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Ⅳ. 研究成果の刊行物・別刷

13 結 核 菌

【病原体の特性，BSL】（表 1）

結核は古代から人類を脅かしてきた感染症であるが，未だ制圧されていない．結核菌既感染者は全世界で約 20 億人（日本：2,500 万人），年間新規患者数は 880 万人（日本：2.6 万人），罹患率（対人口 10 万人）は 140（日本：20.6），年間死亡者数は 200 万人（日本：0.23 万人）であり，現在においても甚大な健康被害を提供している．病変部位により，肺結核，肺外結核，粟粒（全身播種性）結核に分類される．

結核菌は *Mycobacterium* 属（抗酸菌）に属し，非運動性，芽胞や莢膜を形成しない好気性グラム陽性桿菌（長さ：2～4 μm，幅：0.3～0.6 μm）である．ゲノムサイズは約 4.4 Mb で GC 比 65.6 % である．遺伝学的にきわめて近縁の *M. bovis*, *M. africanum* や *M. microti* とともに結核菌群 *Mycobacterium tuberculosis* complex を形成している．結核菌群は生化学的にナイアシンを産生することで，非結核性抗酸菌やらい菌等の他の抗酸菌と区別される．抗酸菌細胞壁は脂質に富むため（乾燥菌量の 10～40%，細胞壁の 20～60%），通常のグラム染色法では染色されにくく，Ziehl-Neelsen 法や蛍光等の特殊染色（抗酸性染色）によって染色される．しかし，いったん染色されると塩酸等でも脱色されにくいことから抗酸菌（acid-fast bacilli）と呼ぶ．細胞壁糖脂質，特に，ミコール酸（mycolic acid）は染色性や細胞壁の疎水性に最も関与している．菌体表層の強い疎水性のため，乾燥，凍結や酸・アルカリ，消毒剤等に強い抵抗性を示し，宿主体内外で長期間にわたり生存する．

発育には適度の酸素（20%）を必要とし，炭酸ガス（5%）は発育を促進させる．しかし，嫌気状態でも完全に死滅することはなく，休眠状態（dormancy）として生存する．37～38℃ を発育至適温度とし，至適 pH は pH6.8～7.0 である．固形培地として，小川培地や Lowenstein-Jensen 培地等の卵培地，あるいは，Middlebrook 7H10 や 7H11 培地が使用されるが，遅発育性であり（倍加時間：16～18 時間），コロニーの肉眼的な検出に 2～3 週間以上を要する．遅発育性は検体培養による結核の診断を遅らせる一因となる．小川培地表面では「ロウ状」の光沢を持った R 型コロニーを形成し，乳白色～淡黄色の色調を呈する．界面活性剤を加えずに液体培地に培養した場合，培地表面に肉眼的にコード状に発育する（cord 形成）．

喀痰に含まれる結核菌の飛沫核（空気）感染により，結核はヒト-ヒト感染伝播する．結核菌は病原性の高い細菌であり，わずか数個の菌を吸入することで感染が成立する．感染しても発病は生涯を通じて約 10% で，90% は感染した結核菌が休眠状態で宿主体内に残存する（persister）．

結核菌は外毒素や内毒素を産生せず，その病原性は宿主感染防御機構から逸脱して細胞内生存や増殖をすること，遅延型過敏反応を誘導することにより表現される．結核菌は細胞内寄生菌であり，宿主マクロファージに貪食された後，殺菌されずに増殖する．マクロファージ内では食胞体（ファゴゾーム）内に留まるが，ファゴゾームとリソゾームの融合（ファゴリソゾーム融合）を阻害することで，細胞内殺菌機構から逸脱する．

すべての結核菌株は BSL-3 である．

感染症の予防及び感染症の患者に対する医療に関する法律等の一部を改正する法律（改正感染症法）では，結核菌は特定病原体等に指定され，四種病原体等に，多剤耐性結核菌は三種病原体等に分類されるので，管理・取扱いに際し，同法を遵

表 1　結核菌の特徴

細胞内寄生性	ファゴリソゾーム融合を阻害し，マクロファージ内ファゴソーム中で生存
遅発育性	倍加時間 16～18 時間 コロニー形成に 2～3 週間以上
細胞壁	脂質に富み（乾燥菌量の 10～40%，細胞壁の 20～60%），抗酸性を呈する 種々の環境や化学物質に抵抗性を示す
運動性	非運動性，芽胞非形成性，莢膜なし
遺伝子	ゲノムサイズ約 4.4 Mb，GC 比 65.6%
感染形式	エアロゾル：飛沫核（空気）感染
好気性	発育には酸素を要求し，酸素分圧の高い肺等で増殖 酸素が枯渇しても死滅せず，休眠状態となる
病態	慢性炎症，肉芽腫形成，乾酪壊死，空洞形成，線維化

表 2　結核菌曝露に対する応急措置，及び事故対応

バイオセーフティレベル		BSL-3
感染経路		結核菌を含む飛沫核（空気）感染
応急処置	針刺し・怪我	血液を絞り出し，流水で洗浄後，傷口を消毒
	皮膚	流水で洗浄後，消毒
	口	うがい
	眼	流水あるいは滅菌生理食塩水で洗浄
	鼻	鼻腔洗浄
感染事故対応		応急処置と施設管理者への報告，医療機関を受診
緊急投薬		なし
有効な薬剤		発病予防にはイソニアジド（INH）投与，INH 耐性菌の場合，リファンピシン（RIF）投与
経過観察		定期健診による胸部 X 線撮影検査 結核菌特異抗原による末梢血 interferon-γ 遊離試験 ツベルクリン皮内反応検査 経過観察，曝露後 2 年間
臨床症状		針刺し・外傷局所では感染部位の炎症，所属リンパ節の腫脹 症状は 2 週間以上持続する咳嗽，喀痰や発熱が最も多く，その他，全身倦怠，血痰，胸痛，体重減少，寝汗や食欲低下等

表 3　結核菌感染に対する予防措置

ワクチンの接種		BCG ワクチン
特異免疫反応試験	TST	ツベルクリン皮内反応（Mantoux） 48 時間後判定：遅延型皮内反応
	IGRA：QFT	末梢血細胞 IFN-γ 産生・遊離試験（IGRA，Quantiferon®）：*in vitro* 抗原：ESAT-6，CFP-10
発病予防内服		感染曝露が確認された場合，INH（300 mg/日，6～9 ケ月）投与，INH 耐性菌の場合，リファンピシン（RIF）投与
滅菌法		使用した器具はオートクレーブ 121℃，30 分による滅菌
消毒薬		アルコール類，フェノール類，アルデヒド類による消毒

守する．

【実験室のハザード及び予想されるリスク】(表 2)

結核菌は飛沫核（エアロゾル）により感染伝播するので，実験室においてはエアロゾルの発生及び吸引に留意する．1979～1999 年における実験室感染の原因病原体の中で，結核菌は最も頻度が高かった．針刺し，外傷等により菌が体内に入った可能性のある場合，緊急処置を必要とする．また，有効な抗微生物薬の投与を行う．針刺しや外傷では感染部位の炎症，所属リンパ節の腫脹が生じる．針刺しや外傷に対しては，血液を絞り出し，流水で洗浄後，創部を消毒する．体表面への付着が懸念される場合には流水で洗浄し，可能であれば消毒する．口腔内に入った可能性がある場合には十分にうがいを行う．感染事故対応は，針刺し事故等の場合には応急処置と施設管理者への報告，医療機関の受診を行う．なお，結核予防法は改正感染症法に統合され，結核は二類感染症に位置づけられた．この規程により，結核患者あるいは結核の疑い患者と診断するに足る高度の蓋然性が認められる場合，診察した医師は届出を直ちに行わなければならない．

臨床症状としては 2 週間以上持続する咳嗽，喀痰や発熱が最も多い．全身倦怠，血痰，胸痛，体重減少，寝汗や食欲低下等を伴うこともある．なお，約 20% は自覚症状の有無に関わらず，健康診断で発見される．

本菌の潜伏期間は長く，若年層の一次感染では 4～18 ケ月，既感染者の二次感染では長期経過後の発症もある．そのため，感染が懸念される場合，胸部 X 線撮影検査，感染曝露 6～8 週間後，結核菌特異抗原による末梢血 interferon-γ 遊離試験（Quantiferon® 陽性：0.35IU/mL 以上）やツベルクリン皮内反応検査（陽性，日本：紅斑≧直径 10 mm 以上，欧米：硬結≧直径 5 mm 以上）等を行い，2 年間経過観察する．なお，ツベルクリン皮内反応陽性は結核菌感染のみならず，BCG 接種や非結核性抗酸菌感染でもみられ，逆に，活動性結核の約 25% は陰性である．陰性は真の陰性（結核菌未感染）や偽陰性（結核菌既感染にもかかわらず，陰性）を包含し，偽陰性として，栄養障害，高齢者，免疫疾患，リンパ系悪性腫瘍，副腎皮質ステロイド薬療法，慢性腎不全，サルコイドーシス，HIV 感染者（AIDS を含む）や重症結核（播種性）等がある．従って，ツベルクリン皮内反応は結核の補助診断である．ツベルクリン皮内反応陽性は感染防御の指標とならないことも留意する．

【予防法－消毒・滅菌法－】(表 3)

結核の予防ワクチンとして，弱毒ウシ型結核菌由来生ワクチンの BCG が汎用されている．乳幼児における BCG 接種は有効とされているが，成人への効果は疑問視されている．結核菌を扱う業務に従事する前には，結核菌既感染の有無を確認しておくことが望ましい．確認にツベルクリン皮内反応，特に，結核菌特異抗原による末梢血単核細胞 interferon-γ 遊離試験が推奨される．予防内服にはイソニアジド（INH）が投与される．感染を受けたと判断される場合，INH300 mg を 1 日量として 6～9 ケ月投与する．感染結核菌が INH 耐性の場合，リファンピシン（RIF）を投与する．

結核菌は熱湯による煮沸 10 分間で完全に殺菌できるが，オートクレーブ（121℃，30 分）による滅菌が最も望ましい．消毒薬ではアルコール類，フェノール類，アルデヒド類が有効である．滅菌不可能な器具はグルタールアルデヒドに 30 分以上浸して消毒する．　**［大原直也・小林和夫］**

