results of mouse footpad studies from a laboratory in South India. *Indian J Lepr*, 2002; **74**: 301–312.
- 4 Cambau E, Perani E, Guillemin I *et al*. Multidrug resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. *Lancet*, 1997; **349**: 103–104.
- 5 Maeda S, Matsuoka M, Nakata N *et al*. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob Agents Chemother*, 2001; **45**: 3635–3639.
- 6 Matsuoka M, Kashiwabara Y, Namisato M. A *Mycobacterium leprae* isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. *Int J Lepr*, 2000; **68**: 452–455.
- 7 Cambau E, Bonnafous P, Perani E *et al*. Molecular detection of rifampicin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. *Clin Infect Dis*, 2001; **34**: 39–45.
- 8 Matsuoka M, Kashiwabara Y, Zhang L *et al*. A second case of multidrug resistant *Mycobacterium leprae* isolated from a Japanese patient with relapsed lepromatous leprosy. *Int J Lepr*, 2003; **71**: 240–243.
- 9 Cambau E, Carthagena L, Chaufour A *et al*. Dihydropteroate synthase mutations in the *folP1* gene predict dapsone resistance in relapsed cases of leprosy. *Clin Infect Dis*, 2006; **42**: 238–241.
- 10 Honore N, Cole ST. Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother*, 1993; **37**: 414–418.
- 11 Honore N, Perani E, Telenti A *et al*. A simple and rapid technique for the detection of rifampin resistance in *Mycobacterium leprae*. *Int J Lepr*, 1993; **61**: 600–604.
- 12 Williams DL, Gillis TP. Molecular detection of resistance in *Mycobacterium leprae*. *Lepr Rev*, 2004; **75**: 118–130.
- 13 Williams DL, Waguespack C, Eisenack K *et al*. Characterization of rifampin resistance in pathogenic *Mycobacteria*. *Antimicrob Agents Chemother*, 1994; **38**: 2380–2386.
- 14 Zhang L, Namisato M, Matsuoka M. A mutation at codon 516 in the *rpoB* gene *Mycobacterium leprae* confers resistance to rifampin. *Int J Lepr*, 2004; **72**: 468–472.
- 15 Kai M, Matsuoka M, Nakata N *et al*. Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. *FEMS Microbiol Lett*, 1999; **177**: 231–235.
- 16 Williams DL, Spring L, Harris E *et al*. Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. *Antimicrob Agents Chemother*, 2000; **44**: 1530–1537.
- 17 Cellona RV, Balagon MVF, dela Cruz EC *et al*. Long-term efficacy of 2 year multiple drug therapy (MDT) in multibacillary (MB) leprosy patients. *Int J Lepr*, 2003; **71**: 308–319.
- 18 Rees RJW, Young DB. Microbiology of leprosy. In: Hastings CR (ed). *Leprosy*, 2nd edn. Churchill Livingstone, Edinburgh, 1994; pp. 49–83.
- 19 Shepard CC. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. *J Exp Med*, 1960; **112**: 45–54.
- 20 Matsuoka M, Zhang L, Budiawan T *et al*. Genotyping of *Mycobacterium leprae* on the basis of polymorphism of TTC repeats for analysis of leprosy transmission. *J Clin Microbiol*, 2004; **42**: 741–745.
- 21 WHO Expert Committee on leprosy, seventh report. WHO, Geneva, 1998; *Tech Rep Ser* 874.
- 22 Gelber RH, Rea TH *et al*. Primary dapsone-resistant Hansen's disease in California. Experience with over 100 *Mycobacterium leprae* isolates. *Arch Dermatol*, 1990; **126**: 1584–1586.
- 23 Guinto RS, Cellona RV *et al*. Primary dapsone-resistant leprosy in Cebu, Philippines. *Int J Lepr*, 1981; **49**: 427–430.
- 24 Jacobson RR, Hastings RC. Primary sulphone resistance in leprosy. *Int J Lepr*, 1978; **46**: 116.
- 25 Warndorff Van Diepen T, Aredath SP, Mengistu G. Dapsone-resistant leprosy in Addis Ababa: a progress report. *Lepr Rev*, 1984; **55**: 149–157.
- 26 Dela Cruz E, Cellona RV, Balagon MVF *et al*. Primary dapsone resistance in Cebu, the Philippines; cause for concern. *Int J Lepr*, 1996; **64**: 253–256.
- 27 Shepard CC, Rees RJ, Levy L *et al*. Susceptibility of strains of *Mycobacterium leprae* isolated prior to 1977 from patients with previously untreated lepromatous leprosy. *Int J Lepr*, 1986; **54**: 11–15.
- 28 Rees RJ. Drug resistance of *Mycobacterium leprae* particularly to DDS. *Int J Lepr*, 1967; **35**: 625–636.
- 29 Peason JMH, Cap JA, Haile GS. Dapsone-resistant leprosy and implications for leprosy control programmes. *Lepr Rev*, 1977; **48**: 83–84.
- 30 Gelber RH, Fukusa K, Byrd S *et al*. A clinical trial of minocycline in lepromatous leprosy. *Br Med J*, 1992; **304**: 91–92.
- 31 Gelber RH, Pardillo FE, Borgos J. Powerful bacterial activity observed in the first clinical trial of Moxifloxacin in leprosy. *Abstracts 46th Interscience Conference on Antimicrobial Agents and Chemotherapy*, 2006; **43**: 371.
- 32 Honore N, Roche P, Grosset J, Cole ST. A method for rapid detection of rifampicin resistant isolates of *Mycobacterium leprae*. *Lepr Rev*, 2001; **72**: 441–448.

- ³³ Musser JM. Antimicrobial agent resistance in Mycobacteria: molecular genetic insights. *Clin Microbiol Rev*, 1995; **8**: 496–514.
- ³⁴ Grosset JH, Guelpa-Lauras CC, Bobin P *et al*. Study of 39 documented relapses of multibacillary leprosy after treatment with rifampicin. *Int J Lepr*, 1989; **57**: 607–614.
- ³⁵ Ellard GA, Pannikar V, Jesudasan K, Christian M. Clofazimin and dapsone compliance in leprosy. *Lepr Rev*, 1988; **59**: 205–213.
- ³⁶ Kyaw K, Khin Saw Aye A. Case of multi-drug resistant leprosy – relapse or re-infection. *Myan J Cur Med Pract*, 2006; **10**: 41–43.
- ³⁷ Cambau E, Jarlier V. Resistance to quinolones in mycobacteria. *Res Microbiol*, 1996; **147**: 52–59.
- ³⁸ Ji B. Rifampicin resistant leprosy: a review and research proposal of a pilot study. *Lepr Rev*, 2002; **73**: 2–8.

Evaluation of major membrane protein-II as a tool for serodiagnosis of leprosy

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Introduction

Leprosy represents a broad-spectrum disease caused by *Mycobacterium leprae*, with lepromatous leprosy at one pole and tuberculoid leprosy at the other end of the pole, depending on the clinical manifestation, which is an ultimate effect of the immunity of the host (Ridley & Jopling, 1996). In all forms of the disease, *M. leprae* induces skin lesions and a chronic progressive peripheral nerve injury, to a lesser or greater extent, which leads to systemic deformity (Stoner, 1979; Job, 1989). Therefore, early detection of *M. leprae* infection is the key to avoiding deformities. The diagnosis of leprosy is based on microscopic detection of acid-fast bacteria (AFB) in skin smears or biopsies, along with clinical and histopathological evaluation. Acid-fast staining requires at least a thousand organisms per gram of tissue for reliable detection (Shepard & McRae, 1968), resulting in an extremely low sensitivity, especially for the tuberculoid form of the disease, where AFB are rare or

Abstract

As serodiagnosis is the easiest way of diagnosing a disease, the utility of *Mycobacterium leprae*-derived major membrane protein-II (MMP-II), one of the immuno-dominant antigens, in the serodiagnosis of leprosy was examined. The percent positivity by an enzyme-linked immunosorbent assay for anti-MMP-II antibody was 82.4% for multi-bacillary leprosy, and the specificity of the test was 90.1%. For pauci-bacillary leprosy where cell-mediated immunity predominates, 39.0% showed positive results. These percentage values were significantly higher than these values obtained for existing phenolic glycolipid-I based methods, suggesting that MMP-II antibody detection would facilitate the diagnosis of leprosy.

absent. Recently, real-time PCR-based methods have been developed (Martinez *et al.*, 2006), but the sensitivity of the test for clinical specimens is still problematic. In developing countries, where leprosy is endemic, diagnosis still relies on clinical observations and easy inexpensive tests. Serodiagnosis is the most easy and tangible way of diagnosing a disease. For leprosy, the only antigen currently used is phenolic glycolipid-I (PGL-I), which is supposed to be *M. leprae* specific. Since the discovery of PGL-I in 1981 by Hunter and Brennan, considerable progress has been made in the development of serological tools (Hunter & Brennan, 1981). In this process, simple user-friendly assays such as Serodia leprae[®], a simple lateral flow test, and dipstick assays, based on PGL-I antigen, have been developed to detect leprosy patients in leprosy-endemic areas (Izumi *et al.*, 1990; Buhner-Sekula *et al.*, 2003). However, these tests seem to be insufficient to detect both multi-bacillary and pauci-bacillary patients, as well as for early diagnosis, and have not been used as widely as would be expected, in the field situations. Therefore, there is a need to look for more sensitive

antigens. To date, various antigens of *M. leprae* have been studied (Hunter *et al.*, 1990), but due to a lack of either specificity or sensitivity, their use has been limited. Major membrane protein-II (MMP-II, #ML2038c, gene name *bfrA*, also known as bacterioferritin) had been identified previously from the cell membrane fraction of *M. leprae* as an antigenic molecule capable of activating both antigen-presenting cells and T cells (Pessolani *et al.*, 1994; Maeda *et al.*, 2005). These findings prompted examination of the role of MMP-II in the humoral responses of patients. Here, MMP-II was expressed and purified in *Escherichia coli* and the use of MMP-II as an antigen for the serodiagnosis of leprosy was evaluated.

Materials and methods

Study population

Sera were obtained with informed consent from healthy individuals, leprosy patients, and tuberculosis patients from Japan. The samples were frozen at -30°C before use. The population studied included multi-bacillary ($n=74$) and pauci-bacillary ($n=77$) leprosy patients, either treated or untreated, from the National Sanatorium Oshimaseishoen, and tuberculosis patients ($n=55$) from Fukujiji Hospital. Classification of leprosy was performed according to WHO recommendations. The home page is available at <http://www.who.int/lep/classification/en/index.html>. Individuals who have not been vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) are unavailable in Japan, due to compulsory vaccination at least once in childhood. Therefore, sera from BCG-vaccinated healthy volunteers ($n=81$) residing in Japan were used as negative controls in the enzyme-linked immunosorbent assay (ELISA) to determine the cut-off value for positivity. The age and sex of normal individuals may not be fully matched with those of patients because some of the details of leprosy patients as well as normal individuals are unknown. This study was approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo.

