図説

結核菌・抗酸菌感染防御機構

Protective immunity to mycobacterial infection

河村伊久雄

はじめに

結核菌は宿主に感染後速やかにマクロファージに貪食されるが，その細胞内殺菌機構を制御して細胞内で増殖する．また，結核菌は感染細胞の細胞死およびシグナル伝達経路を修飾することで，その細胞内環境を生存増殖に適したものにする能力を有し，宿主防御免疫に抵抗して持続感染を成立させる．一方，結核菌が感染した宿主では感染3-4週間後には強い細胞性免疫が誘導され，感染初期にみられる菌の増殖は抑えられる．しかし，結核菌に対して過剰な免疫応答が誘導された場合には，正常組織が破壊され，結核症に特有な病像が現れる．このように，結核に対する感染防御では宿主免疫応答を適切に制御することが重要な要素となる．

1. 食細胞による異物識別と自然免疫応答の誘導

マクロファージや樹状細胞(dendritic cell: DC)は細胞表面のtoll-like receptor(TLR)を介して結核菌を識別する[1](**図1**)．TLR2は結核菌のリポ多糖体成分であるlipoarabinomannan(LAM)やphosphatidylinositol-mannoside(PIM)，あるいはリポタンパク質を認識する．また，結核菌感染ではTLR4およびTLR9経路も活性化することから，これらレセプターに対するリガンドが存在することが示されている．更に，Cタイプレクチンであるdectin-1やmincle，更に細胞質内のnucleotide-binding oligomerization domain 2(NOD2)が結核菌の侵入を感知するセンサーとして働く．これらレセプターからのシグナルはマクロファージやDCを活性化し，アポトーシスやオートファジーを誘導する．また，結核菌感染はインフラマソーム形成を介したカスパーゼ1の活性化を誘導する．その結果，産生された炎症性サイトカインやケモカインは感染局所への炎症性細胞の動因や，肉芽腫形成に関与する．

2. 肺におけるT細胞への抗原提示

肺に侵入した結核菌を取り込んだ肺胞マクロファージは，所属リンパ節に移動する能力が低い．このためT細胞への抗原提示には，感染局所で産生されるケモカインや炎症性サイトカインにより動員されるDCが必要になる(**図2**)．一方，結核菌は感染マクロファージのリポキシンA4産生を誘導し，プロスタグランジンE_2産生を抑制する．このため，感染マクロファージのミトコンドリア内膜が傷害され，細胞がネクローシスに陥り，菌の増殖を許す結果となる[2]．これに対して弱毒株の感染ではリポキシンA4産生が誘導されないため，感染細胞ではアポトーシス経路が活性化され，菌の増殖は阻害される．この菌の病原性に関連した感染細胞の細胞死制御のメカニズムは，結核菌が持続感染を成立させるために重要であると考えられる．

Ikuo Kawamura: Department of Microbiology, Kyoto University Graduate School of Medicine 京都大学大学院医学研究科 微生物感染症学

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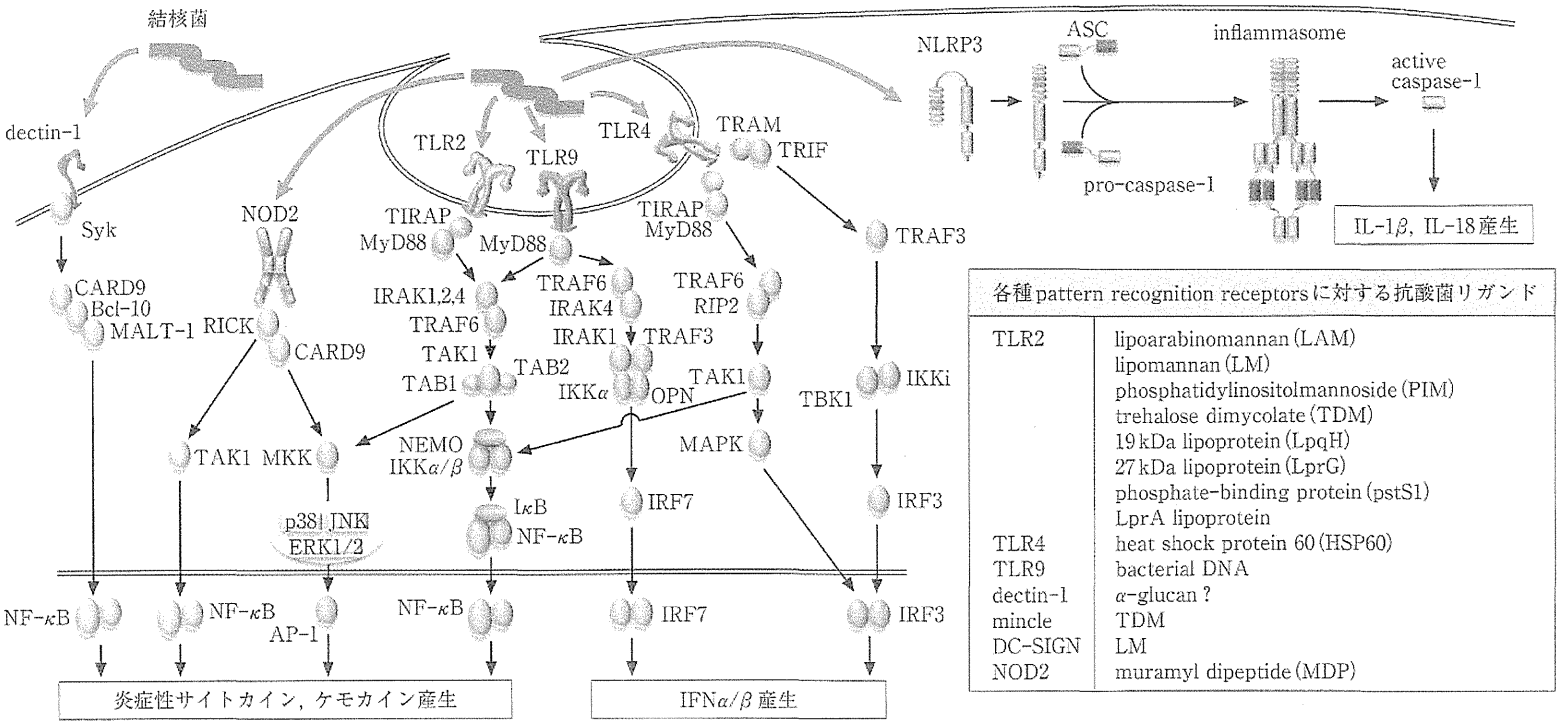

各種pattern recognition receptorsに対する抗酸菌リガンド	
TLR2	lipoarabinomannan (LAM) lipomannan (LM) phosphatidylinositolmannoside (PIM) trehalose dimycolate (TDM) 19 kDa lipoprotein (LpqH) 27 kDa lipoprotein (LprG) phosphate-binding protein (pstS1) LprA lipoprotein
TLR4	heat shock protein 60 (HSP60)
TLR9	bacterial DNA
dectin-1	α-glucan ?
mincle	TDM
DC-SIGN	LM
NOD2	muramyl dipeptide (MDP)

図1　結核菌感染により活性化されるシグナル伝達経路と結核菌リガンド

マクロファージやDCはTLR2，TLR4およびTLR9で結核菌体成分を認識する．また，Cタイプレクチンであるdectin-1やmincleも菌体成分の識別に関与する．更に，細胞質内に存在するNOD2はMDPを認識するセンサーとして働く．これらレセプターとリガンドの結合はそれぞれのシグナル経路を活性化し，炎症性サイトカイン，ケモカインあるいはI型インターフェロン産生を誘導する．また，結核菌はNLRP3インフラマソームの形成を誘導してカスパーゼ1を活性化する．その結果，感染細胞ではピロプトーシス経路が刺激されてIL-1βおよびIL-18産生が誘導される．

AP-1: activator protein 1, ASC: apoptosis-associated speck-like protein containing a CARD, Bcl: B cell lymphoma, CARD: caspase recruitment domain, ERK: extracellular signal-regulated kinase, IκB: inhibitor of κB, IKK: IκB kinase, IRAK: IL-1R-associated kinase, IRF: interferon regulatory factor, JNK: c-jun N-terminal kinase, MALT: mucosa-associated lymphoid tissue, MAPK: mitogen-activated protein kinase, MKK: MAPK kinase, MyD88: myeloid differentiation primary response gene 88, NEMO: NF-κB essential modifier, NF-κB: nuclear factor kappa B, NLRP: NOD like receptor family, pyrin domain containing protein, OPN: osteopontin, RICK: RIP-like interacting caspase-like apoptosis regulatory protein kinase, RIP: receptor-interacting protein, Syk: spleen tyrosine kinase, TAB: TAK1-binding protein, TAK: transforming growth factor β-activated kinase, TIRAP: TIR domain-containing adapter protein, TRAF: TNFR-associated factor, TRAM: TRIF-related adapter molecule, TRIF: TIR-containing adapter inducing IFNβ.

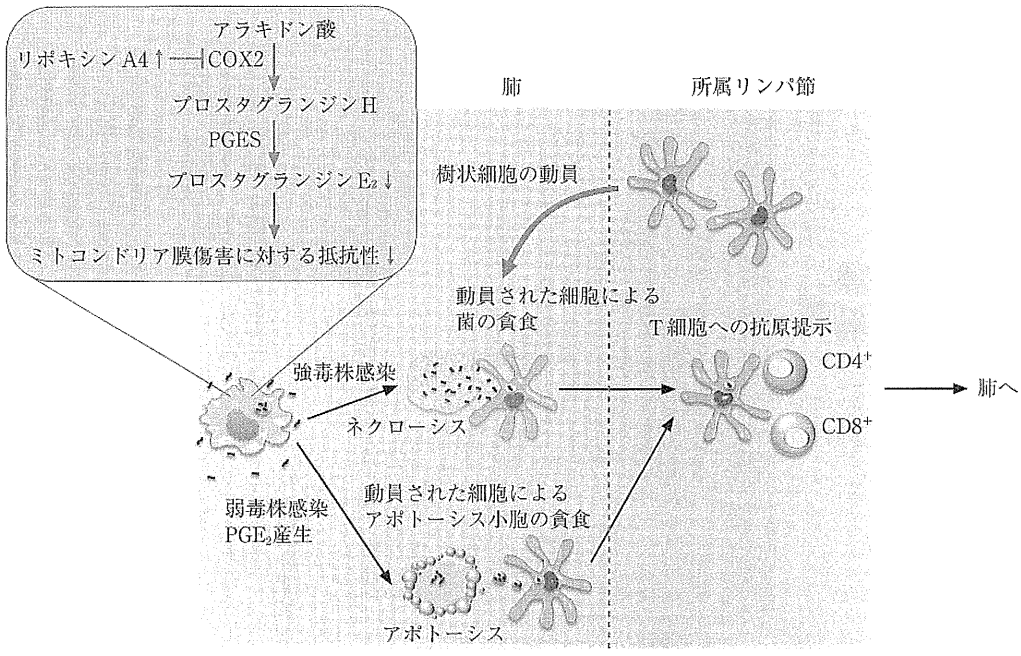

図2　結核菌感染後の肺における抗原提示

結核菌を貪食した肺胞マクロファージは，所属リンパ節に移行する能力が低い．そこで抗原特異的T細胞を誘導するためには，感染の刺激で肺に動員されるDCが結核菌抗原や菌体を取り込み，所属リンパ節に存在するT細胞に抗原提示する必要がある．また，病原性の強い結核菌は感染細胞のリポキシンA4合成を誘導してミトコンドリア膜傷害に対する抵抗性を下げる．その結果，感染細胞はネクローシスに陥り，菌の増殖が可能になる．一方，弱毒株の感染ではリポキシンA4合成は誘導されず，感染細胞にはアポトーシスが誘導されて菌の増殖が阻害される．