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COLUMN **多剤耐性結核菌（MDR-TB）と超多剤耐性結核菌（XDR-TB）**

結核菌においても抗微生物薬耐性が問題となっているが，薬剤耐性結核菌で，INH と RIF に同時耐性菌を多剤耐性結核菌（Multidrug-resistant TB：MDR-TB），多剤耐性結核菌でフルオロキノロン耐性，かつカナマイシン，アミカシン，カプレオマイシン等，注射可能薬の少なくとも 1 剤以上に耐性の菌を超多剤耐性結核菌（Extensively drug-resistant TB：XDR-TB）という．薬剤耐性，特に，XDR-TB は抗結核化学療法がほとんど無効であり，生命予後も不良である．薬剤耐性結核菌に有効な新規抗結核薬の開発が希求されている．

Serodiagnosis of *Mycobacterium avium*–Complex Pulmonary Disease Using an Enzyme Immunoassay Kit

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***Rationale*: The diagnosis of *Mycobacterium avium*–complex pulmonary disease (MAC-PD) and/or its discrimination from pulmonary tuberculosis (TB) is sometimes complicated and time consuming.**

***Objectives*: We investigated in a six-institution multicenter study whether a serologic test based on an enzyme immunoassay (EIA) kit was useful for diagnosing MAC-PD and for distinguishing it from other lung diseases.**

***Methods*: An EIA kit detecting serum IgA antibody to glycopeptidolipid core antigen specific for MAC was developed. Antibody levels were measured in sera from 70 patients with MAC-PD, 18 with MAC contamination, 37 with pulmonary TB, 45 with other lung diseases, and 76 healthy subjects.**

***Measurements and Main Results*: Significantly higher serum IgA antibody levels were detected in patients with MAC-PD than in the other groups ($P < 0.0001$). Setting the cutoff point at 0.7 U/ml resulted in a sensitivity and specificity of the kit for diagnosing MAC-PD of 84.3 and 100%, respectively. Significantly higher antibody levels were also found in patients with nodular-bronchiectatic disease compared with fibrocavitary disease in MAC-PD ($P < 0.05$). There was a positive correlation between the extent of disease on chest computed tomography scans and the levels of antibody ($r = 0.43$, $P < 0.05$) in patients with MAC-PD.**

***Conclusions:* The EIA kit is useful for the rapid diagnosis of MAC-PD and for differentiating MAC-PD from pulmonary TB and, if validated by studies in other populations, could find wide application in clinical practice.**

Keywords: nontuberculous mycobacteria; immunocompetence; sensitivity and specificity

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The diagnosis of pulmonary disease due to ubiquitous *Mycobacterium avium* complex (MAC) is complicated, and requires clinical findings together with repeatedly positive sputum culture.

What This Study Adds to the Field

An enzyme immunoassay kit for measuring human serum antibody to glycopeptidolipid core antigen specific for MAC was developed. The kit is useful for the serodiagnosis of MAC pulmonary disease and could find wide application in clinical practice.

The prevalence of disease due to nontuberculous mycobacteria has been increasing recently (1–5). In Japan, *Mycobacterium avium* complex (MAC) accounts for approximately 70% of nontuberculous mycobacterial disease (6). MAC is now widely recognized as an important pathogen that causes chronic and progressive pulmonary disease even in immunocompetent patients and not only in those who are immunosuppressed. The diagnosis of MAC-PD is complicated because, in contrast to *Mycobacterium tuberculosis*, MAC contamination of clinical specimens can come from environmental sources such as water, dust, and soil, and because this organism may colonize the respiratory tract without any accompanying invasive disease (4). Thus, isolation of MAC from sputa is often of no clinical significance. Diagnosis of pulmonary disease due to MAC is complicated and time consuming when made according to the guidelines of the American Thoracic Society (ATS) (1), because MAC is ubiquitous in nature and the diagnosis requires clinical findings and its repeated isolation from sputum. In addition, it is also difficult to discriminate MAC-PD from infection due to other mycobacteria in the absence of culture results, because clinical features, such as symptomatic or radiographic findings, are very similar in mycobacterial diseases. In the context of infection control, it is particularly important to distinguish between MAC-PD and pulmonary tuberculosis (TB).

To overcome these difficulties, we have developed a serologic test for the glycopeptidolipid (GPL) antigen specific for MAC, and have reported its clinical usefulness (7–9). The levels of antibody to GPL core were measured by an enzyme immunoassay (EIA) using sera of immunocompetent patients with MAC-PD. MAC-PD could be discriminated from pulmonary TB, *Mycobacterium kansasii* pulmonary disease and MAC colonization/contamination using this serologic test. Healthy subjects were seronegative. Of the different immunoglobulin (Ig) subclasses, best results were obtained by the measurement of IgA, with a sensitivity of 92.5% and specificity of 95.1%. These results suggest that the test is useful as a diagnostic aid. In the present study, to apply this test widely in clinical practice, we

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developed an EIA kit detecting serum IgA antibody specific for GPL core and investigated its usefulness in a multicenter study.

METHODS

See the online supplement for additional methodologic details.

Patients and Serum Samples

Six institutions participated in this study. Between June 2003 and December 2005, serum samples were collected from 70 patients with MAC-PD, 18 with MAC contamination, 36 with pulmonary TB, 45 with other lung diseases, and 76 healthy subjects. All patients with MAC-PD met the ATS guidelines (1). Of the 70 patients with MAC-PD, 64 had previously received combination chemotherapy for mycobacterial diseases recommended by the ATS guidelines, but had MAC-positive cultures at the time of serum collection. Pulmonary TB was confirmed by culture positivity for *M. tuberculosis*. Patients with pulmonary TB who had an underlying pulmonary disease or past history of treatment for pulmonary TB were excluded. Individuals with MAC contamination showed a single culture positive for MAC in small amounts, but were asymptomatic and had no significant chest computed tomography (CCT) findings indicating active mycobacterial disease. The other lung diseases included chronic obstructive pulmonary disease (n = 15), idiopathic interstitial pneumonia (n = 11), lung cancer (n = 11), bacterial pneumonia (n = 4), pulmonary sarcoidosis (n = 2), and bronchiectasis (n = 2). All sera were stored at −20°C until assayed for IgA GPL core antibody. None of the patients was seropositive for HIV type 1 or 2. The patients with MAC-PD were classified into two groups on the basis of the chest radiography: fibrocavitary disease and nodular-bronchiectatic (NBE) disease (1).

Fibrocavitary disease was defined as the presence of cavitary forms in upper lobes. NBE disease was defined as the presence of bronchiectasis and multiple nodular shadows on CCT. Disease conforming to neither of these types was considered unclassifiable. Forty-five patients underwent CCT and serodiagnosis at the same time. A correlation between the extent of disease and antibody levels was investigated. The extent of disease was expressed as the number of MAC-involved CCT segments, as described in the previous study (9).

The studies in human subjects were approved by the research and ethical committees of the NHO National Toneyama Hospital, and written, informed consent was obtained from all subjects.

EIA Kit

The EIA kit was developed by Tauns Laboratories, Inc. (Shizuoka, Japan), with a slight modification of the method described previously (8). Results are given as arbitrary U/ml in relation to a standard curve that was constructed by mixing sera from three patients with MAC-PD as a reference. The intra- and interplate coefficients of variation were 2.27–9.29% and 0.57–8.86%, respectively, which indicated good reproducibility. The linearity of measurement was confirmed. The influence of blood elements and temperature was examined, and revealed good stability. The assay was performed by a technologist with no prior knowledge of the clinical data.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA). Antibody levels in patient groups are expressed as means ± SD. For comparison of the mean values of multiple groups, data were compared by analysis of variance and nonparametric analysis. A probability value of less than 0.05 was regarded as significant.

RESULTS

Study Subjects

The characteristics of the subjects are shown in Table 1. Patients with pulmonary TB and healthy subjects were younger than patients with MAC-PD ($P < 0.001$), and there was a larger proportion of females in the latter group ($P < 0.001$). Of the 70 patients with MAC-PD, 15 had underlying pulmonary disease, all of which were the sequelae of pulmonary TB. Of the 18 individuals with MAC contamination, 15 had underlying pulmonary diseases (8 patients with the sequelae of pulmonary TB, 2 with lung cancer, 2 with chronic obstructive pulmonary disease, 1 with emphysema, 1 with pneumoconiosis, and 1 with sarcoidosis. Of the patients with MAC-PD, 19 were classified as having fibrocavitary disease, and 35 as having NBE disease, with 16 patients unclassifiable. The MAC-PD group included infections with *M. avium* (n = 56), *Mycobacterium intracellulare* (n = 12), or both (n = 2). The MAC contamination group included *M. avium* (n = 16) and *M. intracellulare* (n = 2).

Level of GPL Core IgA Antibody

The level of serum IgA antibody to GPL core was quantified using the EIA kit (Figure 1). As expected, patients with MAC-PD had significantly higher levels than patients with MAC contamination, those with pulmonary TB, those with other lung diseases, and healthy subjects—namely, 10.7 ± 7.9, 0.2 ± 0.1, 0.1 ± 0.1, 0.0 ± 0.1, and 0.0 ± 0.0 U/ml, respectively ($P < 0.0001$). A receiver operating characteristic (ROC) curve was constructed for MAC-PD and the other groups to establish the best cutoff value (Figure 2). Setting the cutoff value at 0.7 U/ml resulted in 100% specificity, at a sensitivity of 84.3% (Table E1). Using the EIA kit allowed clear discrimination between patients with MAC-PD and MAC contamination, pulmonary TB, and other lung diseases, as well as healthy subjects.

Next, we compared levels of serum IgA antibody to GPL core in fibrocavitary disease and NBE disease of MAC-PD. Significantly higher levels were found in NBE ($P < 0.05$) (Figure 3). With the cutoff value set at 0.7 U/ml, positivity in NBE and fibrocavitary disease was 91.4 and 63.2%, respectively. In contrast, in patients with MAC-PD, no significant differences between *M. avium* and *M. intracellulare* as causative agents were observed ($P = 0.403$). The erythrocyte sedimentation rate in MAC-PD was 32.6 ± 28.6 mm/hour and there was a significant positive correlation between the erythrocyte sedimentation rate and antibody levels in patients with MAC-PD ($r = 0.294$, $P < 0.05$).