Expression and purification of protein

The MMP-II gene (ML2038c, *bfrA*) was expressed in *E. coli* as a fusion construct using a pMAL-c2X expression vector (New England Biolabs). The protein was affinity purified to almost homogeneity using an amylose column (data not shown), and used as the antigen for the detection of anti-MMP-II antibody levels in the leprosy patients. The synthetic bovine serum albumin (BSA)-conjugated trisaccharide-phenyl propionate (NTP-BSA) for the detection of PGL-I antibodies was kindly provided by Dr T. Fujiwara, Institute for Natural Sciences, Nara University. The procedure for synthesis of the antigen is described elsewhere (Fujiwara *et al.*, 1984).

Assay method for the detection of antibodies

The ELISA for the detection of anti-MMP-II immunoglobulin G (IgG) antibodies or anti-PGL-I IgM antibodies was performed as described previously, with modifications (Izumi *et al.*, 1990). Ninety-six well plates (Immunosorb, Nunc) were coated overnight, with MMP-II at a concentration of $2\ \mu\text{g mL}^{-1}$ in 0.1 M carbonate buffer (pH 9.5). After blocking with 2% skim milk, the plates were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBST), and human sera (normal or patient's sera) diluted 100-fold were added and incubated at 37°C for 2 h. After washing with PBST, biotinylated anti-human IgG (Vector Laboratories) was added at a concentration of $0.5\ \mu\text{g mL}^{-1}$ and incubated for 1 h. The plates were incubated with reagents from a Vectastain ABC Kit (Vector Laboratories) for 30 min. After further washing with PBST, a substrate solution consisting of $0.2\ \text{mg mL}^{-1}$ of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.02% H_2O_2 in 0.1 M citrate buffer was added until a blue color developed and the OD was measured at 405 nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a common standard serum with an OD value of 0.350. For detecting anti-PGL-I antibody, NTP-BSA was coated at a concentration of $0.5\ \mu\text{g mL}^{-1}$, and the same procedure used to detect anti-MMP-II antibody was followed, except that the secondary antibody used was biotinylated anti-human IgM at a concentration of $1\ \mu\text{g mL}^{-1}$. The volume of all solutions used in the 96-well plate was $100\ \mu\text{L well}^{-1}$.

Statistical analyses

The data were analyzed using MEDCALC software (MedCalc, Belgium). A receiver operator characteristics (ROC) curve was drawn to calculate the cut-off levels. The McNemar test was applied (MMP-II vs. PGL-I test) to determine the *P* value. The *P* value of <0.05 was considered to be statistically significant. The κ value was calculated to determine the agreement between the two tests.

Results and discussion

MMP-II (*bfrA*) has been previously identified as one of the components of *M. leprae* capable of stimulating CD4^+ and CD8^+ T cells (Maeda *et al.*, 2005). In multi-bacillary leprosy, *M. leprae* is widely disseminated and abundant antibody production is observed due to polyclonal B cell activation, while in pauci-bacillary leprosy, the bacilli are usually localized in skin lesions and type 1 T cells are predominantly activated, and so the level of antibodies to *M. leprae* antigens is usually low. However, preliminary experiments showed that the pooled pauci-bacillary leprosy sera reacted to MMP-II on polyvinylidene difluoride (PVDF) membranes.

Table 1. Positivity rates of MMP-II and PGL-I tests in various groups of subjects by an enzyme-linked immunosorbent assay

| | MMP-II | | | | PGL-I | | | | McNemar test (MMP-II vs. PGL) | Rate difference (95% CI) | Inter-rater agreement (κ) |
|-------------------------|--------|----------|------|-----------|--------|----------|------|-----------|----------------------------------|-----------------------------|--|
| | Tested | Positive | % | 95% CI | Tested | Positive | % | 95% CI | | | |
| Multi-bacillary leprosy | 74 | 61 | 82.4 | 71.8–90.3 | 74 | 51 | 68.9 | 57.1–79.2 | $P=0.063$ | 13.5% (-0.7 to 24.2) | 0.14 |
| Pauci-bacillary leprosy | 77 | 30 | 39 | 28.8–50.1 | 77 | 15 | 19.5 | 12.2–29.7 | $P=0.007$ | 19.5% (5.4 to 28.9) | 0.189 |
| Tuberculosis | 55 | 9 | 16.4 | 8.9–28.3 | ND | ND | ND | ND | – | – | – |
| Healthy subjects | 81 | 8 | 9.9 | 5.1–18.3 | 81 | 8 | 9.9 | 5.1–18.3 | – | – | – |

ND, not detected.

Therefore, a systematic study was conducted, measuring the anti-MMP-II antibody IgG levels in the leprosy patients' sera. The study population consisted of multi-bacillary leprosy, pauci-bacillary leprosy, tuberculosis patients, and normal healthy BCG-vaccinated volunteers from Japan. Some of the leprosy patients were already under treatment, so that all patients were not active leprosy patients. The cut-off value of OD 0.130 was defined by an ROC curve analysis (MEDCALC software) using the OD titers from 81 normal individuals and 74 multi-bacillary leprosy patients. Using this cut-off value, it was observed that 61 (82.4%, 95% CI; 71.8–90.3) out of 74 multi-bacillary patients had positive results, and 30 (39.0%, 95% CI; 28.8–50.1) out of 77 pauci-bacillary patients had positive titers (Table 1). The only serological test for leprosy that is currently available is the detection of antibodies to PGL-I of *M. leprae*. The haptenic trisaccharide of PGL-I is known to be *M. leprae* specific, and this trisaccharide unit could be chemically synthesized (Fujiwara et al., 1987). Several reports show the performance of PGL-I for serodiagnosis (Agis et al., 1988; Cho et al., 1991). When the anti-PGL-I IgM antibody levels were examined in the same Japanese leprosy patients by ELISA, it was found that only 68.9% (95% CI; 57.1–79.2) of multi-bacillary patients ($n=74$) and 19.5% (95% CI; 12.2–29.7) of pauci-bacillary patients ($n=77$) showed positive values (Table 1). These percentages were far lower than expected, which may be due to the influence of chemotherapy. However, the percent positivity for anti-MMP-II antibodies was significantly higher than that for anti-PGL-I antibodies of the same sera ($P=0.0008$, McNemar test, $n=152$) when both multi-bacillary and pauci-bacillary leprosy were considered together. When multi-bacillary sera were taken separately, the significance of the MMP-II test was statistically marginal compared with that of the PGL-I test ($P=0.06$), but there was a significant difference ($P=0.007$) between the two tests for pauci-bacillary leprosy (Table 1). The agreement between these two tests was low (κ value for multi-bacillary leprosy: 0.140, κ value for pauci-bacillary leprosy: 0.189), so that when positive titers for either or both PGL-I and MMP-II were considered, the percent positivity increased to 91.9% in multi-bacillary patients and 48.7% in pauci-bacillary patients. The specificity of both the MMP-II and PGL-I tests was 90.1%.

When the anti-MMP-II IgG levels were measured in the normal individuals (BCG-vaccinated), a low level of seropositivity was found, 9.9% (95% CI; 5.1–18.3) ($n=81$). As the amino acid homology between MMP-II protein from *M. leprae* and its homologue in *M. bovis* BCG is 90.6%, it was expected that a higher percentage of normal individuals would be positive. But this result was to the contrary and advantageous to the assay system. Also, tuberculosis patients showed a low positive result of 16.4% (95% CI; 9.9–28.3) ($n=55$). As, the genes encoding MMP-II were conserved between *M. leprae* and *M. tuberculosis*, the low positivity in tuberculosis was unexpected. The exact reason for the low positivity in tuberculosis patients is not clear, but might be due to slight conformational differences in the antibody recognition site on MMP-II between *M. leprae* and *M. tuberculosis*. More intensive studies need to be carried out using active tuberculosis patients and native MMP-II protein derived from *M. tuberculosis* to fully realize the significance of MMP-II homologue in the detection of tuberculosis.

There is only one report showing that the sera from leprosy patients had a higher IgG titer to MMP-II, regardless of the clinical type of leprosy (Deshpande et al., 1995). However, their study was carried out with a limited number of individuals ($n=10$ in each group), and so, here, a more systematic study has been conducted for evaluating the expediency of measuring anti-MMP-II antibody levels. In addition, when monitoring of patients on multidrug treatment was conducted ($n=4$) for a period of 2 years, there was a definite decline in the MMP-II antibody levels after chemotherapy (data not shown). Further intensive studies have to be carried out with a larger cohort of patients to fully realize the significance of MMP-II in the monitoring of chemotherapy.

This report showed that MMP-II could contribute to the sero-detection of multi-bacillary as well as pauci-bacillary leprosy patients. Further study will be pursued to evaluate its efficacy for serodiagnosis in developing countries and for the development of user-friendly tools.

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References

- Agis F, Schlich P, Cartel JL, Guidi C & Bach MA (1988) Use of anti-*M. leprae* phenolic glycolipid-I antibody detection for early diagnosis and prognosis of leprosy. *Int J Lepr Other Mycobact Dis* **56**: 527–535.
- Buhrer-Sekula S, Smits HL, Gussenhoven GC, Leeuwen van J, Amador S, Fujiwara T, Klatser PR & Oskam L (2003) Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. *J Clin Microbiol* **41**: 1991–1995.
- Cho SN, Cellona RV, Fajardo TT Jr, Abalos RM, dela Cruz EC, Walsh GP, Kim JD & Brennan PJ (1991) Detection of phenolic glycolipid-I antigen and antibody in sera from new and relapsed lepromatous patients treated with various drug regimens. *Int J Lepr Other Mycobact Dis* **59**: 25–31.
- Deshpande RG, Khan MB, Bhat DA & Navalkar RG (1995) Immunoaffinity chromatographic isolation of a high molecular weight seroreactive protein from *Mycobacterium leprae* cell sonicate. *FEMS Immunol Med Microbiol* **11**: 163–169.
- Fujiwara T, Hunter SW, Cho SN, Aspinall GO & Brennan PJ (1984) Chemical synthesis and serology of disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. *Infect Immun* **43**: 245–252.
- Fujiwara T, Aspinall GO, Hunter SW & Brennan PJ (1987) Chemical synthesis of the trisaccharide unit of the species-specific phenolic glycolipid from *Mycobacterium leprae*. *Carbohydr Res* **163**: 41–52.
- Hunter SW & Brennan PJ (1981) A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. *J Bacteriol* **147**: 728–735.
- Hunter SW, Rivoire B, Mehra V, Bloom BR & Brennan PJ (1990) The major native proteins of the leprosy bacillus. *J Biol Chem* **265**: 14065–14068.
- Izumi S, Fujiwara T, Ikeda M, Nishimura Y, Sugiyama K & Kawatsu K (1990) Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field. *J Clin Microbiol* **28**: 525–529.
- Job CK (1989) Nerve damage in leprosy. *Int J Lepr Other Mycobact Dis* **57**: 532–539.
- Maeda Y, Mukai T, Spencer J & Makino M (2005) Identification of an immunomodulating agent from *Mycobacterium leprae*. *Infect Immun* **73**: 2744–2750.
- Martinez AN, Britto CFPC, Nery JAC, Sampaio EP, Jardim MR, Sarno EN & Moraes MO (2006) Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. *J Clin Microbiol* **44**: 3154–3159.
- Pessolani MC, Smith DR, Rivoire B, McCormick J, Hefta SA, Cole ST & Brennan PJ (1994) Purification, characterization, gene sequence, and significance of a bacterioferritin from *Mycobacterium leprae*. *J Exp Med* **180**: 319–327.
- Ridley DS & Jopling WH (1996) Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis* **34**: 255–273.
- Shepard CC & McRae DH (1968) A method for counting acid-fast bacteria. *Int J Lepr Other Mycobact Dis* **36**: 78–82.
- Stoner GL (1979) Importance of the neural predilection of *Mycobacterium leprae* in leprosy. *Lancet* **2**: 994–996.

