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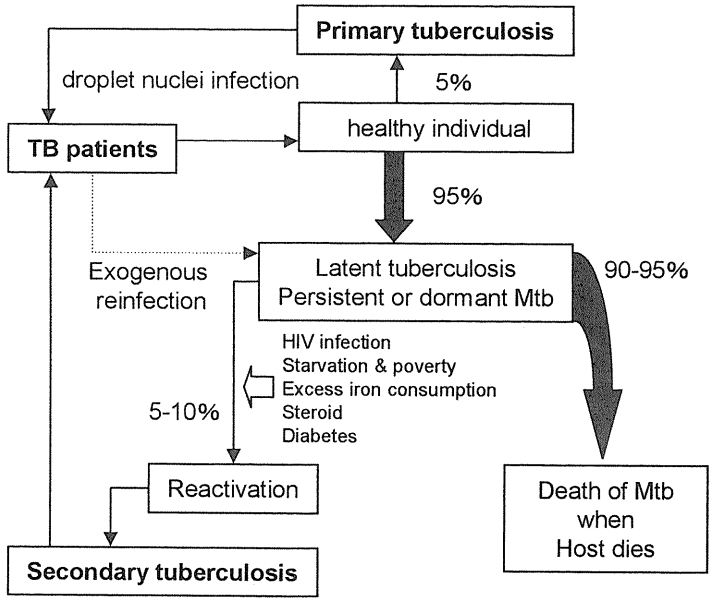

Figure 1. Life cycle of *M. tuberculosis* (Mtb).
M. tuberculosis resides in human beings. Currently, one-third of the world's human population is estimated to be *M. tuberculosis*-infected. Approximately 95% of individuals infected with *M. tuberculosis* do not have progressive disease. During latent infection, most *M. tuberculosis* bacilli are dormant and do not replicate. After a lengthy persistence, *M. tuberculosis* dies upon host death. However, in approximately 5−10% of latently infected persons, the infection will reactivate and cause active TB. Airborne droplets containing bacilli are infectious if they are deposited in alveoli in humans. HIV infection, starvation, poverty, excess iron consumption, steroid treatment, and diabetes are risk factors for reactivation.

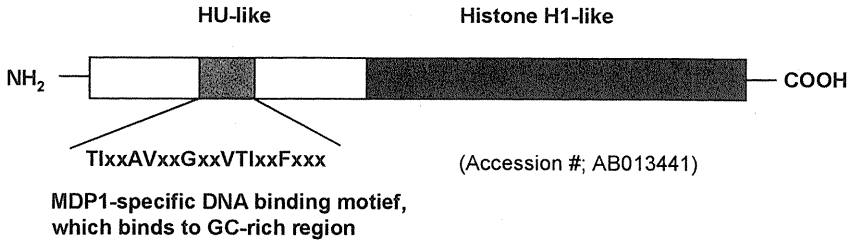

Figure 2. Structure of MDP1.

MDP1 is a histone-like protein distributed in mycobacteria. It possesses the chimerical structure of the bacterial histone-like protein IHF or HU and eukaryotic histone H1 but also possesses a unique DNA-binding motif that interacts with GC-rich DNA. The N-terminal region is relatively conserved among MDP1 orthologs, while the C-terminal region is variable. The C-terminal region contains the PAKK repetitive sequence, which is observed in a mycobacterial adhesin, heparin-binding hemagglutinin.[88,89]