Radiographic Severity and the Level of GPL Core Antibody

Forty-five patients with MAC-PD (10 with fibrocavitary disease, 26 with NBE disease, and 9 with unclassifiable type disease) underwent CCT and serodiagnosis at the same time. Four patients with unclassifiable type disease were excluded from the investigation because it was hard to discriminate between MAC lesions and underlying pulmonary disease. There was a positive correlation between the extent of disease and the

TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS

	MAC-PD	MAC Contamination	Pulmonary TB	Other Lung Disease	Healthy Subjects
Number	70	18	36	45	76
Age, mean yr ± SD	68.0 ± 9.6	64.6 ± 11.6	52.9 ± 16.6*	66.3 ± 10.9	38.1 ± 12.0*
Age range, yr	50–90	28–78	24–76	29–82	20–65
Sex, no. male/no. female	25/45	10/8	26/10*	34/11*	41/35*
Duration of disease, mean yr ± SD	4.8 ± 4.6		0.3 ± 0.2	2.2 ± 2.4	

* $P < 0.001$.

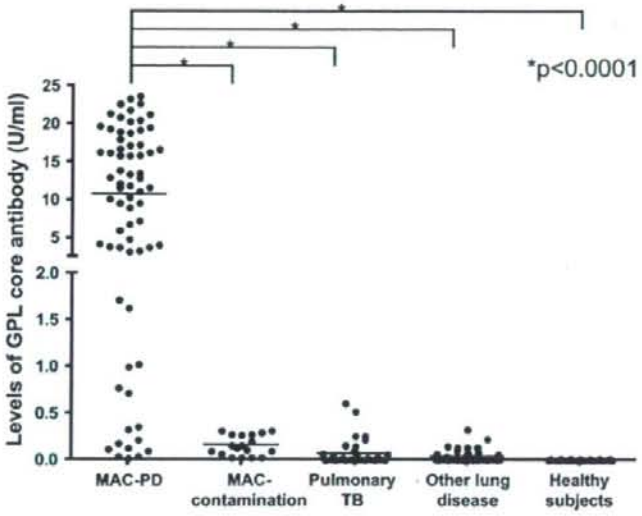

Figure 1. The level of serum IgA antibody to glycopeptidolipid (GPL) core antigen. Serum samples from six different institutions included 70 patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD), 18 with MAC contamination, 37 with pulmonary tuberculosis (TB), 45 with other lung diseases, and 76 healthy subjects. Antibody levels in MAC-PD were significantly higher than in the other groups ($P < 0.0001$). All results are expressed as individual data, and *horizontal bars* indicate geometric means.

levels of the antibody ($r = 0.43$, $P < 0.05$) (Figure 4). The total numbers of involved segments were not different (7.8 ± 4.9 and 7.9 ± 4.2 in fibrocavitary and NBE disease, respectively). Of 26 patients with NBE disease, 9 had small thin wall cavities. A tendency toward elevated GPL core antibody levels was found in NBE patients with cavities compared with those without, but this trend was not statistically significant ($P = 0.08$).

DISCUSSION

We previously established a serologic test for MAC-PD using a mixture of GPLs and GPL core antigen, and reported the clinical application of the EIA method for quantifying antibody levels (7, 8). GPL is an antigen located on the surface of the MAC cell wall and determines the serotype. At present, 31 distinct serotype-specific GPLs have been identified, of which the complete structures of 14 have been identified (10–12). GPL consists of a core common to all MAC serotypes and a serotype-specific oligosaccharide. In the initial study to establish the serodiagnosis of MAC-PD, we used the whole GPL antigen, a mixture of 11 serotype-specific GPLs (7). We then found that the GPL core was the dominant antigenic epitope of GPL, and subsequently developed a serologic test using GPL core antigen (8). In the previous study, GPL core antibody (IgG, IgA, and IgM) levels were found to be elevated in sera of patients with MAC-PD, but not pulmonary TB, *M. kansasii*–PD, MAC colonization/contamination, and healthy subjects. The study showed that this serologic test was useful for diagnosing MAC-PD and for differentiating it from pulmonary TB and *M. kansasii*–PD. Consistent with this, Fujita and colleagues (13) reported elevated levels of antibody against the GPL core antigen in patients with MAC-PD but not in those with pulmonary TB. In our previous study (8), of the different Ig classes, best results were obtained by IgA, including an association with CCT findings. Thus, a higher level of serum IgA antibody to GPL core indicated a wider extent of MAC disease and larger nodule formation on CCT (9). Therefore, we have attempted to develop and to assess an EIA kit for quantifying serum IgA antibody to GPL core in the present study. Optical density levels were converted to U/ml using standard serum samples, which provided reliable and reproducible results. In this multicenter study, using the EIA kit, it was confirmed that patients with MAC-PD could be clearly differentiated from those with pulmonary TB, those with MAC contamination, those with other lung diseases, and healthy subjects. Similar to our previous studies (7–9), the sensitivity and specificity for diagnosing MAC-PD by the kit was high and the level of the antibody correlated with the extent of MAC-PD assessed using CCT.

Distinguishing pulmonary TB from MAC-PD in clinical practice using the EIA kit has proven useful. Differentiating TB from MAC is difficult because symptoms and radiographic findings are often similar among patients with pulmonary mycobacterial diseases. Patients with pulmonary TB require immediate treatment and isolation, whereas the diagnosis of MAC-PD does not necessitate rapidly starting antimicrobial therapy (1), and isolation is not required. GPL antigens, which are major cell surface antigens of MAC, are not present in the cell wall of *M. tuberculosis* complex (11). On the basis of this observation, patients with TB do not produce anti-GPL antibody. Indeed most patients with TB did not possess serum antibodies against GPLs (Figure 1) (7, 8). However, we cannot exclude the possibility that disease in patients with TB was of too short duration (MAC-PD, 4.8 ± 4.6 yr, vs. TB, 0.3 ± 0.2 yr) to have allowed immune responses and shed mycobacterial antigen. In this present study, with a cutoff level of 0.7 U/ml, all patients with TB were classified as seronegative. The levels of GPL core antibody in patients with pulmonary TB were very low or absent

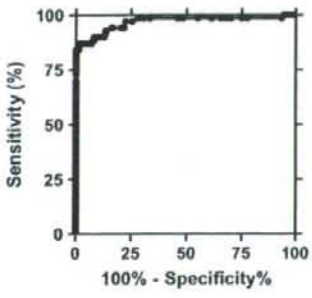

Figure 2. Receiver operating characteristic curve constructed for patients with *Mycobacterium avium*-complex pulmonary disease and the other groups.

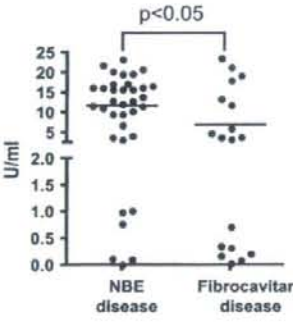

Figure 3. Levels of IgA antibody to glycopeptidolipid core antigens in nodular-bronchiectatic (NBE) and fibrocavitary subtypes of patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD). Significantly higher levels were found in patients with MAC-PD with NBE compared with fibrocavitary disease ($P < 0.05$).

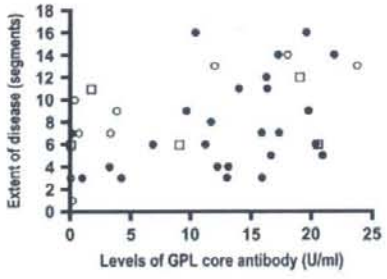

Figure 4. Correlation between antibody levels and radiographic severity using chest computed tomography in 41 patients with *Mycobacterium avium*-complex pulmonary disease. There was a positive correlation between the extent of disease and the levels of antibody ($r = 0.43$, $P < 0.05$). *Closed circles* represent patients with nodular-bronchiectatic disease, *open circles* represent patients with fibrocavitary disease, and *open squares* represent patients with unclassifiable type disease.

(0.1 ± 0.1 U/ml). In contrast, in previous studies (7, 8, 13), GPL seropositivity in patients with pulmonary TB ranged between 5.2 and 25%. One possible explanation for this previously reported lack of specificity may be that there was latent coinfection of MAC in patients with pulmonary TB. In the present study, however, we attempted to exclude patients with such latent coinfection because the entry criteria precluded patients having underlying lung disease or past history of pulmonary TB. Patients with lung diseases such as chronic obstructive pulmonary disease associated with smoking, bronchiectasis, previous mycobacterial disease, cystic fibrosis, and pneumoconiosis are prone to have MAC coinfection (1). In addition, future studies are needed to verify the cutoff value obtained from the ROC analysis using another sample of cases and controls on a much larger scale.

MAC-PD has recently been classified into two distinct subtypes: fibrocavitary disease and NBE disease (1). Fibrocavitary disease, the most common manifestation of MAC-PD, is usually seen in middle-aged or elderly men predisposed to lung disease due to smoking and alcohol drinking. This subtype of disease, generally progressive, is similar to pulmonary TB on chest radiography. If left untreated, it can lead to extensive lung destruction and death. In contrast, NBE disease is mostly seen in nonsmoking middle-aged or elderly women without predisposing lung disease. The clinical course is usually slower and less dramatic. Patients with NBE are presumed to have had a long subclinical period before appearance of disease manifestations. Significantly higher levels of GPL core antibody were seen in NBE than in fibrocavitary disease ($P < 0.05$) and higher seropositivity was found in patients with the former (91.4% compared with 63.2%). There were no significant differences of extent of disease between the two groups in patients who underwent CCT and serodiagnosis at the same time. Therefore, the results suggested the possibility that the antibody levels tend not to elevate in patients with fibrocavitary disease. This may reduce the utility of serodiagnosis for discriminating cavitary MAC from cavitary TB. However, the antibody would probably be present at high levels in patients with extensive lesions in fibrocavitary disease as was indeed found in three patients (17.9 ± 5.9 U/ml) who had extensive lesions (more than 13 segments) (Figure 4). Further investigations are required for confirmation of this notion in a larger study.

