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結核慢性感染の成立・維持における肺環境内恒常性に関する研究

分担研究報告書

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研究要旨.

活性化T細胞上に発現する抑制性補助因子PD-1は、その特異的リガンドであるPD-L1およびPD-L2との会合を介してT細胞活性を抑制し、ウイルスや細菌感染の慢性化に関与することが報告されている。そこで本研究では、結核菌感染においてPD-1シグナル経路が果す役割について解析を行った。Wild type (WT)マウスとPD-1欠損マウスに結核菌を感染させたところ、WTマウスに比べてPD-1欠損マウスは結核菌感染に対して感受性を示し、感染後早期に死亡した。また、感染後のPD-1欠損マウスの肺ではマクロファージと好中球を中心とした炎症細胞の著明な浸潤と、各種炎症性サイトカインおよびケモカイン産生の亢進が認められ、壊死を伴う炎症性病変が観察された。さらに、PD-1欠損マウスの肺では感染21日目以降著明な菌数の増加が観察された。WTマウス由来T細胞上のPD-1の発現は結核菌感染後17- 21日目に明らかな増加が認められ、I-A^b陽性抗原提示細胞上のPD-L1の発現は感染21日目以降に増加した。PD-1欠損マウスの肺における激しい炎症反応と菌数の増加は感染3週目以降に認められることから、結核菌に対する宿主の防御免疫が発現する感染3週目以降にPD-1シグナル経路が機能することが、宿主応答を正常に制御するために重要である事が示された。これまでの報告から、PD-1欠損マウスで観察される激しい炎症反応の原因が、抗原特異的T細胞応答に起因する可能性が考えられる。そこで、結核菌感染後のWTおよびPD-1欠損マウスの肺における抗原特異的T細胞の出現時期を調べた。PD-1欠損マウスでは、結核菌の主要T細胞抗原であるESAT-6やAg85Bを認識する$CD4^+$ T細胞は感染17日目に認められ、21日目にはWTの約2倍に増加した。また、この$CD4^+$ T細胞は抗原刺激に対して強いIFN-γ産生能を有することが示された。さらに、結核菌感染21日目のPD-1欠損マウスの肺ではIFN-γ産生の増強に伴うNOの強い産生とHMGB-1の放出を伴う細胞死の亢進が観察された。以上の結果から、結核菌感染したPD-1欠損マウスの肺では$CD4^+$ T細胞を中心とした過剰な免疫応答が誘導され、逆に菌の増殖を許す結果となっていることが示された。

A. 研究目的

WHOの報告では、現在年間930万人の新規結核患者があり、1年間に130万人が結核で死亡している。この点から、結核は現在でも人類の脅威となる重要な感染症であるといえる。結核の原因菌である結核菌は感染しても多くの場合結核を発症せず、そのまま長期間に渡り体内で生存し続ける。その後、宿主の抵抗性が低下すると菌は再び増殖をはじめ、結核を発症する。一方、結核菌が感染した宿主では、感染数週間後には抗原特異的Th1型$CD4^+$ T細胞が誘導され、防御免疫が発現する。しかし、防御免疫が発現しても菌の増殖を抑えることはで

きるが、菌を体内から排除することは容易ではない。また、現在使用されている BCG ワクチンによっても防御免疫は亢進するが、感染した菌を排除することはできない。これらの事実は、結核菌が感染した宿主の感染防御機構を制御する機序を有することを示唆するものである。

抑制性補助因子である PD-1 は活性化 T 細胞上に発現し、その特異的リガンドである PD-L1 および PD-L2 との会合を介して T 細胞レセプターからのシグナル伝達を阻害し、T 細胞の活性化を抑制することが知られている。また、これまでに PD-1 シグナル経路はウイルス感染防御に重要な $CD8^+$ T 細胞の機能抑制に関与することが報告されている。実際に、HIV、SIV、B 型肝炎ウイルス、C 型肝炎ウイルスまたは LCMV の感染実験では、PD-1 シグナル経路を遮断することで宿主感染抵抗性の亢進が認められている。一方、この PD-1 シグナル経路は self-tolerance に関与し、自己免疫病の発症を抑えるための重要な役割を果たすことが知られている。そのため HSV 感染実験では、PD-1 シグナル経路を阻害することにより防御に働くべき T 細胞応答によって逆に感染病態の悪化を招く結果になることが報告されている。また、BCG 感染実験系では PD-L1 の発現が上昇する感染 3 週間目以降に PD-1 経路が活性化され、BCG に対する感染抵抗性 T 細胞の機能が阻害されることが示されている。そこで本年度は、結核菌のマウス感染モデルを用いて、結核菌感染後に誘導される免疫応答の制御に PD-1/PD-L1 シグナル経路が関与するか否かについて解析を行った。