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*.

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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 observa-

tions 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.

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 H₂SO₄ 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/H₂SO₄ (10:20:1, by vol.) [14]. Mycolic acid methyl esters from each

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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

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 μ l of TDM or TMM samples (5 μ g) in chloroform/methanol (2:1 v/v) solution and 5 μ l of the matrix solution were mixed, and 1.5 μ l 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

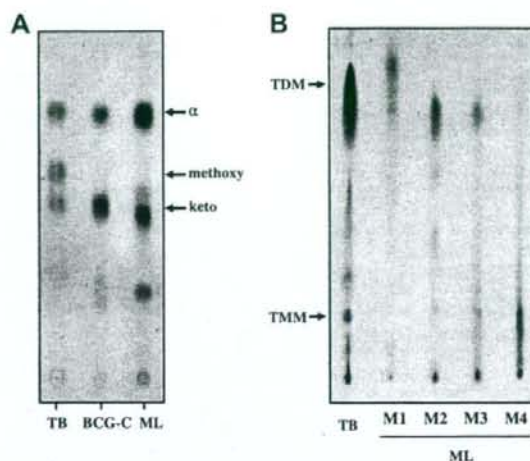


Fig. 1. Thin-layer chromatograms (TLC) of solvent extracts from tetrahydrofuran soluble fraction and mycolic acid methyl ester subclasses of *M. tuberculosis*, *M. bovis* BCG Connaught, and *M. leprae*. (A) TLC of mycolic acid methyl ester subclasses with the solvent system of benzene/methanol/ H_2SO_4 (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.

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.

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

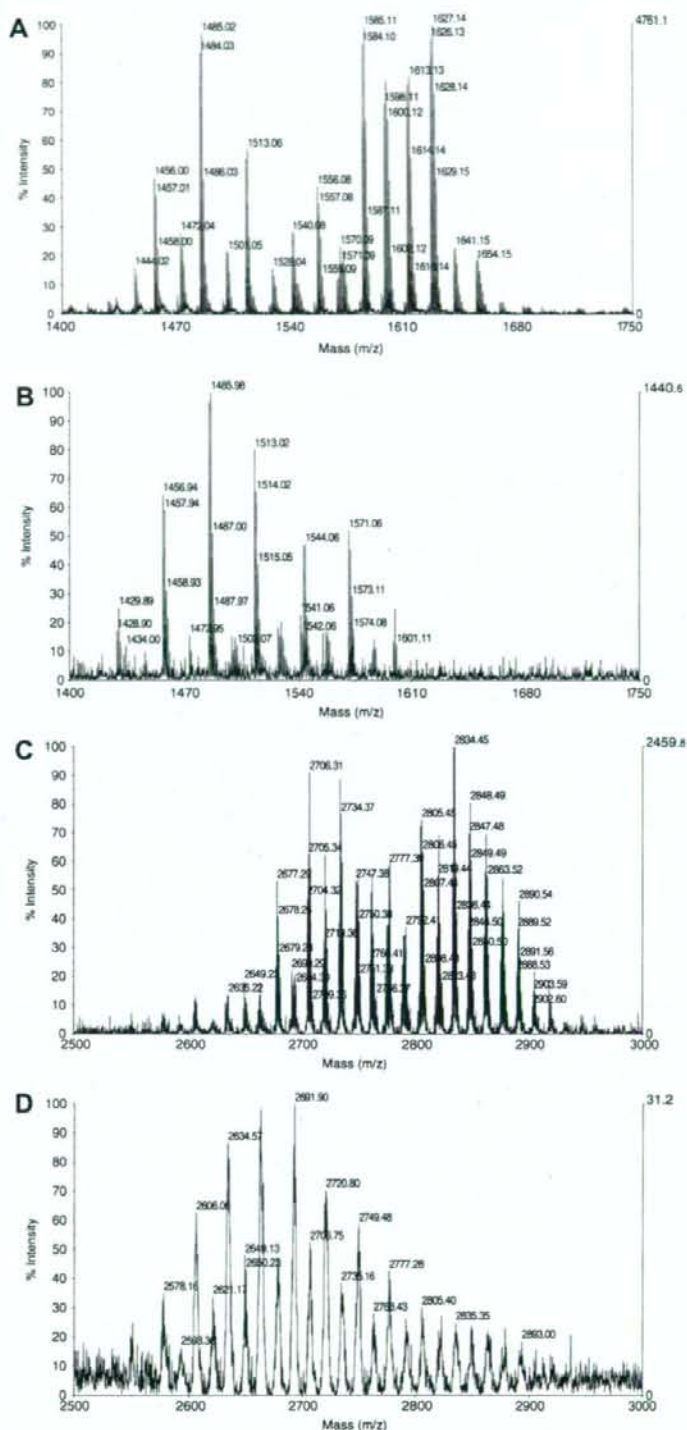


Fig. 2. MALDI-TOF-MS spectra of TMM and TDM. (A) TMM of *M. bovis* BCG Connaught, (B) TMM of *M. leprae* Thai 53, (C) TDM of *M. bovis* BCG Connaught, (D) TDM of *M. leprae* Thai 53.

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 |
| <i>M. leprae</i> (Thai 53) | I | 1427 | 1455 | 1483 | 1497 | 1511 | 1525 | 1539 | 1553 | | | | | | | | | | |
| | II | | 1471 | | 1499 | | 1527 | 1541 | 1555 | 1569 | 1583 | 1597 | | | | | | | |
| <i>M. bovis</i> BCG (Connaught) | I | | 1455 | 1469 | 1483 | | 1511 | | 1539 | | | | | | | | | | |
| | II | | | | | | | | 1555 | 1569 | 1583 | 1597 | 1611 | 1625 | 1639 | 1653 | 1667 | 1681 | |

^aI; 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) | <i>M. leprae</i> (Thai 53) | <i>M. bovis</i> BCG (Connaught) |
|---------------------------|---|---|
| 2573 | α 76: α 78 | |
| 2601 | α 76: α 80, α 78: α 78 | |
| 2617 | α 74:k82, α 76:k80, α 78:k78, α 80:k76 | |
| 2629 | α 78: α 80 | |
| 2645 | α 74:k84, α 76:k82, α 78:k80, α 80:k78, α 82:k76 | |
| 2659 | α 76:k83, α 78:k81 | |
| 2673 | α 76:k84, α 78:k82, α 79:k81, α 80:k80, α 82:k78 | α 76:k84, α 78:k82 |
| 2687 | α 78:k83, α 80:k81 | |
| 2701 | α 78:k84, α 79:k83, α 80:k82, α 81:k81, α 82:k80 | α 76:k86, α 78:k84, α 80:k82 |
| 2715 | α 80:k83 | α 76:k87, α 78:k85 |
| 2729 | | α 78:k86, α 80:k84 |
| 2731 | k80:k83, k81:k82 | |
| 2743 | | α 78:k87, α 80:k85 |
| 2745 | k81:k83 | k82:k82 |
| 2757 | | α 80: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 $\geq 30\%$ of the highest intensity observed are listed.

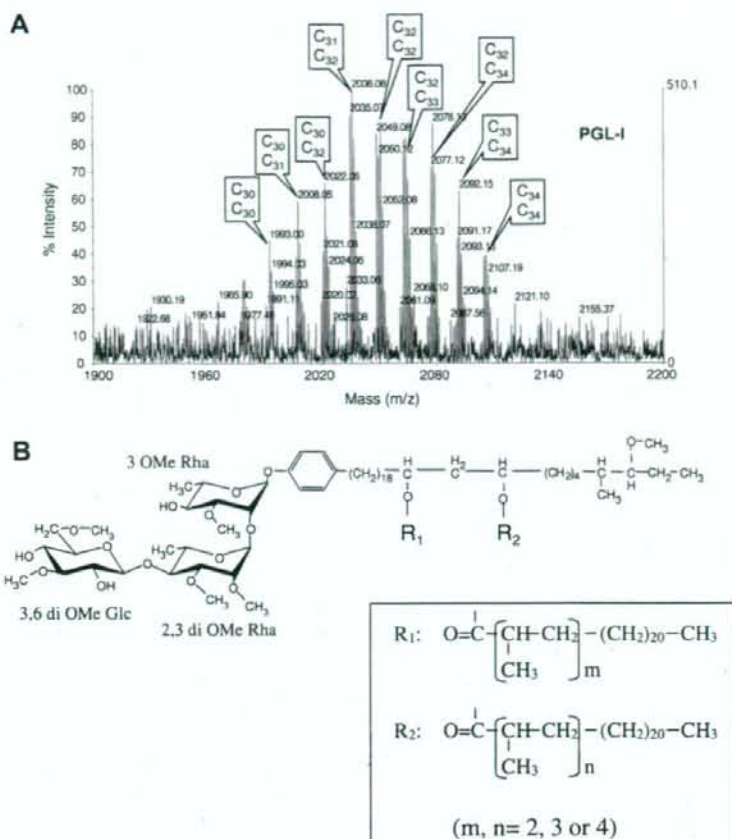


Fig. 3. MALDI-TOF mass spectra of *M. leprae* PGL-I and the deduced chemical structure. (A) The mass spectra of the major cluster ions ranging from around m/z 1997 (C30:C30) to 2103 (C34:C34) due to PGL-I separated by solvent fractionation, followed by TLC. C30–C34 shows carbon numbers of two sets of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