Of the 70 patients with MAC-PD, 64 had previously received combination chemotherapy, as recommended by the ATS guidelines (1). However, all had MAC-positive cultures at the time of serum collection, and were considered to have active MAC-PD. Thus, antibody levels were not changed by the failure of chemotherapy—that is, there was no conversion to seronegative from seropositive status (8); therefore, effects of the previous treatment on antibody levels were limited. Obviously, it would nonetheless be better to enroll chemotherapy-naive patients from diverse ethnic and racial populations and different geographic areas in future studies.

At present, the diagnosis of MAC-PD is usually made according to the ATS guidelines, which include clinical, radiographic, and microbiological criteria (1). The latter requires multiple positive cultures for MAC from sputum, a positive culture from bronchial lavage or a lung biopsy specimen, together with the other diagnostic features. Although it is easy to meet the criteria in advanced-stage MAC-PD, it is often difficult in early-stage disease. In clinical routine, it is impractical to obtain multiple sputum samples or perform bronchoscopy to obtain bronchial washings or lung tissue in all patients. It is also time consuming, because a long duration is required before the results of multiple cultures are available. There are several rapid methods for identification of MAC, but they have some limitations. The liquid culture–based system using radiometry and fluorometry allows the detection of mycobacterial growth at an early stage, fewer than 7 days for nontuberculous mycobacteria. However, limitations of this system include the inability to observe colony morphology, difficulty in recognizing mixed cultures, overgrowth by contaminations, cost, and radioisotope disposal. Rapid identification of MAC is also possible using DNA hybridization, nucleic acid amplification, or high-pressure liquid chromatography (1). The use of molecular biological technology has shortened the time required to identify mycobacteria from several weeks to as little as 1 day. The overall sensitivity for detecting MAC varies between 70 and 100%, with a specificity greater than 98%. However, the inability to distinguish live and dead organisms precludes nucleic acid amplification for definite diagnosis of active disease (14).

The EIA kit is a rapid (within a few hours) and noninvasive assay with high sensitivity (84.3%) and specificity (100%) for diagnosing MAC-PD. Using the EIA kit, as reported here, MAC-PD could be efficiently differentiated from MAC contamination. "MAC contamination" defined in the present study was considered to represent contamination from the environment, because patients were asymptomatic and revealed no significant CCT findings indicating active mycobacterial disease. Most of those people classified into the MAC contamination group were so categorized based on a single positive MAC culture by chance during the follow-up period after completion of chemotherapy for pulmonary TB or at routine examination on admission for other diseases. It is difficult to be certain that MAC contamina-

tion, as defined here, does not indicate subclinical infection because no confirmatory pathology was obtained. However, if MAC contamination does reflect subclinical infection, it is of little clinical importance and does not mandate therapy.

There were 15.7% false-negative EIA determinations in patients with MAC-PD. In such cases, diagnosis of MAC-PD should be made according to the ATS guidelines, as previously described. There are several possible explanations for these false-negative results, including the following: (*1*) recently diagnosed disease; (*2*) change of GPL core antigenicity after chemotherapy; or (*3*) diversity of immune responses to GPL core in individual patients, potentially governed by HLA genes (15). Therefore, it might be expected that not all patients with MAC-PD are capable of producing antibody to GPL core. Although the specificity determined here for the EIA kit was high, there remains also the possibility of false-positive results in patients with disease due to other mycobacteria, such as *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, and *Mycobacterium scrofulaceum*, because these organisms also possess GPL on their cell wall surface (10, 11, 16). Indeed, we have detected seropositivity in several patients with culture-positive *M. fortuitum* (data not shown). The incidence of pulmonary disease due to these other mycobacteria is relatively low (<5%) in Japan and the United States (6, 17), but a report from South Korea documented a high incidence of pulmonary infection by *M. abscessus* or *M. fortuitum* (33 and 11%, respectively (18). Therefore, caution is necessary when interpreting the results of the EIA kit in locations where other mycobacterial infections are endemic.

A recent study using high-resolution CT documented that characteristic findings with multiple small nodular shadows combined with bronchiectasis are predictive for culture-positive MAC with a relatively high probability. Swenson and colleagues (19) reported that, of 15 patients with these characteristic findings, 8 (53%) had cultures positive for MAC. Tanaka and coworkers (20) reported that, of 26 similar patients, 13 (50%) had positive cultures for MAC in bronchial washings. Therefore, combining positive results obtained by the EIA and the characteristic findings of high-resolution CT should yield a definitive diagnosis of MAC-PD even in patients with sputum culture-negative results for MAC. This approach may be useful especially in elderly patients with complications, in whom bronchoscopy cannot be performed.

In summary, the EIA kit for detection of serum IgA antibody specific for GPL core antigen is useful for rapid and accurate serodiagnosis of MAC-PD. Taken together with clinical, radiographic, and microbiological criteria, the kit may be a valuable tool for the diagnosis of MAC-PD. Validation of the EIA kit in the diagnosis of MAC-PD requires a larger controlled study in diverse populations.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Identification and Characterization of Two Novel Methyltransferase Genes That Determine the Serotype 12-Specific Structure of Glycopeptidolipids of *Mycobacterium intracellulare*▽

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The *Mycobacterium avium* complex is distributed ubiquitously in the environment. It is an important cause of pulmonary and extrapulmonary diseases in humans and animals. The species in this complex produce polar glycopeptidolipids (GPLs); of particular interest is their serotype-specific antigenicity. Several reports have described that GPL structure may play an important role in bacterial physiology and pathogenesis and in the host immune response. Recently, we determined the complete structure of the GPL derived from *Mycobacterium intracellulare* serotype 7 and characterized the serotype 7 GPL-specific gene cluster. The structure of serotype 7 GPL closely resembles that of serotype 12 GPL, except for O methylation. In the present study, we isolated and characterized the serotype 12-specific gene cluster involved in glycosylation of the GPL. Ten open reading frames (ORFs) and one pseudogene were observed in the cluster. The genetic organization of the serotype 12-specific gene cluster resembles that of the serotype 7-specific gene cluster, but two novel ORFs (*orfA* and *orfB*) encoding putative methyltransferases are present in the cluster. Functional analyses revealed that *orfA* and *orfB* encode methyltransferases that synthesize *O*-methyl groups at the C-4 position in the rhamnose residue next to the terminal hexose and at the C-3 position in the terminal hexose, respectively. Our results show that these two methyltransferase genes determine the structural difference of serotype 12-specific GPL from serotype 7-specific GPL.

The *Mycobacterium avium* complex (MAC) consists of two species, *M. avium* and *Mycobacterium intracellulare*, which are opportunistic pathogens of humans and animals. Human exposure to the MAC is common because organisms of this complex are ubiquitous in the environment: they have been isolated from water, soil, plants, house dust, and other sources. In fact, the MAC is the most common cause of disease attributable to nontuberculous mycobacteria in humans (9). The majority of MAC infections are acquired environmentally, and person-to-person transmission is considered to be rare. The treatment of MAC infection is difficult because the organisms are often resistant to standard antituberculosis drugs.

Many antigenic or immunoregulatory glycolipids with structural diversity are expressed on the mycobacterial cell wall. These molecules are considered to be involved in bacterial virulence through host immune responses (5, 14, 22, 23). It is necessary to elucidate the molecular structure, biochemical characteristics, and biological functions of the lipid components to better understand the mechanisms of pathogenesis and drug resistance of the MAC. The most prominent feature of the MAC is the presence of antigenic glycolipids, the glycopeptidolipids (GPLs), which are present on the cell surface (1). The standard method for differentiation of MAC strains is serologic typing based on the oligosaccharide (OSE) residue of the GPL. GPLs contain a tetrapeptide-amino alcohol core, D-phenylalanine-D-*allo*-threonine-D-alanine-L-alaninol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol), with an amido-linked 3-hydroxy or 3-methoxy C_{26}-to-C_{34} fatty acid at the N terminus of D-Phe (4). The D-*allo*-Thr and terminal L-alaninol are further linked with 6-deoxy-talose (6-d-Tal) and 3,4-di-*O*-methyl-rhamnose (3,4-di-*O*-Me-Rha), respectively. This core GPL is present in all species of the MAC and shows a common antigenicity (1). In the serotype-specific GPLs, a haptenic OSE is linked with the 6-d-Tal residue. To date, 31 distinct serotype-specific polar GPLs have been identified biochemically; the complete structures of GPLs are partly defined for serotype 1 to 4, 7, 8, 9, 12, 14, 17, 19 to 21, 25, and 26 GPLs (7, 10). On the other hand, it has been reported that serotype-specific GPLs participate in pathogenesis and immunomodulation in the host (2, 13). Modification of the GPL structure might play an important role not only in antigenicity but also in host immune responses and bacterial physiology (18). Recently, chemical synthesis of various haptenic OSEs was demonstrated, and the genes encoding glycosylation pathway enzymes for the biosynthesis of GPLs were identified and characterized (8, 12, 19, 21). However, genes responsible for serotype-specific glycosylation have yet to be analyzed for most of the serotypes.

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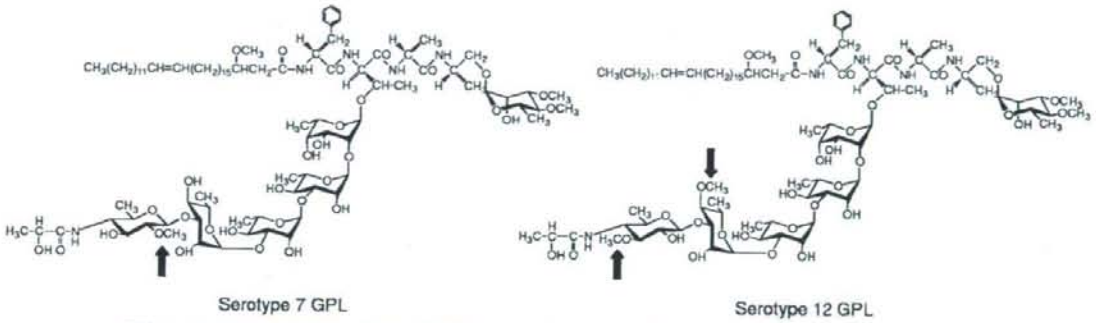

FIG. 1. Structures of serotype 7 and 12 GPLs. *O*-methyl groups specific to the serotypes are indicated by arrows.