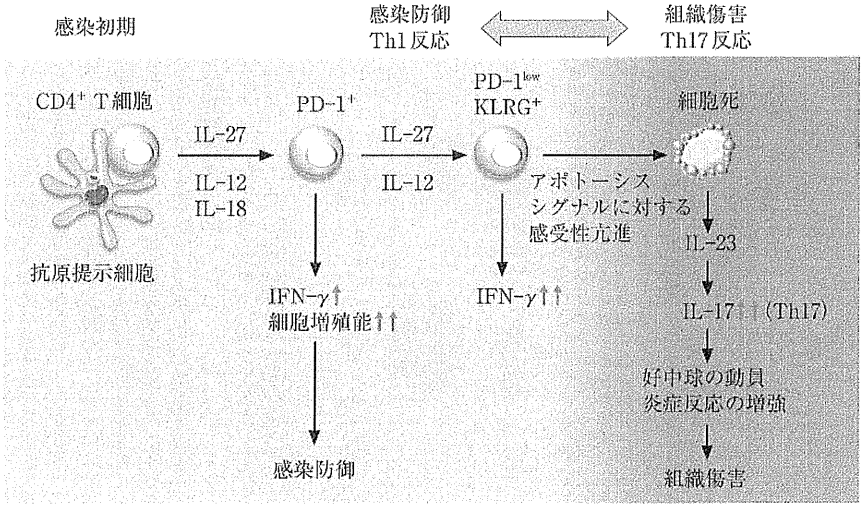

図3　感染防御免疫における Th1/Th17 バランスの重要性

抗原提示を受けた$CD4^+$T細胞は防御免疫を担当する$PD\text{-}1^+CD4^+$T細胞および$PD\text{-}1^{low}KLRG^+CD4^+$T細胞に分化する．一方，これらT細胞が減少した場合には感染局所でIL-23およびIL-17産生が亢進し，Th17型の組織傷害が引き起こされる．結核の慢性感染をコントロールするためには，Th1/Th17のバランスが重要である．

3. 抗原特異的T細胞応答

$CD4^+$T細胞は抗原提示を受け，programmed death-1^+($PD-1^+$) killer cell lectin-like receptor $G1^-$($KLRG^-$) $CD4^+$T細胞に分化する．更にIL-12やIL-27の持続的な刺激を受けると$PD-1^{low}$ $KLRG^+$ $CD4^+$T細胞が出現する[3,4]（**図3**）．$PD-1^{low}$ $KLRG^+$ $CD4^+$T細胞は強いサイトカイン産生能を有するが，アポトーシスシグナルに感受性で増殖能が低い．一方，これら防御免疫に関与するT細胞が減少した場合には，感染局所でIL-23やIL-17産生が増加し，好中球の浸潤と強い炎症反応を伴うTh17型の組織傷害が誘導される．したがって，これら抗原特異的$CD4^+$T細胞（Th1/Th17）のバランスを保つことが感染防御の観点から重要になる．

また，結核に対する感染防御には$CD4^+$T細胞以外に異なる機能を有する様々な細胞が関与することがわかっている（**図4**）．これら免疫担当細胞の機能を適切にコントロールすることが，結核に対する感染防御能を高めるために必要である．

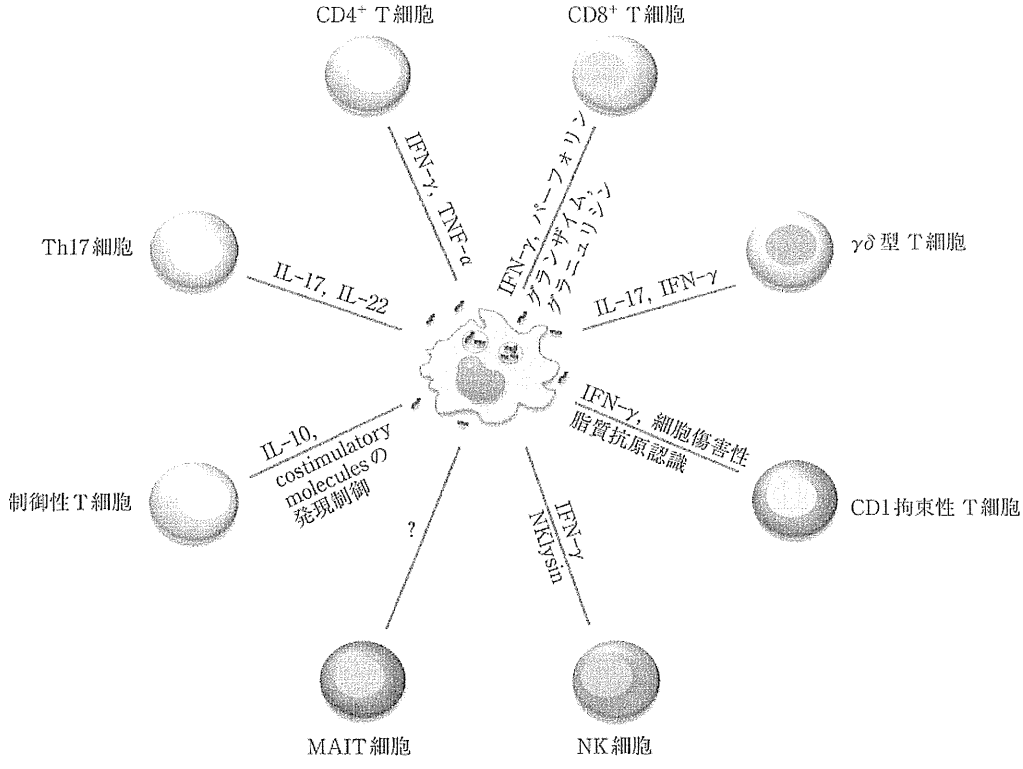

図4 結核菌に対する感染防御に関与する免疫担当細胞

結核に対する感染防御には$CD4^+$T細胞以外に様々な細胞が関与する．$CD8^+$T細胞はIFN-γやグラニュリシンを産生するとともにキラー活性を発揮して感染防御にかかわる．IL-17を産生するγδT細胞は肉芽腫形成に重要であり，natural killer T細胞や一部の$CD8^+$T細胞はCD1拘束性に脂質抗原を認識する．NK細胞はIFN-γ産生細胞として初期防御に関与する．また最近，mucosally associated invariant T（MAIT）細胞が感染防御に関与することが示されている．一方，Th17細胞は組織傷害を引き起こし，制御性T細胞は宿主防御免疫を抑制する．これら免疫担当細胞の機能を適切に制御することが，結核に対する感染防御に重要である．

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RESEARCH LETTER

Biochemical characteristics among *Mycobacterium bovis* BCG substrains

Daisuke Hayashi[1], Takemasa Takii[1], Tetsu Mukai[2], Masahiko Makino[2], Emi Yasuda[1], Yasuhiro Horita[1], Ryuji Yamamoto[1], Akiko Fujiwara[1], Keita Kanai[1], Maki Kondo[1], Aya Kawarazaki[1], Ikuya Yano[3], Saburo Yamamoto[3] & Kikuo Onozaki[1]

[1]Department of Molecular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan; [2]Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan; and [3]Japan BCG Laboratory, Tokyo, Japan

Correspondence: Takemasa Takii, Department of Molecular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-Dori, Mizuho-Ku, Aichi, Nagoya 467-8603, Japan. Tel.: +81 52 836 3421; fax: +81 52 834 9309; e-mail: ttakii@phar.nagoya-cu.ac.jp

Received 11 November 2009; revised 8 February 2010; accepted 18 February 2010. Final version published online April 2010.

DOI:10.1111/j.1574-6968.2010.01947.x

Editor: Jan-Ingmar Flock

Keywords
BCG; substrain; vaccine; biochemical characteristics; tuberculosis.

Abstract

In order to evaluate the biochemical characteristics of 14 substrains of *Mycobacterium bovis* bacillus Calmette Guérin (BCG) – Russia, Moreau, Japan, Sweden, Birkhaug, Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia and Pasteur – we performed eight different biochemical tests, including those for nitrate reduction, catalase, niacin accumulation, urease, Tween 80 hydrolysis, pyrazinamidase, *p*-amino salicylate degradation and resistance to thiophene 2-carboxylic acid hydrazide. Catalase activities of the substrains were all low. Data for nitrate reduction, niacin accumulation, Tween 80 hydrolysis, susceptibility to hydrogen peroxide and nitrate, and optimal pH for growth were all variable among these substrains. These findings suggest that the heterogeneities of biochemical characteristics are relevant to the differences in resistance of BCG substrains to environmental stress. The study also contributes to the re-evaluation of BCG substrains for use as vaccines.

Introduction

Biochemical tests are currently used as a technique for the identification of bacterial species. Recently, several studies have investigated the physiological meaning of the biochemical characters in the genus *Mycobacterium*. Sohaskey and colleagues reported variable nitrate production among *Mycobacterium bovis* bacillus Calmette Guérin (BCG) substrains in relation to survival in host cells (Sohaskey, 2008; Sohaskey & Modesti, 2009). Recycling of NAD and NAD-quinoline reductase relevant to the latent infection of *Mycobacterium tuberculosis* and resistance to oxidative stress, respectively, have also been reported (Boshoff *et al.*, 2008). Mycobacterial phospholipase A (MPLA) catalyses the hydrolysis of lipids including Tween 80 (Parker *et al.*, 2007), and this activity appears to contribute to survival under starvation at the dormant stage of growth (Jackson *et al.*, 1989; Deb *et al.*, 2009). Here, we analysed the biochemical characteristics and their relationship to susceptibility to environmental stress, such as oxidative stress, nitrosative stresses and pH changes, among BCG substrains.

Materials and methods

Bacterial strains

Mycobacterium bovis BCG strains Australia (ATCC 35739), Birkhaug (ATCC 35731), Connaught (ATCC 35745), Danish (ATCC 35733), Glaxo (ATCC 35741), Mexico (ATCC 35738), Montreal (ATCC 35735), Pasteur (ATCC 35734), Phipps (ATCC 35744), Tice (ATCC 35743), Russia (ATCC 35740) and *M. tuberculosis* strain H_{37}Rv (ATCC 25618) were purchased from American Type Culture Collection (ATCC, Manassas, VA). BCG-Moreau, *M. bovis* (JATA) and *Mycobacterium smegmatis* were provided by Dr M. Takahashi (The Research Institute of Tuberculosis Japan Anti-tuberculosis Association, Kiyose, Tokyo, Japan). BCG-Japan (Tokyo 172) was purchased from Japan BCG Laboratory (Kiyose, Tokyo, Japan). BCG-Sweden (vaccine seed) was provided by Dr S. Yamamoto (Japan BCG Laboratory). *Mycobacterium avium* strains 724S and 2151SmO were kindly provided by Drs J. Inamine and E. Torsten (Colorado State University, Fort Collins, CO).

