厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）
分担研究報告書

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

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研究要旨.
これまでの PD-1 欠損マウスを用いた解析から、PD-1 を介したシグナルが結核菌感染初期の宿主感染防御応答の制御に重要であること。また、BCG に対する感染防御が PD-1 シグナル経路の阻害により増強することが示されている。さらに、これらの結果は、PD-1 シグナル経路を適切に制御することで、結核に対する BCG ワクチン効果を亢進させうる可能性を示している。そこで本年度は、抗 PD-1 抗体を用いて PD-1 シグナル経路を阻害した場合の BCG ワクチン効果について検討した。正常マウスに結核菌を経鼻感染させると、感染約１年後にマウスはすべて死亡した。結核菌感染後に抗 PD-1 抗体を投与すると、結核菌感染のみの群と比較して感染初期の炎症性サイトカインやケモカイン産生および臓器内菌数の増加が認められ、感染後約 10 ヶ月でマウスはすべて死亡した。一方、予めマウスを BCG 免疫し、その 4 週後に結核菌を感染させると、結核菌単独感染の場合に比較して菌数の明らかな減少と炎症性サイトカインやケモカイン産生量の低下が認められた。また、マウスの生存率は結核菌感染 13 ヶ月後には 60%であったが、17 ヶ月後では 20%になった。一方、BCG 感染後に抗 PD-1 抗体を投与し、その 4 週後に結核菌を感染させた場合、BCG 免疫のみの群と比較するとサイトカインおよびケモカイン産生は同程度に認められたが、結核菌感染 17 ヶ月後のマウスの生存率は 80%であった。この結果から、BCG 免疫に抗 PD-1 抗体を併用することにより BCG ワクチン効果が亢進することが示唆された。

A. 研究目的

結核は世界的にみて今なお単一の病原体による最大の感染症である。WHO はこれまでに世界人口の３割が結核の感染を受け、2008 年にはおよそ 940 万人の結核患者が発生し、約 180 万人が結核で死亡したと推定している。結核患者の発生はアフリカおよびアジアなどの発展途上国に多くみられるが、多剤耐性結核菌の出現頻度の増加や、結核蔓延国からの人的流入などにより、先進国においても今後結核の増加が懸念される。また、現在わが国の結核罹患率は米国の約５倍、欧米主要国の数倍あり、結核は今もなお我々の脅威であることを忘れてはならない。

結核の原因菌である結核菌は感染しても多くの場合発症せず、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増殖をはじめ、結核を発症するのである。一方、結核菌が感染した宿主では、感染後数週間経つと Th1 型 $CD4^+$ T 細胞が誘導され、特異的感染防御免疫が成立する。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 ワクチンによって特異的防御免疫を誘導することはできるが、感染した菌を排除することは簡単ではない。これらの事実は、結核菌が宿主の感染防御機構に対する強い抵抗性を有す

ることを示している。

PD-1は、アポトーシスに陥ったT細胞の表面抗原として同定された。その後の解析から、PD-1は活性化したT細胞やB細胞に発現すること。また、機能不全に陥った$CD4^+$ T細胞に恒常的に発現することが示されている。さらに、PD-1はレセプターとしての機能を有し、2種類の特異的リガンド(PD-L1とPD-L2)が存在することが明らかにされている。これらリガンドのうち、PD-L1は多様な細胞に発現が認められるが、PD-L2は活性化した樹状細胞やマクロファージに発現する。さらに、抗原提示細胞上のPD-L1の発現は、IFN-γやTLRリガンドの刺激により亢進することが報告されている。PD-1経路の機能解析から、抗原提示細胞がT細胞に抗原を提示する場合、PD-1とPD-1特異的なリガンドの結合がT細胞に抑制性シグナルを伝達することが明らかにされている。この抑制性シグナルは、末梢組織において自己免疫反応を抑制し、免疫寛容を維持するために必要である。一方、リンパ球性脈絡髄膜炎ウイルス(lymphocytic choriomeningitis virus)、ヒト免疫不全ウイルス(human immunodeficiency virus)やB型肝炎ウイルス(hepatitis B virus)などの慢性ウイルス感染では、PD-1を介した抑制性シグナルは病原体の排除を阻害することが示されている。従って、PD-1シグナル経路の活性化はT細胞機能を制御して組織傷害を抑えるために重要であるが、逆に慢性感染した病原体の排除を困難にする原因になる。

昨年度、抗結核感染防御におけるPD-1シグナル経路の役割を明らかにするため、PD-1欠損マウスを用いて結核菌感染実験を行った。その結果、正常マウスに比較してPD-1欠損マウスは結核菌感染に感受性であり、感染3週目以降の肺における菌の増殖をコントロールできないことがわかった。組織学的解析の結果、結核菌感染4週後のPD-1欠損マウスの肺では、多数の結節が観察され、その内部ではマクロファージや好中球を中心とした炎症性細胞の浸潤と壊死を伴う広範な炎症反応、および各種サイトカイン(IFN-γ、TNF-α、IL-6、IL-17A)やケモカイン(MCP-1、MIP-1、CXCL1)の産生増強が認められた。これらの結果から、結核感染後の急性期の肺ではPD-1を介したシグナルが過剰な炎症性反応を抑制して正常レベルの防御免疫を誘導するために重要な役割を果たしていることが明らかとなった。一方、BCGを正常およびPD-1欠損マウスに感染させ、経時的に抗原特異的T細胞によるサイトカイン産生と臓器内生菌数を測定したところ、PD-1欠損マウスではBCG感染後の持続的なIFN-γおよびTNF-αの強い産生が認められ、正常マウスよりも菌の排除が亢進することが示された。これらの結果は、PD-1シグナル経路の制御が結核菌に対する感染防御の発現を調節する上で重要な要素となり、その適切な制御が有効な抗結核感染防御の発現を可能にすることを示唆するものである。

そこで本年度は、抗PD-1抗体を用いてPD-1シグナル経路を阻害した場合の結核菌に対する防御免疫応答や、BCGワクチン効果への影響について解析した。

B. 研究方法

結核菌感染実験

抗PD-1抗体の抗結核感染防御への影響を解析するため、C57BL/6マウスに結核菌(200 cfu)を経鼻感染させた。抗PD-1抗体(100 μg)またはコントロール抗体(100 μg)を感染14、17および20日後に静脈内投与し、その後のマウスの生存率を調べた。また、マウスをBCG(10^4 cfu)皮下免疫し、その4週後に結核菌を経鼻感染させた。結核菌感染14、17および20日後に抗PD-1抗体を投与し、その後のマウスの生存率を調べた。さらに、マウスをBCGで免疫し、その14、17および20日後に抗PD-1抗体(100 μg)を静脈内投与した。BCG免疫4週後に結核菌を経鼻感染させ、その後のマウスの生存率を調べた。

菌数測定

結核菌感染2および4週間後に感染マウスより肺および脾臓を回収し、ホモジネー

トを作製した。段階希釈したホモジネートを Middlebrook7H10 寒天培地に塗抹し、3 週間培養後にコロニー数を数え、臓器内生菌数を算出した。

サイトカインおよびケモカイン産生応答

結核菌感染 2 および 4 週間後に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。

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

抗 PD-1 抗体投与による BCG ワクチン効果への影響

正常マウスに結核菌を経鼻感染させると、感染後 9 ヶ月で死亡するマウスが観察されるようになり、感染後約 1 年で全てのマウスが死亡した。結核菌感染後に抗 PD-1 抗体を投与したマウスでは、結核菌感染のみの群と比較して感染 4 週後の肺内生菌数の増加が観察された。また、肺における IFN-γ および TNF-α産生も結核菌感染のみの場合と比較して高い傾向にあった。さらに、抗 PD-1 抗体を投与した結核菌感染マウスは感染約 7 ヶ月後より死亡が認められ、感染後 10 ヶ月で全てのマウスが死亡した。一方、マウスを予め BCG 免疫した後結核菌を感染させた群では、結核菌のみを感染させた群と比較して感染 4 週後の肺内生菌数は明らかに少なく、感染後 1 年を経過した時点でマウス生存率は 100%であった。また、結核菌感染 2 週後より IFN-γや TNF-αなどのサイトカインおよび各種ケモカイン（MCP-1、MCP-3、IP-10）産生が誘導され、感染 4 週後でもそれらの産生は持続して認められた。しかし、そのレベルは結核菌単独感染群および結核菌感染後に抗 PD-1 抗体を投与した群と比較すると低いことが示された。さらに、BCG 免疫後に抗 PD-1 抗体を投与し、その後結核菌を感染させた群では、BCG 免疫のみの場合と比較して結核菌感染 4 週後の肺内生菌数に有意な違いは認められず、各種サイトカインおよびケモカイン産生においても抗 PD-1 抗体投与の効果は明らかではなかった。しかし、結核菌感染後 17 ヶ月経過した時点での生存率は 80%であり、BCG 免疫単独群（この時点での生存率は 20%）と比較して明らかな違いが認められた。