- [1] Minnikin, D.E. (1982) Presented at the Part I. Physiology of the mycobacteria. In *The Biology of the Mycobacteria*.
- [2] Rastogi, N., Legrand, E. and Sola, C. (2001) The mycobacteria: an introduction to nomenclature and pathogenesis. *Rev. Sci. Tech.* 20, 21–54.
- [3] Ryll, R., Kumazawa, Y. and Yano, I. (2001) Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids – a review. *Microbiol. Immunol.* 45, 801–811.
- [4] Fujiwara, N. (1997) Distribution of antigenic glycolipids among *Mycobacterium tuberculosis* strains and their contribution to virulence. *Kekkaku* 72, 193–205.
- [5] Coelho, E.A., Tavares, C.A., de Melo Lima, K., Silva, C.L., Rodrigues Jr., J.M. and Fernandes, A.P. (2006) *Mycobacterium hsp65* DNA entrapped into TDM-loaded PLGA microspheres induces protection in mice against *Leishmania (Leishmania) major* infection. *Parasitol. Res.* 98, 568–575.
- [6] Oiso, R., Fujiwara, N., Yamagami, H., Maeda, S., Matsumoto, S., Nakamura, S., Oshitani, N., Matsumoto, T., Arakawa, T. and Kobayashi, K. (2005) Mycobacterial trehalose 6,6'-dimycolate preferentially induces type 1 helper T cell responses through signal transducer and activator of transcription 4 protein. *Microb. Pathog.* 39, 35–43.
- [7] Hunter, R.L., Olsen, M.R., Jagannath, C. and Actor, J.K. (2006) Multiple roles of cord factor in the pathogenesis of primary, secondary, and cavity tuberculosis, including a revised description of the pathology of secondary disease. *Ann. Clin. Lab. Sci.* 36, 371–386.
- [8] Dhariwal, K.R., Yang, Y.-M., Fales, H.M. and Goren, M.B. (1987) Detection of trehalose monomycolate in *Mycobacterium leprae* grown in armadillo tissues. *J. Gen. Microbiol.* 133, 201–209.
- [9] Prome, J.C., Lacave, C.A., Ahibo-Coffy and Savagnac, A. (1976) Separation of structural studies of the molecular species of monomycolates and dimycolates of alpha-D-trehalose present in *Mycobacterium phlei*. *Eur. J. Biochem.* 63, 543–552.
- [10] Wang, L., Izumi, S., He, H., Fujiwara, N., Saita, N., Yano, I., Kobayashi, K. and Tatsumi, N. (1999) Serodiagnosis of Hansen's disease/leprosy by enzyme-linked immunosorbent assay using cord factor (trehalose 6,6'-dimycolate) as an antigen. *Nihon Hansenbyo Gakkai Zasshi* 68, 165–174.
- [11] Goren, M.B., Brokl, O. and Roller, P. (1979) Cord factor (trehalose-6,6'-dimycolate) of in vivo-derived *Mycobacterium lepraemurium*. *Biochim. Biophys. Acta* 574, 70–78.
- [12] Fujita, Y., Ogata, H. and Yano, I. (2005) Clinical evaluation of serodiagnosis of active tuberculosis by multiple-antigen ELISA using lipids from *Mycobacterium bovis* BCG Tokyo 172. *Clin. Chem. Lab. Med.* 43, 1253–1262.
- [13] Sasaki, N., Kawatsu, K., Tsutsumi, S., Gidoh, M., Nakagawa, H., Kashiwabara, Y., Matsuki, G. and Endo, H. (1997) Pathological investigation of armadillos infected with *Mycobacterium leprae*. *Nihon Hansenbyo Gakkai Zasshi* 66, 227–235.
- [14] Fujita, Y., Naka, T., Doi, T. and Yano, I. (2005) Direct molecular mass determination of trehalose monomycolate from 11 species of mycobacteria by MALDI-TOF mass spectrometry. *Microbiology* 151, 1443–1452.
- [15] Fujita, Y., Naka, T., McNeil, M.R. and Yano, I. (2005) Intact molecular characterization of cord factor (trehalose 6,6'-dimycolate) from nine species of mycobacteria by MALDI-TOF mass spectrometry. *Microbiology* 151, 3403–3416.
- [16] Minnikin, D.E. and Polgar, N. (1967) Synthesis of methyl 3,12,15-trimethylidocosanoate and 3,15-dimethylidocosanoate. *J. Chem. Soc.* 7, 575–577.
- [17] Yuan, Y., Crane, D.C., Musser, J.M., Sreevatsan, S. and Barry 3rd., C.E. (1997) MMAS-1, the branch point between *cis*- and *trans*-cyclopropane-containing oxygenated mycolates in *Mycobacterium tuberculosis*. *J. Biol. Chem.* 272, 10041–10049.
- [18] Draper, P., Dobson, G., Minnikin, D.E. and Minnikin, S.M. (1982) The mycolic acids of *Mycobacterium leprae* harvested from experimentally infected nine-banded armadillos. *Ann. Microbiol.* 133, 39–47.
- [19] Takayama, K., Wang, C. and Besra, G.S. (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* 18, 81–101.
- [20] Minnikin, D.E., Dobson, G., Goodfellow, M., Draper, P. and Magnusson, M. (1985) Quantitative comparison of the mycolic and fatty acid compositions of *Mycobacterium leprae* and *Mycobacterium goodii*. *J. Gen. Microbiol.* 131, 2013–2021.
- [21] Watanabe, M., Aoyagi, Y., Mitome, H., Fujita, T., Naoki, H., Ridell, M. and Minnikin, D.E. (2002) Location of functional groups in mycobacterial meromycolate chains; the recognition of new structural principles in mycolic acids. *Microbiology* 148, 1881–1902.
- [22] Watanabe, M., Aoyagi, Y., Ridell, M. and Minnikin, D.E. (2001) Separation and characterization of individual mycolic acids in representative mycobacteria. *Microbiology* 147, 1825–1837.
- [23] Glickman, M.S., Cahill, S.M. and Jacobs Jr., W.R. (2001) The *Mycobacterium tuberculosis* *cmaA2* gene encodes a mycolic acid *trans*-cyclopropane synthetase. *J. Biol. Chem.* 276, 2228–2233.
- [24] Yuan, Y., Zhu, Y., Crane, D.D. and Barry 3rd., C.E. (1998) The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 29, 1449–1458.
- [25] Bhatt, A., Fujiwara, N., Bhatt, K., Gurcha, S.S., Kremer, L., Chen, B., Chan, J., Porcelli, S.A., Kobayashi, K., Besra, G.S. and Jacobs Jr., W.R. (2007) Deletion of *kasB* in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proc. Natl. Acad. Sci. USA* 104, 5157–5162.
- [26] Hunter, S.W. and Brennan, P.J. (1981) A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. *J. Bacteriol.* 147, 728–735.
- [27] Daffe, M. and Laneelle, M.A. (1989) Diglycosyl phenol phthiocerol diester of *Mycobacterium leprae*. *Biochim. Biophys. Acta* 1002, 333–337.

Characterization of the Fucosylation Pathway in the Biosynthesis of Glycopeptidolipids from *Mycobacterium avium* Complex[†]

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The cell envelopes of several species of nontuberculous mycobacteria, including the *Mycobacterium avium* complex, contain glycopeptidolipids (GPLs) as major glycolipid components. GPLs are highly antigenic surface molecules, and their variant oligosaccharides define each serotype of the *M. avium* complex. In the oligosaccharide portion of GPLs, the fucose residue is one of the major sugar moieties, but its biosynthesis remains unclear. To elucidate it, we focused on the 5.0-kb chromosomal region of the *M. avium* complex that includes five genes, two of which showed high levels of similarity to the genes involved in fucose synthesis. For the characterization of this region by deletion and expression analyses, we constructed a recombinant *Mycobacterium smegmatis* strain that possesses the *rfA* gene of the *M. avium* complex to produce serovar 1 GPL. The results revealed that the 5.0-kb chromosomal region is responsible for the addition of the fucose residue to serovar 1 GPL and that the three genes *mdhA*, *merA*, and *gfd* are indispensable for the fucosylation. Functional characterization revealed that the *gfd* gene encodes a glycosyltransferase that transfers a fucose residue via 1→3 linkage to a rhamnose residue of serovar 1 GPL. The other two genes, *mdhA* and *merA*, contributed to the formation of the fucose residue and were predicted to encode the enzymes responsible for the synthesis of fucose from mannose based on their deduced amino acid sequences. These results indicate that the fucosylation pathway in GPL biosynthesis is controlled by a combination of the *mdhA*, *merA*, and *gfd* genes. Our findings may contribute to the clarification of the complex glycosylation pathways involved in forming the oligosaccharide portion of GPLs from the *M. avium* complex, which are structurally distinct.

Mycobacteria have a unique multilayer cell envelope composed of peptidoglycan, arabinogalactan, mycolic acids, and an outer layer that contains abundant species-specific glycolipids. It is thought that these cell wall characteristics allow mycobacteria to survive in host cells (8, 11). Among the glycolipids present in the outer layer of the cell envelope, glycopeptidolipids (GPLs) are recognized as highly antigenic surface molecules in several species of nontuberculous mycobacteria, including the *Mycobacterium avium* complex (29). The common structure in which the fatty acyl-tetrapeptide core is glycosylated with both 6-deoxy-talose (6-d-Tal) and *O*-methyl-rhamnose (*O*-Me-Rha), termed core GPLs, is present in all types of GPLs (2, 4, 12). The core GPLs are further glycosylated with a Rha residue or various oligosaccharides linked to the 6-d-Tal residue. These glycosylation events give rise to structural variations, especially in the GPLs from the *M. avium* complex, that are responsible for the creation of serotypes, and they are also known as serovar-specific GPLs (ssGPLs). The ssGPLs define approximately 30 serotypes, some of which are found predominantly in isolates from patients. For example, the serotypes 1, 4, 6, and 8 are frequently isolated from AIDS patients coinfecting with the *M. avium* complex (17, 30). In addition, ssGPLs are associated with host responses to infection and func-

tion as agonists of cell surface receptors such as Toll-like receptor 2 (28).