B. 研究方法

結核菌感染実験

C57BL/6 (wild type)および PD-1 欠損マウスに結核菌(約 300 cfu)を経鼻感染させ、その後のマウス生存数を調べた。さらに、感染後経時的に肺および脾臓をホモジナイズし、Middlebrook7H10 寒天培地に塗抹後、3 週間培養して得られたコロニー数より臓器内菌数を算出した。また、感染後経時的に肺および脾臓を採取し、その肉眼所見を比較した。さらに、HE 染色および Ziehl-Neelsen 染色を施して、炎症の程度を観察した。肺への炎症性細胞の浸潤を調べるため、感染後経時的に採取した肺をコラゲナーゼ処理した後、回収された細胞の表面抗原を FACS で解析した。

サイトカインおよびケモカイン産生応答

結核菌感染後経時的に WT および PD-1 欠損マウスの肺を採取し、ホモジネートを作製した。ホモジネート中の各種サイトカイン量を ELISA で測定した。また、感染後経時的に採取した肺より RNA を抽出し、各種ケモカインの発現量を real-time RT-PCR で解析した。さらに、感染マウスの肺における抗原特異的 Th1 型 T 細胞の反応を調べるため、感染後経時的に肺より T 細胞を回収し、抗原提示細胞と共に特異的ペプチド抗原で刺激した。培養後、上清中に産生された IFN-γ量を ELISA で測定し、FACS で IFN-γ産生細胞の表面抗原を解析した。

倫理面への配慮

本研究は、マウスを用いた感染動物実験を含み、実験は京都大学動物実験指針に基づいて行われた。

C. 研究結果

結核菌感染後のマウス生存率と臓器内菌数におよぼす PD-1 シグナル経路の影響

PD-1 欠損マウスは結核菌感染 50 日後には全例死亡した。しかし、WT マウスに死亡例は認められなかった。この結果から、PD-1 欠損マウスは結核菌感染に対して感受性であることが示された。感染 32 日後、WT マウスの肺には目立った所見は見当たらないのに対して、PD-1 欠損マウスの肺には肉眼で観察できる多数の病変が認められた。また、HE 染色の結果、PD-1 欠損マウスの肺ではネクローシスを伴う広範な炎症像が認められた。さらに、Ziehl-Neelsen 染色して観察したところ、PD-1 欠損マウスの肺には多数の結核菌が認められた。結核菌感染後経時的に肺への浸潤細胞について調べたところ、WT および PD-1 欠損マウスの肺では、感染後 14-21 日目にかけて浸潤してくる細胞数の増加が認められた。しかし、その数

は PD-1 欠損マウスの肺で明らかに多いことが示された。浸潤してくる細胞のうち、B 細胞、αβT 細胞やγδT 細胞数に目立った違いはなかったが、マクロファージおよび好中球数は PD-1 欠損マウスの肺で著明に増加することが示された。特にマクロファージは全浸潤細胞の半数以上を占めることが明らかとなった。

サイトカイン産生応答

PD-1 欠損マウスでは結核菌感染後にマクロファージや好中球の著しい浸潤がみられたことから、PD-1 欠損マウスの肺におけるケモカイン産生が WT よりも亢進していることが予想された。そこで、感染後経時的に肺における各種ケモカイン mRNA 発現量を調べた。その結果、感染後 17 日目から 28 日目にかけて KC、MCP-1、MIP-1α、RANTES、Eotaxin、MIP-2、MIG、IP-10 の発現が PD-1 欠損マウスで亢進していることが示された。また、炎症性サイトカインである IFN-γ、TNF-α、IL-17A および IL-6 の強い産生が PD-1 欠損マウスの肺で認められた。一方、抑制性サイトカインである IL-10 産生については WT と PD-1 欠損マウスの間で明らかな差は認められなかった。この結果、PD-1 欠損マウスでは、結核菌感染後に炎症性サイトカイン産生とマクロファージや好中球の浸潤を伴う強い炎症反応が誘導されていることが明らかとなった。この炎症反応の惹起には IL-17 が関与する可能性が考えられたので、IL-17 に対する中和抗体の影響を調べた。その結果、抗 IL-17 抗体は産生された IL-17 を完全に中和することができたが、PD-1 欠損マウスの肺でみられる細胞浸潤や菌数の増加を抑制することはできなかった。従って、IL-17 は、PD-1 欠損マウスの肺における炎症反応の直接の原因ではないことが示された。