D. 考察

これまでの研究成績から、正常マウスに比べて PD-1 欠損マウスは結核菌感染に感受性を示し、結核菌感染早期の肺では炎症性サイトカインやケモカイン産生の亢進、マクロファージや好中球を中心とした炎症性細胞の著明な浸潤、および壊死を伴う広範な炎症性病変が認められる。また、PD-1 欠損マウスの肺では IFN-γ 産生能を有する抗原特異的 $CD4^+$ T 細胞数が増加し、この細胞を移入されたマウスは結核菌に対する感受性が亢進することが示されている。これらの結果は、PD-1 を介した抑制性シグナルが働かないと、結核菌感染後の肺では抗原特異的 $CD4^+$ T 細胞が過剰な炎症反応を惹起するため、感染した菌を制御することができなくなることを示しており、PD-1 経路は結核菌感染初期に誘導される $CD4^+$ T 細胞の機能を適度に制御し、過剰な炎症反応を引き起こさないための重要な役割を果たしていると考えられる。また、結核感染の慢性期に抗 PD-1 抗体を投与しても菌の感染動態には影響がないことが報告されていることから（IAI 77: 4621-4630, 2009）、結核菌感染では PD-1 を介した抑制性シグナルはナイーブ $CD4^+$ T 細胞のエフェクター細胞への分化を正常に誘導するために必要であり、すでにエフェクター/メモリー細胞に分化した細胞の機能制御にはその効果を発揮しないことが考えられる。

一方、結核菌感染とは異なり、BCG 感染の場合には PD-1 経路の阻害は菌の排除を亢進させることが示されている。また、本研究成果から、抗 PD-1 抗体投与により結核菌感染後の生存率が改善することが示されたことから、PD-1 を介したシグナル制御が

BCG ワクチン効果の増強に繋がると考えられる。さらに、PD-1 欠損マウスに BCG 免疫すると、その後の結核菌の攻撃感染に対する抵抗性が増強する。また、少なくとも BCG 感染では結核菌感染でみられる炎症反応による肺の組織傷害は観察されず、PD-1 シグナル経路の阻害が感染の増悪には結びつかないことが示されている。今後、PD-1 を介したシグナル制御のワクチンへの応用を可能にするため、PD-1 シグナルの阻害によるメリット・デメリットを分子レベルで明らかにすることで結核に対するワクチン開発に有用な情報を提供していく。

E. 結論

PD-1 欠損マウスに BCG を感染させると、正常マウスに比較して菌の排除が亢進し、BCG 免疫後の結核菌による攻撃感染に対して強い抵抗性を示したことから、PD-1 シグナル経路を制御することで BCG ワクチンの効果を増強できる可能性が考えられた。そこで本研究では、抗 PD-1 抗体を用いて PD-1 シグナルを阻害した場合の BCG ワクチン効果への影響を検討した。結核菌感染後に抗 PD-1 抗体を投与すると、感染マウスの肺では炎症性サイトカインやケモカイン産生が増強し、結核菌感染のみの群と比較して早期に死亡することが示された。この結果から、PD-1 欠損マウスでみられる結核菌に対する感受性の亢進は抗 PD-1 抗体投与により再現できることが示された。一方、マウスを BCG 免疫し、その後抗 PD-1 抗体を投与して結核菌の攻撃感染に対する宿主応答を調べたところ、抗 PD-1 抗体投与による BCG ワクチンの増強効果が認められた。この結果を踏まえ、更に効果的な PD-1 経路の制御方法について検討していく予定である。

G. 研究発表

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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

別紙 5-1

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

別紙 5-1

雑誌

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NOTE *Bacteriology*

Mycobacterium pseudoshottsii Isolated from 24 Farmed Fishes in Western Japan

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ABSTRACT. Mycobacteria isolated from epizootics of farmed fishes in western Japan were examined for the first time using multigenotypic analysis. By analysis of the sequences of the internal transcribed spacer between the 16S and 23S rRNA genes (ITS) region and the partial 16S rRNA, *hsp65* and *rpoB* genes, *M. pseudoshottsii* was identified as the causative agent in these infections. Prior to this study, only *M. marinum* has been known as the causative agent of lethal mycobacterial disease in marine fishes in Japan.
KEY WORDS: lethal fish infection, *Mycobacterium pseudoshottsii*, mycolactone.

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Mycobacterium (M.) marinum, M. salmoniphilum, M. fortuitum, M. chelonae and *M. abscessus* are the most commonly identified mycobacterial fish pathogens [1, 4]. In particular, *M. marinum* is found in a wide range of saltwater species [2]. Molecular and phylogenetic analyses have facilitated the worldwide recovery of novel mycobacterial species, strains and isolates, such as *M. shottsii* [9] and *M. pseudoshottsii*, from wild marine fishes [3, 10].

M. pseudoshottsii, a slow-growing, photochromogenic mycobacterium, was initially isolated in 2005 from striped bass [10]. Its biochemical reactions, growth characteristics and mycolic acid profiles resemble those of *M. shottsii*, a nonpigmented mycobacterium that was isolated during the same epizootic outbreak [10]. However, the sequences of the 16S rRNA gene and the gene encoding the 65 kDa heat shock protein (hsp65) revealed that the isolate was unique [9, 10]. Initially, *M. pseudoshottsii* was found only in wild Chesapeake Bay striped bass; however, both the range of host species and the area of disease distribution have expanded to a variety of fishes and locations [13, 15]. In Japan, molecular and genotypic examinations of piscine-related nontuberculous mycobacteria (NTM) are rare. Here, we report on the genotypic analysis of mycobacteria isolated from infected fishes raised on farms in western Japan.

Twenty-four isolates were recovered from moribund yellowtails (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerili*), striped jack (*Pseudocaranx dentex*), sevenband grouper (*Epinephelus septemfasciatus*), and yellowtail amberjack (*Seriola lalandi*) at fish farms in the western part of Japan from 1999 to 2008 (Table 1). The diseased fish generally showed lethargy, anorexia, emaciation and abdominal distension with ascites. Sometimes, mass culling of the same fish group at a farm was needed because of mass mortality. In some cases, skin ulceration and eye corneal ulceration were observed. White nodules were often found in several internal organs especially in enlarged spleens and kidneys. Isolation was attempted with the affected organ, kidneys, spleen, liver and gills of each fish. These tissues were aseptically dissected, homogenized in phosphate buffered saline, inoculated on 2% Ogawa egg slant (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) or homogenized with 4% NaOH for 10 min and inoculated on 1% Ogawa egg slant (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Incubation was performed at 23 to 25°C for 2 to 3 months. Subculture were performed for colony purification with 2% Ogawa egg slant and/or Middlebrook 7H11 agar supplemented with 10% OADC enrichment (Becton, Dickinson and Company, Fukushima, Japan).

Multigenotypic analysis was used to identify the resulting isolates. One loopful of mycobacterial colonies on Ogawa egg slant or 7H11 agar was suspended in 400 μl sterilized phosphate-buffered saline supplemented with 0.05% Tween 80 and was stored at –80°C until DNA was extracted. A frozen bacterial suspension was crushed in a bead-beating instrument (Magnalizer; Roche Diagnostics Japan, Tokyo, Japan) at 3,000 rpm for 90 sec with zirconia beads (diameter, 2 mm). Total genomic DNA was purified from the crashed suspension using a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics Japan, Tokyo, Japan) and was stored at –20°C.