The biosynthesis of GPLs, including methylation, acetylation, and peptide synthesis, has been investigated mainly with *M. smegmatis*, but it has not been fully elucidated (6, 15, 21, 23). We have recently identified the glycosyltransferase genes involved in the formation of core GPLs in *M. smegmatis* and *M. avium* (19), but the glycosylation pathways of ssGPLs have not been clarified, except for that of serovar 1 GPL (14). The presence of a Rha residue linked to 6-d-Tal, as observed with serovar 1 GPL, is structurally required for the formation of all types of ssGPLs. The Rha residue is further extended by the subsequent addition of one of the three different sugars, Rha, fucose (Fuc), or glucose, to form the structural group of ssGPLs (9). In these groups, the Fuc-containing ssGPLs, such as serovar 2, 3, 4, and 9 GPLs, shown in Table 1, comprise the major group in the ssGPLs of the *M. avium* complex, and serovar 2 GPL is the basic structure for these ssGPLs (9). The biosynthetic pathway for the Fuc-containing ssGPLs is still unclear. In the *M. avium* complex, a 22- to 25-kb chromosomal region with the ability to synthesize the serovar 2 GPL has been identified and cloned from two strains (4, 5). However, the key genes responsible for the formation of the Fuc residue of serovar 2 GPL have not been identified in this chromosomal region. In this study, we have focused on a 5.0-kb segment that includes the genes predicted to be associated with Fuc synthesis and have characterized their functions in the biosynthesis of GPLs in the *M. avium* complex.

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TABLE 1. Oligosaccharide structures of Fuc-containing ssGPLs

| Serotype | Oligosaccharide ^a |
|----------|--|
| 2 | 2,3-di-O-Me- α -L-Fuc-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)-L-6-d-Tal |
| 4 | 4-O-Me- α -L-Rha-(1 \rightarrow 4)-2-O-Me- α -L-Fuc-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)-L-6-d-Tal |
| 3 | 2,3-di-O-Me- α -L-Fuc-(1 \rightarrow 4)- β -D-GlcA-(1 \rightarrow 4)-2,3-di-O-Me- α -L-Fuc-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)-L-6-d-Tal |
| 9 | 4-O-Ac-2,3-di-O-Me- α -L-Fuc-(1 \rightarrow 4)- β -D-GlcA-(1 \rightarrow 4)-2,3-di-O-Me- α -L-Fuc-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)-L-6-d-Tal |

^a The oligosaccharide structures are from reference 9. GlcA, glucuronic acid; Ac, acetyl.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation. The bacterial strains and vectors used and constructed in this study are listed in Table 2. *M. avium*, used for the isolation of chromosomal DNA, was grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 supplemented with 10% Middlebrook ADC enrichment broth (BBL). Recombinant *M. smegmatis* strains for GPL production were cultured in Luria-Bertani broth with 0.2% Tween 80. The isolation of DNA, transformation, and PCR were carried out as previously described (20). *Escherichia coli* strain DH5 α was used for the routine manipulation and propagation of plasmid DNA. The following antibiotics were added as required: kanamycin, 50 μ g/ml for *E. coli* and 25 μ g/ml for *M. smegmatis*; hygromycin B, 150 μ g/ml for *E. coli* and 75 μ g/ml for *M. smegmatis*. Oligonucleotide primers used for PCR are available on request.

Construction of the integrating mycobacterial vector (pYM301). The site-specific integrating mycobacterial vector pYM301 was constructed from parts of pYUB854, pMV306kan, and pMV261 (3, 24, 27). To replace the *oriM* region of pMV261 with the region needed for integration, a fragment containing *attP* and *int* was amplified from pMV306kan DNA using the primers INT-S and INT-A, digested with the respective restriction enzyme, and cloned into the XbaI-MluI site of pMV261 to give pMV301kan. The hygromycin-resistant cassette, a selective marker of integration, was excised from pYUB854 with XbaI and NheI and inserted into the NheI-SpeI site of pMV301kan to obtain *hyg* instead of *kan*. Because the resulting plasmid had restriction sites for EcoRI, BamHI, and PstI outside of the multicloning site, it was disrupted in turn by PCR to create pYM301 by using the following primers: 301ECO-D and 301ECO-U for disruption of the EcoRI site, 301PST-D and 301PST-U for disruption of the PstI site, and 301BAM-D and 301BAM-U for disruption of the BamHI site.

Construction of expression vectors. Previous studies have shown that the clustered *gfc-gfd* region is about 5.0 kb long and contains five genes, designated *gfc*, *mdhA*, *merA*, *mtfF*, and *gfd* (13). To express these five genes as one operon, the 5.0-kb segment was obtained as a PstI-EcoRI fragment to be inserted

into the expression cassette of pMV261. Prior to cloning into pMV261, it was necessary to clone the 5.0-kb segment into pUC18 to confirm the DNA sequence. Since we could not directly clone the 5.0-kb segment as one PCR-amplified fragment, three divided fragments were amplified from the genomic DNA of *M. avium* JATA51-01 by using following primers: GTFC-S and HA for the 2.0-kb PstI-HindIII fragment, HS and KA for the 1.0-kb HindIII-KpnI fragment, and KS and GTFD-A for the 2.0-kb KpnI-EcoRI fragment (Fig. 1A). These three fragments then were combined in pUC18 as a PstI-EcoRI fragment to give pUCgtfCD (Fig. 1A). A 5.0-kb PstI-EcoRI fragment was excised from pUCgtfCD and inserted into the PstI-EcoRI site of pMV261 to create pMVgtfCD, which expressed the above-described five genes (Fig. 1B).

Deletion of each gene from the *gfc-gfd* region was performed as follows. The expression vectors were constructed from pUCgtfCD by PCR using following primers: 18PST-U and MDHTA-S for the deletion of *gfc*, MDHTA-U and MDHTA-D for the deletion of *mdhA*, MERA-U and MERA-D for the deletion of *merA*, MTFE-U and MTFE-D for the deletion of *mtfF*, and MTFE-A and 18ECO-D for the deletion of *gfd*. The PCR products were digested with each restriction enzyme and were ligated. The PstI-EcoRI fragment was excised from each resulting plasmid and was cloned into the same restriction sites of pMV261 to give pMV Δ gtfC, pMV Δ mdhA, pMV Δ merA, pMV Δ mtfF, and pMV Δ gfd (Fig. 1B).

The two *M. avium* genes *rfA* and *gfd* were amplified from genomic DNA of *M. avium* JATA51-01 by using the following primers: RTFA-S and RTFA-A for *rfA* and GTFD-S and GTFD-PA for *gfd*. The PCR products were digested with each restriction enzyme and were cloned into the corresponding site of pYM301 and pMV261 to give pYMrfA-int and pMVgfd, respectively.

To construct the vector for the simultaneous expression of *rfA*, *mdhA*, and *merA*, the *Hpa*I site of pYMrfA-int was replaced with an *Afl*III site by PCR using AFL-U and AFL-D to give pYMrfA-int-Afl. The *mdhA* and *merA* genes were amplified as one operon from genomic DNA of *M. avium* JATA51-01 by using the primers MDHTA-S2 and MERA-A. The PCR product was digested with

TABLE 2. Bacterial strains and vectors used in this study

| Strain or vector | Characteristic(s) | Source or reference |
|---|--|---------------------|
| Bacterial strains | | |
| <i>E. coli</i> DH5 α | Cloning host | 25 |
| <i>M. smegmatis</i> mc ² 155 | Expression host | |
| <i>M. avium</i> JATA51-01 (ATCC 25291) | Source of the 5.0-kb region containing <i>gfc</i> , <i>mdhA</i> , <i>merA</i> , <i>mtfF</i> , and <i>gfd</i> | |
| Vectors | | |
| pUC18 | <i>E. coli</i> cloning vector | This study |
| pBluescript II SK(+) | <i>E. coli</i> cloning vector | |
| pYM301 | Site-specific integrating mycobacterial vector carrying the <i>hsp60</i> promoter cassette | 3 |
| pYUB854 | Source of pYM301 | 24 |
| pMV306kan | Source of pYM301 | 27 |
| pMV261 | <i>E. coli</i> - <i>Mycobacterium</i> shuttle vector carrying the <i>hsp60</i> promoter cassette | This study |
| pMVgtfCD | pMV261 with <i>gfc</i> , <i>mdhA</i> , <i>merA</i> , <i>mtfF</i> , and <i>gfd</i> | This study |
| pMV Δ gtfC | pMV261 with <i>mdhA</i> , <i>merA</i> , <i>mtfF</i> , and <i>gfd</i> | This study |
| pMV Δ mdhA | pMV261 with <i>gfc</i> , <i>merA</i> , <i>mtfF</i> , and <i>gfd</i> | This study |
| pMV Δ merA | pMV261 with <i>gfc</i> , <i>mdhA</i> , <i>mtfF</i> , and <i>gfd</i> | This study |
| pMV Δ mtfF | pMV261 with <i>gfc</i> , <i>mdhA</i> , <i>merA</i> , and <i>gfd</i> | This study |
| pMV Δ gfd | pMV261 with <i>gfc</i> , <i>mdhA</i> , <i>merA</i> , and <i>mtfF</i> | This study |
| pMVgfd | pMV261 with <i>gfd</i> | This study |
| pYMrfA-int | pYM301 with <i>rfA</i> | This study |
| pYMrfA-mdhA-merA-int | pYM301 with <i>rfA</i> , <i>mdhA</i> , and <i>merA</i> | This study |

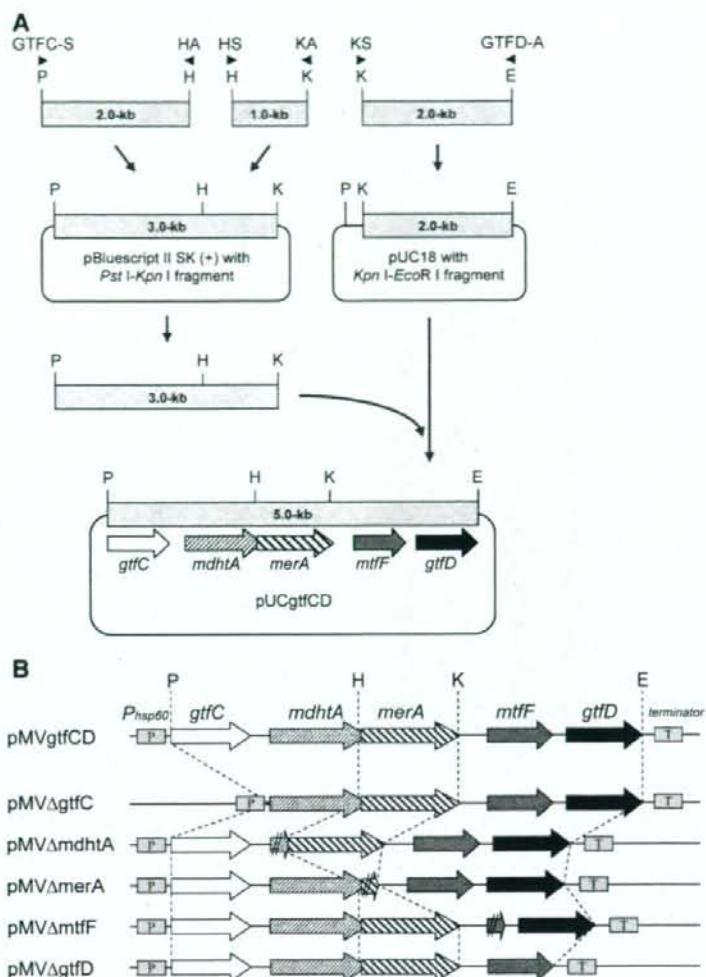


FIG. 1. Schematic presentation of the cloning procedure for the 5.0-kb *gtfC-gtfD* region (A) and its gene-deleted constructs, which were inserted into an expression cassette made up of the *hsp60* promoter and the terminator of pMV261 (B). The primers used for PCR amplification of the three fragments are indicated by filled triangles. In pMVΔgtfC and pMVΔgtfD, the genes *gtfC* and *gtfD* were completely deleted from the *gtfC-gtfD* region of pMVgtfCD. In-frame deletions were designed for the construction of the expression cassettes of pMVΔmdhA, pMVΔmerA, and pMVΔmtfF to prevent the polar effect on each downstream gene. P, PstI; H, HindIII; K, KpnI; E, EcoRI.

each restriction enzyme and cloned into the PstI-AflIII site of pYMrfA-int-Afl to give pYMrfA-mdhA-merA-int.