In a previous study, we determined the complete structure of the GPL derived from *M. intracellulare* serotype 7 and characterized the serotype 7-specific gene cluster for GPL synthesis (10). The structure of serotype 7 GPL closely resembles that of serotype 12 GPL, except for O methylation (Fig. 1). In the present study, we determined the nucleotide sequence of the serotype 12-specific gene cluster involved in the glycosylation of the GPL and characterized two novel open reading frames (ORFs) encoding *O*-methyltransferases that determine the difference of serotype 12 GPL from serotype 7 GPL.

MATERIALS AND METHODS

Bacterial strains and construction of *M. intracellulare* cosmid library. *M. intracellulare* serotype 12 strain ATCC 35762 (NF 103), *M. intracellulare* serotype 7 strain ATCC 35847 (NF 027), and *M. intracellulare* serotype 7 strain NF 112 were used for this study. A cosmid library of *M. intracellulare* NF 103 was constructed as described previously (10). Briefly, genomic DNA of *M. intracellulare* NF 103 was prepared by mechanical disruption of bacterial cells in phosphate-buffered saline containing 50 mM EDTA, followed by phenol-chloroform extraction and precipitation with ethanol. Genomic DNA fragments randomly sheared to 30-kb to 50-kb fragments during the extraction process were fractionated and electroeluted from agarose gels. These DNA fragments were ligated to dephosphorylated arms of pYUB412 (XbaI-EcoRV and EcoRV-XbaI). After in vitro packaging using Gigapack III Gold extracts (Stratagene, La Jolla, CA), recombinant cosmids were introduced into *Escherichia coli* STBL2.

Isolation of cosmid clones carrying the GPL biosynthesis gene cluster and sequence analysis. PCR was used to isolate cosmid clones carrying the rhamnosyltransferase gene (*rtfA*), using primers rtfA-F (5′-TTTTGGAGCGACGAGTTCAT C-3′) and rtfA-R (5′-GTGTAGTTGACCACGCCGAC-3′). The insert of cosmid clone 161 was sequenced using a kit (BigDye Terminator cycle sequencing kit, version 3.1; Applied Biosystems, Foster City, CA) and a sequence analyzer (ABI Prism 310; Applied Biosystems). The putative function of each ORF was identified by similarity searches between the deduced amino acid sequences and those of known proteins, using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and FramePlot (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) with the DNASIS computer program (Hitachi Software Engineering, Yokohama, Japan).

Transformation of *M. intracellulare*. PCR was used to amplify and clone *orfA* and *orfB* into the plasmid vector pVV16. *M. intracellulare* NF 027 and NF 112 were transformed with the resultant plasmids by electroporation. Primers used to amplify *orfA*, *orfB*, and *orfA-orfB* were orfA-F (5′-GCGGATCCAGTGTGCAG ACGAGCGGAACT-3′), orfA-R (5′-GCGAATTCTTATCGAGAAAAAATA AAAG-3′), orfB-F (5′-GCGGATCCACTGCTAGACTCCGCCACCAT-3′), and orfB-R (5′-GCGAATTCCTACACCTTCACGGCGAGTC-3′).

Preparation of GPLs and OSE moieties. GPL 7 and GPL 12 were purified from *M. intracellulare* NF 027 and NF 103, respectively. The preparation of GPLs was performed as described previously (10, 15, 17). Briefly, each strain was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) with 0.5% glycerol and 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment (Difco Laboratories) at 37°C for 2 to 3 weeks. The heat-killed bacteria were sonicated and extracted using chloroform-methanol (2:1 [vol/vol]). The extractable lipids were hydrolyzed with 0.2 N sodium hydroxide in methanol at 37°C for 2 h. After neutralization using 6 N hydrochloride, chloroform-methanol (2:1 [vol/vol]) and water were added. The organic phase containing alkaline-stable lipids was recovered and evaporated, with subsequent addition of acetone to remove any acetone-insoluble components. The supernatant was dried up. It was then treated using a Sep-Pak silica cartridge (Waters Corp., Milford, MA) with washing (chloroform-methanol [95:5 {vol/vol}]) and elution (chloroform-methanol [1:1 {vol/vol}]) for partial purification. The GPL was then purified completely by preparative thin-layer chromatography (TLC) with silica gel G (Uniplate; 20 cm × 20 cm × 250 μm; Analtech, Inc., Newark, DE). The TLC was developed repeatedly, using chloroform-methanol-water (60:16:2 [vol/vol/vol]), until a single spot was obtained. To prepare the OSE moiety, purified GPL was processed using β-elimination with alkaline borohydride, and then the carbohydrate chain moiety

TABLE 1. Similarity of Orfs in *M. intracellulare* serotype 12 strain ATCC 35762 to known protein sequences

Orf	Predicted molecular mass (Da)	Predicted pI	Similar protein	Identity (no. of matched amino acids/total no. of amino acids)	E value	GenBank accession no.
GtfB	45,830	6.87	Glycosyltransferase GtfB	412/418	0.0	BAF45360
Orf1	45,203	6.10	Putative glycosyltransferase	414/417	0.0	BAF45361
OrfA	28,904	7.42	Putative methyltransferase	182/224	5e−88	NP_218045
OrfB	29,930	5.15	Putative methyltransferase	102/204	1e−19	EAZ88812
Orf3	32,151	10.41	Putative glycosyltransferase	196/223	1e−108	BAF45363
Orf4	40,742	5.41	Putative aminotransferase	338/374	0.0	BAF45364
Orf5	35,812	5.26	Hypothetical protein	303/329	4e−162	BAF45365
Orf7	27,693	5.99	Putative metallophosphoesterase	223/241	1e−122	BAF45367
Tn	28,538	11.85	Putative transposase	213/255	6e−107	AAL61662
Orf8	80,044	9.16	Putative acyltransferase	689/747	0.0	BAF45368
Orf9	37,797	8.26	Putative glycosyltransferase	310/337	7e−169	BAF45369
DrrC	28,549	12.01	Daunorubicin resistance protein C	261/263	3e−141	BAF45370

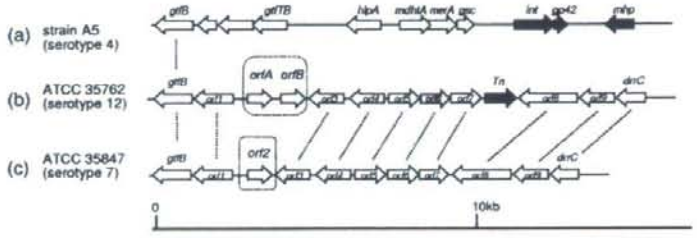

FIG. 2. Comparison of genetic organization of GPL biosynthesis clusters. (a) *M. avium* strain A5 organization, based on the annotated sequence obtained from GenBank (accession no. AY130970). (b) *M. intracellulare* ATCC 35762 (NF 103), sequenced in this study. (c) *M. intracellulare* ATCC 35847 (NF 027), sequenced in our previous study (GenBank accession no. AB274811). The orientation of each gene is shown by the arrow direction. The black arrows represent mobile elements, and the gray arrow represents a pseudogene. Mutually homologous ORFs and sequences are indicated with dotted lines.

elongated from D-*allo*-Thr was released as described previously (10, 15). Briefly, GPL was treated with 5 mg/ml sodium borohydride or borodeuteride in 0.5 N sodium hydroxide-ethanol (1:1 [vol/vol]) at 60°C for 16 h, with stirring. The reaction mixture was decationized with Dowex 50W X8 beads (The Dow Chemical Company, Midland, MI). The supernatant was collected and evaporated under nitrogen to remove boric acid. The dried residue was partitioned into two layers, using chloroform-methanol (2:1 [vol/vol]) and water. The upper aqueous phase was recovered and evaporated. In these processes, the OSE was purified as an oligoglycosyl alditol.

MALDI-TOF MS and MALDI-TOF/TOF MS analyses. The molecular species of the intact GPLs were detected using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) with an Ultraflex II spectrophotometer (Bruker Daltonics, Billerica, MA). Each GPL was dissolved in chloroform-methanol (2:1 [vol/vol]) at a concentration of 1 mg/ml; 1 μl of a sample was then applied directly to the sample plate, followed by the addition of 1 μl of 10-mg/ml 2,5-dihydroxybenzoic acid in chloroform-methanol (1:1 [vol/vol]) as a matrix. The intact GPL was analyzed in the reflectron mode, with an accelerating voltage operating in positive mode at 20 kV (3). The OSE was analyzed by the fragment pattern with MALDI-TOF/TOF MS to determine the glycosyl composition. The OSE was dissolved with ethanol-water (3:7 [vol/vol]); the matrix was 10 mg/ml 2,5-dihydroxybenzoic acid in ethanol-water (3:7 [vol/vol]). The OSE and matrix were added to the sample plate by the same method as that for intact GPL. They were then analyzed in the lift-lift mode.