An approximately 1,500-bp fragment of the 16S rRNA gene, the partial sequences of the *hsp65* and *rpoB* genes and the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region) were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, U.S.A.) with the primers listed in Table 2. The amplicons of the isolates were sequenced using an ABI

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Table 1. Origin of the mycobacterial strains used in this study

Strain	Host fish	Isolation date	Site isolated	Location of fish farm (Prefecture)
MF01	Yellow tail (*Seriola quinqueradiata*)	Oct/19/2004	Kidney	Kagoshima
MF06	Yellow tail (*Seriola quinqueradiata*)	Sep/08/2008	Kidney	Kagoshima
MF09	Yellow tail (*Seriola quinqueradiata*)	Jul /19/2001	Kidney	Oita
MF10	Yellow tail (*Seriola quinqueradiata*)	Oct/31/2001	Kidney	Oita
MF12	Yellow tail (*Seriola quinqueradiata*)	Aug/19/2008	Kidney	Ehime
MF14	Yellow tail (*Seriola quinqueradiata*)	Aug/29/2008	Kidney	Ehime
MF31	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Spleen	Kagoshima
MF32	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF33	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF34	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF35	Yellow tail (*Seriola quinqueradiata*)	Feb/02/2005	Kidney	Kagoshima
MF36	Yellow tail (*Seriola quinqueradiata*)	Feb/02/2005	Kidney	Kagoshima
MF44	Yellow tail (*Seriola quinqueradiata*)	Jul /19/2001	NC[a)]	Oita
MF45	Yellow tail (*Seriola quinqueradiata*)	Oct/01/2001	NC	Oita
MF46	Yellow tail (*Seriola quinqueradiata*)	NC/– /2004	NC	Ehime
MF02	Greater amberjack (*Seriola dumerili*)	Jan/06/2005	Kidney	Kagoshima
MF05	Greater amberjack (*Seriola dumerili*)	Nov/15/2005	Kidney	Kagoshima
MF07	Greater amberjack (*Seriola dumerili*)	NC/– /2006	Miliary nodule	Miyazaki
MF40	Greater amberjack (*Seriola dumerili*)	Jan/05/2004	NC	Kagoshima
MF13	Sevenband grouper (*Epinephelus septemfasciatus*)	Aug/18/2008	Kidney	Ehime
MF15	Sevenband grouper (*Epinephelus septemfasciatus*)	Oct/– /2008	Kidney	Ehime
MF04	Striped jack (*Pseudocaranx dentex*)	Nov/15/2005	Kidney	Kagoshima
MF08	Striped jack (*Pseudocaranx dentex*)	Sep/06/1999	Kidney	Oita
MF11	Yellowtail amberjack (*Seriola lalandi*)	Aug/09/2007	Spleen	Oita

a) Not clear.

Table 2. Primers used in this study

Primer	Sequence (positions)	PCR target (fragment size)	Reference
8F16S	5'-AGAGTTTGATCCTGGCTCAG- 3' (8–27)	16S rRNA gene (app. 1,500 bp)	12
1047R16S	5'-TGCACACAGGCCACAAGGGA- 3' (1,047–1,028)		
830F16S	5'-GTGTGGGTTTCCTTCCTTGG- 3' (830–849)		
1542R16S	5'-AAGGAGGTGATCCAGCCGCA- 3' (1,542–1,523)		
ITSF	5'-TTGTACACACCGCCCGTC- 3' (16S, 1,390–)	16S-23S ITS region (app. 340 bp)	11
ITSR	5'-TCTCGATGCCAAGGCATCCACC- 3' (23S, 44–)		
TB11	5'-ACCAACGATGGTGTGTCCAT- 3' (398–417)	*hsp65* (439bp)	16
TB12	5'-CTTGTCGAACCGCATACCCT- 3' (836–817)		
MF	5'-CGACCACTTCGGCAACCG- 3'	*rpoB* (342 bp)	5
MR	5'-TCGATCGGGCACATCCGG- 3'		

Prism 310 PCR Genetic Analyzer (Applied Biosystems) [6] and compared to the sequences of six strains of mycobacteria: "*M. ulcerans* subsp. *shinshuense*" ATCC33728 [6], *M. ulcerans* ATCC19423 (type strain), *M. ulcerans* Agy99 [14], *M. marinum* ATCC 927 (type strain), *M. marinum* clinical isolate strain 112509 (the preceding 5 strains originated in humans) and *M. pseudoshottsii* JCM15466 (type strain). The JCM strain was distributed by the Microbe Division of the Riken BioResource Center (BRC; Saitama, Japan). Isolate and reference sequences were deposited into the DNA Data Bank of Japan (DDBJ) under accession numbers AB548704 to AB548734 and AB642161 to AB642165.

The sequences of the 1,475-bp fragment of 16S rRNA gene from the piscine isolates showed almost complete identity with the *M. pseudoshottsii* reference strain (99.93–100% identity). Only a single mismatch was found at nucleotide position 487 or 488 in 9 of 24 piscine isolates compared with the DNA sequence of *M. pseudoshottsii* JCM15466. However, conserved mismatches with the 5 strains that originated in humans were found at nucleotide positions 95, 969, 1,007 and 1,215 (Table 3). *M. ulcerans* Agy99 had sum upped 3-base pair insertion (TTT) at nucleotide position 1,449–1,451. Similarly, the ITS regions of the piscine isolates and the *M. pseudoshottsii* reference strain were either identical or differed at position 57, while conserved mismatches with the strains originating in humans were at nucleotide positions 30 and 62. All of the sequences of *hsp65* and *rpoB* gene fragments from the iso-

Table 3. Alignment of the 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences from 24 piscine isolates and 6 reference strains[a)]

Strain	Prefecture/ Country	Nucleotide sequence positions																			
		16S rRNA[b)]								ITS region				*hsp65*[c)]						*rpoB*[d)]	
		95	487–8	492	969	1007	1215	1247	1288	30	57	62	83	455	571	637	639	647	797	92	143
M. shinshuense ATCC 33728	Nagano/Japan	T	GG	G	A	G	T	G	G	G	G	T	A	C	T	C	C	A	T	C	C
M. ulcerans ATCC 19423^T	NC[e)]/ Australia	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C	T	C
M. ulcerans Agy99	NC / Ghana	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C	T	C
M. marinum ATCC 927^T	NC / USA	T	GG	A	A	G	T	A	A	G	G	T	A	C	C	C	T	G	C	C	G
M. marinum 112509	Tokyo/Japan	T	GG	A	A	G	T	A	A	G	G	T	G	C	C	C	T	G	C	C	G
M. pseudoshottsii JCM 15466^T	NC / USA	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF01 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF06 (yellow tail)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF09 (yellow tail)	Oita/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF10 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF12 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF14 (yellow tail)	Ehime/Japan	C	AA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF31 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF32 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF33 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF34 (yellow tail)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF35 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF36 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF44 (yellow tail)	Oita/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF45 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF46 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF02 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF05 (greater amberjack)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF07 (greater amberjack)	Miyazaki /Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF40 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF13 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF15 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF04 (striped jack)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF08 (striped jack)	Oita/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF11 (yellowtail amberjack)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C

a) Only nucleotide differences are noted. Nucleotide positions were based on the b) *E. coli* 16S rRNA gene (accession No. J01859). c) *M. tuberculosis hsp65* gene. (accession No. M15467) and d) *rpoB* gene (accession No. AF057454). e) Not clear.

lates showed complete identity with those of the *M. pseudoshottsii* sequences (Table 3). A conserved mismatch between piscine and human isolates in *hsp65* gene fragments was only found at nucleotide position 637. The results showed that the 24 piscine isolates were all identified as *M. pseudoshottsii* rather than *M. marinum*.