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Bacterial culture and freeze stock

Bacterial culture and freeze stocking were performed as reported by Hayashi *et al.* (2009).

Biochemical tests

Tests for nitrate reduction, catalase, Tween 80 hydrolysis, urease, pyrazinamidase and resistance to thiophene 2-carboxylic acid hydrazide (TCH) were performed by standard procedures except as described below (Gangadharam & Jenkins, 1998). Nitrate reduction was performed by the classical procedure with liquid reagent. Pyrazinamidase activity was tested on Middlebrook 7H11 broth (BD, Franklin Lakes, NJ) instead of Dubos broth. Resistance to TCH was determined on solid Ogawa medium containing 1 or 10 $\mu g\,mL^{-1}$ TCH. Niacin accumulation was detected using the Kyokuto Niacin Test (Kyokuto Pharmaceutical Industries, Tokyo, Japan) in accordance with the manufacturer's instruction. Degradation of *p*-amino salicylate (PAS) was determined according to Tsukamura (1961). *Mycobacterium tuberculosis*, *M. bovis*, *M. avium* and *M. smegmatis* were used as controls. In the urease test, urease-deficient recombinant BCG (Mukai *et al.*, 2008) was used as a negative control.

Culture and differentiation of THP-1 cells

The human monocytic cell line THP-1 (ATCC TIB202) was purchased from ATCC and maintained in RPMI 1640 medium containing 100 $U\,mL^{-1}$ penicillin G and 5% heat-inactivated fetal bovine serum (FBS). THP-1 cells were stimulated with 10 nM phorbol 12-myristate 13-acetate (PMA; Wako Pure Chemical Industries, Osaka, Japan) for 24 h to be differentiated to macrophages. Cells were washed three times with culture medium and used for the assays.

Isolation and culture of bone marrow-derived macrophages (BMMs)

Bone marrow was isolated from the tibias and femurs of C57BL/6J female mice at 4–8 weeks of age. Bone marrow cells haemolysed in 0.83% NH_4Cl–Tris buffer were cultured in RPMI 1640 supplemented with 10% FBS, 100 $U\,mL^{-1}$ penicillin G, 50 μM 2-mercaptoethanol and 10 $ng\,mL^{-1}$ granulocyte-macrophage colony-stimulating factor (Wako) in 24-well culture plates; the culture medium was refreshed every 2 days. On day 7, adherent cells were collected and used for the assays.

Macrophage infection

Macrophages infected with bacilli at a multiplicity of infection (MOI) of 20 were incubated at 37 °C for 6 h. Extracellular bacilli were washed out three times and killed by 100 $\mu g\,mL^{-1}$ amikacin treatment for 6 h. Interferon (IFN)-γ (final concentration of 100 $U\,mL^{-1}$) was added to some of the wells as a stimulator. Following incubation, cells were washed three times and ruptured with 100 μL of sterile distilled water. To determine the number of intracellular live bacteria, the lysates were diluted and plated on 7H11 agar in triplicate. Colonies were counted after 3 weeks' incubation.

Tolerance test for hydrogen peroxide and nitric oxide

Bacilli (2×10^6 CFU) were incubated in 7H9 broth containing albumin, dextrose (without catalase) and 0–10 mM H_2O_2 for 6 h. In the same manner, bacilli were incubated in 7H9 broth supplemented with ADC (albumin, dextrose, catarase) and containing 0–10 mM $NaNO_2$, as an NO donor, at pH 6.6, 6.0 or 5.5 for 3 days. Following incubation, bacilli were washed with 7H9 medium three times, diluted and plated on 7H11 agar. Plates were incubated for 3 weeks and the percentage of live bacilli relative to control (0 mM H_2O_2 or $NaNO_2$) was calculated.

Determination of permissive pH range for growth of bacilli

Bacterial log-phase cultures in Middlebrook 7H9 (BD) supplemented with 10% ADC (BD) were adjusted to an OD of 0.1 at 530 nm and mixed with 100-fold volume of various pH-adjusted broths (pH 3, 4, 5, 5.4, 5.7, 6.2, 6.6, 7, 8, 9, 10, 11 and 12, adjusted with HCl or NaOH). Following incubation at 37 °C for 21 days, bacterial growth was evaluated by measuring OD at 530 nm.

Statistical analysis

Each experiment was repeated three times. Statistically significant differences between two series were assessed by Student's *t*-test or Aspin–Welch's *t*-test following an *F*-test assessment of variance.

Results and discussion

Eight different biochemical tests, nitrate reduction, niacin, catalase, Tween 80 hydrolysis, urease, pyrazinamidase, PAS degradation and resistance to TCH, were applied to 14 substrains of BCG, BCG-Russia, -Moreau, -Japan, -Sweden, -Birkhaug, -Danish, -Glaxo, -Mexico, -Tice, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur (Table 1). BCG-Birkhaug was positive for nitrate reduction whereas BCG-Mexico, -Australia and -Pasteur were negative; the other BCG strains were weakly positive, although *M. bovis*, the parental strain of BCG, was negative. The nitrate respiration system may be responsible for the survival of *M. tuberculosis* under anaerobic conditions (Sohaskey, 2008), and the nitrate reductase gene *narGHJI* contributes to the virulence of BCG in immunodeficient mice (Weber *et al.*, 2000). BCG-Russia and -Japan survived better both in THP-1 and in mouse BMMs than other substrains (Fig. 1 and Table 1). Although host *M. bovis* was negative for nitrate reduction,

Table 1. Summary of characteristics of BCG substrains *in vitro*[*]

Organism	Nitrate reduction	Niacin accumulation	Tween 80 hydrolysis	Urease	Pyrazinamidase Day 4	Pyrazinamidase Day 7	PAS degradation	Resistance to TCH (μg mL^{-1}) 1	Resistance to TCH (μg mL^{-1}) 10	Catalase (room temperature) Bubble column (mm)	Activity	68 °C catalase	H_2O_2 tolerance	NO tolerance	Optimal pH	Viability in THP-1 −IFN-γ	Viability in THP-1 +IFN-γ	Viability in BMM −IFN-γ	Viability in BMM +IFN-γ
BCG[‡]																			
Russia	±	+	−	+	−	−	−	+	−	9.3 ± 2.4	Low	−	+	−	6.6	+	+	+	+
Moreau	±	+	±	+	−	−	−	+	−	7.1 ± 1.8	Low	−	ND	ND	ND	ND	ND	ND	ND
Japan	±	+	−	+	−	−	−	+	−	14.8 ± 2.3	Low	−	+	+	6.6	+	+	+	+
Sweden	±	+	±	+	−	−	−	+	−	6.7 ± 1.7	Low	−	−	−	8–9	−	−	+	+
Birkhaug	+	+	−	+	−	−	−	+	−	11.8 ± 2.3	Low	−	+	−	8–9	+	+	−	−
Danish	±	−	±	+	−	−	−	+	−	9.4 ± 2.4	Low	−	−	+	7–8	+	−	−	+
Glaxo	±	−	−	+	−	−	−	+	−	7.4 ± 1.1	Low	−	−	+	7–8	−	−	−	−
Mexico	−	−	−	+	−	−	−	+	−	6.4 ± 1.8	Low	−	ND	ND	ND	ND	ND	ND	ND
Tice	±	−	−	+	−	−	−	+	−	6.3 ± 1.6	Low	−	ND	ND	ND	ND	ND	ND	ND
Connaught	±	−	±	+	−	−	−	+	−	7.9 ± 1.9	Low	−	+	−	7–8	−	−	−	+
Montreal	±	+	±	+	−	−	−	+	−	6.0 ± 2.3	Low	−	ND	ND	ND	ND	ND	ND	ND
Phipps	±	+	±	+	−	−	−	+	−	6.0 ± 2.2	Low	−	−	−	6.6	−	−	−	−
Australia	−	−	±	+	−	−	−	+	−	6.1 ± 2.1	Low	−	ND	ND	ND	ND	ND	ND	ND
Pasteur	−	−	±	+	−	−	−	+	−	7.3 ± 2.6	Low	−	−	+	6.6	+	+	−	+
M. bovis	−	−	−	+	−	−	−	+	−	5.4 ± 0.7	Low	−	+	+	6.6	+	+	+	+
M. tuberclosis																			
H37Rv	+	+	±	+	+	+	−	+	+	8.4 ± 1.1	Low	−	ND	ND	ND	ND	ND	ND	ND
H37Ra	+	+	±	+	+	+	−	+	+	10.0 ± 1.6	Low	−	ND	ND	ND	ND	ND	ND	ND
M. avium																			
724S	−	−	−	−	+	+	−	+	+	35.8 ± 13.0	Low	−	ND	ND	ND	ND	ND	ND	ND
2151SmO	−	−	−	−	+	+	−	+	+	27.6 ± 3.5	Low	±	ND	ND	ND	ND	ND	ND	ND
M. smegmatis	+	−	+	−	+	+	−	+	+	14.0 ± 1.3	Low	−	ND	ND	ND	ND	ND	ND	ND

*Summarizing the data from biochemical tests, tolerance to oxidative stress (Fig. 1) and survival activities in host cells (Fig. 2).

†Scores indicate the numbers that are positive (+) and slightly positive (±).

‡BCG substrains, which were historically distributed from the Pasteur Institute, are given in chronological order.

Methods for conventional biochemical tests for mycobacteria are described in Materials and methods. Experiments were conducted more than three times. Representative results are indicated.

ND, no data

the viability in host cells was higher than BCG (Table 1 and Fig. 1). According to the standard method for the nitrate reductase test, the assay period was 2 h. Under different conditions, for example longer incubation times and anaerobic conditions, nitrite production has been found in some BCG strains (Weber *et al.*, 2000; Sohaskey & Wayne, 2003; Stermann *et al.*, 2003; Sohaskey & Modesti, 2009). Therefore, different incubation times could explain the discrepancy observed between nitrate reductase test results and intercellular survival. Nitrate reductase activity is not the sole explanation, but we believe it is partly responsible for the survival in host cells, as shown in previous reports (Weber *et al.*, 2000; Sohaskey, 2008) and the present study.

Heterogeneity of niacin accumulation was also observed among BCG substrains (Table 1). Recycling of NAD favours the latent infection of *M. tuberculosis* (Boshoff *et al.*, 2008), and NAD-quinoline reductase is responsible for resistance to oxidative stress (Akhtar *et al.*, 2006). These reports suggest that the activity of NAD metabolism is associated with the survival of BCG in macrophages or host cells. Whether the long or short survival of BCG in host cells favours the effectiveness of BCG has not been determined. However, the different characteristics of BCG substrains as reported here provide the basic information for further investigation of immunological characteristics and evaluation.

Parker *et al.* (2007) purified and characterized MPLA. MPLA is associated with cutinase, a serine esterase and catalyses the hydrolysis of lipids including Tween 80. MPLA activity was observed not only in pathogenic *M. tuberculosis*, but also in BCG-Pasteur. BCG-Pasteur was weakly positive for Tween 80 hydrolysis (Table 1). In fact, eight of the 14 substrains, namely BCG-Moreau, -Sweden, -Danish, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur, were weakly positive. Mycobacteria are known to use this fatty acid as carbon source at the dormant stage. Therefore, this activity could contribute to survival under starvation conditions during dormancy (Jackson *et al.*, 1989; Deb *et al.*, 2009).