Isolation and purification of GPLs. Whole-lipid extracts were isolated from harvested bacterial cells that had been mixed with $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1, vol/vol) for several hours at room temperature. The extracts obtained from the organic phase were separated from the aqueous phase and evaporated to dryness. To remove the lipid components except for GPLs, the whole-lipid extracts were subjected to mild alkaline hydrolysis as previously described (20, 21). For analytical thin-layer chromatography (TLC), crude GPLs from equal amounts of harvested bacterial cells were spotted on silica gel 60 plates (Merck) using $\text{CHCl}_3\text{-CH}_3\text{OH}$ (9:1, vol/vol) as the solvent and were visualized by spraying with 10% H_2SO_4 and then charring. Purified GPLs were prepared from crude GPLs by preparative TLC on the same plates and were extracted from the bands corresponding to each GPL. Perdeuteriomethylation for determination of the linkage position of sugar moieties was carried out as previously described (7, 10, 14).

GC-MS and MALDI-TOF analyses. For the monosaccharide analysis, crude GPLs from equal amounts of harvested cells were hydrolyzed in 2 M trifluoroacetic acid (2 h, 120°C), and the released sugars were reduced with sodium tetradeuterborate and then were acetylated with pyridine-acetic anhydride (1:1, vol/vol) at room temperature overnight. The resulting alditol acetates were separated and analyzed by gas chromatography-mass spectrometry (GC-MS) on a TRACE DSQ (Thermo Electron) equipped with an SP-2380 column (SUPELCO) using helium gas. The temperature program was from 52 to 172°C with 40°C/min increments and then from 172 to 250°C with 3°C/min increments. To determine the total mass of the purified GPLs, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra (in the positive mode) were acquired on a QSTAR XL (Applied Biosystems) with a pulse laser emitting at 337 nm. Samples mixed with 2,5-dihydroxybenzoic acid as the matrix were analyzed in the reflectron mode with an accelerating voltage of 20 kV and operating in positive-ion mode.

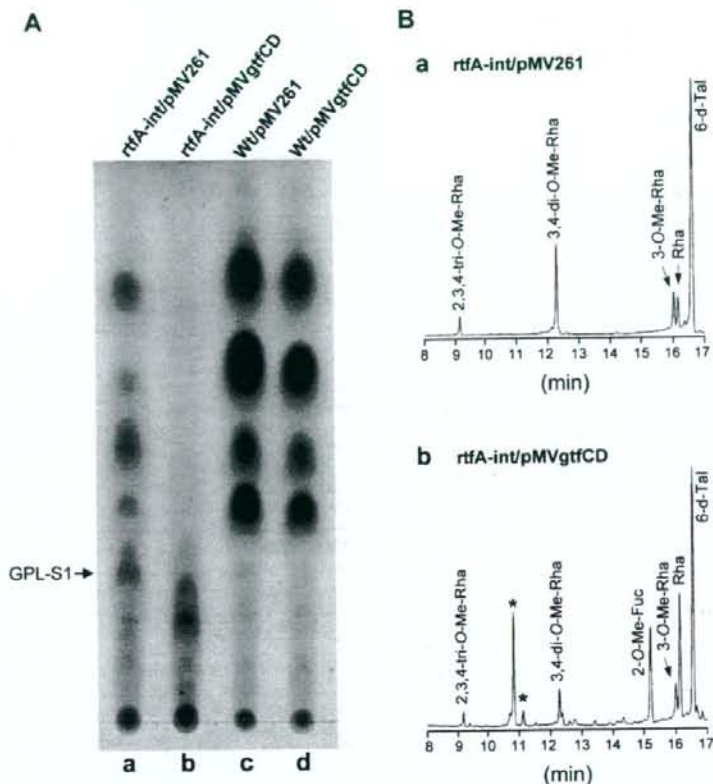


FIG. 2. Functional analyses of the *gtfC-gtfD* region. (A) TLC analysis of crude GPL extracts from *rtfA-int/pMV261* (lane a), *rtfA-int/pMVgtfCD* (lane b), *Wt/pMV261* (lane c), and *Wt/pMVgtfCD* (lane d). The total lipid fraction after mild alkaline hydrolysis was spotted on plates and was developed in $\text{CHCl}_3\text{-CH}_3\text{OH}$ (9:1 [vol/vol]). (B) GC-MS analyses of alditol acetates of sugars released from crude GPL extracts of *rtfA-int/pMV261* (a) and *rtfA-int/pMVgtfCD* (b). Alditol acetate derivatives were prepared from the total lipid fraction after mild alkaline hydrolysis. Asterisks indicate noncarbohydrates.

RESULTS

Identification of the genes involved in the formation of the Fuc residue in serovar 2 GPL. To reveal the genes responsible for the fucosylation pathway that lead to the formation of serovar 2 GPL, we focused on the 5.0-kb segment designated the *gtfC-gtfD* region (GenBank accession no. AF125999.1). This region contains five genes: *mdhA* and *merA*, whose deduced amino acid sequences show a high level of similarity to those of enzymes involved in Fuc synthesis; *mtfF*, previously identified as the fucosyl 2-O-methyltransferase gene; and *gtfC* and *gtfD*, putative glycosyltransferase genes whose functions remain unknown (13, 18). Since *M. smegmatis* only produces core GPLs, we introduced the chromosomal integrating vector pYMrta-int possessing the *M. avium* gene *rtfA*, whose gene product transfers the Rha residue to 6-d-Tal of core GPLs, and obtained the recombinant strain *rtfA-int*, which produces GPL with a terminal Rha residue (termed GPL-S1) that could be a substrate for the synthesis of serovar 2 GPL. The expression vector pMVgtfCD, harboring the *gtfC-gtfD* region (Fig. 1B), then was introduced into the GPL-S1-producing strain (*rtfA-int*) and wild-type *mc*²¹⁵⁵, and GPL production was analyzed

by TLC (Fig. 2A). Although there were no differences between the TLC profiles of *Wt/pMV261* and *Wt/pMVgtfCD* (Fig. 2A, lanes c and d), new spots appeared in *rtfA-int/pMVgtfCD* (Fig. 2A, lane b), indicating that the *gtfC-gtfD* region contains genes with the ability to convert GPL-S1 into structurally different compounds, but it can do so only in the presence of the *rtfA* gene. To confirm that the products formed in *rtfA-int/pMVgtfCD* contained Fuc, a characteristic of serovar 2 GPL, alkaline-stable extracts from *rtfA-int/pMVgtfCD* were hydrolyzed, and the released monosaccharides were analyzed by GC-MS (Fig. 2B). The results showed that 2-O-Me-Fuc, which is structurally related to serovar 2 GPL, was present together with Rha, 6-d-Tal, 3-O-Me-Rha, 3,4-di-O-Me-Rha, and 2,3,4-tri-O-Me-Rha in *rtfA-int/pMVgtfCD* (Fig. 2B, graph b). 2-O-Me-Fuc was not detected in strain *rtfA-int/pMV261* (vector control) (Fig. 2B, graph a) or recombinant wild-type *mc*²¹⁵⁵ (data not shown). These results indicated that the *gtfC-gtfD* region is responsible for the transfer of the Fuc residue to serovar 1 GPL. Additionally, for identification of the individual genes involved in this fucosylation, we constructed various plasmids that have one of the genes deleted from the *gtfC-gtfD* region, as shown in

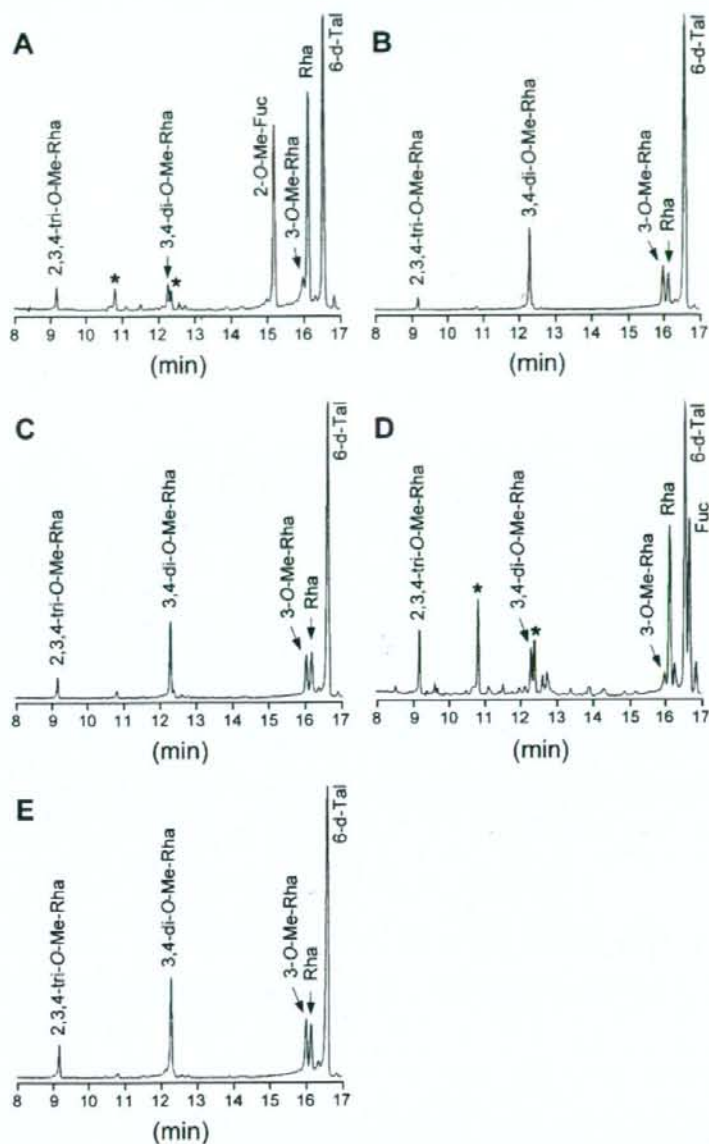


FIG. 3. GC-MS analyses of alditol acetates of sugars released from crude GPLs. GPLs were extracted from *rtfA-int/pMVΔgtfC* (A), *rtfA-int/pMVΔmdhA* (B), *rtfA-int/pMVΔmerA* (C), *rtfA-int/pMVΔmtfF* (D), and *rtfA-int/pMVΔgtfD* (E). Alditol acetate derivatives were prepared from the total lipid fraction after mild alkaline hydrolysis. Asterisks indicate noncarbohydrates.