A lethal case of *M. marinum* in cultured yellowtails, which was identified using biological, biochemical and 16S rRNA sequence analyses, has been reported in Japan [17]. In our study, *M. pseudoshottsii* was identified as an additional source of atypical piscine mycobacteriosis and (the bacteria) had been distributed in farmed fisheries in the west part of Japan since 1999. Further studies are needed to develop an easier method to distinguish *M. pseudoshottsii* from *M. marinum* because both strains might have not been differentiated before in Japan. Their differences in susceptibility to antimicrobial agents and in capacity for human pathogenesis should be elucidated. In addition, *M. pseudoshottsii* produces a unique plasmid-encoded toxic macrolide, mycolactone F [7], suggesting that *M. pseudoshottsii* provides a reservoir in aquatic environments for the horizontal transfer of the plasmid-borne genes that encode mycolactone F. Interestingly the potency of mycolactone F with regard to apoptosis in a mammalian cell line was significantly less than that of mycolactone A/B, which is produced by *M. ulcerans*, a causative agent of Buruli ulcer [17]. Further molecular, biochemical and drug susceptibility studies are needed to understand the possible role of mycolactone F in mycobacteriosis and to fully characterize piscine mycobacterial infections in Japan.

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Mutation Analysis of Mycobacterial *rpoB* Genes and Rifampin Resistance Using Recombinant *Mycobacterium smegmatis*

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Rifampin is a major drug used to treat leprosy and tuberculosis. The rifampin resistance of *Mycobacterium leprae* and *Mycobacterium tuberculosis* results from a mutation in the *rpoB* gene, encoding the β subunit of RNA polymerase. A method for the molecular determination of rifampin resistance in these two mycobacteria would be clinically valuable, but the relationship between the mutations and susceptibility to rifampin must be clarified before its use. Analyses of mutations responsible for rifampin resistance using clinical isolates present some limitations. Each clinical isolate has its own genetic variations in some loci other than *rpoB*, which might affect rifampin susceptibility. For this study, we constructed recombinant strains of *Mycobacterium smegmatis* carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without mutation and disrupted their own *rpoB* genes on the chromosome. The rifampin and rifabutin susceptibilities of the recombinant bacteria were measured to examine the influence of the mutations. The results confirmed that several mutations detected in clinical isolates of these two pathogenic mycobacteria can confer rifampin resistance, but they also suggested that some mutations detected in *M. leprae* isolates or rifampin-resistant *M. tuberculosis* isolates are not involved in rifampin resistance.

Leprosy and tuberculosis persist as important global public health concerns. Rifampin, a major drug used to treat these two infectious diseases, has a molecular mechanism of activity involving the inhibition of DNA-dependent RNA polymerase (15). In *Escherichia coli*, this enzyme is a complex oligomer comprised of four subunits, α, β, β′, and σ, encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively. Rifampin binds to the β subunit of RNA polymerase and results in transcription inhibition (15). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, reportedly result in resistance to rifampin in several mycobacterial species, including *Mycobacterium leprae* and *Mycobacterium tuberculosis* (9, 21). The former has not yet been cultured on artificial media; it requires 11 to 14 days to double in experimentally infected mice. Therefore, it is difficult to determine the rifampin susceptibilities of *M. leprae* isolates. The standardized method using a mouse footpad takes more than half a year to determine the rifampin susceptibility of *M. leprae* isolates and requires 5×10^3 *M. leprae* bacilli (3), which require almost a year to prepare. *In vitro* drug susceptibility testing for *M. leprae* using a radioactive reagent requires more (10^7) *M. leprae* cells (7). In contrast, mutations in the *rpoB* gene of *M. leprae* can be detected in a few days or less. It would be very helpful if mutations responsible for rifampin resistance could be determined without performing mouse footpad testing. The main mutations that confer rifampin resistance to *M. tuberculosis* are located in the 81-bp core region of the *rpoB* gene, encompassing codons 507 to 533, known as the rifampin resistance-determining region (RRDR) (17, 18). About 95% of rifampin-resistant *M. tuberculosis* strains have a mutation in this region (18, 20). Four mutations, D516V, H526Y, H526D, and S531L, are most commonly associated with the high-level rifampin resistance of *M. tuberculosis* strains (4, 10, 19), but some other mutations in the 81-bp region have not yet been confirmed completely as being responsible for rifampin resistance.

We have established a method to determine the mutations responsible for the dapsone resistance of *M. leprae* using recombinant *Mycobacterium smegmatis* strains (16). In the present study, we assessed the applicability of the determination of rifampin resistance for analysis. We then analyzed *rpoB* mutations conferring rifampin resistance to *M. leprae* and *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5α was used for DNA cloning. *M. smegmatis* mc^2155 was used as a mycobacterial host to produce strains for drug susceptibility testing. Plasmids pYUB854 and phAE87 were kindly provided by W. R. Jacobs, Jr. (Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY). *M. smegmatis* mc^2155 and its transformants were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.5% bovine serum albumin (fraction V), 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80.

Site-directed mutagenesis. The wild-type *rpoB* genes of *M. leprae* and *M. tuberculosis* were amplified from *M. leprae* Thai-53 and *M. tuberculosis* H37Rv by PCR and cloned into pMV261. Site-directed mutagenesis was performed by using PCR with DNA polymerase (Takara PrimeStar HS; Takara Bio Inc., Kyoto, Japan) and the primers presented in Table 1. PCR products were purified and phosphorylated with T4 kinase and ATP and were then ligated to make them circular. The ligation mixture was used to transform *E. coli* DH5α cells, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants. The mutated sequences were then confirmed by sequencing. The inserts of the plasmids were also cloned into pNN301 (16). Mutations introduced into the *M. leprae rpoB* or *M. tuberculosis rpoB* gene are listed in Table 2.

Disruption of the *rpoB* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc^2155 cells were transformed with plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allel-

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TABLE 1 Primers used for this study