GC-MS analyses of alditol acetate derivatives. Gas chromatography (GC) and GC-MS analyses of partially methylated alditol acetate derivatives were performed to determine glycosyl compositions and linkage positions. Perdeuteromethylation was conducted using a modified procedure of Hakomori, as described previously (10, 11). Briefly, the dried OSE was dissolved with a mixture of dimethyl sulfoxide and sodium hydroxide, and deuteromethyl iodide was added. The reaction mixture was stirred at room temperature for 15 min, followed by the addition of water and chloroform. After centrifugation at 2,400 × *g* for 15 min, the upper water layer was discarded. The chloroform layer was washed twice with water and evaporated completely. To prepare partially deuteromethylated alditol acetates, perdeuteromethylated OSE was hydrolyzed using 2 N trifluoroacetic acid at 120°C for 2 h, reduced with 10 mg/ml sodium borodeuteride at 25°C for 2 h, and acetylated with acetic anhydride at 100°C for 1 h (6, 10, 16). GC-MS was then performed using a benchtop ion-trap mass spectrometer (Trace DSQ GC/MS; Thermo Electron Corporation, Austin, TX) equipped with a fused capillary column (30 m; 0.25-mm internal diameter) (Equity-1 or SP-2380; Supelco, Bellefonte, PA). Helium was used as the carrier gas, and the flow rate was 1 ml/min. The SP-2380 column was used for the analysis of alditol acetate derivatives. The temperature program was started at 60°C, with an increase of 40°C/min to 260°C and a hold at 260°C for 25 min. The Equity-1 column was used for analysis of perdeuteromethylated alditol acetate derivatives. The temperature program was 80°C for 1 min, with an increase of 20°C/min to 180°C followed by an increase of 8°C/min to 280°C.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the NCBI GenBank database under accession number AB353739.

RESULTS

Cloning and sequence of the serotype 12 GPL biosynthesis cluster. To isolate the serotype 12-specific GPL biosynthesis gene cluster, a genomic cosmid library of an *M. intracellulare* serotype 12 strain, NF 103, was constructed. DNA was extracted from each clone by boiling. Using colony PCR with *rftA* primers, the positive clone 161 was isolated from the *E. coli* transductants. Sequencing analysis revealed that cosmid clone 161 carried the DNA region from *gtfB* to *drrC*. Ten ORFs and one pseudogene other than *gtfB* and *drrC* were observed in the cluster (Table 1 and Fig. 2). The genetic organization between the *gtfB* and *drrC* genes (15.6 kb) of *M. intracellulare* NF 103 (serotype 12) closely resembled that of

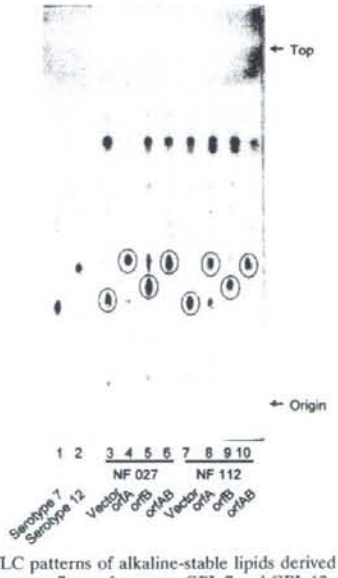

FIG. 3. TLC patterns of alkaline-stable lipids derived from *M. intracellulare* serotype 7 transformants. GPL 7 and GPL 12 were purified from *M. intracellulare* serotype 7 strain ATCC 35847 (NF 027) and serotype 12 strain ATCC 35762 (NF103). TLC was developed with a solvent system of chloroform-methanol-water (65:25:4 [vol/vol/vol]). Circled spots indicate prominent GPLs.

the same region of *M. intracellulare* NF 027 (serotype 7), except for three loci (Fig. 2). The first difference between them was an additional ORF encoding a transposase between *orf7* and *orf8* in NF 103 (Fig. 2). The second difference was that the *orf6* homologous sequence in NF 103 had a frame shift, indicating that this locus does not encode a protein. The third difference is that two novel ORFs (*orfA* and *orfB*) instead of *orf2* were found between *orf1* and *orf3* in NF 103.

Functional analysis of the two unique ORFs found in the serotype 12 GPL biosynthesis cluster. Based on sequence homology, *orfA* and *orfB* were able to encode methyltransferases responsible for producing serotype 12 GPLs (Table 1). We constructed three plasmids carrying *orfA* and/or *orfB* downstream of the *hsp-60* promoter to test this. These plasmids and a control vector plasmid were introduced individually into *M. intracellulare* serotype 7 (NF 027 and NF 112), and transformants were obtained. The GPLs produced from each transformant were analyzed.

The alkaline-stable lipids derived from six transformants of NF 027 and NF 112 in addition to the control strains (vector only) were developed by TLC, and the produced GPLs were compared to the spots of GPL 7 and GPL 12 (Fig. 3). The R_f values for GPLs synthesized in NF 027 transformed with *orfA* and NF 027 transformed with *orfA* and *orfB* (GPL 7-orfA and GPL 7-orfAB, respectively) were almost identical to that for GPL 12; the R_f value for the GPL synthesized in NF 027 transformed with *orfB* (GPL 7-orfB) was intermediate between those of GPL 7 and GPL 12, although the GPL synthesized in the control strain (GPL vector) was not changed from GPL 7. These results suggest that *orfA*, *orfB*, and *orfA-orfB* introduced into serotype 7 strain NF 027 were expressed and that they functioned for the modification of GPLs. We investigated the structural definition of these modified GPLs.

The GPLs produced in the transformants were purified using preparative TLC; their molecular weights were measured using MALDI-TOF MS (Fig. 4). The main molecularly related ions of GPL 7 and GPL 12 were detected as *m/z* 1,897 and 1,911, respectively, for $[M + Na]^+$ (Fig. 4a and b). The predominant *m/z* values were 1,911 for GPL 7-orfA, 1,897 for GPL 7-orfB, and 1,911 for GPL7-orfAB (Fig. 4c to e). The molecular weight of GPL 7-orfB was the same as that of GPL 7, and those of GPL 7-orfA and GPL 7-orfAB were equal to that of GPL 12. Next, MALDI-TOF/TOF MS analysis was performed to determine the glycosyl pattern, using fragment ions of glycosyl cleavage. The fragment ions of the GPL vector (equal to GPL 7) showed *m/z* 254, 400, 546, and 692 for cleavage in turn from terminal 4*N*-acyl-hexose (Hex) and 336, 482, and 628 for cleavage in the opposite direction from 6-d-Tal (Fig. 5a). The fragment ions of GPL 7-orfA, *m/z* 414 and 642, were different from those of GPL 7, i.e., *m/z* 400 and 628, respectively; they demonstrated that the mass number of the sugar next to the terminal Hex increased 14 mass units (Fig. 5b). This result suggests that the second sugar from the terminal one was changed from Rha to *O*-methyl rhamnose (*O*-Me-Rha). Similarly, the fragment pattern of GPL 7-orfAB was identical to that of GPL 7-orfA, although that of GPL 7-orfB was the same as that of GPL 7 (Fig. 5c and d). Altogether, GPL 7-orfAB was predicted to have a modification of the *O*-Me position in the terminal Hex along with the substitution of *O*-Me-Rha for Rha in the sugar next to the terminal Hex; GPL 7-orfB was modified only at the *O*-Me position in the terminal Hex.

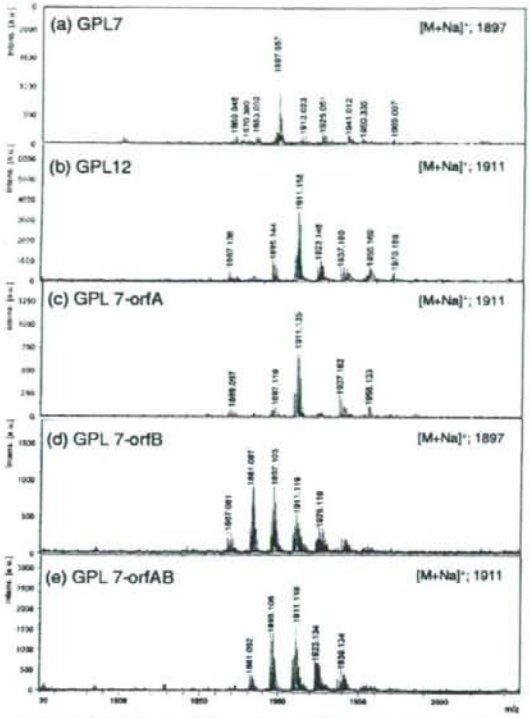

FIG. 4. MALDI-TOF MS spectra of GPLs derived from *M. intracellulare* serotype 7, serotype 12, and serotype 7 transformants. a.u., absorbance units.

GC-MS analyses of alditol acetate and perdeuteromethyl alditol acetate derivatives were performed to assign the linkage position of *O*-Me. As portrayed in Fig. 6a and b, the alditol acetate derivatives of the second sugar from the terminal 4*N*-acyl-Hex in GPL 7-orfA and GPL 7-orfB were assigned to 1,2,3,5-tetra-acetyl-4-*O*-methyl-rhaminitol (*m/z* 99, 131, 159, 201, and 261) and 1,2,3,4,5-penta-acetyl-rhaminitol (*m/z* 115, 157, 187, 217, 231, 289, and 303), respectively. The perdeuteromethyl alditol acetate derivatives of the terminal sugar in GPL 7-orfA and GPL 7-orfB were assigned to 3-*O*-deuteromethyl-1,5-di-*O*-acetyl-4-2′-*O*-deuteromethyl-propanoyl-deuteromethylamido-4,6-dideoxy-2-*O*-methyl-hexitol (*m/z* 105, 118, 165, 209, 222, 269, and 300) and 2-*O*-deuteromethyl-1,5-di-*O*-acetyl-4-2′-*O*-deuteromethyl-propanoyl-deuteromethylamido-4,6-dideoxy-3-*O*-methyl-hexitol (*m/z* 105, 121, 165, 206, 222, 266, and 300), respectively (Fig. 6c and d). In particular, the fragment ions of *m/z* 118 and 269 (Fig. 6c) versus *m/z* 121 and 266 (Fig. 6d) strongly indicated the different positions of linkages 2-*O*-Me and 3-*O*-Me. The alditol acetate and perdeuteromethyl alditol acetate derivatives in GPL 7-orfAB were detected with the same patterns of 4*N*-acyl-4,6-dideoxy-3-*O*-Me-Hex and 4-*O*-Me-Rha. According to these results, all OSE structures in GPLs derived from three serotype 7 transfor-