Fig. 1B, and examined the sugar moieties of the alkaline-stable GPL extracts from each recombinant strain by GC-MS analysis (Fig. 3). The results show that the profile of *rtfA-int/pMVΔgtfC* was the same as that of *rtfA-int/pMVΔgtfCD*, indicating that the *gtfC* gene does not participate in the formation of 2-*O*-Me-Fuc (Fig. 2B, graph b, and 3A). In *rtfA-int/pMVΔmtfF*, Fuc was detected instead of 2-*O*-Me-Fuc, demonstrating that the *mtfF* gene encodes fucosyl 2-*O*-methyltrans-

ferase (Fig. 3D). On the other hand, no Fuc derivatives were detected in the recombinant strains *rtfA-int/pMVΔmdhA*, *rtfA-int/pMVΔmerA*, and *rtfA-int/pMVΔgtfD* (Fig. 3B, C, and E). These results indicated that the three genes *mdhA*, *merA*, and *gtfD* are all indispensable for the formation of the Fuc residue in serovar 2 GPL.

Functional analysis of the *gtfD* gene. Although the *gtfD* gene was predicted to encode a type of glycosyltransferase based on

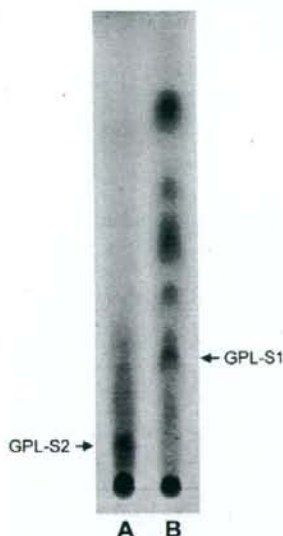


FIG. 4. TLC analyses of crude GPL extracts from *rtfA-mdhA-merA-int/pMVgtfD* (lane A) and *rtfA-mdhA-merA-int/pMV261* (lane B). The total lipid fraction after mild alkaline hydrolysis was spotted onto plates and developed in $\text{CHCl}_3\text{-CH}_3\text{OH}$ (9:1 [vol/vol]).

a homology search of its deduced amino acid sequences, it is not clear whether the product of *gtfD* functions as the glycosyltransferase that transfers the Fuc residue via 1 \rightarrow 3 linkage to the Rha residue of serovar 1 GPL to form the oligosaccharide part of serovar 2 GPL. To elucidate the function of *gtfD*, we constructed a recombinant strain by introducing a chromosomal integrating vector expressing *rtfA*, *mdhA*, and *merA* (*pYMrtfA-mdhA-merA-int*). We then characterized the product formed when *gtfD* was expressed solely by the plasmid vector (*pMVgtfD*). TLC analysis of the recombinant strains showed that the *gtfD*-expressing strain (*rtfA-mdhA-merA-int/pMVgtfD*) caused the appearance of a new spot, termed GPL-S2, in connection with the disappearance of GPL-S1 (Fig. 4, lane A), while the vector control (*rtfA-mdhA-merA-int/pMV261*) did not produce GPL-S2 (Fig. 4, lane B). To determine the structure of sugar moieties of GPL-S2, perdeuterio-methylation was performed on purified GPL-S2, and derived alditol acetates were analyzed by GC-MS. The GC-MS profile yielded four peaks corresponding to 6-d-Tal, Rha, Fuc, and 2,3,4-tri-*O*-Me-Rha (data not shown). The characteristic spectra of Fuc, Rha, and 6-d-Tal are shown in Fig. 5. The spectrum of Fuc gave fragment ions at *m/z* of 121, 134, and 168, which represent the presence of deuteriomethyl groups at positions C-2, C-3, and C-4 (Fig. 5A). In contrast, the detection of fragment ions at *m/z* of 121, 134, 193, and 240 from Rha indicated that a deuteriomethyl group was introduced at positions C-2 and C-4 of Rha, whose C-3 position was acetylated (Fig. 5B). Additionally, positions C-3 and C-4 of 6-d-Tal were found to be deuteriomethylated, with the detection of fragment ions at *m/z* of 134, 181, and 193 (Fig. 5C). These observations demonstrated that position C-1 of Fuc is linked to position C-3 of Rha but not position C-2 of 6-d-Tal, because it

was previously determined that position C-1 of Rha is linked to position C-2 of 6-d-Tal in the oligosaccharide of serovar 1 GPL through the catalytic reaction of *RtfA* (14). Furthermore, we compared the molecular mass of GPL-S2 to that of GPL-S1 purified from the vector control strain by MALDI-TOF (mass spectrometry). The main pseudomolecular ion $[M + \text{Na}]^+$ from both compounds revealed that the difference between GPL-S2 (*m/z*, 1,479.9) and GPL-S1 (*m/z*, 1,333.8) was 146 mass units, suggesting that a Fuc residue was further added to the GPL-S1 (data not shown). Accordingly, the structure of GPL-S2 was determined to have Fuc-(1 \rightarrow 3)-Rha-(1 \rightarrow 2)-6-d-Tal at *D*-*allo*-Thr and 2,3,4-tri-*O*-Me-Rha at *D*-alaninol (Fig. 6), demonstrating that *gtfD* encodes the glycosyltransferase that transfers a Fuc residue via 1 \rightarrow 3 linkage to the Rha residue of serovar 1 GPL.

DISCUSSION

The gene cluster involved in synthesizing ssGPLs has been cloned from *M. avium* strains. In this cluster, the functions of several genes responsible for the biosynthesis of serovar 1 GPL, such as *rtfA*, *gtfA*, *gtfB*, *mtfB*, *mtfC*, and *mtfD*, have been elucidated (14, 16, 19). On the other hand, the genes associated with the conversion of serovar 1 GPL to other serotypes, including serovar 2 GPL, have not been identified. In this study, we focused on the five genes assumed to encode the enzymes associated with fucosylation in the biosynthesis of serovar 2 GPL and experimentally showed that *GtfD* is responsible for the transfer of the Fuc residue to the Rha residue of serovar 1 GPL (Fig. 6). Gene deletion experiments revealed that *mdhA* and *merA* also contribute to the formation of the Fuc residue in serovar 2 GPL. The deduced amino acid sequences of *mdhA* and *merA* showed high levels of similarity to GDP-D-mannose-4,6-dehydratase and GDP-6-deoxy-4-keto-D-mannose-3,5-epimerase-4-reductase, respectively. These are enzymes involved in the synthesis of L-Fuc from D-mannose and are highly conserved among other bacteria (1, 26). For mycobacteria, there are no homologues of *mdhA* and *merA* in the genome databases for *M. bovis*, *M. leprae*, and *M. smegmatis*. However, for *M. tuberculosis*, the deduced amino acid sequences of Rv1511 and Rv1512 show 89 and 84% homology to those of *mdhA* and *merA*, respectively. This observation is supported by the fact that several strains of *M. tuberculosis* produce the Fuc-containing phenolic glycolipid, whereas *M. bovis* and *M. leprae* lack the Fuc residue, and other carbohydrate components having the Fuc residue have not been reported from the above-mentioned three species. Thus, it is strongly suggested that *mdhA* and *merA* encode synthetases involved in the conversion of D-mannose to L-Fuc that can be transferred by *GtfD* to form the Fuc residue of serovar 2 GPL (Fig. 6). Before performing the functional analyses of these genes, we speculated that the glycosyltransferase involved in the fucosylation was encoded by *gtfC*, but not *gtfD*, from the observations that *gtfC* includes a putative glycosyltransferase motif and its homologue in *M. tuberculosis*, Rv1514c, is adjacent to Rv1511 and Rv1512, which are predicted to be responsible for the Fuc synthesis, while a *gtfD* homologue, Rv2957, is located far from Rv1511, Rv1512, and Rv1514c. However, the deletion analysis demonstrated that *gtfC* does not contribute to the fucosylation of the GPLs. This result raises the possibility