Primer	Sequence[a]	Application
M. smegmatis		
MSRBUF	GCCTTAAGGAGGAGAAGGACGAGGCCAC	*rpoB* disruption, upstream forward
MSRBUR	GCTCTAGACAAGATGCATCCTTCCAGCA	*rpoB* disruption, upstream reverse
MSRBDF	GCAAGCTTTCGCGCAACGAATCCGCGTC	*rpoB* disruption, downstream forward
MSRBDR	GCACTAGTAGCGCACGCAGCTTCTTCTG	*rpoB* disruption, downstream reverse
MSRBF	TGGTCAAGCAGTTCCTCAAC	Detection of *rpoB* disruption, forward
MSRBR	CGTTGTTGACGATGATCTCG	Detection of *rpoB* disruption, reverse
M. leprae		
MLRBWTF	GCGGATCCGTGCTGGAAGGATGCATCTT	Cloning of *M. leprae rpoB*, forward
MLRBWTR	GCGTTAACCTAAGCCAGATCTTCTATGG	Cloning of *M. leprae rpoB*, reverse
MLRBWTF1	CAGTTCATGGATCAGAACAACCCTC	Introduction of point mutation at codons 507 and 508
MLRBWTF2	TGTCGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation at codon 526
MLRBWTF3	TTCGCACTACGGCCGGATGTGCCCG	Introduction of point mutation at codon 547
MLRBWTR1	CGACAGCTGGCTGGTGCCGAAGAAT	Introduction of point mutation at codons 513, 516, and 517
MLRBWTR2	GCCGGCGCTTGTGGGTCAGGCCCGA	Introduction of point mutation at codons 531, 532, and 533
MLRB507GGG	CGACAGCTGGCTGGTCCCGAAGAAT	Introduction of point mutation GGC507→GGG
MLRB507AGC	CGACAGCTGGCTGGTGCTGAAGAAT	Introduction of point mutation GGC507→AGC
MLRB508ACA	CGACAGCTGGCTTGTGCCGAAGAAT	Introduction of point mutation ACC508→ACA
MLRB513GTG	GTGTTCATGGATCAGAACAACCCTC	Introduction of point mutation CAG513→GTG
MLRB516AAT	CAGTTCATGAATCAGAACAACCCTC	Introduction of point mutation GAT516→AAT
MLRB517CAT	CAGTTCATGGATCATAACAACCCTC	Introduction of point mutation CAG517→CAT
MLRB526TAC	GCCGGCGCTTGTAGGTCAGGCCCGA	Introduction of point mutation CAC526→TAC
MLRB531TTG	TGTTGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TTG
MLRB531TGG	TGTGGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TGG
MLRB532TCG	TGTCGTCGCTGGGCCCGGGTGGTTT	Introduction of point mutation GCG532→TCG
MLRB533CCG	TGTCGGCGCCGGGCCCGGGTGGTTT	Introduction of point mutation CTG533→CCG
MLRB547ATC	GGGTGCACGTCACGGATCTCTAGCC	Introduction of point mutation GTC547→ATC
M. tuberculosis		
MTRBWTF	GCGAATTCTTGGCAGATTCCCGCCAGAG	Cloning of *M. tuberculosis rpoB*, forward
MTRBWTR	GCAAGCTTTTACGCAAGATCCTCGACAC	Cloning of *M. tuberculosis rpoB*, reverse
MTRBWTF1	AATTCATGGACCAGAACAACCCGCT	Introduction of point mutation at codons 507, 508, 510, 511, 512, and 513 and deletion of codons 506-508
MTRBWTF2	CTGTCGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation at codons 522, 523, 526, and 531
MTRBWTR1	GGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of mutation at codons 514, 516, 518, 519, and 521; deletion of codon 518; and insertion of TTC between codons 514 and 515
MTRBWTR2	TCGGCGCTTGTGGGTCAACCCCGAC	Introduction of point mutations TCG531→TTC and TCG531→TTG
MTRB507AGC	GGCTCAGCTGGCTGGTGCTGAAGAA	Introduction of point mutation GGC507→AGC
MTRB507GAT	GGCTCAGCTGGCTGGTATCGAAGAA	Introduction of point mutation GGC507→GAT
MTRB508CAC	GGCTCAGCTGGCTGTGGCCGAAGAA	Introduction of point mutation ACC508→CAC
MTRB508GCC	GGCTCAGCTGGCTGGCGCCGAAGAA	Introduction of point mutation ACC508→GCC
MTRB510CAT	GGCTCAGATGGCTGGTGCCGAAGAA	Introduction of point mutation CAG510→CAT
MTRB511CCG	GGCTCGGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CTG511→CCG
MTRB513AAT1	TGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→AAT
MTRB513AAT2	ATTTCATGGACCAGAACAACCCGCT	Introduction of point mutation CAA513→AAT
MTRB513GAA	CGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→GAA
MTRB516GAG	AATTCATGGAGCAGAACAACCCGCT	Introduction of point mutation GAC516→GAG
MTRB516CAC	AATTCATGCACCAGAACAACCCGCT	Introduction of point mutation GAC516→CAC
MTRB516GTC	AATTCATGGTCCAGAACAACCCGCT	Introduction of point mutation GAC516→GTC
MTRB521ATG	AATTCATGGACCAGAACAACCCGAT	Introduction of point mutation CTG521→ATG
MTRB522TTG	TCGGCGCTTGTGGGTCAACCCCAAC	Introduction of point mutation TCG522→TTG
MTRB523GCG	TCGGCGCTTGTGGGTCAACGCCGAC	Introduction of point mutation GGG523→GCG
MTRB523GGC	TCGGCGCTTGTGGGTCAAGCCCGAC	Introduction of point mutation GGG523→GGC
MTRB526CTC	TCGGCGCTTGAGGGTCAACCCCGAC	Introduction of point mutation CAC526→CTC
MTRB526TAC	TCGGCGCTTGTAGGTCAACCCCGAC	Introduction of point mutation CAC526→TAC
MTRB526GAC	TCGGCGCTTGTCGGTCAACCCCGAC	Introduction of point mutation CAC526→GAC
MTRB526TTC	TCGGCGCTTGAAGGTCAACCCCGAC	Introduction of point mutation CAC526→TTC
MTRB526AAC	TCGGCGCTTGTTGGTCAACCCCGAC	Introduction of point mutation CAC526→AAC
MTRB526CGC	TCGGCGCTTGCGGGTCAACCCCGAC	Introduction of point mutation CAC526→CGC
MTRB526CAA	TCGGCGCTTTTGGGTCAACCCCGAC	Introduction of point mutation CAC526→CAA
MTRB529AAA	TTTGCGCTTGTGGGTCAACC	Introduction of point mutation CGA529→AAA
MTRB531TTC	CTGTTCGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTC
MTRB531TTG	CTGTTGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTG
MTRB506d	GGCTCAGCTGGCTGAACTCCTTGAT	Introduction of mutation 506-508del
MTRBin514TTC	AATTCTTCATGGACCAGAACAACCC	Introduction of mutation 514insTTC
MTRBd518	AATTCATGGACCAGAACCCGCTGTC	Introduction of mutation 518del

[a] Restriction sites are underlined.

TABLE 2 Rifampin and rifabutin susceptibilities of the recombinant *M. smegmatis* strains

Mutation	Rifampin		Rifabutin		Reference(s)
	MIC (μg/ml)	Fold increase[a]	MIC (μg/ml)	Fold increase	
M. leprae					
Wild type	1		0.25		
GGC507→GGG (silent)	1	1	0.25	1	This study
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	3
ACC508→ACA (silent)	1	1	0.25	1	This study
CAG513→GTG (Q513V)	32	32	8	32	3
GAT516→AAT (D516N)	32	32	2	8	14
CAG517→CAT (Q517H)	1	1	0.25	1	11
CAC526→TAC (H526Y)	32	32	8	32	14
TCG531→TTG (S531L)	32	32	4	16	3, 14
TCG531→TGG (S531W)	32	32	8	32	14
GCG532→TCG (A532S)	1	1	0.25	1	11
CTG533→CCG (L533P)	32	32	4	16	14
GTC547→ATC (V547I)	1	1	0.25	1	This study
M. tuberculosis					
Wild type	1		0.25		
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	1
GGC507→GAT (G507D)	0.5	0.5	0.125	0.5	1
ACC508→CAC (T508H)	0.5	0.5	0.125	0.5	1
ACC508→GCC (T508A)	1	1	0.25	1	1
CAG510→CAT (Q510H)	1	1	0.25	1	22
CTG511→CCG (L511P)	16	16	1	4	1, 12
CAA513→AAT (Q513N)	8	8	0.5	2	1
CAA513→GAA (Q513E)	32	32	2	8	1
GAC516→GAG (D516E)	8	8	0.5	2	12
GAC516→CAC (D516H)	1	1	0.25	1	1
GAC516→GTC (D516V)	32	32	2	8	12, 21, 22
CTG521→ATG (L521M)	1	1	0.125	0.5	21
TCG522→TTG (S522L)	>32	>32	8	32	21
GGG523→GCG (G523A)	1	1	0.125	0.5	1
GGG523→GGC (silent)	1	1	0.25	1	1
CAC526→CTC (H526L)	32	32	4	16	12, 22
CAC526→TAC (H526Y)	>32	>32	8	32	12, 22
CAC526→GAC (H526D)	>32	>32	8	32	12, 22
CAC526→TTC (H526F)	>32	>32	4	16	1
CAC526→AAC (H526N)	32	32	2	8	8
CAC526→CGC (H526R)	32	32	8	32	12, 22
CAC526→CAA (H526Q)	8	8	0.5	2	1
CGA529→AAA (R529K)	32	32	4	16	22
TCG531→TTC (S531F)	32	32	4	16	1
TCG531→TTG (S531L)	32	32	8	32	21, 22
506-508del[b]	16	16	0.5	2	5
514insTTC[c]	>32	>32	8	32	12 22
518del[d]	32	32	2	8	22

[a] Fold increase in MIC compared to the wild-type sequence.
[b] Deletion of codons 506 to 508.
[c] Insertion of TTC between codons 514 and 515.
[d] Deletion of codon 518.