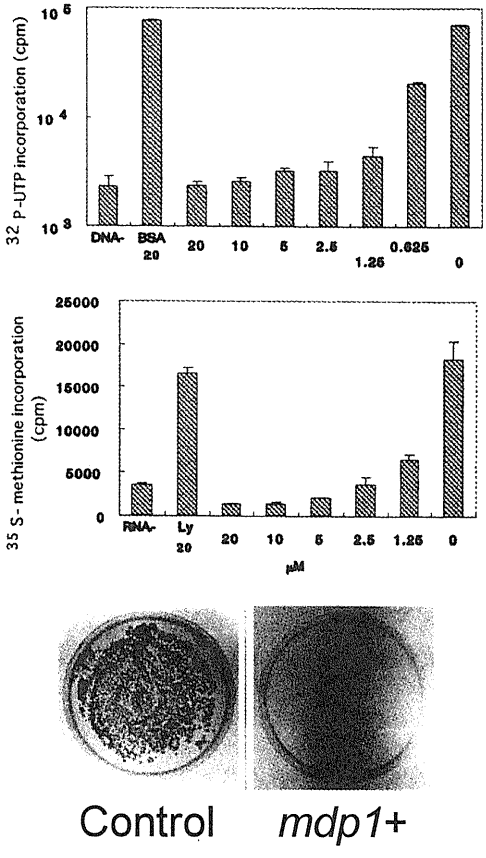

Figure 3. MDP1 suppresses *in vitro* macromolecular biosyntheses and bacterial growth. In the upper figure, the *in vitro* effect of MDP1 on DNA synthesis is evaluated by Klenow fragment production. DNA synthesis was performed in the presence of different concentrations of MDP1 as indicated in mM below the bars. "DNA-" indicates the negative control without template DNA. TCA-insoluble [alpha-^{32}P]-dTTP incorporation (cpm; vertical axis) was quantified. In the middle figure, the effect of MDP1 on transcription by T7 RNA polymerase was evaluated *in vitro*. "DNA-" indicates the negative control without template DNA. TCA-insoluble [a-^{32}P]-dTTP incorporation was quantified (vertical axis). In the lower figure, *M. smegmatis* transformed with pSO246 (Control) or pSO246 including the MDP1 gene (*mdp1+*) was cultured at 37°C for 7 days on 7H10 agar. There figures are from reference[64].

Review:

Global Threats and the Control of Multidrug-Resistant Tuberculosis

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[Received January 5, 2011; accepted February 15, 2011]

About one-third of the world's population has been infected with *Mycobacterium tuberculosis*. Active disease develops in about 9 million people per year, and tuberculosis is responsible for 2 million deaths per year. The disease caused by this *bacterium*, tuberculosis (TB), remains one of the leading causes of mortality caused by infection worldwide and is a major threat to global health. The situation of TB is recently exacerbated by the emergence of highly drug-resistant forms of the disease-causing pathogen and synergy with human immunodeficiency virus/acquired immune deficiency syndrome, which greatly increases the risk of latent *M. tuberculosis* infection progressing to active disease. Multidrug-resistant (MDR) tuberculosis is defined as disease caused by strains of *M. tuberculosis* that are at least resistant to isoniazid and rifampicin; extensively drug-resistant (XDR) tuberculosis refers to disease caused by MDR strains that are also resistant to any fluoroquinolone and any of the injectable drugs used in treatment with second-line anti-tuberculosis drugs (amikacin, capreomycin, and kanamycin). MDR- and XDR-TB are serious threats to the progress that has been made in the control of tuberculosis worldwide over the past decade. In this review, we focus on threats of MDR-TB and the research and development of improved diagnostics, new chemotherapeutic agents, and vaccine candidates for MDR-TB.

Keywords: surveillance, diagnostics, control strategy, chemotherapeutic intervention, vaccine research and development

1. Introduction

Tuberculosis (TB), one of the oldest diseases known to affect humans, is a major cause of death worldwide. This disease, which is caused by acid-fast bacteria of the *Mycobacterium tuberculosis* complex, usually affects the lungs, although other organs are involved in up to one-third of cases. Characteristic features include man-to-man airborne transmission, a prolonged subclinical latency period between the initial infection and overt disease, a granulomatous response that is a compact organized collection of macrophages, associated with intense tissue inflammation and damage. If properly treated, TB caused by drug susceptible strains is curable in virtually all cases. If untreated, the disease may be fatal within 5 years in 50-65% of cases. Transmission usually takes place through the airborne spread of droplet nuclei produced by patients with infectious pulmonary TB [1].

About 15 million (>25%) of 57 million annual deaths worldwide are estimated to be related directly to infectious diseases. Among them, TB is still a major threat to global health, recently exacerbated by the emergence of drug-resistant strains of *M. tuberculosis* and coinfection with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS). The global plan to stop "TB: 2006-2015," which set out a vision of halving the prevalence of and mortality caused by the disease by 2015, followed by eliminating the disease as a public health problem by 2050 declared by the Stop TB Partnership in 2006 [2]. This vision depends on the development of improved diagnostics, better treatment by using novel anti-TB agents, and more effective vaccination. Recently, active translational research pipelines directed toward these goals have progressed, but improved understanding of the fundamental biology of this complex disease, including the causative pathogen and host responses, will promise to be the key to radical advances in TB control [1].

TB is one of the most devastating re-emerging diseases. The re-emergence of TB was fueled by the immune deficiencies of people with HIV/AIDS, which greatly increases the risk of latent *M. tuberculosis* infection progressing to active disease, and being transmitted to other. Inadequate courses of anti-TB chemotherapy compound the problem, leading to the emergence and spread of drug-resistant and multidrug-resistant (MDR) strains of *M. tuberculosis*, and a need for more expensive treatment strategies such as directly observed therapy. It has been known for over a century that TB is a disease of poverty, associated with crowding and inadequate hygiene. The continuing expansion of global populations living in poverty makes TB more difficult to control [3].