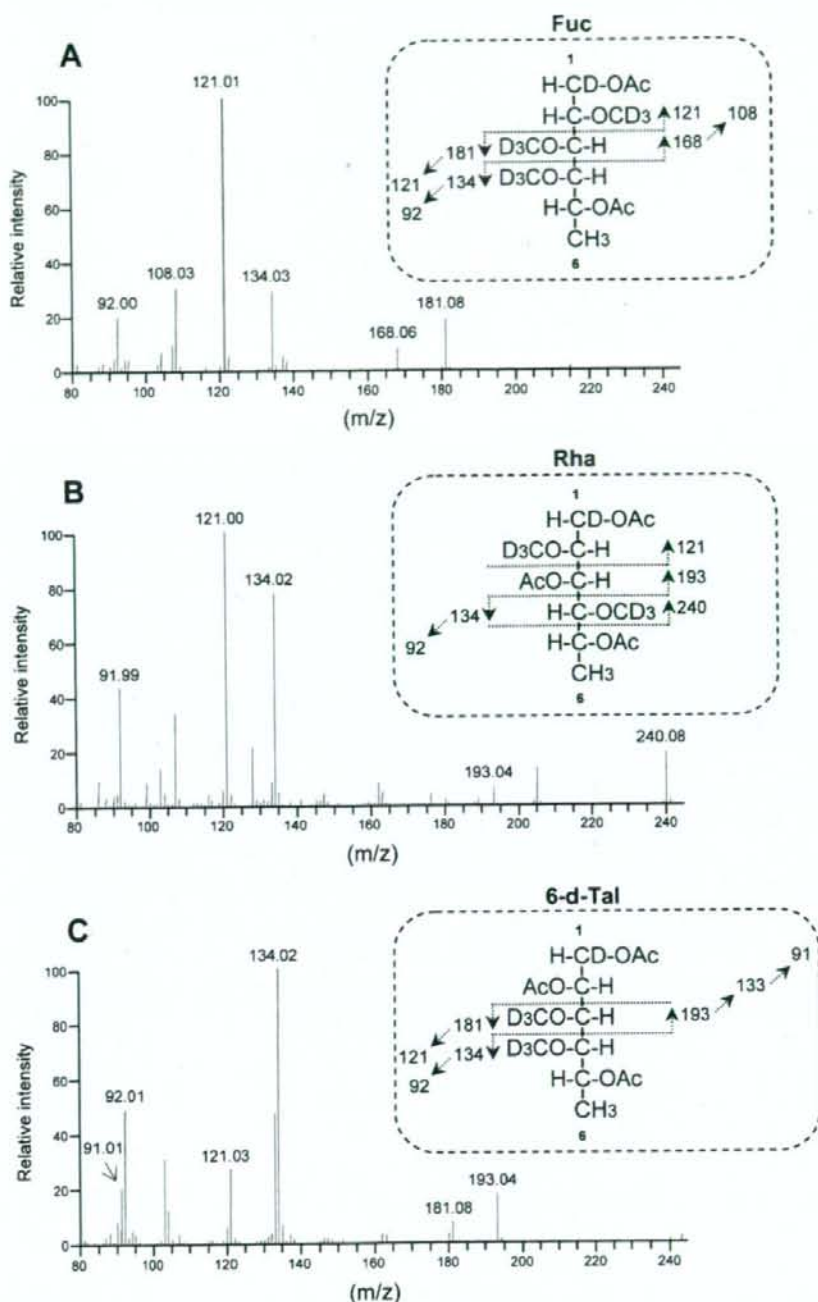


FIG. 5. GC mass spectra and fragment ion assignment of Fuc (A), Rha (B), and 6-d-Tal (C), which are derived from alditol acetates of sugars released from deuteriomethylated GPL-S2. Ac, acetate; D, deuterium.

that *gtfC* is involved in the transfer of another sugar moiety, such as glucuronic acid and Rha, followed by fucosylation, as observed for the serovar 3, 9, and 4 GPLs (9). As for *gtfD*, Rv2957 is reported to be one of the glycosyltransferase genes

involved in the biosynthesis of phenolic glycolipid in *M. tuberculosis*, but its catalytic functions, such as sugar substrate and glycosidic linkage, are not clear (22). Thus, our findings implied that Rv2957 is the fucosyltransferase gene responsible for

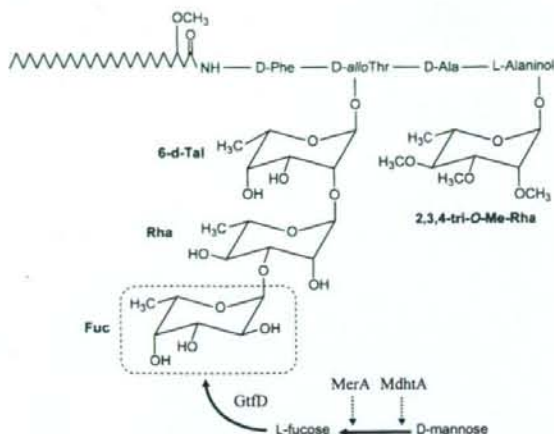


FIG. 6. Proposed structure and biosynthetic pathway of fucosylated GPL (GPL-S2).

the transfer of the Fuc residue in the phenolic glycolipid. Moreover, morphological observations showed that the surface of colony of the GPL-S2-producing strain was rougher than that of the vector control strain, suggesting that the presence of a Fuc residue in the GPL structure affected the cell surface properties (data not shown). Taking the results together, our study is the first report identifying the genes involved in the fucosylation pathway in mycobacteria and might provide a clue to understanding its role in the biosynthesis of glycolipids, including GPLs.

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REFERENCES

- Andrianopoulos, K., L. Wang, and P. R. Reeves. 1998. Identification of the fucose synthetase gene in the colanic acid gene cluster of *Escherichia coli* K-12. *J. Bacteriol.* **180**:998-1001.
- Aspinall, G. O., D. Chatterjee, and P. J. Brennan. 1995. The variable surface glycolipids of mycobacteria: structures, synthesis of epitopes, and biological properties. *Adv. Carbohydr. Chem. Biochem.* **51**:169-242.
- Bardarov, S., S. Bardarov, Jr., M. S. Pavelka, Jr., V. Sambandamurthy, M. Larsen, J. Tufariello, J. Chan, G. Hatfull, and W. R. Jacobs, Jr. 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG, and *M. smegmatis*. *Microbiology* **148**:3007-3017.
- Belisle, J. T., K. Klaczekiewicz, P. J. Brennan, W. R. Jacobs, Jr., and J. M. Inamine. 1993. Rough morphological variants of *Mycobacterium avium*. Characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. *J. Biol. Chem.* **268**:10517-10523.
- Belisle, J. T., L. Pascepola, J. M. Inamine, P. J. Brennan, and W. R. Jacobs, Jr. 1991. Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of *Mycobacterium avium*. *J. Bacteriol.* **173**:6991-6997.
- Billman-Jacobe, H., M. J. McConville, R. E. Haines, S. Kovacevic, and R. L. Coppel. 1999. Identification of a peptide synthetase involved in the biosynthesis of glycopeptidolipids of *Mycobacterium smegmatis*. *Mol. Microbiol.* **33**:1244-1253.
- Bjorndal, H., C. G. Helleqvist, B. Lindberg, and S. Svensson. 1970. Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. *Angew. Chem. Int. Ed. Engl.* **9**:610-619.
- Brennan, P. J., and H. Nikaido. 1995. The envelope of mycobacteria. *Annu. Rev. Biochem.* **64**:29-63.
- Chatterjee, D., and K. H. Khoo. 2001. The surface glycopeptidolipids of mycobacteria: structures and biological properties. *Cell. Mol. Life Sci.* **58**:2018-2042.
- Ciucanu, I., and F. Kerek. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **131**:209-217.
- Daffe, M., and P. Draper. 1998. The envelope layers of mycobacteria with reference to their pathogenicity. *Adv. Microb. Physiol.* **39**:131-203.
- Daffe, M., M. A. Lanelle, and G. Puzo. 1983. Structural elucidation by field desorption and electron-impact mass spectrometry of the C-mycosides isolated from *Mycobacterium smegmatis*. *Biochim. Biophys. Acta* **751**:439-443.
- Eckstein, T. M., J. T. Belisle, and J. M. Inamine. 2003. Proposed pathway for the biosynthesis of serovar-specific glycopeptidolipids in *Mycobacterium avium* serovar 2. *Microbiology* **149**:2797-2807.
- Eckstein, T. M., F. S. Silbaugh, D. Chatterjee, N. J. Kelly, P. J. Brennan, and J. T. Belisle. 1998. Identification and recombinant expression of a *Mycobacterium avium* rhamnosyltransferase gene (*rfA*) involved in glycopeptidolipid biosynthesis. *J. Bacteriol.* **180**:5567-5573.
- Jeevarajah, D., J. H. Patterson, M. J. McConville, and H. Billman-Jacobe. 2002. Modification of glycopeptidolipids by an O-methyltransferase of *Mycobacterium smegmatis*. *Microbiology* **148**:3079-3087.
- Jeevarajah, D., J. H. Patterson, E. Taig, T. Sargeant, M. J. McConville, and H. Billman-Jacobe. 2004. Methylation of GPLs in *Mycobacterium smegmatis* and *Mycobacterium avium*. *J. Bacteriol.* **186**:6792-6799.
- Julander, I., S. Hoffner, B. Petrin, and L. Ostlund. 1996. Multiple serovars of *Mycobacterium avium* complex in patients with AIDS. *APMIS* **104**:318-320.
- Mills, J. A., M. R. McNeil, J. T. Belisle, W. R. Jacobs, Jr., and P. J. Brennan. 1994. Loci of *Mycobacterium avium* *ser2* gene cluster and their functions. *J. Bacteriol.* **176**:4803-4808.
- Miyamoto, Y., T. Mukai, N. Nakata, Y. Maeda, M. Kai, T. Naka, I. Yano, and M. Makino. 2006. Identification and characterization of the genes involved in glycosylation pathways of mycobacterial glycopeptidolipid biosynthesis. *J. Bacteriol.* **188**:86-95.
- Miyamoto, Y., T. Mukai, F. Takeshita, N. Nakata, Y. Maeda, M. Kai, and M. Makino. 2004. Aggregation of mycobacteria caused by disruption of fibronectin-attachment protein-encoding gene. *FEMS Microbiol. Lett.* **236**:227-234.
- Patterson, J. H., M. J. McConville, R. E. Haines, R. L. Coppel, and H. Billman-Jacobe. 2000. Identification of a methyltransferase from *Mycobacterium smegmatis* involved in glycopeptidolipid synthesis. *J. Biol. Chem.* **275**:24900-24906.
- Perez, E., P. Constant, A. Lemassu, F. Laval, M. Daffe, and C. Guilhot. 2004. Characterization of three glycosyltransferases involved in the biosynthesis of the phenolic glycolipid antigens from the *Mycobacterium tuberculosis* complex. *J. Biol. Chem.* **279**:42574-42583.
- Recht, J., and R. Kolter. 2001. Glycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. *J. Bacteriol.* **183**:5718-5724.
- Sambandamurthy, V. K., X. Wang, B. Chen, R. G. Russell, S. Derrick, F. M. Collins, S. L. Morris, and W. R. Jacobs, Jr. 2002. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nat. Med.* **8**:1171-1174.
- Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr. 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol. Microbiol.* **4**:1911-1919.
- Stevenson, G., K. Andrianopoulos, M. Hobbs, and P. R. Reeves. 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J. Bacteriol.* **178**:4885-4893.
- Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom. 1991. New use of BCG for recombinant vaccines. *Nature* **351**:456-460.
- Sweet, L., and J. S. Schory. 2006. Glycopeptidolipids from *Mycobacterium avium* promote macrophage activation in a TLR2- and MyD88-dependent manner. *J. Leukoc. Biol.* **80**:415-423.
- Vergne, I., and M. Daffe. 1998. Interaction of mycobacterial glycolipids with host cells. *Front. Biosci.* **3**:d865-876.
- Yakrus, M. A., and R. C. Good. 1990. Geographic distribution, frequency, and specimen source of *Mycobacterium avium* complex serotypes isolated from patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **28**:926-929.