ic-exchange mutants were constructed by using a temperature-sensitive mycobacteriophage method described in a previous report (2). Using the *M. smegmatis* mc^{2}155 genome sequence (GenBank accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *rpoB* gene (MSMEG_1367). To disrupt the *rpoB* gene, DNA segments from 1,119 bp upstream through 21 bp downstream of the initiation codon of *M. smegmatis rpoB* and from 39 bp upstream through 941 bp downstream of the termination codon were cloned directionally into the cosmid vector pYUB854, which contains a *res-hyg-res* cassette and a *cos* sequence for lambda phage assembly. The plasmids thus produced were digested with PacI and ligated into PH101 genomic DNA excised from the phage-plasmid hybrid (phasmid) phAE87 by PacI digestion. The ligated DNA was packaged (GigaPackIII Gold packaging extract; Stratagene, La Jolla, CA). The resultant mixture was used for the transduction of *E. coli* STBL2 cells (Life Technologies Inc., Carlsbad, CA) to yield cosmid DNA. After *E. coli* was transduced and the transductants were plated onto hygromycin-containing medium, phasmid DNA was prepared from the pooled antibiotic-resistant transductants and electroporated into *M. smegmatis* mc^{2}155. Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M. smeg-*

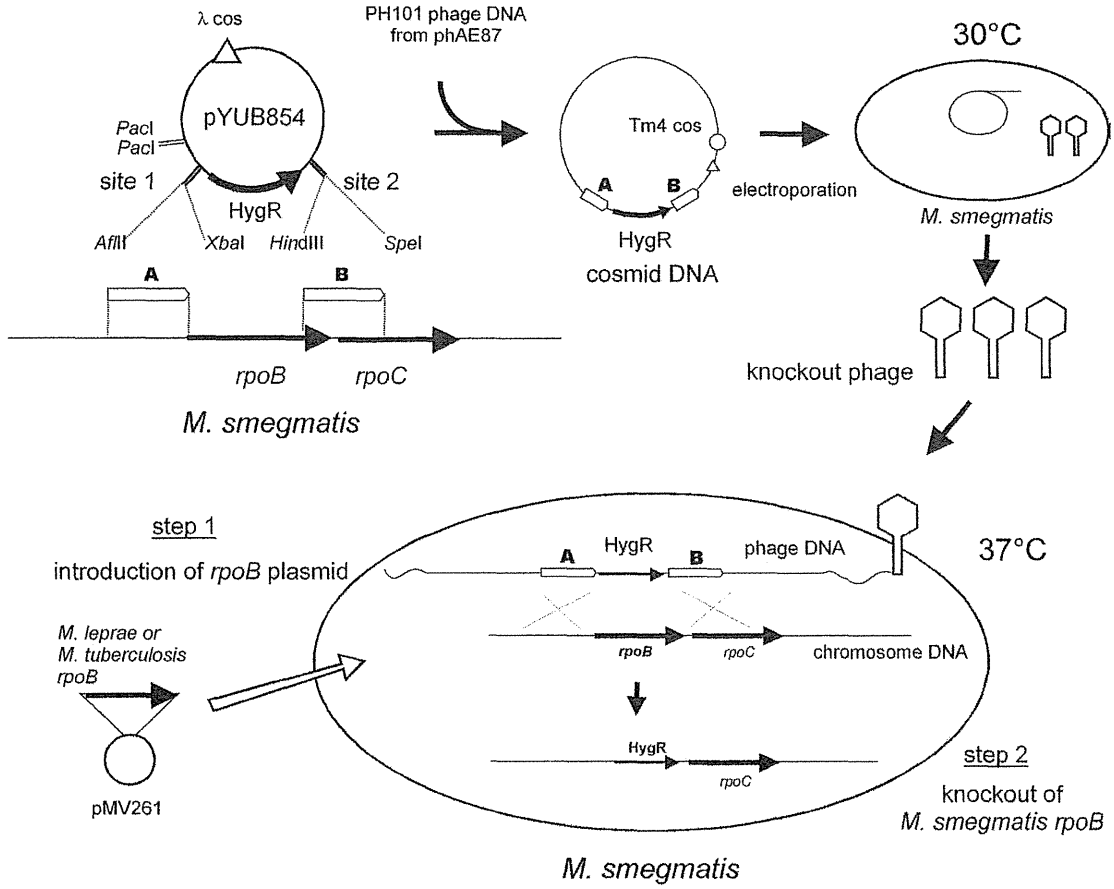

FIG 1 Construction of recombinant *M. smegmatis* strains for rifampin susceptibility testing.

matis transformant carrying the *M. leprae* or *M. tuberculosis rpoB* gene was infected with the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin-resistant and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

Drug susceptibility testing. The MIC values for *M. smegmatis* recombinant clones were determined by culture on Middlebrook 7H10 agar plates containing 2-fold serial dilutions of rifampin (0.25 to 32 μg/ml) or rifabutin (0.0625 to 8 μg/ml). The MIC value for each strain was defined as the lowest concentration of the drug necessary to inhibit bacterial growth.

RESULTS

Construction of recombinant *M. smegmatis* strains. In our previous study, we sequenced the *rpoB* regions of *M. leprae* clinical samples isolated in Vietnam and detected several mutations (11). In addition to these mutations, we detected some mutations (GGC→GGG at codon 507, ACC→ACA at codon 508, and GTC→ATC at codon 547) in clinical specimens from Vietnam and other countries (our unpublished data). We prepared plasmids with mutations in the *M. leprae* and *M. tuberculosis rpoB* genes. Each plasmid has one of 40 mutations (12 for *M. leprae rpoB* and 28 for *M. tuberculosis rpoB*) presented in Table 2. The mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation were introduced individually into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *rpoB* gene on their own chromosome (Fig. 1). The isolation of *rpoB*-disrupted mutants carrying the pNN301-*rpoB* constructs was unsuccessful. Consequently, the recombinant strains with pMV261-*rpoB* constructs were used for subsequent tests. PCR analysis confirmed that the *M. smegmatis rpoB* sequences in the recombinant strains with pMV261-*rpoB* constructs were replaced by hygromycin resistance gene sequences (see Fig. S1 in the supplemental material). All strains showed growth rates comparable to that of wild-type *M. smegmatis*.

Drug susceptibility. The rifampin susceptibilities and rifabutin susceptibilities of the recombinant *M. smegmatis* strains were tested (see Fig. S2 in the supplemental material). The MIC values of rifampin and rifabutin for the recombinant *M. smegmatis* strains and the fold increases in MIC compared to the wild-type sequences are presented in Table 2. It should be noted that the MIC values for the *M. smegmatis* strains might be shifted from those for *M. leprae* or *M. tuberculosis* because of their differences in cell wall permeability and other factors. The MIC value of rifampin for the recombinant *M. smegmatis* strain with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene was 1 μg/ml. Most strains that had a mutation at codon 511, 513, 516, 522, 526, 531, or 533 showed rifampin resistance. In contrast, strains that had a mutation at codon 507, 508, 517, 521, 523, or 532 showed MIC values of rifampin comparable to those for the wild-type sequence. The MIC values of rifabutin for the recombinant