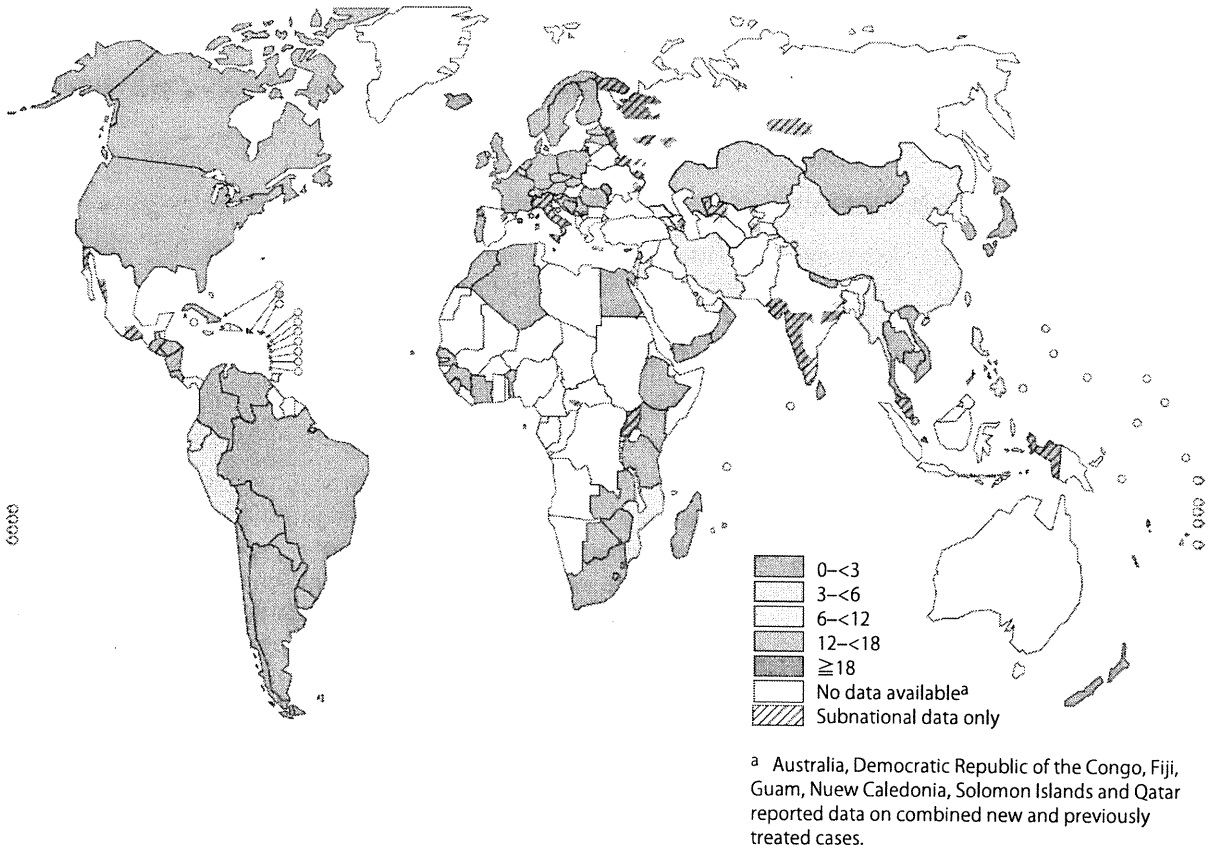

Fig. 1. Proportion of MDR-TB among new TB cases, 1994-2009. The proportion of MDR-TB among new TB cases reported ranges from 0% to 28%. The following 27 countries are responsible for 85% of the world's estimated cases of MDR-TB and are classified as countries with a high burden of MDR-TB: China, India, Russia, Pakistan, Bangladesh, South Africa, Ukraine, Indonesia, Philippines, Nigeria, Uzbekistan, Democratic Republic of Congo, Kazakhstan, Vietnam, Ethiopia, Myanmar, Tajikistan, Azerbaijan, Moldova, Kyrgyzstan, Belarus, Georgia, Bulgaria, Lithuania, Armenia, Latvia, and Estonia [9].

2. Definition of Multidrug- and Extensively Drug-Resistant Tuberculosis

Multidrug-resistant (MDR)-TB is defined as disease caused by strains of *M. tuberculosis* that are at least resistant to both isoniazid (INH) and rifampicin (RIF). Extensively drug-resistant (XDR)-TB refers to disease caused by MDR strains that are also resistant to any fluoroquinolone and any of the injectable drugs used in treatment with second-line anti-TB drugs, such as amikacin, capreomycin, and kanamycin. MDR- and XDR-TB are serious threats to the progress that has been made in the control of TB worldwide over the past decade [4].