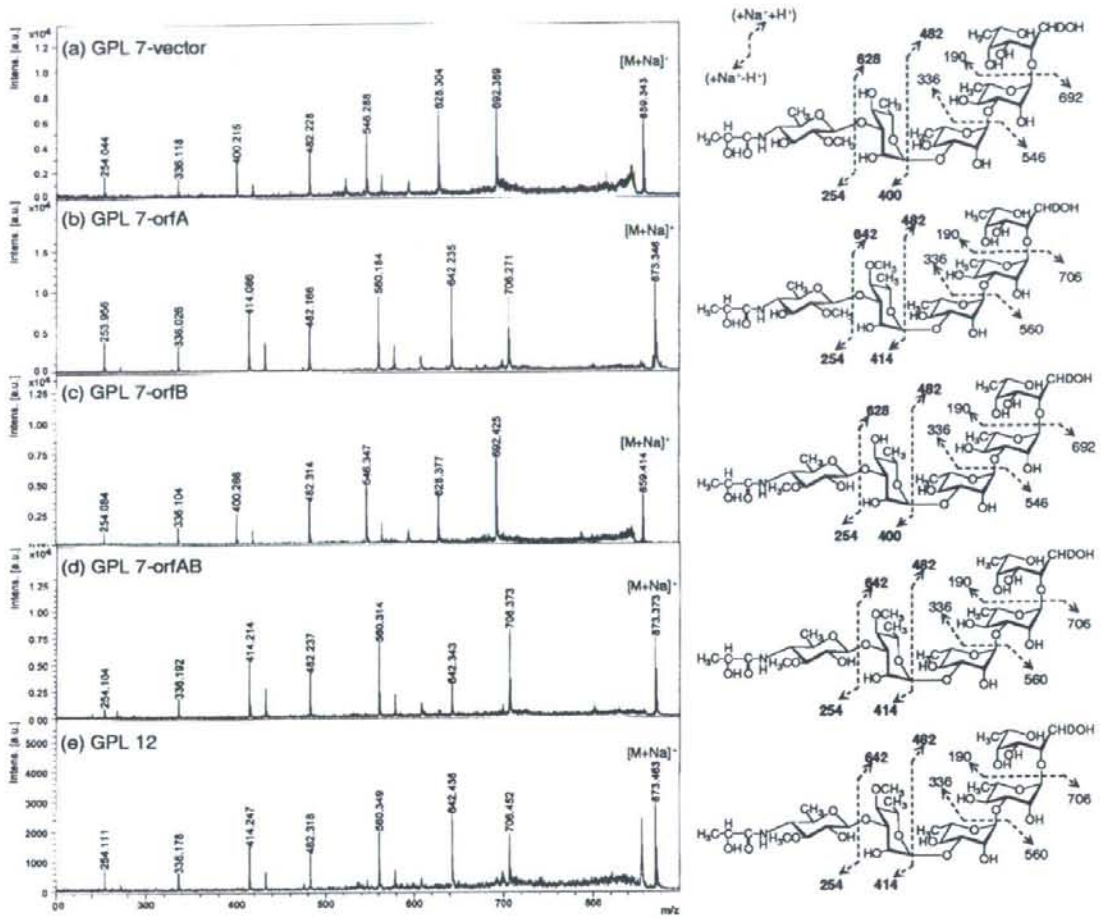

FIG. 5. Fragment patterns of MALDI-TOF/TOF MS spectra of OSEs in GPLs derived from *M. intracellulare* serotype 7, serotype 12, and serotype 7 transformants. The MALDI-TOF/TOF MS spectra were acquired using 10 mg/ml 2,5-dihydroxybenzoic acid in ethanol-water (3:7 [vol/vol]) as the matrix; the molecularly related ions were detected as $[M + Na]^+$ in lift-lift mode. The assigned fragment patterns of glycosyl residues are depicted. a.u., absorbance units.

mants were assigned as listed in Table 2. Altogether, the functions of the two genes were defined. The *orfA* product transfers a methyl group to the C-4 position of Rha next to the terminal sugar, and the *orfB* product transfers a methyl group to the C-3 position of the terminal sugar (Fig. 7). The results demonstrated that GPL 7 in the serotype 7 strain was changed completely to GPL 12 by introduction of the *orfA-orfB* gene cluster.

DISCUSSION

Nontuberculous mycobacteria, including the pathogenic species belonging to the MAC, have serotype-specific GPLs that are important components of the outer layer of the lipid-rich cell walls (5). Structural analyses of some serotype-specific GPLs derived from predominant clinical isolates have been reported (20). We recently determined the complete structure of serotype 7 GPL and the nucleotide sequence of the serotype 7-specific GPL biosynthesis cluster (10). In this cluster, Orfs 1, 3, and 9 might engender transfer of the two molecules of L-Rha and the terminal Hex of serotype 7 GPL (10). Orfs 4, 5, 7, and 8 are homologous to an aminotransferase, a carbamoyl phosphate synthase protein, a metallophosphoesterase, and an acyltransferase, respectively, and possibly relate to the biosynthesis of 2′-hydroxypropanoylamido in the terminal Hex. Based on analysis of sequence homology, these ORFs are probably responsible for the glycosylation of serotype 7 GPL. Serotype 12 GPL has a similar structure to that of serotype 7 GPL, except for O methylation (Fig. 1). In the present study, we cloned the serotype 12 GPL biosynthesis cluster and analyzed its sequence. Although the genetic organization of the *gtfB*-to-*drrC*

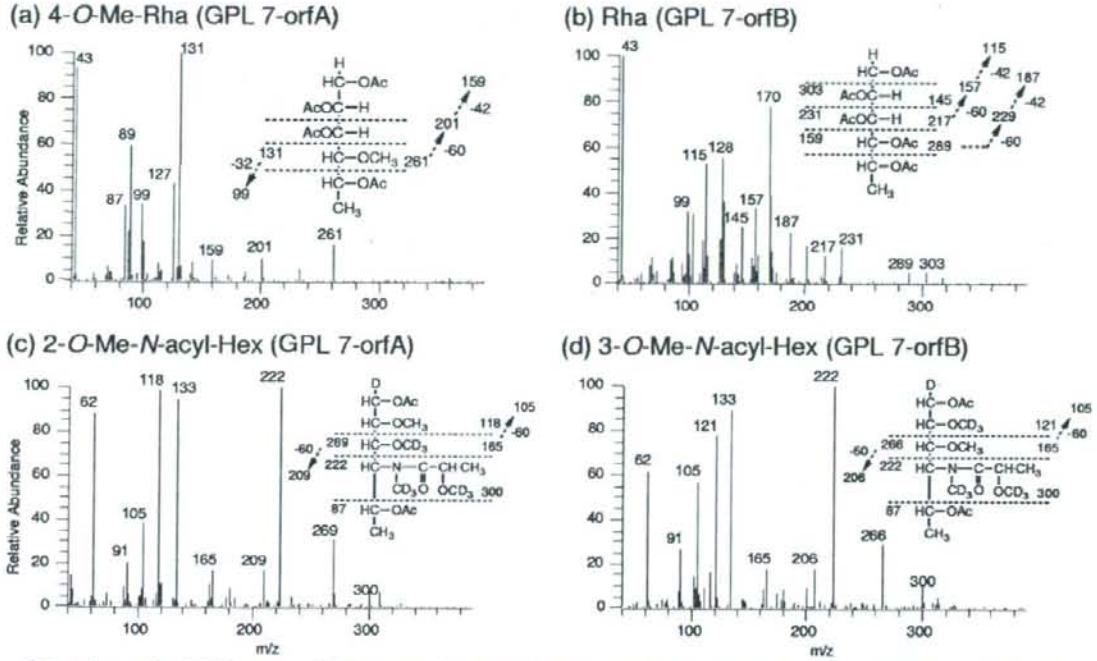

FIG. 6. Preparative GC-MS spectra of alditol acetate (a and b) and perdeuteromethylated alditol acetate (c and d) derivatives. The patterns of prominent fragment ions are presented. An SP-2380 column was used for the analysis of alditol acetate derivatives. The temperature program was started at 60°C, with an increase of 40°C/min to 260°C and a hold at 260°C for 25 min. An Equity-1 column was used for the perdeuteromethylated alditol acetate derivatives. The temperature program was 80°C for 1 min, with an increase of 20°C/min to 180°C followed by an increase of 8°C/min to 280°C.

region of the serotype 12 GPL biosynthetic cluster closely resembled that of serotype 7, significant differences were found in three loci (Fig. 2). The *M. intracellulare* serotype 12 strain NF 103 had one ORF encoding a transposase between *orf7* and *orf8* and had an *orf6* homologous sequence with frameshift inactivation. Orf6 in *M. intracellulare* serotype 7 exhibits sequence similarity to nucleotide sugar epimerases/dehydrogenases, but NF 112, one of the *M. intracellulare* serotype 7 isolates, had an interrupted *orf6* (10). These findings suggest that *orf6* is not involved in biosynthesis of either serotype 7 GPL or serotype 12 GPL. The most important difference between the two serotypes is that *M. intracellulare* serotype 12 had two unique ORFs, *orfA* and *orfB*, instead of *orf2* in *M. intracellulare* serotype 7. Actually, Orf2 in *M. intracellulare* serotype 7 was assigned to a methyltransferase and might be responsible for synthesis of the *O*-methyl group at the C-2 position in the terminal Hex. That possibility suggests that the two unique ORFs for serotype 12 encode *O*-methyltransferases that produce the serotype 12-specific structure. NF 027 (serotype 7) transformed with *orfA* produced 4*N*-acyl-4,6-dideoxy-2-*O*-Me-Hex→4-*O*-Me-Rha→Rha→Rha→6-d-Tal, indicating that the product from *orfA* had activity to synthesize an *O*-methyl group at C-4 in L-Rha next to the terminal Hex (Table 2 and Fig. 7). NF 027 transformed with *orfB* produced 4*N*-acyl-4,6-dideoxy-3-*O*-Me-Hex→Rha→Rha→Rha→6-d-Tal, indicating that the product from *orfB* had activity to synthesize an *O*-methyl group at C-3 in the terminal Hex. NF 027 transformed with *orfA* and *orfB* produced serotype 12-specific GPL,