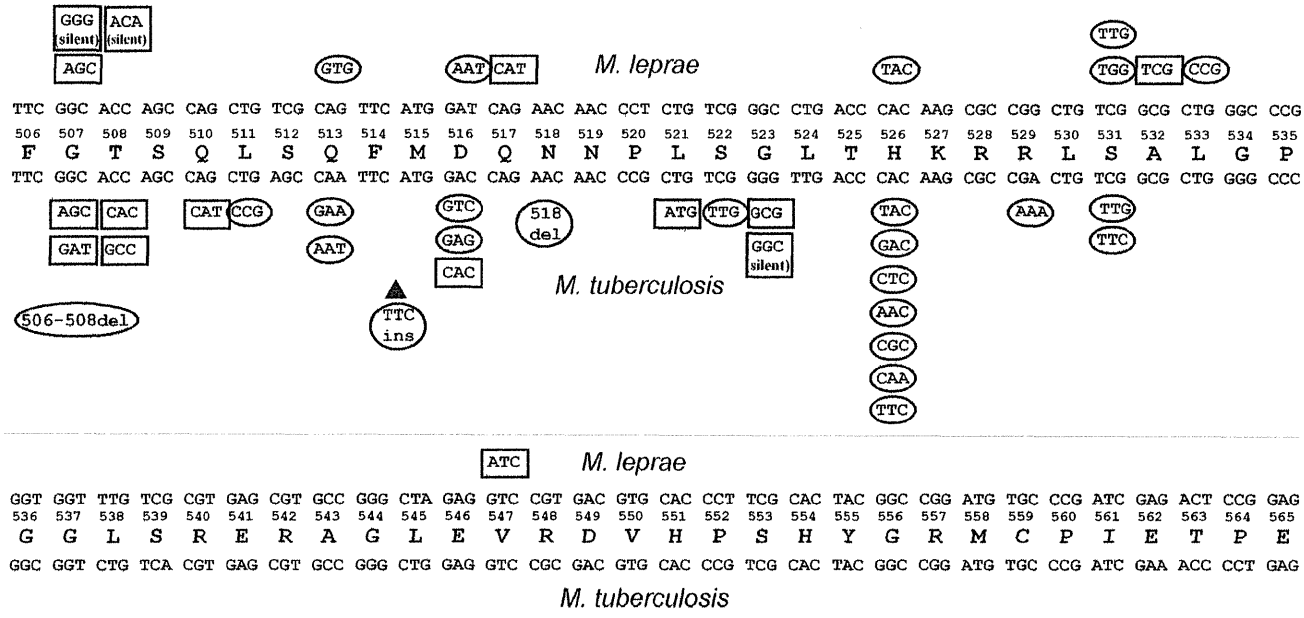

FIG 2 Mutations introduced into the *M. leprae rpoB* gene or *M. tuberculosis rpoB* gene and rifampin susceptibility. The consensus amino acid sequence of *M. leprae* RpoB and *M. tuberculosis* RpoB between codons 506 and 565 is shown. The *M. leprae rpoB* sequence and codons are shown above the consensus amino acid sequence. The *M. tuberculosis rpoB* sequence and codons are shown below the consensus sequence. Mutated codons that gave rise to rifampin resistance are surrounded by ovals. Mutated codons that showed levels of rifampin susceptibility comparable to those of the wild-type sequences are surrounded by rectangles.

M. smegmatis strains with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene were 0.25 μg/ml. Generally, rifabutin was more efficacious than rifampin in terms of concentration.

DISCUSSION

To functionally replace the *rpoB* gene of *M. smegmatis* with the *M. leprae* or *M tuberculosis* counterpart, we used a method established in our previous study (16). Because *rpoB* is a necessary gene for bacterial growth, this genetic locus cannot be disrupted without compensating for its activity. Therefore, we first introduced the *rpoB* gene of *M. leprae* or *M. tuberculosis* into *M. smegmatis* using vector plasmids of two types before disrupting the *rpoB* gene on the *M. smegmatis* chromosome. One vector was pMV261, a multicopy shuttle plasmid. The other was a single-copy integrative shuttle plasmid, pNN301. However, the isolation of *rpoB*-disrupted mutants carrying pNN301-*rpoB* constructs was unsuccessful, probably because of insufficient RpoB expression.

We tested 2 silent mutations and 10 mutations that change amino acid residues for *M. leprae* (Fig. 2). Codons 516, 526, 531, and 533 in the *M. leprae rpoB* gene are known to be codons responsible for rifampin resistance. However, it remains unclear whether or not mutations that have not been reported previously can confer rifampin resistance. Our results show that not all mutations in the *rpoB* gene detected in *M. leprae* clinical samples confer rifampin resistance. *M. leprae* is not cultivable. Therefore, it has been very difficult to analyze the mutation-susceptibility relationship. Using recombinant *M. smegmatis*, however, we can analyze it in a few weeks. We also tested 1 silent mutation, 24 mutations that change amino acids, 2 deletions, and 1 insertion for *M. tuberculosis*. Some mutations did not confer rifampin resistance, which is inconsistent with the susceptibility of the *M. tuberculosis* clinical isolates reported previously. Most mutations at codon 516, 526, or 531 showed rifampin resistance. It is interesting that the strains with the mutation GAC516→CAC for D516H were not rifampin resistant. All other mutations at codon 516 showed rifampin resistance. The mutation GAC516→CAC in *M. tuberculosis* was reported for a strain with multiple mutations and should not be involved in rifampin resistance.

Rifabutin, a spiropiperidyl rifampin, is a rifamycin derivative that is more active than rifampin against slow-growing mycobacteria, including *M. tuberculosis* and *M. avium-M. intracellulare* complex strains, *in vitro* and *in vivo*. It is also active against some rifampin-resistant strains of *M. tuberculosis* (6, 13). Our results indicate that some mutations (e.g., GAT516→AAT of *M. leprae* and GAC516→GAG of *M. tuberculosis*) show weak resistance to rifabutin.

Molecular methods designed to detect drug resistance have some limitations. In some cases, the identified mutations are not related to the acquisition of resistance. Caution is necessary when considering mutations, especially if the mutation detected in clinical isolates is not reported very often. For example, Q510H and L521M mutations were detected in rifampin-resistant *M. tuberculosis* isolates (21, 22), but our results suggest that these mutations are not responsible for rifampin resistance (Table 2). The method used for this study can directly assess the influence of designated mutations in *rpoB*. If the mutations can confer rifampin resistance, we can eliminate the possibility that genetic variation in some region other than *rpoB* on the chromosome of the clinical isolates is responsible for the resistance. Bahrmand et al. previously reported the high-level rifampin resistance of *M. tuberculosis* isolates with multiple mutations within the *rpoB* gene (1). Our method might also be useful for analyzing multiple mutations

detected in the *rpoB* gene of clinical isolates to determine the contribution of each single mutation to rifampin resistance.

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Essential role of hormone-sensitive lipase (HSL) in the maintenance of lipid storage in *Mycobacterium leprae*-infected macrophages

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ABSTRACT

Mycobacterium leprae (M. leprae), the causative agent of leprosy, parasitizes within the foamy or enlarged phagosome of macrophages where rich lipids accumulate. Although the mechanisms for lipid accumulation in the phagosome have been clarified, it is still unclear how such large amounts of lipids escape degradation. To further explore underlying mechanisms involved in lipid catabolism in *M. leprae*-infected host cells, we examined the expression of hormone-sensitive lipase (HSL), a key enzyme in fatty acid mobilization and lipolysis, in human macrophage THP-1 cells. We found that infection by live *M. leprae* significantly suppressed HSL expression levels. This suppression was not observed with dead *M. leprae* or latex beads. Macrophage activation by peptidoglycan (PGN), the ligand for toll-like receptor 2 (TLR2), increased HSL expression; however, live *M. leprae* suppressed this increase. HSL expression was abolished in the slit-skin smear specimens from patients with lepromatous and borderline leprosy. In addition, the recovery of HSL expression was observed in patients who experienced a lepra reaction, which is a cell-mediated, delayed-type hypersensitivity immune response, or in patients who were successfully treated with multi-drug therapy. These results suggest that *M. leprae* suppresses lipid degradation through inhibition of HSL expression, and that the monitoring of HSL mRNA levels in slit-skin smear specimens may be a useful indicator of patient prognosis.

1. Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*). Although its prevalence has declined over the last several decades due to the introduction of multi-drug therapy (MDT), leprosy still remains a major public health problem in many developing countries. In 2009, 244,796 new cases were registered worldwide [1]. *M. leprae* is a typical intracellular pathogen that parasitizes tissue macrophages (histiocytes) and Schwann cells of the peripheral nerves of the dermis. In 1966 Ridley and Jopling used clinical, histological and immunological criteria to classify leprosy patients across the spectrum, and suggested five member groups: Tuberculoid (TT), Borderline Tuberculoid (BT), Borderline (BB), Borderline Lepromatous (BL) and Lepromatous (LL) [2]. Lepromatous leprosy is a stable condition (patient status does not shift from these polar positions), while borderline lepromatous leprosy is immunologically unstable. Lepromatous leprosy is characterized by widespread skin lesions that form due to an impaired cellular immune response. The lesions consist of numerous bacilli that live in the foamy or enlarged lipid-filled phagosome within macrophages. Although lipid-laden macrophages are observed in other mycobacterial infections, including tuberculosis [3,4], the amount of lipid and the number of infected macrophages are most prominent in cases of lepromatous leprosy.