抗原特異的 T 細胞応答

結核に対する感染防御は抗原特異的 $CD4^+$ T 細胞によって担われている。この T 細胞を中心とした免疫応答が防御の主体となるが、その反応が過剰に誘導された場合には強い炎症反応が起こり、それが組織障害の原因となる。そこで、PD-1 欠損マウスでみられる強い炎症反応が抗原特異的 T 細胞応答に起因するか否かを調べる目的で、結核菌を感染した WT および PD-1 欠損マウスの肺において、抗原特異的 $CD4^+$ T 細胞の出現状況を調べた。その結果、ESAT-6_{1-20} や $Ag85B_{240-254}$ を認識する $CD4^+$ T 細胞の IFN-γ 産生能には明らかな違いは認められなかったが、その細胞数は PD-1 欠損マウスで感染 21 日目には WT の２倍程度に増加していることが明らかとなった。さらに、結核菌感染後の WT マウスの肺に動員される $CD4^+$ T 細胞上の PD-1 の発現は感染 17-21 日目には誘導されることが示された。これらの結果から、感染 2-3 週目に誘導される PD-1 を介したシグナルは、抗原特異的 $CD4^+$ T 細胞の誘導あるいは肺への動員を制御することで防御免疫を正常に機能させていることが示唆された。さらに、結核菌感染 21 日目の PD-1 欠損マウスの肺では、NO の強い産生と HMGB-1 の放出を伴う細胞死の亢進が観察された。これらは PD-1 欠損マウスでみられる強い IFN-γ産生によって誘導されたものと考えられるが、産生された NO はさらに感染局所の細胞死を誘導し、細胞死によって遊離した HMGB1 はさらに炎症性サイトカイン産生を増強させることで組織傷害の悪化を促進するものと考えられた。

D. 考察

本研究では、結核菌感染後の防御免疫誘導における PD-1 シグナル経路の役割について解析を行った。WT マウスと PD-1 欠損マウスに結核菌を経鼻感染させたところ、WT マウスに比較して PD-1 欠損マウスの抵抗性は著しく弱く、肺における菌数の著明な増加と肺の壊死を伴う広範な組織傷害が認められた。PD-1 欠損マウスでは、感染後に強い炎症性サイトカインやケモカインの産生、マクロファージや好中球の浸潤および抗原特異的 T 細胞応答の亢進が観察されており、これら過剰な免疫応答が組織傷害を招き、防御免疫応答を正常に維持できなくなっているものと考えられる。PD-1 欠損マウスに結核菌を感染させると、肺に動員される IFN-γ産生性 $CD4^+$ T 細胞数は増加す

るが、個々のT細胞のIFN-γ産生能には明らかな違いは認められなかった。PD-1は抑制性補助因子であるPD-L1のレセプターであり、PD-1を介したシグナルがT細胞抗原受容体からのシグナルを抑制することで抗原特異的T細胞のサイトカイン産生応答を阻害することが示されている。結核菌感染ではなぜPD-1の有無が$CD4^+$ T細胞のIFN-γ産生能に影響しないのかは今のところ説明できないが、この点は菌の病原性あるいは宿主の抵抗性を決める重要な点であり、今後明らかにしなければならない課題であると考えている。

PD-1シグナル経路は、自己免疫疾患の発症を抑えるための免疫抑制システムとして見いだされ、最近の研究では、長期間宿主体内で生存可能なウイルスや細菌感染の成立にもPD-1シグナル経路が関与していることが報告されている。これまでの解析から我々は、BCG感染実験系ではPD-1経路を阻害することで菌の排除が亢進することを明らかにした。この結果は、PD-1シグナル経路の制御がBCGのワクチン効果を亢進させる可能性を示唆している。しかし、PD-1欠損マウスは結核菌感染に対して感受性を示したことから、PD-1シグナル経路の阻害が結核に対する宿主感染防御を低下させる可能性も示された。一方、予備実験の結果から、PD-1欠損マウスにBCGを接種しておくと、その後の結核菌感染に対して抵抗性になることが示されている。この機序については現在解析を行っているところであるが、少なくともPD-1シグナル経路を制御することでBCGによる予防接種の効果が亢進することは間違いなく、本研究で結核感染防御における負の面を分子レベルで解明でき、その対策が可能になれば、本格的にワクチンへの応用が期待できると考えている。

E. 結論

結核菌を感染したPD-1欠損マウスの肺では細胞死を伴った過剰な炎症反応が惹起され、感染した菌の増殖をコントロールすることができない。これは、本来感染防御を担うべき抗原特異的$CD4^+$ T細胞が正常に機能しないためであることが示された。従って、PD-1シグナル経路は結核菌の初感染後に誘導される宿主感染防御を正常に維持するために重要な役割を果たしていることが明らかになった。