All BCG strains belong to the low-catalase group, although there were variations in the height of bubble column among them (Table 1). It was over 10 mm in BCG-Japan (14.8 mm) and -Birkhaug (11.8 mm) (Table 1). No mutation in the coding region of the *ahpC* gene among was observed among the substrains (data not shown). The

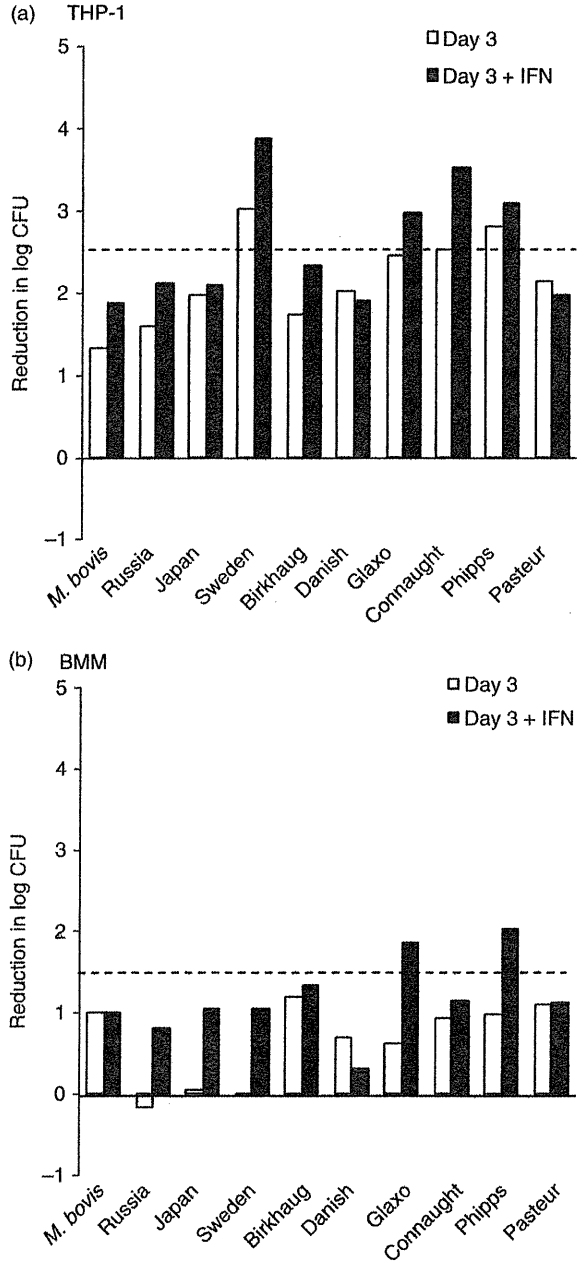


Fig. 1. Viability of BCG strains in THP-1 and mouse BMMs. PMA-differentiated THP-1 (a) or mouse BMMs (b) were infected with BCG at an MOI of 20 with (solid) or without (open) 100 U mL^{-1} of IFN-γ as a stimulator. After 6 h of infection, BCG CFU counts were determined from infected cell lysates and were monitored on days 0, 3 and 7. The data are expressed as the reduction in $\log_{10}$ CFU compared with control at day 0. Error bars represent means+SD for triplicate results from one of two similar experiments. Statistically significant differences between BCG group Russia, Japan, Birkhaug, Danish and Pasteur and BCG group Sweden, Glaxo, Connaught and Phipps were observed in (a) (Student's *t*-test, $P < 0.05$). In (b) there were statistically significant differences between BCG group Russia, Japan and Sweden and BCG group Birkhaug, Danish, Glaxo, Connaught, Phipps and Pasteur in the absence of IFN-γ (open column) (Aspin–Welch's *t*-test, $P < 0.05$). In the presence of IFN-γ (solid column) there were statistically significant differences between BCG group Russia, Japan, Sweden, Birkhaug, Danish, Connaught and Pasteur and BCG group Glaxo and Phipps (b) (Aspin–Welch's *t*-test, $P < 0.05$).

differences between transcription of the genes and the activities have not yet been analysed. Catalase (*katG*) and peroxidase (*ahpC*) activities of *M. tuberculosis* are related to resistance to oxidative killing in human monocytes *in vitro* (Manca *et al.*, 1999). The expression of *katG* is partially regulated by ferric uptake regulators (*fur*), and contributes to the virulence of *M. tuberculosis* (Lucarelli *et al.*, 2008). Resistance to hydrogen peroxide of *M. bovis*, BCG-Russia and -Japan was higher than that of other BCG substrains (Fig. 1). This resistance relates well to survival in host cells, THP-1 and BMMs (Fig. 1). These findings suggest that resistance to H_2O_2 contributes to survival of BCG substrains in host cells and that enzyme activities other than of catalase could be relevant to the resistance to oxidative stress from host cells.

We next investigated the susceptibility of BCG substrains to nitrosative stress by exposing them to sodium nitrite for 3 days (Fig. 2b). BCG-Pasteur was tolerant to nitric oxide, and moderate susceptibility was observed in BCG-Japan, -Danish and -Glaxo. BCG-Russia, -Sweden, -Birkhaug, -Connaught and -Phipps were sensitive to NO. The parental strain of BCG, *M. bovis*, was able to tolerate NO. To assess NO production from the bacilli, reduction of pH of the media is required to generate NO from sodium nitrate (Darwin *et al.*, 2003; MacMicking *et al.*, 2003). Intriguingly, optimal pH levels were found to be different among the BCG substrains (Table 2). The optimal pH of BCG-Russia, -Moreau, -Japan, -Phipps, -Pasteur and *M. bovis* was 6.6. Optimal pH of BCG-Sweden and -Birkhaug was 8–9, and that of BCG-Danish, -Glaxo and -Connaught was 7–8. According to maturation state, pH in phagosomes decreases from about 6 to 4. All BCG strains were positive for urease (Table 1). The changes in pH of the culture broths for each BCG strain were not significantly different (data not shown). Therefore, these data indicate that the increasing pH of the culture broth, such as by generating ammonium, is not responsible for the tolerance of BCG strains to a reduction of pH. The precise mechanisms of adaptability to pH changes have not been elucidated.

In summary, we have evaluated the usefulness of various biochemical tests currently used for identifying mycobacterial species. Surprisingly, there were differences in the results of these tests among BCG substrains. These differences could be generated during the long time of passage of BCG vaccine strains. Their characteristics are quality controlled by lyophilizing techniques. A good correlation between oxidative and nitrosative stress and survival in host cells were observed among BCG substrains. The relationship between antigen presentation and viability in host cells is not clear. The longer persistence of the bacilli in the host cells may favour antigen presentation by continuous supply of the antigens, while short persistent bacilli may stimulate antigen presentation through a different pathway (Grode L

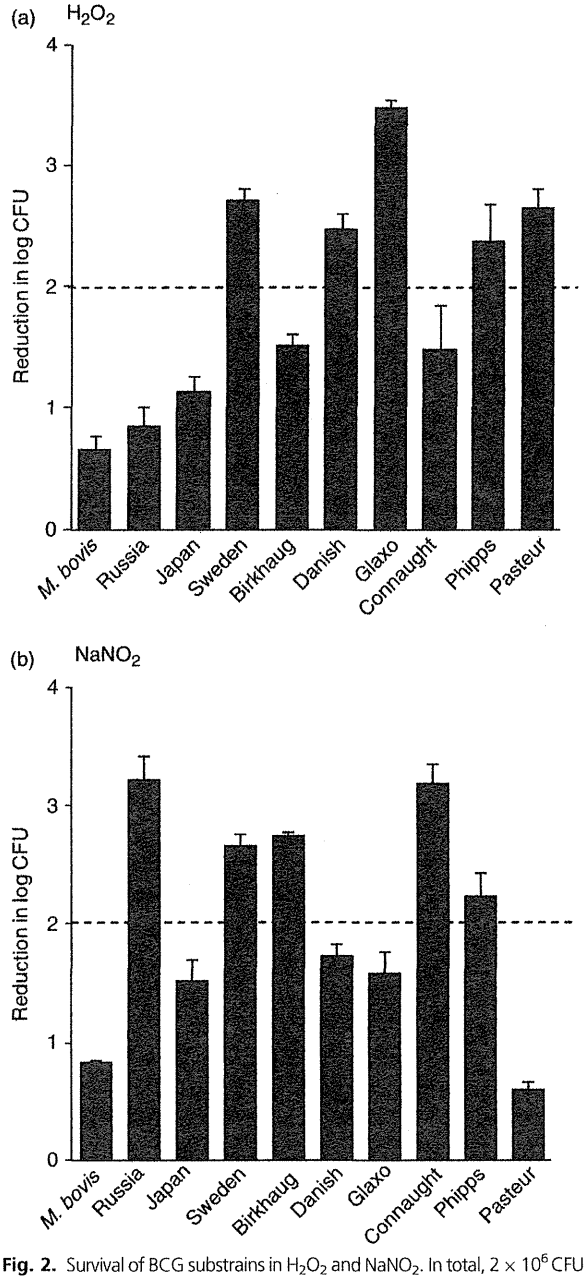
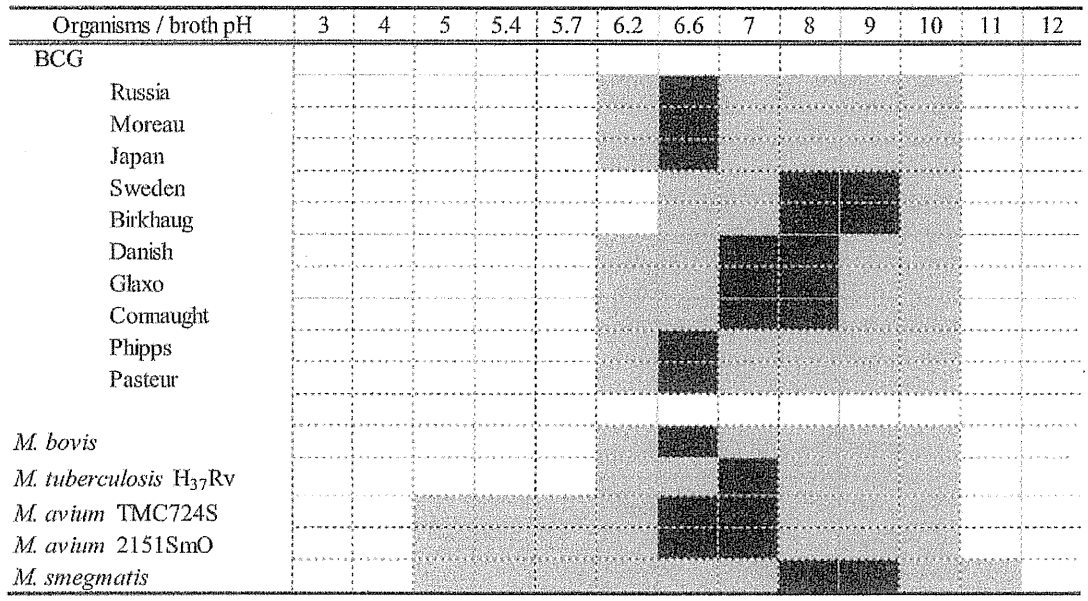


Fig. 2. Survival of BCG substrains in H_2O_2 and $NaNO_2$. In total, 2×10^6 CFU of *Mycobacterium bovis* or BCG substrains were treated with (a) 10 mM H_2O_2 for 6 h or (b) 10 mM $NaNO_2$ for 3 days. Treated and washed cells were serially diluted, and aliquots from four serial dilutions were plated in duplicate on 7H11 agar. The results are expressed as the reduction in $\log_{10}$ CFU compared with control at day 0. Error bars show means+SD of triplicate results from one of three similar experiments. BCG substrains, which were historically distributed from the Pasteur Institute, are aligned in chronicle order. In (a), statistically significant differences were found between BCG group Russia, Japan, Birkhaug and Connaught and BCG group Sweden, Danish, Glaxo, Phipps and Pasteur (Student's *t*-test, $P < 0.05$). In (b), statistically significant differences were found between BCG group Japan, Danish, Glaxo and Pasteur and BCG group Russia, Sweden, Birkhaug, Connaught and Phipps (Student's *t*-test, $P < 0.05$).