The PAT protein family is named after perilipin, adipophilin/adipose differentiation-related protein (ADRP) and the tail-interacting protein of 47 kDa (TIP47). Members of the PAT family are responsible for lipid transportation and lipid droplet formation in a variety of tissues and cultured cell lines, including adipocytes [5–8]. We previously reported that ADRP and perilipin play important roles in lipid accumulation in *M. leprae*-infected macrophages [9]. ADRP and perilipin localized to the phagosomal membrane of histiocytes, which contained numerous *M. leprae*, in the skin lesions of patients with lepromatous leprosy. *M. leprae* infection increased mRNA and protein expression of ADRP and perilipin in cultured human THP-1 monocytes. The results suggested that ADRP and perilipin contribute to the creation of

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a lipid-rich environment that is favorable for *M. leprae* parasitization and survival in the host.

However, accumulated lipids are supposed to undergo degradation and reutilization by cells over time. In fact, fatty acids mobilized from stored triacylglycerols (TAG) are a major energy source in humans. Mobilization occurs through the consecutive action of three lipases: adipose triglyceride lipase (ATGL), monoacylglycerol lipase (MGL) and hormone-sensitive lipase (HSL) [10]. Among these, HSL was the first enzyme identified in the induction of lipo-catabolic action initiated by hormones and is the predominant lipase effector of catecholamine-stimulated lipolysis in adipocytes [11]. Therefore, ADRP/perilipin and HSL have opposing functions, *i.e.* lipid accumulation vs. its degradation. In addition to adipocytes, HSL is expressed in the cytoplasm of macrophages, pancreatic β cells, skeletal muscle cells, steroid producing cells, the intestine, and spleen [10]. HSL serine residues are phosphorylated by enzymes such as protein kinase A (PKA), 5′ AMP-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPK) to regulate the process of hormone-induced lipolysis [11].

To date, the molecular mechanism(s) that allows the phagosome of *M. leprae*-infected macrophages to escape lipolytic activities is not known. In this study, we investigate the expression and phosphorylation of HSL in *M. leprae*-infected cultured macrophages. We also examine clinical samples from leprosy patients and explore the impact of *M. leprae* on lipid metabolism in infected host cells.

2. Materials and methods

2.1. M. leprae isolation and cell culture

Hypertensive nude rats (SHR/NCrj-*rnu*), in which the Thai53 strain of *M. leprae* was actively grown [12,13], were kindly provided by Dr. Y. Yogi of the Leprosy Research Center, National Institute of Infectious Diseases, Japan. *M. leprae* was isolated as previously described [14,15]. The human premonocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in 10 cm tissue culture dishes in RPMI medium supplemented with 10% charcoal-treated fetal bovine serum (FBS), 2% non-essential amino acids and 50 mg/ml penicillin/streptomycin at 37 °C in 5% CO_2 [9,16]. Typically, 3×10^7 bacilli were added to 3×10^6 THP-1 cells, for a multiplicity of infection (MOI) of 10. Peptidoglycan (PGN) and lipopolysaccharide (LPS) were purchased from Sigma (St Louis, MO) and added at final concentrations of 2 μg/ml and 1 μg/ml, respectively. TLR2 antibody (sc-21759; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a final concentration of 5 μg/ml.

2.2. Immunohistochemistry and lipid staining

THP-1 cells were grown on glass coverslips in 24-well plates for 24 h before the culture medium was exchanged with RPMI 1640 containing *M. leprae*. Control and *M. leprae*-infected THP-1 cells were fixed in 10% paraformaldehyde for 10 min. They were then washed with Dulbecco's phosphate buffered saline (DPBS) containing 0.4% Triton-X 100 (DPBST), incubated with anti-HSL antibody (Cell Signaling Technology, Danvers, MA) diluted to 1:100 for 24 h at 4 °C and washed again with DPBST. The signal was detected using peroxidase-labeled streptavidin-biotin (LSAB2 Kit; DAKO, Carpinteria, CA) and 3,3-diaminobenzidine tetrahydrochloride (DAB) [9]. Cells were counterstained with methylene blue. Lipid staining was performed with oil red O (Muto Pure Chemicals, Tokyo, Japan) for 10 min, and counterstained with hematoxylin for another 5 min.

2.3. RNA preparation and RT-PCR

RNA from cultured cells was prepared using RNeasy Mini Kits (Qiagen Inc., Valencia, CA) as described previously [9,16]. RNA preparation from slit-skin smear samples was performed as described [9]. Briefly, stainless steel blades (Feather Safety Razor Co., LTD, Osaka, Japan) used to obtain slit-skin smear specimens were rinsed in 1 ml of sterile 70% ethanol, then the tube was and centrifuged at 20,000 × g for 1 min at 4 °C. After removing the supernatant, RNA was purified with the same protocol used for cultured cells. RNA was eluted in 20 μl of elution buffer and treated with 0.1 U/μl of DNase I (TaKaRa Bio, Kyoto, Japan) at 37 °C for 60 min in order to degrade any contaminating genomic DNA. RNA concentration and purity were assessed using a NanoVue spectrophotometer (GE Healthcare, Little Chalfont, UK). Total RNA from each sample was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) [9]. The following primers were used to amplify specific cDNAs: HSL: 5′-CTCCTCATGGCTCAACTCCTTCC-3′ (forward) and 5′-AGGGGTTCTTGACTATGGGTG-3′ (reverse); ADRP: 5′-TGTGGAGAAGACCAAGTCTGTG-3′ (forward) and 5′-GCTTCTGAACCAGATCAAATCC-3′ (reverse); and actin: 5′-AGCCATGTACGTAGCCATCC-3′ (forward) and 5′-TGTGGTGGTGAAGCTGTAGC-3′ (reverse). Touchdown PCR was performed using a PCR thermal cycler DICE (TaKaRa Bio) as previously described [9]. The products were analyzed by 2% agarose gel electrophoresis.

Slit-skin smear samples from leprosy patients were used according to the guidelines approved by the National Institute of Infectious Diseases, Tokyo, Japan.

2.4. Protein preparation and Western blot analysis

Cellular protein was extracted and analyzed as previously described [9,17]. Briefly, cells were lysed in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 20% glycerol, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN) for 1 h. After centrifugation, the supernatant was transferred and 10 μg of protein was used for analysis. Samples were heated in SDS sample loading buffer at 95 °C for 5 min and loaded on a polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membrane was washed with PBST (PBS with 0.1% Tween 20), blocked in blocking buffer (PBST containing 5% nonfat milk) overnight, and then incubated with either anti-HSL, anti-phospho-HSL (Ser^{563}) or anti-phospho-HSL (Ser^{565}) antibody (Cell Signaling Technology, 1:2000 dilution). After washing with PBST, the membrane was incubated for 1 h with biotinylated donkey anti-rabbit antibody (GE Healthcare, 1:2000 dilution) and streptavidin-HRP (GE Healthcare, 1:10,000 dilution) according to the manufacturer's protocol. The signal was developed using ECL Plus Reagent (GE Healthcare).

3. Results

3.1. HSL expression is suppressed in macrophages infected with M. leprae

To confirm the possible relationship between lipid accumulation and HSL expression in macrophage, we infected *M. leprae* in THP-1 cells and performed oil red O staining and HSL and ADRP immunostaining. Lipid droplets were not evident in control THP-1 cells (Fig. 1A), but accumulation was clearly demonstrated in cells 24 h after *M. leprae* infection (Fig. 1B). ADRP expression, which contributes to lipid intake, was not evident in control cells, but was significantly increased following *M. leprae* infection as previously reported (Fig. 1C and D, respectively) [9]. Conversely, HSL expression