G. 研究発表

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西支部総会　2010 年 11 月　枚方

H. 知的財産権の出願・登録状況

1. 特許取得　　なし
2. 実用新案登録 なし
3. その他　　なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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K. Nakanaga, Y. Hoshino, Y. Era, K. Matsumoto, Y. Kanazawa, A. Tomita, M. Furuta, M. Washizu, M. Makino, N. Ishii.	Multiple Cases of Cutaneous *Mycobacterium massiliense* Infection in a "Hot Spa" in Japan.	J. Clin. Microbiol	in press		

RESEARCH LETTER

Biochemical characteristics among *Mycobacterium bovis* BCG substrains

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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).

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)	Catalase (room temperature) 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

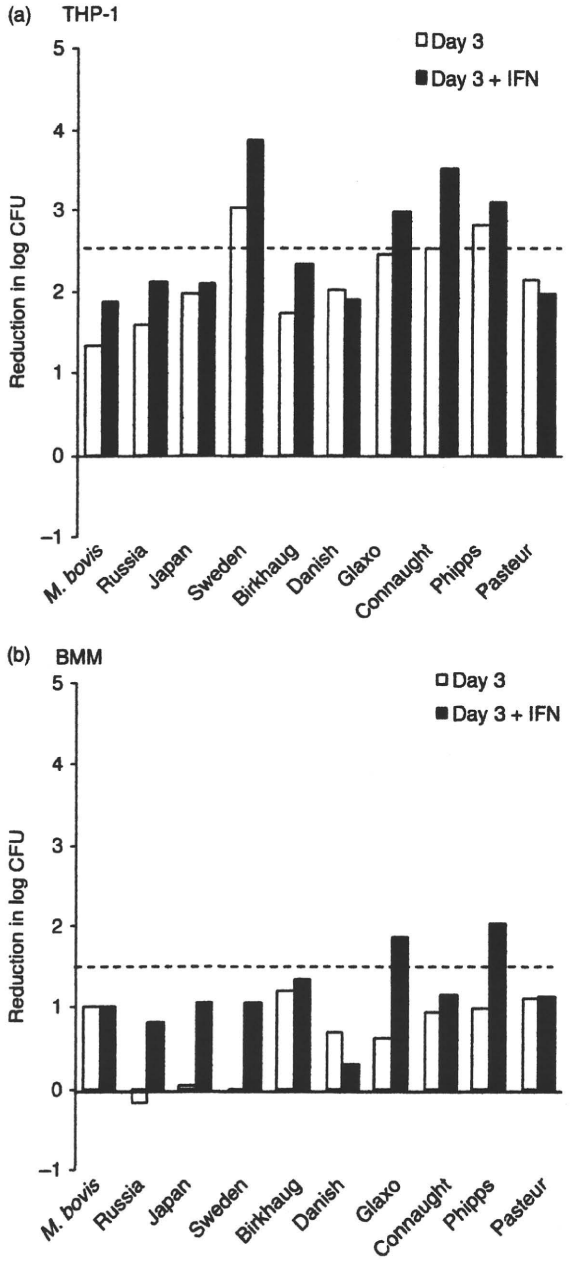


Fig. 1. Viability of BCG strains in THP-1 and mouse BMMs. PMA-differentiated THP-1 (a) or mouse BMMs (b) were infected with BCG at an MOI of 20 with (solid) or without (open) 100 U mL^{-1} of IFN-γ as a stimulator. After 6 h of infection, BCG CFU counts were determined from infected cell lysates and 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