Table 2. The range of pH permissive for growth of BCG and other mycobacteria



BCG substrains, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium smegmatis* were cultured in 7H9 broth at the indicated pH for 21 days and OD at 530 nm was monitored every 3 days.
Grey, pH ranges that the broth OD was above 0.1; black, maximal pH.

et al., 2005). Comparative analysis of BCG substrains on acquired immunity should be undertaken. This and our previous studies provide basic information on the biological characteristics and the effect on the innate immunological characteristics of BCG substrains, and these studies could contribute to the re-evaluation of BCG vaccine.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Sciences, a grant for Research on Publicly Essential Drugs and Medical Devices, No. KHC1021, from the Japan Health Sciences Foundation, and a Grant-in-Aid for Scientific Research of the US–Japan Cooperative Medical Sciences Program, Ministry of Health, Labour and Welfare, Japan.

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JOURNAL OF BACTERIOLOGY, Nov. 2010, p. 5700–5708 Vol. 192, No. 21
0021-9193/10/$12.00 doi:10.1128/JB.00554-10

Novel Rhamnosyltransferase Involved in Biosynthesis of Serovar 4-Specific Glycopeptidolipid from *Mycobacterium avium* Complex[▽]

Yuji Miyamoto,[1]* Tetsu Mukai,[1] Takashi Naka,[2,3] Nagatoshi Fujiwara,[2] Yumi Maeda,[1] Masanori Kai,[1] Seiko Mizuno,[2,4] Ikuya Yano,[5] and Masahiko Makino[1]

Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan[1]; Department of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan[2]; MBR Co., Ltd., 7-7-15 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan[3]; Faculty of Human Development, Soai University, 4-4-1 Nanko-naka, Suminoe-ku, Osaka 559-0033, Japan[4]; and Japan BCG Laboratory, 3-1-5 Matsuyama, Kiyose, Tokyo 204-0022, Japan[5]

Received 14 May 2010/Accepted 24 August 2010

Glycopeptidolipids (GPLs) are one of the major glycolipid components present on the surface of *Mycobacterium avium* complex (MAC) that belong to opportunistic pathogens distributed in the natural environment. The serovars of MAC, up to around 30 types, are defined by the variable oligosaccharide portions of the GPLs. Epidemiological studies show that serovar 4 is the most prevalent type, and the prognosis of pulmonary disease caused by serovar 4 is significantly worse than that caused by other serovars. However, little is known about the biosynthesis of serovar 4-specific GPL, particularly the formation of the oligosaccharide portion that determines the properties of serovar 4. To investigate the biosynthesis of serovar 4-specific GPL, we focused on one segment that included functionally unknown genes in the GPL biosynthetic gene cluster of a serovar 4 strain. In this segment, a putative hemolytic protein gene, *hlpA*, and its downstream gene were found to be responsible for the formation of the 4-*O*-methyl-rhamnose residue, which is unique to serovar 4-specific GPL. Moreover, functional characterization of the *hlpA* gene revealed that it encodes a rhamnosyltransferase that transfers a rhamnose residue via 1→4 linkage to a fucose residue of serovar 2-specific GPL, which is a key pathway leading to the synthesis of oligosaccharide of serovar 4-specific GPL. These findings may provide clues to understanding the biological role of serovar 4-specific GPL in MAC pathogenicity and may also provide new insights into glycosyltransferase, which generates structural and functional diversity of GPLs.

The genus *Mycobacterium* has a unique feature in the cell envelope that contains a multilayered structure consisting of peptidoglycan, mycolyl-arabinogalactan complex, and surface glycolipids (8, 12). It is known that these components play a role in protection from environmental stresses, such as antimicrobial agents and host immune responses (8, 12). Some of them are recognized as pathogenic factors related to mycobacterial diseases, such as tuberculosis and leprosy (8, 12). In case of nontuberculous mycobacteria that are widely distributed in the natural environment as opportunistic pathogens, glycopeptidolipids (GPLs) are abundantly present on the cell envelope as surface glycolipids (34). GPLs have a core structure in which a fatty acyl-tetrapeptide is glycosylated with 6-deoxy-talose (6-d-Tal) and *O*-methyl-rhamnose (*O*-Me-Rha) (2, 5, 13). This structure is common to all types of GPLs, and GPLs with this structure that have not undergone further glycosylation are termed non-serovar-specific GPLs (nsGPLs) (2, 5, 13). Structural diversity generated by further glycosylations, such as rhamnosylation, fucosylation, and glucosylation, is observed for the oligosaccharide portion linked to the 6-d-Tal residue of nsGPLs from *Mycobacterium avium* complex (MAC), a member of the nontuberculous mycobacteria consisting of two species, *M. avium* and *M. intracellulare* (2, 5, 34). Consequently, these nsGPLs with varied oligosaccharides lead to the formation of the serovar-specific GPLs (ssGPLs) that define around 30 types of MAC serovars (10).

The properties of MAC serovars are known to be notably different from each other and also to be closely associated with the pathogenicity of MAC (3, 6, 18, 30, 31, 32). Various epidemiological studies indicate that serovar 4 is the most prevalent type and is also one of the serovars frequently isolated from AIDS patients (1, 20, 33, 36). Additionally, pulmonary MAC disease caused by serovar 4 is shown to exhibit a poorer prognosis than that caused by other serovars (23). With respect to host immune responses to MAC infection, serovar 4-specific GPL is reported to have characteristic features that are in contrast to those of other ssGPLs (21, 30). Structurally, serovar 4-specific GPL contains a unique oligosaccharide in which the oligosaccharide of serovar 2-specific GPL is further glycosylated with 4-*O*-methyl-rhamnose (4-*O*-Me-Rha) residue through a 1→4 linkage (Table 1) (24). Therefore, it is thought that the presence of 4-*O*-Me-Rha and its linkage position are important in exhibiting the specificity of biological activities. The biosynthesis of the oligosaccharide portion in several ssGPLs is currently being clarified (15, 16, 17, 25, 26), while that of serovar 4-specific GPL is still unresolved. In this study, we have focused on the genomic region predicted to be associated with GPL biosynthesis in the serovar 4 strain and explored the key genes responsible

* Corresponding author. Mailing address: Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-394-9092. E-mail: yujim@nih.go.jp.
▽ Published ahead of print on 3 September 2010.

TABLE 1. Oligosaccharide structures of serovar 2- and 4-specific GPLs

Serovar	Oligosaccharide	Reference
2	2,3-di-*O*-Me-α-L-Fuc-(1→3)-α-L-Rha-(1→2)-L-6-d-Tal	9
4	4-*O*-Me-α-L-Rha-(1→4)-2-*O*-Me-α-L-Fuc-(1→3)-α-L-Rha-(1→2)-L-6-d-Tal	24

for the formation of 4-*O*-Me-Rha that might determine the specific properties of MAC serovar 4.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation. Table 2 indicates the bacterial strains and vectors used in this study. MAC strains were grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 supplemented with 10% Middlebrook ADC enrichment (BBL). For GPL production, *Mycobacterium smegmatis* strains were cultured in 2× YT broth (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) with 0.2% Tween 80. DNA manipulation of *M. smegmatis* strains was conducted as previously described (27). PCR amplification was done by two-step PCR using TaKaRa LA *Taq* with GC buffer, with the following program: denaturation at 98°C for 20 s and annealing-extension at 68°C for an appropriate time depending on the length of the targeted region. *Escherichia coli* strain DH5α was used for the routine manipulation and propagation of plasmid DNA. Antibiotics were added as required: kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for *M. smegmatis*; hygromycin B, 150 μg/ml for *E. coli* and 75 μg/ml for *M. smegmatis*. Oligonucleotide primers used in this study are listed in Table 3.

Construction of expression vectors. For generation of the serovar 2-specific GPL (GPL-S2)-producing strain, the vector possessing *rtfA*, *mdhtA*, *merA*, and *gtfD* genes was constructed. The *rtfA* gene was amplified from genomic DNA of *M. avium* strain JATA51-01 using primers RTFA-S and RTFA-A. The *mdhtA*, *merA*, and *gtfD* genes were amplified as one operon from the previously constructed vector pMVΔmtfF using primers MDHTA-S2 and GTFD-A2 (26). After construction of pMV261a, in which an AflII site was introduced into pMV261, the above two PCR products were digested with each restriction enzyme and cloned into the BamHI-PstI and PstI-AflII sites of pMV261a to give pMV-rtfA-mdhtA-merA-gtfD (Table 2). The fragments for construction of expression vectors were amplified from genomic DNA of MAC serovar 4 strain (ATCC 35767) using the following primers: HLPA-S and HLPA-A for *hlpA*, HLPA-S and ORF2-A for *hlpA-orf2*, and ORF3-S and ORF5-A for *orf3-orf4-orf5*. These PCR products were digested with each restriction enzyme and cloned into the EcoRI-ClaI, EcoRI-HindIII, and PstI-EcoRI sites of pYM301a to give pYM-hlpA, pYM-hlpA-orf2, and pYM-orf3-orf4-orf5, respectively (Table 2).

Isolation and purification of GPLs. To isolate whole-lipid extracts, harvested bacterial cells were mixed with $CHCl_3/CH_3OH$ (2:1 [vol/vol[) for several hours at room temperature. The extracts in organic phase were separated by adding water and evaporated until dry. To remove the lipid components except for GPLs, the whole-lipid extracts were subjected to mild alkaline hydrolysis to prepare the crude GPLs as previously described (27, 28). For analytical thin-layer chromatography (TLC), crude GPLs on silica gel 60 plates (Merck) were developed with $CHCl_3/CH_3OH/H_2O$ (30:8:1 [vol/vol/vol]), followed by spraying with 10% H_2SO_4 and charring. Purified GPLs were prepared from crude GPLs by preparative TLC on the same plates and extracted from the bands corresponding to each GPL. To determine the linkage position of sugar moieties, perdeuteriomethylation was performed for purified GPLs as previously described (7, 11, 15).