TABLE 2. Summarized structures of OSEs derived from serotype 7 transformants

GPL	Molecular weight of OSE	Fragment ions in MALDI-TOF/TOF MS	*O*-Methyl group		Structure of OSE
			Terminal sugar	Residue next to terminal sugar	
GPL 7 vector	859	254, 400, 546, 692	2-*O*-Met		4*N*-acyl-4,6-dideoxy-2-*O*-Me-Hex→Rha→Rha→Rha→6-d-Tal
GPL 7-orfA	873	254, 414, 560, 706	2-*O*-Met	4-*O*-Met	4*N*-acyl-4,6-dideoxy-2-*O*-Me-Hex→4-*O*-Me-Rha→Rha→Rha→6-d-Tal
GPL 7-orfB	859	254, 400, 546, 692	3-*O*-Met		4*N*-acyl-4,6-dideoxy-3-*O*-Me-Hex→Rha→Rha→Rha→6-d-Tal
GPL 7-orfAB	873	254, 414, 560, 706	3-*O*-Met	4-*O*-Met	4*N*-acyl-4,6-dideoxy-3-*O*-Me-Hex→4-*O*-Me-Rha→Rha→Rha→6-d-Tal

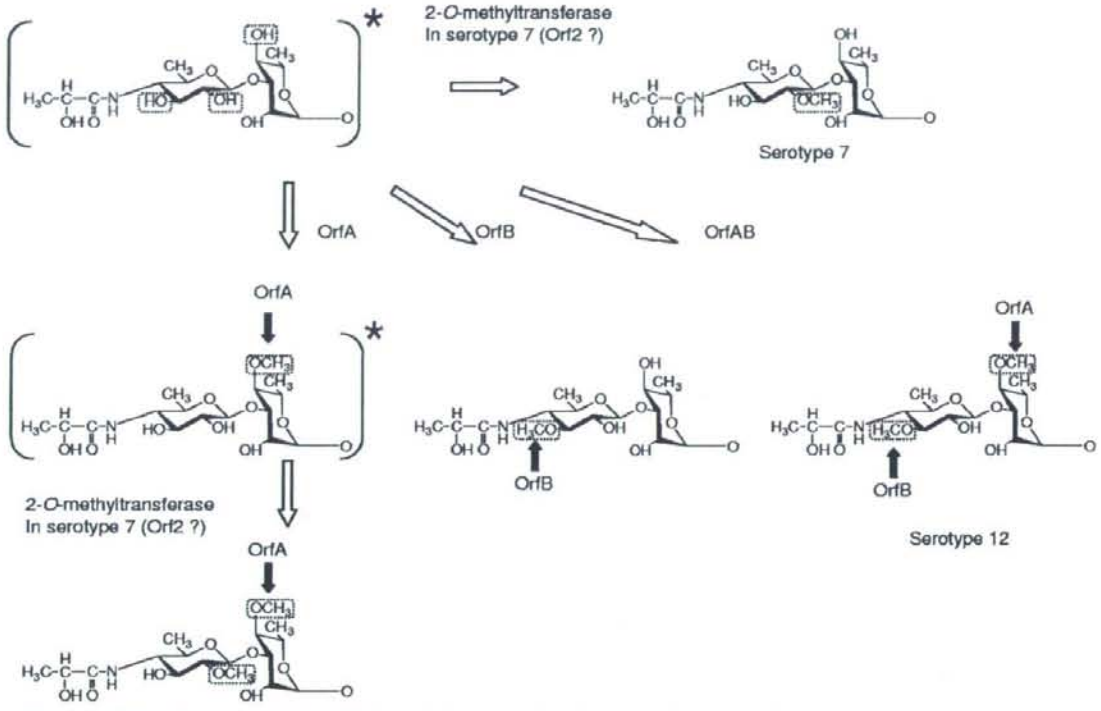

FIG. 7. Synthesis of *O*-methyl groups specific for GPL 7 and GPL 12 in the terminal disaccharide. The structures asterisked in the figure were not detected in this study. Serotype 12-specific O methylations and ORFs responsible for their syntheses are indicated by black arrows.

indicating that these two ORFs were responsible for producing the serotype 12-specific structure. The TLC patterns showed that the migration of GPL 7-orfB was different from that of GPL 7, although the MS data showed that they had the same molecular weight and the same number of methyl groups. A possible explanation for this is that a difference in the position of O methylation could influence hydrogen bond formation and the polarity of the whole molecule and consequently result in a different TLC migration pattern. GPL 7-orfB had an *O*-methyl group at C-3 but not at C-2 in the terminal Hex, indicating that the reaction of O methylation at C-2 by the 2-*O*-methyltransferase in serotype 7 is strongly inhibited by *O*-methylation at C-3. In addition, NF 027 transformed with *orfA* produced a trace of serotype 7-specifc GPL (Fig. 3, lane 4), and NF 027 transformed with *orfA* and *orfB* produced only serotype 12 GPL (Fig. 3, lane 6), suggesting that O methylation at C-2 in the terminal Hex might hinder the reaction of O methylation at C-4 in Rha next to the terminal Hex or that O methylation at C-3 in the terminal Hex might promote the reaction of O methylation at C-4 in Rha.

Because it is not likely that *M. intracellulare* serotypes 7 and 12 independently acquired different methyltransferase genes in the same genetic location between *orf1* and *orf3*, the common ancestor for these two serotypes possibly had all three genes and activated them as the occasion demanded. However, our results showed that reactions of O methylation at C-3 and C-2 in the terminal Hex were competitive (Fig. 3, lane 5, and Table 2). Tsang et al. (26) reported that the frequency of isolation of MAC organisms from AIDS or non-AIDS patients varied among serotypes and that *M. intracellulare* serotype 12 was isolated more often than serotype 7. These two serotypes of *M. intracellulare* might have evolved to adapt to certain environments by losing *orf2* or *orfA-orfB*.

Actually, GPLs are among the immunogenic molecules of the MAC. Tassel et al. reported that the core GPL seems to play a role in suppression of a mitogen-induced blastogenic response of spleen cells (25); furthermore, our previous study showed that sera of patients with MAC disease contain antibodies against GPLs and that the antibody level reflects disease activity (17). In addition, the immunomodulating activity of GPLs on macrophage functions is serotype dependent (13, 24). Elucidation of the structure-activity relationship of GPLs is necessary to better understand the pathogenesis of MAC infection.

ACKNOWLEDGMENTS

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Structural Analysis and Biosynthesis Gene Cluster of an Antigenic Glycopeptidolipid from *Mycobacterium intracellulare*▽†

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***Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) is the most common isolate of nontuberculous mycobacteria and causes pulmonary and extrapulmonary diseases. MAC species can be grouped into 31 serotypes by the epitopic oligosaccharide structure of the species-specific glycopeptidolipid (GPL) antigen. The GPL consists of a serotype-common fatty acyl peptide core with 3,4-di-*O*-methyl-rhamnose at the terminal alaninol and a 6-deoxy-talose at the *allo*-threonine and serotype-specific oligosaccharides extending from the 6-deoxy-talose. Although the complete structures of 15 serotype-specific GPLs have been defined, the serotype 16-specific GPL structure has not yet been elucidated. In this study, the chemical structure of the serotype 16 GPL derived from *M. intracellulare* was determined by using chromatography, mass spectrometry, and nuclear magnetic resonance analyses. The result indicates that the terminal carbohydrate epitope of the oligosaccharide is a novel *N*-acyl-dideoxy-hexose. By the combined linkage analysis, the oligosaccharide structure of serotype 16 GPL was determined to be 3-2′-methyl-3′-hydroxy-4′-methoxy-pentanoyl-amido-3,6-dideoxy-β-hexose-(1→3)-4-*O*-methyl-α-L-rhamnose-(1→3)-α-L-rhamnose-(1→3)-α-L-rhamnose-(1→2)-6-deoxy-α-L-talose. Next, the 22.9-kb serotype 16-specific gene cluster involved in the glycosylation of oligosaccharide was isolated and sequenced. The cluster contained 17 open reading frames (ORFs). Based on the similarity of the deduced amino acid sequences, it was assumed that the ORF functions include encoding three glycosyltransferases, an acyltransferase, an aminotransferase, and a methyltransferase. An *M. avium* serotype 1 strain was transformed with cosmid clone no. 253 containing *gtfB-drrC* of *M. intracellulare* serotype 16, and the transformant produced serotype 16 GPL. Together, the ORFs of this serotype 16-specific gene cluster are responsible for the biosynthesis of serotype 16 GPL.**

Mycobacterial diseases, such as tuberculosis and infection due to nontuberculous mycobacteria (NTM), are still among the most serious infectious diseases in the world. The incidence is increasing because of the spread of drug-resistant mycobacteria and the human immunodeficiency virus (HIV) infection/AIDS epidemic (16, 17, 30). *Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) is the most common among isolates of NTM and is distributed ubiquitously in the environment. MAC causes pulmonary and extrapulmonary diseases in both immunocompromised and immunocompetent hosts. It affects primarily patients with advanced HIV infection. MAC includes at least two mycobacterial species, *M. avium* and *M. intracellulare*, that cannot be differentiated on the basis of traditional physical and biochemical tests (1, 41).

The cell envelope of mycobacteria is a complex and unusual structure. The key feature of this structure is an extraordinarily high lipid concentration (6, 10). To better understand the pathogenesis of MAC infection, it is necessary to elucidate the molecular structure and biochemical features of the lipid components. Among MAC lipids, the glycopeptidolipid (GPL) is of particular importance, because it shows not only serotype-specific antigenicity but also immunomodulatory activities in the host immune responses (2, 9, 23). Structurally, GPLs are composed of two parts, a tetrapeptide-amino alcohol core and a variable oligosaccharide (OSE). C_{26}-C_{34} fatty acyl-D-phenylalanine-D-*allo*-threonine-D-alanine-L-alaninol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol) is further linked with 6-deoxy talose (6-d-Tal) and 3,4-di-*O*-methyl rhamnose (3,4-di-*O*-Me-Rha) at D-*allo*-Thr and the terminal L-alaninol, respectively. This type of core GPL is found in all subspecies of MAC, shows a common antigenicity, and is further glycosylated at 6-d-Tal to form a serotype-specific OSE.

At present, 31 distinct serotype-specific GPLs have been identified serologically and chromatographically (9). Although the standard technique for differentiation of MAC subspecies

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