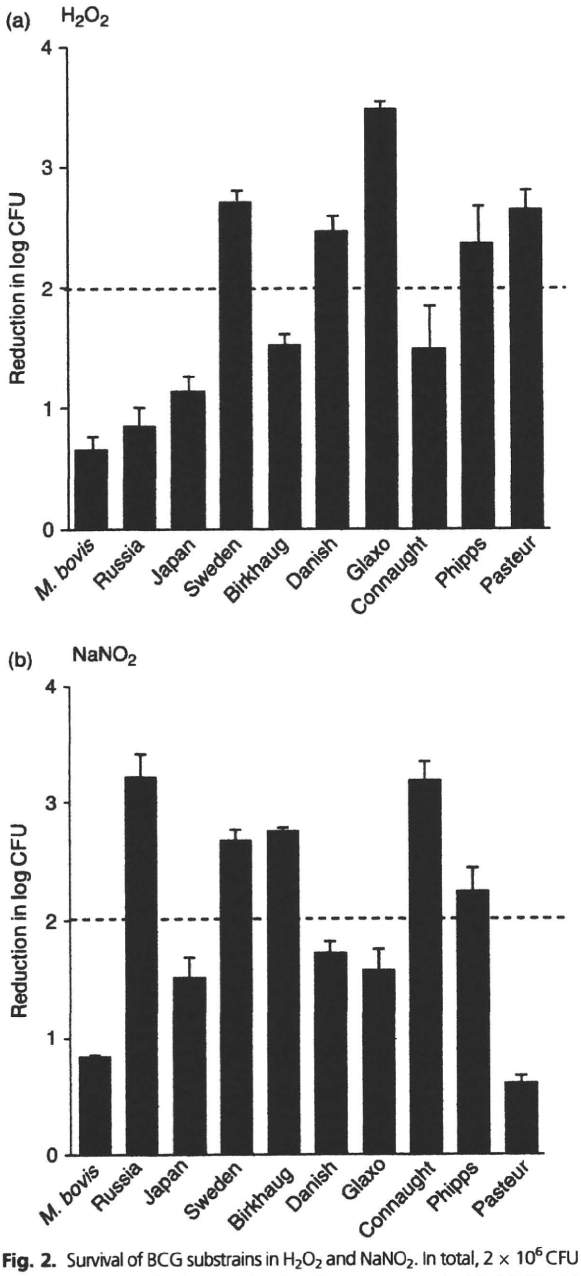
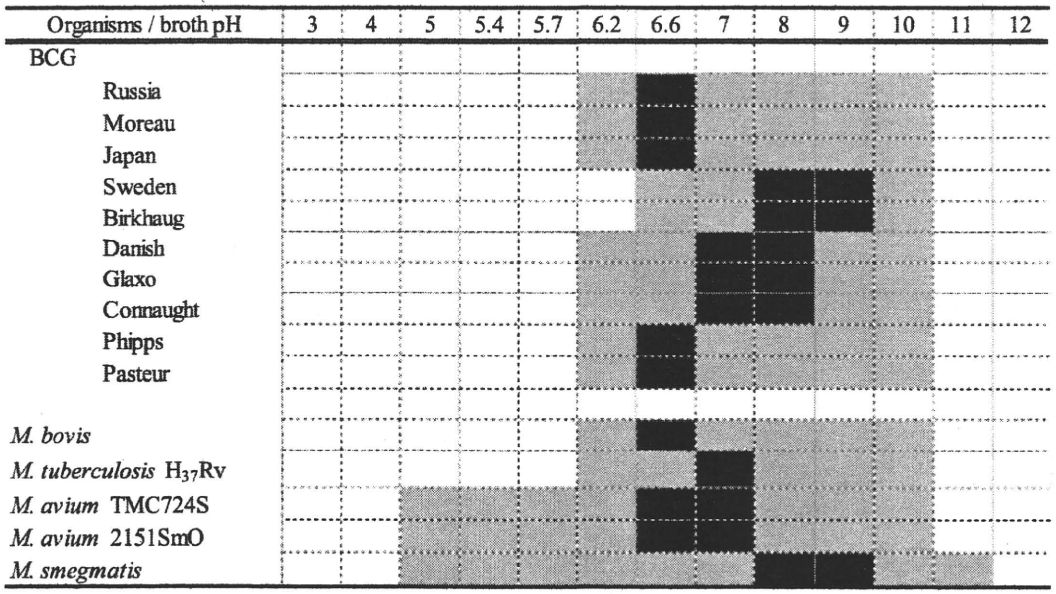


Fig. 2. Survival of BCG substrains in H_2O_2 and $NaNO_2$. In total, 2×10^6 CFU of *Mycobacterium bovis* or BCG substrains were treated with (a) 10 mM H_2O_2 for 6 h or (b) 10 mM $NaNO_2$ for 3 days. Treated and washed cells were serially diluted, and aliquots from four serial dilutions were plated in 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.

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

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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

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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 *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.

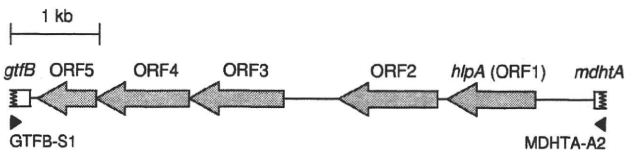

FIG. 1. Organization of the 6.8-kb genomic segment isolated from MAC serovar 4 strain (ATCC 35767). Filled triangles indicate the primers used for PCR amplification.

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