GC-MS and MALDI-TOF MS analysis. Purified and perdeuteriomethylated GPLs were hydrolyzed in 2 M trifluoroacetic acid (2 h, 120°C), and the released sugars were reduced with $NaBD_4$ and then acetylated with pyridine/acetic anhydride (1:1 [vol/vol]) at room temperature overnight. The resulting alditol acetates were analyzed by gas chromatography-mass spectrometry (GC-MS) with a GCMS-QP2010 (Shimadzu) equipped with a SP-2380 column (Supelco) using helium gas. The temperature program was from 52 to 172°C with an increase in temperature of 40°C/min, 172 to 223°C at 3°C/min, and then 223 to 270°C at 40°C/min. To determine the total mass of the purified GPLs, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were acquired with an Ultraflex II (Bruker Daltonics). Samples were dissolved in chloroform-methanol (2:1 [vol/vol]) at a concentration of 1 mg/ml, 1 μl was applied directly to the sample plate, and then 1 μl of 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform/methanol (1:1 [vol/vol]) was added as a matrix. The purified GPL was analyzed in the reflectron mode with an accelerating voltage operating in a positive mode of 20 kV (17).

Nucleotide sequence accession number. The 6.8-kb genomic region amplified from the MAC serovar 4 strain (ATCC 35767) by using primers GTFB-S1 and MDHTA-A2 has been deposited in the DDBJ nucleotide sequence database under accession no. AB550236.

RESULTS

Previously, the A5 strain, one of the MAC serovar 4 strains, was reported to contain a genomic region similar to the GPL biosynthetic gene cluster identified in other serovars (22). However, to date, there are no studies clarifying the biosynthetic pathways involved in the formation of 4-*O*-Me-Rha, which is unique to serovar 4-specific GPL. To explore this glycosylation pathway, we focused on one segment interposed with the *gtfB* and *mdhtA* genes whose organization was shown to vary in strains of other serovars (14, 22). In this study, using another serovar 4 strain, ATCC 35767, whose genomic information is unknown, we designed various primers for PCR amplification of a focused segment based on the sequences from other serovar strains. After the testing of primer pairs, a

TABLE 2. Bacterial strains and vectors used in this study

Strain or vector	Characteristic(s)	Source or reference
Bacterial strains		
E. coli DH5α	Cloning host	TaKaRa
M. smegmatis mc^{2}155	Expression host	29
M. intracellulare ATCC 35767	MAC serovar 4 strain	35
M. avium JATA51-01	Source of the *rtfA* gene	26
Vectors		
pYM301a	Site-specific integrating mycobacterial vector carrying an *hsp60* promoter cassette	25
pMV261a	*E. coli-Mycobacterium* shuttle vector carrying an *hsp60* promoter cassette with an AflII site	This study
pMVΔmtfF	Source of *mdhtA*, *merA*, and *gtfD* genes	26
pMV-rtfA-mdhtA-merA-gtfD	pMV261a carrying *rtfA*, *mdhtA*, *merA*, and *gtfD* genes	This study
pYM-hlpA	pYM301a carrying the *hlpA* gene	This study
pYM-hlpA-orf2	pYM301a carrying the *hlpA* gene and ORF2	This study
pYM-orf3-orf4-orf5	pYM301a carrying ORF3, ORF4, and ORF5	This study

TABLE 3. Oligonucleotide primers used in this study

Primer	Sequence[a]	Restriction site
RTFA-S	5′-CGGGATCCCATGAAATTTGCTGTGGCAAG-3′	BamHI
RTFA-A	5′-AACTGCAGCTCAGCGACTTCGCTGCGCTTC-3′	PstI
MDHTA-S2	5′-GCTCTAGACTGCAGAAAAACCAACTTCTACTGCCTGACCTG-3′	PstI
GTFD-A2	5′-GGAATTCTTAAGTCTACGGTTCTGCGCTTCGTTCTTTG-3′	AflII
HLPA-S	5′-GGAATTCGTGACAACGACGCCACCAGT-3′	EcoRI
HLPA-A	5′-CCATCGATACTACGCTGCCGCGCTAGGCG-3′	ClaI
ORF2-A	5′-CCCAAGCTTCTCAGACTCTAACGTACAGTTC-3′	HindIII
ORF3-S	5′-CACCTGCAGAAATGACCGCCACAACCAGGGC-3′	PstI
ORF5-A	5′-GCAGAATTCCTACGGCGCCAATTCGATGAG-3′	EcoRI
GTFB-S1	5′-GGAACTCCTGCACCTTGGGGCCGT-3′	
MDHTA-A2	5′-GGTGCGGGTCAACGTAGAGGTG-3′	

[a] Underlining indicates the restriction site.

6.8-kb fragment was amplified with primers GTFB-S1 and MDHTA-A2 (Fig. 1). Nucleotide sequences of the amplified fragments were similar to that of the GPL biosynthetic gene cluster from the A5 strain (94% identity in nucleotide sequences) (GenBank accession no. AY130970.1). This segment contains five complete open reading frame (ORF) genes (Fig. 1): the ORF1 gene, similar to a putative hemolytic protein gene (*hlpA*) previously found in the GPL biosynthetic gene cluster of the serovar 2 strain (69% identity in amino acid sequences) (GenBank accession no. AF125999.1) (14); the ORF2 gene, an undefined gene showing low similarity to some *O*-methyltransferases; and the ORF3, ORF4, and ORF5 genes, with amino acid sequences almost identical to those of three proteins, including GtfTB, which were previously identified as biosynthetic enzymes for serovar 8-specific GPL (GenBank accession no. AB437139.1) (25).

Prior to functional analysis of each ORF, it was necessary to prepare a strain producing the substrate for the enzymes participating in the biosynthesis of serovar 4-specific GPL. Since serovar 4-specific GPL has a structure in which the terminal Fuc residue of serovar 2-specific GPL is further glycosylated with 4-*O*-Me-Rha, we created a recombinant *M. smegmatis* strain (termed MS-S2) by introducing the plasmid vector pMV-rtfA-mdhtA-merA-gtfD possessing *M. avium rtfA*, *mdhtA*, *merA*, and *gtfD* genes, which were previously shown to convert nsGPLs to serovar 2-specific GPL with a terminal Fuc residue (termed GPL-S2) (26). For five ORFs, we first examined the function of the ORF1 (termed *hlpA*) and its downstream ORF2 gene by TLC analysis of recombinant strains, because these have not been functionally defined and it is difficult to predict the role of each gene. In comparison with the profile of the control strain (MS-S2/pYM301a) (Fig. 2, lane A), the new products (GPL-S4) were observed for the strain with the *hlpA* gene introduced (MS-S2/pYM-hlpA) (Fig. 2, lane B). Moreover, when the expression vector covering both

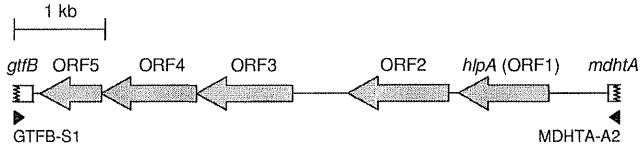

FIG. 1. Organization of the 6.8-kb genomic segment isolated from MAC serovar 4 strain (ATCC 35767). Filled triangles indicate the primers used for PCR amplification.

hlpA and ORF2 was introduced into MS-S2 (MS-S2/pYM-hlpA-orf2), another new product (GPL-S4M) appeared (Fig. 2, lane C). These observations indicated that GPL-S2 was converted to structurally different compounds by the expression of *hlpA* and that the compounds generated by *hlpA* were further modified by ORF2. As for the ORF3, ORF4, and ORF5 genes, which show a high similarity to the biosynthetic genes for serovar 8-specific GPL, we further generated a strain having three ORFs (MS-S2/pYM-orf3-orf4-orf5) and examined the GPL production by TLC analysis (Fig. 2, lane D). The results indicated the appearance of known product GPL-S8, previously shown to have a sugar residue of serovar 8-specific GPL, with no GPL-S4 and GPL-S4M (25), demonstrating that the enzymes encoded by three ORFs might act on the serovar 1-specific GPL which was produced as a precursor of GPL-S2 and subsequently yielded GPL-S8.

Because the compounds produced by *hlpA* and ORF2 were structurally unidentified, we performed a GC-MS analysis of the products GPL-S2, GPL-S4, and GPL-S4M, which were purified from recombinant strain MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively. Although two spots were seen for each product, this might be due to a different methylation pattern for the fatty acid portion, which is often observed with GPL biosynthesis of *M. smegmatis* and does not affect oligosaccharide structure (19, 25). In GC-MS profiles of GPL-S2 and GPL-S4, the classes of the detected sugar residues, Fuc, 6-d-Tal, Rha, and 2,3,4-tri-*O*-Me-Rha, were found to be identical to each other (Fig. 3A and B). However, it was observed that the intensity of the Rha residue in GPL-S4 was higher than that of the other sugars, while in GPL-S2, the intensity of the Rha residue was lower than that of Fuc, indicating that the proportion of Rha content in GPL-S4 was relatively large compared to that in GPL-S2. These results implied that the *hlpA* gene mediates the transfer of an additional Rha residue to GPL-S2. In contrast, the profiles of GPL-S4M showed the presence of 4-*O*-Me-Rha that is specifically observed for serovar 4-specific GPL (Fig. 3C), demonstrating that ORF2 encodes a rhamnosyl 4-*O*-methyltransferase and that both genes are responsible for the formation of the unique sugar residue of serovar 4-specific GPL. Furthermore, we confirmed the molecular masses of products GPL-S2, GPL-S4, and GPL-S4M by MALDI-TOF MS analysis (Fig. 4). Each product contained two main pseudomolecular ions ($[M + Na]^+$) with 14 mass unit differences, indicating the

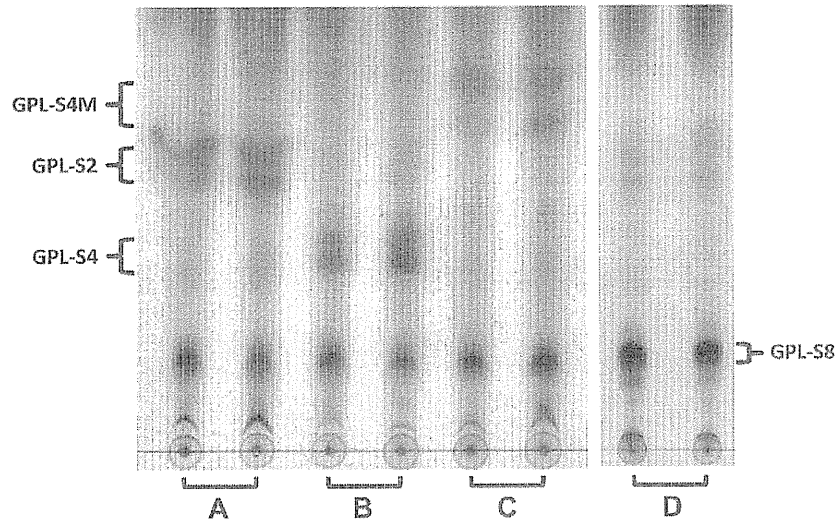

FIG. 2. TLC analysis of crude GPL extracts from recombinant *M. smegmatis* strains MS-S2/pYM301a (A), MS-S2/pYM-hlpA (B), MS-S2/pYM-hlpA-orf2 (C), and MS-S2/pYM-orf3-orf4-orf5 (D). GPL extracts were prepared from the total lipid fraction after a mild alkaline hydrolysis step. Each recombinant strain was tested by two samples derived from independent colonies. Samples were spotted and developed in $CHCl_3$-CH_3OH-H_2O (30:8:1 [vol/vol/vol]).

presence or absence of the methyl group in the fatty acid portion as described above. Thus, the results revealed that the mass unit difference between GPL-S2 (*m/z* 1,465.80, 1,479.82) and GPL-S4 (*m/z* 1,611.84, 1,625.85) was 146 and that between GPL-S2 and GPL-S4M (*m/z* 1,625.89, 1,639.90) was 160, demonstrating that the Rha and 4-*O*-Me-Rha residues were further added to the GPL-S2 to yield GPL-S4 and GPL-S4M, respectively.

The results from TLC, GC-MS, and MALDI-TOF MS analyses strongly suggested that *hlpA* and ORF2 are involved in the formation of 4-*O*-Me Rha. However, it is not clear whether the *hlpA* gene product functions as a glycosyltransferase that transfers a Rha via 1→4 linkage to a Fuc residue, which is observed only for serovar 4-specific GPL. To elucidate the function of *hlpA*, we determined the linkage of sugar moieties of GPL-S4 produced by recombinant strain MS-S2/pYM-hlpA (Fig. 2, lane B). The purified GPL-S4 was subjected to perdeuteriomethylation followed by GC-MS and gave four peaks corresponding to Fuc, 6-d-Tal, Rha, and 2,3,4-tri-*O*-Me-Rha (data not shown). The spectra of Rha and 6-d-Tal demonstrated that the linkage position between these two sugar residues is commonly observed in the oligosaccharide of all ssGPLs, and position C-3 of Rha is linked to the next one, which is consistent with the data previously reported (Fig. 5B and C) (25, 26). In addition, the detection of fragment ions at *m/z* 121, 168, and 206 in spectra of Fuc indicated that its positions C-2 and C-3 were deuteriomethylated (Fig. 5D), meaning that position C-1 of Fuc is linked to position C-3 of Rha and position C-4 of Fuc is linked to the next one. These observations were supported by the fact that GPL-S4 was structurally based on the oligosaccharide of serovar 2-specific GPL. The peak of 2,3,4-tri-*O*-Me-Rha was found to include mixed fragment ions (Fig. 5A). A group of fragment ions corresponding to the spectra of 2,3,4-tri-*O*-Me-Rha linked to alaninol of tetrapeptide was observed. The remaining fragment ions at *m/z* 121, 134, 168, and 181 indicate the presence of deuteriomethyl groups at positions C-2, C-3, and C-4 of the other Rha that is linked at the terminus of oligosaccharide in GPL-S4. These results indicate that position C-1 of terminal Rha is linked to position C-4 of Fuc. Accordingly, the oligosaccharide structures of GPL-S4 were determined to have Rha-(1→4)-Fuc-(1→3)-Rha-(1→2)-6-d-Tal at D-*allo*-Thr, demonstrating that *hlpA* encodes a rhamnosyltransferase that transfers a Rha residue via 1→4 linkage to a Fuc residue of serovar 2-specific GPL (Fig. 6).

DISCUSSION

It is known that serovar 4 is the most prevalent type, and serovar 4-specific GPL, particularly its oligosaccharide portion, plays a role in exhibiting the specific properties that belong to pathogenic factors. However, to date, the biosynthesis of its oligosaccharide portion has not been clarified. In this study, structural determination of three recombinant products, GPL-S2, GPL-S4, and GPL-S4M, revealed that *hlpA* and its downstream gene (ORF2) in the GPL biosynthetic gene cluster are involved in the formation of 4-*O*-methyl Rha, which is unique to serovar 4-specific GPL (Fig. 6). Previously, it was reported that the GPL biosynthetic gene cluster of MAC serovar 2 strains contained one gene whose amino acid sequences are similar to that of *hlpA* with 69% identity (14). This has been regarded as the gene not associated with GPL biosynthesis, because its amino acid sequences are similar to those of hemolytic proteins distributed in some species of bacteria (4). Thus, as shown in Fig. 6, it was surprising that *hlpA* from serovar 4 was found to encode a rhamnosyltransferase that plays a critical role in the pathway leading from serovar 2-specific GPL to serovar 4-specific GPL. For mycobacterium species, a BLAST analysis of HlpA revealed that its homologues are seen only in MAC serovar 2 and not in other species, including *Mycobacterium tuberculosis*. When we tested the

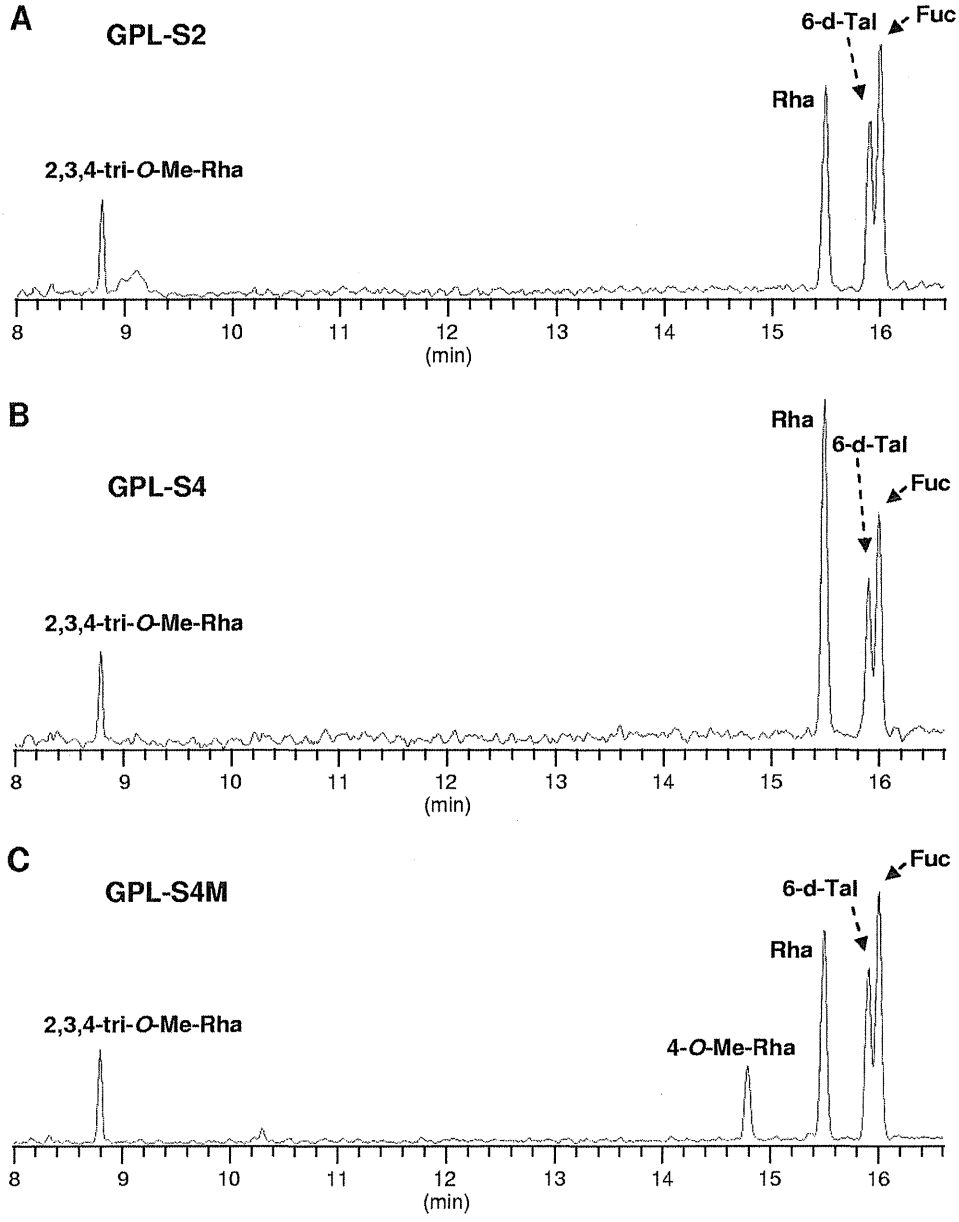

FIG. 3. GC-MS of alditol acetate derivatives from GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

function of HlpA from serovar 2, it did not serve as a glycosyltransferase like HlpA from serovar 4 (data not shown). At present, the function of HlpA from serovar 2 is still unclear, because the biosynthesis of the oligosaccharide portion in serovar 2-specific GPL has been fully elucidated (14, 26). The oligosaccharide structure of serovar 2-specific GPL is basic for several ssGPLs, including serovar 4-specific GPL. In the biosynthetic gene cluster of serovar 2-specific GPL, several insertion sequence (IS) elements are observed, raising the possibility that the HlpA from serovar 2 is retained through genomic alterations that induce biosynthetic changes from other ssGPLs to serovar 2-specific GPL. Therefore, HlpA in the serovar 2 strain originally might function as a glycosyltransferase in the biosynthesis of oligosaccharides of other serovars.

Most HlpA homologues are putatively categorized as hemolytic proteins because they are similar to one protein from *Prevotella intermedia*, which is actually proved to have hemolytic activity (4). Since the amino acid sequences of HlpA show 38% identity and 54% similarity to the above protein of *P. intermedia*, we predicted that HlpA also possesses hemolytic activity as an additional function. However, none was detected when *hlpA* was expressed in *M. smegmatis* and *E. coli* by plate assay using a sheep blood agar plate (data not shown). A BLAST analysis of HlpA homologues showed that they also

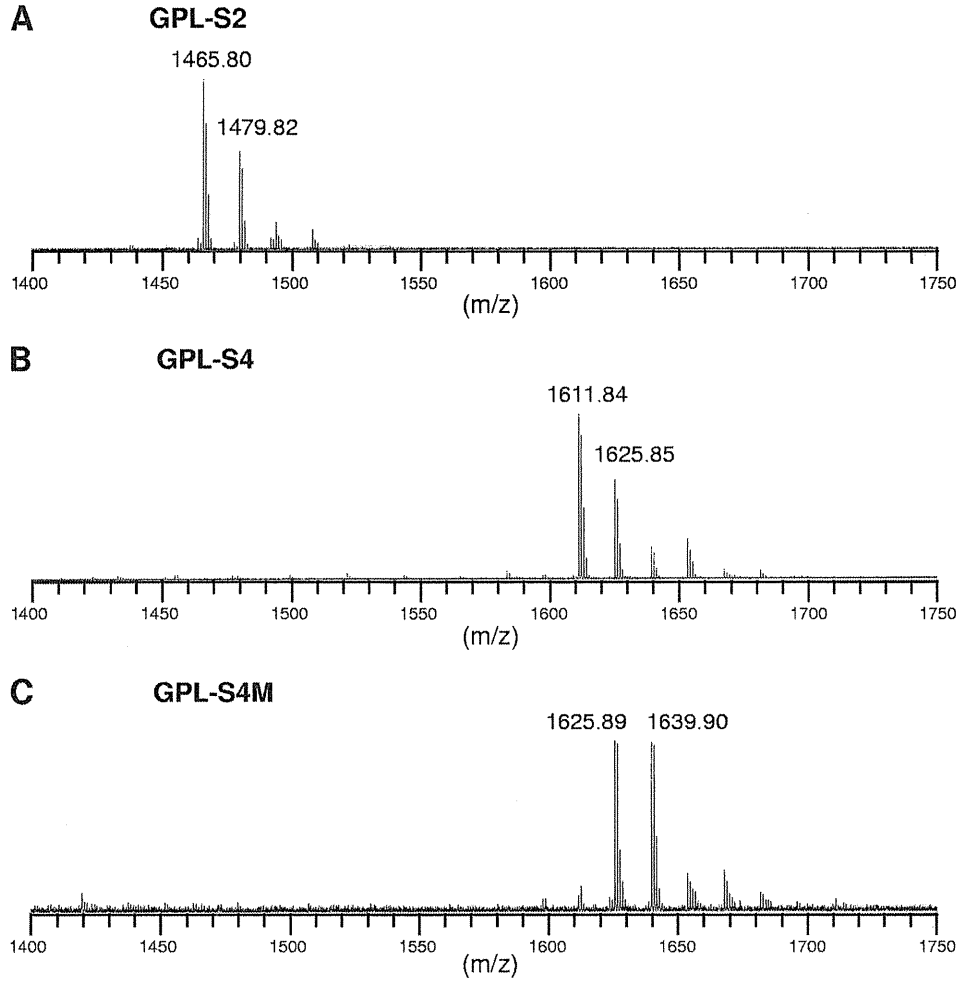

FIG. 4. MALDI-TOF MS of GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

contained a partial motif of some glycosyltransferases and methyltransferases. Therefore, it is envisaged that the evolutionary ancestor of HlpA might have lost hemolytic activity in MAC or, conversely, have been altered to retain it in *P. intermedia* in the course of phylogenetic evolution between these bacterial species.

Serovar 4 strains, including ATCC 35767, have been recognized as strains producing the serovar 4-specific GPL but not the serovar 8-specific GPL (24, 35). However, as shown in Fig. 1, we found that the GPL biosynthetic gene cluster contains three known genes (the ORF3, ORF4, and ORF5 genes) previously identified as biosynthetic genes responsible for the formation of 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl glucose residue in the oligosaccharide of serovar 8-specific GPL (25). TLC analysis showed that overexpression of three ORFs potentially produces the serovar 8-specific GPL, including the 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl glucose residue (Fig. 2, lane D), demonstrating that in the serovar 4 strain, there is inefficient expression of three genes, which might be caused by genomic alterations affecting their transcription, resulting in the loss of serovar 8-specific GPL. Moreover, HlpA homologues are often found in several species of cyanobacteria but not in other bacterial groups and mycobacterium species, implying the occurrence of a certain kind of "horizontal gene transfer" between these environmental bacteria. Thus, MAC seemed to incorporate foreign genes or realign preexisting genes to modify the oligosaccharide structures of GPLs for their survival in a varied environment. In terms of sugar composition and linkage affecting the properties of ssGPLs, the functional aspects of the 4-*O*-methyl-Rha residue, which influence the interactions with the host cell, are still unclear. In addition, the sugar linkage Rha-(1→4)-Fuc is seen only in serovar 4-specific GPL and not in other ssGPLs, suggesting that it might generate unique properties that differ notably from those generated by other sugar linkages. Also, the rarity of this sugar linkage could be one of the factors that define the specificity of MAC serovar 4, which would be resolved by further studies, including the generation of an *hlpA* knockout mutant. For functional characterization of *hlpA* and ORF2, we have adopted the gene expression experiment

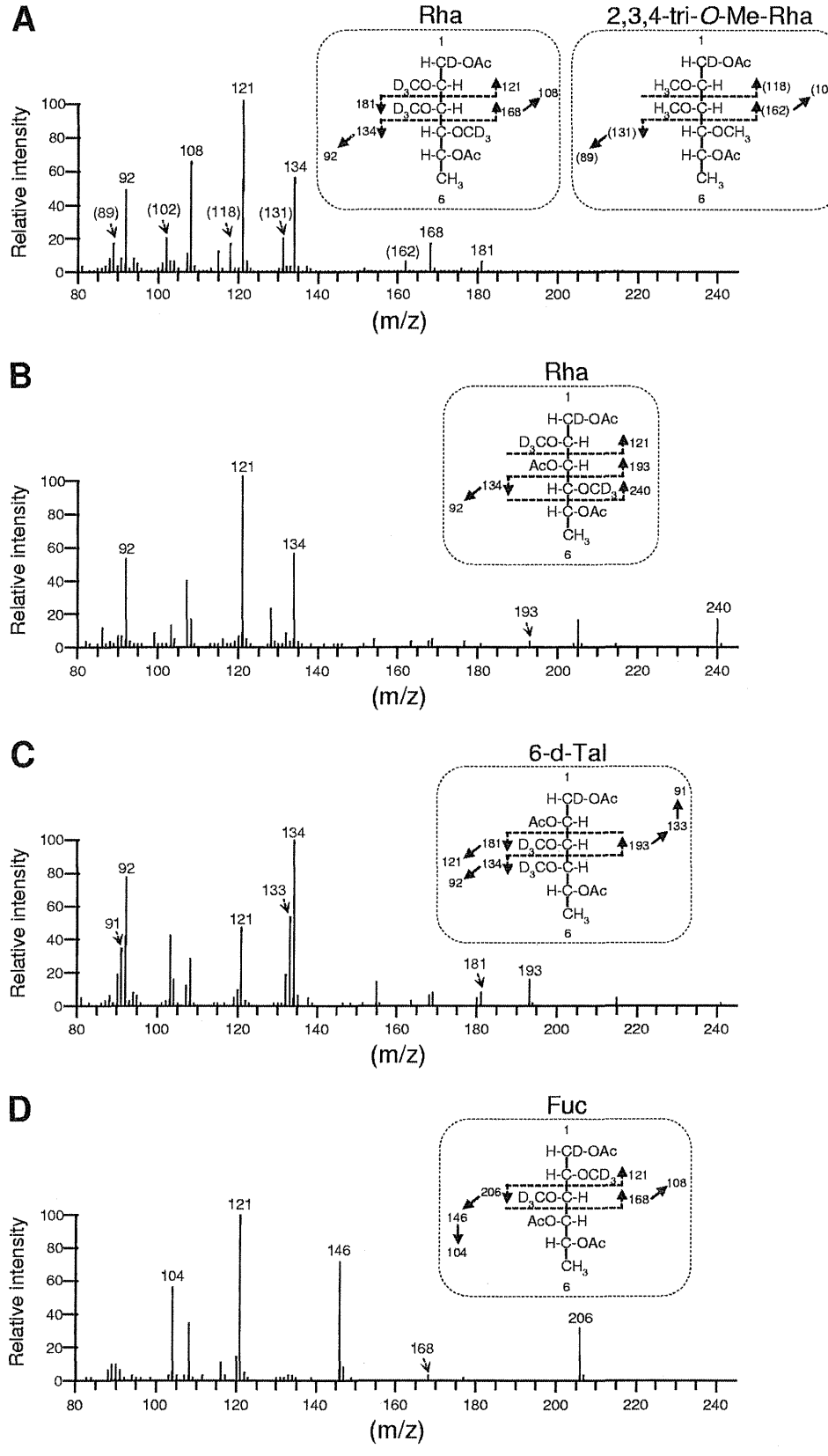

FIG. 5. GC-MS spectra and fragment ion assignments of 2,3,4-tri-*O*-Me-Rha (A), Rha (A and B), 6-*d*-Tal (C), and Fuc (D), which are derived from alditol acetates of sugars released from deuteriomethylated GPL-S4. Ac, acetate; D, deuterium.

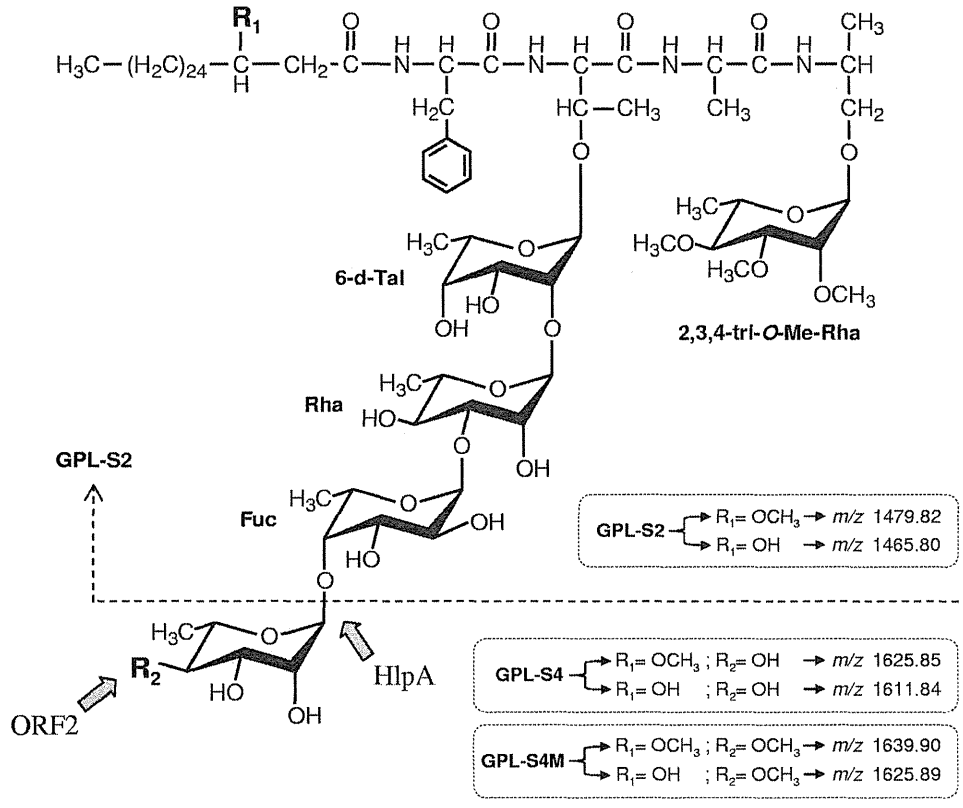

FIG. 6. Proposed structures and biosynthetic pathways for GPL-S2, GPL-S4, and GPL-S4M. Parentheses indicate structural differences between three compounds, which are deduced from MALDI-TOF MS analyses {pseudomolecular ions ($[M + Na]^+$)}.

using the *M. smegmatis* strain. Further enzymatic analyses, such as *in vitro* testing with recombinant proteins, would confirm our results. Taken together, these findings may contribute to understanding the mechanism for generation of structural and functional diversity of GPLs as well as their biological role in MAC.

ACKNOWLEDGMENTS

This research was partially supported by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Science and Technology of Japan and by a Research on Emerging and Re-Emerging Infectious Diseases grant from the Ministry of Health, Labor and Welfare of Japan.

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