3. Epidemiology

About 2 billion people in the world today are latently infected with *M. tuberculosis*. There were 9.4 million new TB cases (139 per 100,000 population) and 1.7 million deaths from TB in 2009 [5]. It has been reported 24 thousand (19 per 100,000 population) of new TB cases and 2.2 thousand deaths by TB in Japan, 2009 [6]. The public health surveillance system for TB in Japan is based on the law regarding infectious disease prevention and medical care for the patients. TB is legally classified as a type 2 disease, and therefore the clinical doctor responsible for the patient must report the information to the regional public health center immediately. The data are collected by the prefecture and then finally by the national center, the Tuberculosis and Infectious Diseases Control Division, Health Service Bureau, Ministry of Health, Labour and Welfare of Japan. National Tuberculosis Treatment Research Unit and laboratory centers of Japan reported and analyzed the results of drug susceptibility testing of *M. tuberculosis* [6, 7].

The proportion of MDR-TB among new TB cases reported ranges from 0% to 28% (**Fig. 1**). In 2009, the annual number of new patients with MDR-TB was estimated to be 0.5 million (320 in Japan, 2005) and XDR-TB was an estimated 50 thousand cases globally (100 in Japan, 2005) [8, 9]. The following 27 countries are responsible for 85% of the world's estimated cases of MDR-TB and are classified as countries with a high burden of MDR-TB: China, India, Russia, Pakistan, Bangladesh, South Africa, Ukraine, Indonesia, Philippines, Nigeria, Uzbekistan, Democratic Republic of Congo, Kazakhstan, Vietnam, Ethiopia, Myanmar, Tajikistan, Azerbaijan, Moldova, Kyrgyzstan, Belarus, Georgia, Bulgaria, Lithuania, Armenia, Latvia, and Estonia [9, 10].

Table 1. Mechanisms of drug action and resistance of mycobacteria.

Drug	MIC (μg/mL)	Genes associated with resistance	Gene function	Role	Mechanisms of action	Mutation frequency (%)
Isoniazid (INH)	0.02-0.2	*kat G*	Catalase peroxidase	Prodrug conversion	Inhibition of mycolic acid synthesis	20-80
		inhA	Enoyl ACP reductase	Drug target	Other multiple effects on lipids,	15-40
		ndh	NADH dehydrogenase	Modulator of INH activity	carbohydrates, NAD metabolism,	10
		ahpC	Alkyl hydroperxidase	Marker of resistance	and cell wall assembly	10-15
Rifampicin (RIF)	0.5-2	*rpoB*	RNA polymerase	Drug target	Inhibition of transcription	95
Pyrazinamide (PZA)	16-50	*pncA*	Nicotinamidase Pyrazinamidase	Prodrug conversion	Acidification of cytoplasm and inhibition of membrane energy	70-95
Ethambutol (EMB)	1-5	*embCAB*	?	Drug target	Inhibition of arabinogalactan synthesis	50-65
Streptomycin (SM)	2-8	*rpsL* *rrs*	12S ribosomal protein	Drug target	Inhibition of protein synthesis	50-60

MIC: minimum inhibitory concentration
ACP: acyl carrier protein
NADH: reduced form of nicotinamide adenine dinucleotide
NAD: nicotinamide adenine dinucleotide
FAS: fatty acid synthase

MDR-TB can be treated and cured. However, treatment regimens are complicated, lengthy, and expensive. Medications that are currently available can produce crippling side effects and are less effective than drugs for non-resistant TB. If left untreated, however, MDR-TB not only kills the patients but can spread to other people, where it may develop additional drug resistance. Strains of XDR-TB that are resistant to three or more of the second-line drugs used to treat MDR-TB have reached epidemic proportions in several areas. One outbreak of XDR-TB in KwaZulu-Natal, South Africa, killed 74 of 78 patients within a matter of weeks, sparking fears that XDR-TB could spread rapidly and lethally, particularly in areas with high prevalence of HIV infection. In September 2006, the World Health Organization issued an alert regarding the emergence of XDR-TB [9].

National TB programs are challenged by MDR-TB. Globally, fewer than 2% of estimated cases of MDR-TB are reported to the World Health Organization (WHO) and managed according to international guidelines [11]. The vast majority of remaining cases are probably never properly diagnosed or treated, further propagating the epidemic of MDR-TB. The situation is further worsened by the epidemic of HIV, especially in Africa [10].

4. Mechanisms of Drug Resistance in Tubercle Bacilli

Drug resistance arises due to the improper use of chemotherapeutic agents of drug-susceptible TB patients. This improper use is a result of a number of actions, including administration of improper treatment regimens by health care workers and failure to ensure that patients complete the whole course of treatment. Essentially, drug-resistance arises in areas with poor TB control programs [4].

Resistance is a phenotype, the ability of pathogens to survive in the presence a drug at a concentration that normally kills and/or inhibits growth. Resistance is caused by mutation of the target gene ("genetic resistance") and can be distinct from tolerance ("phenotypic resistance") that is a conditional phenotype mediated by the physiological/metabolic state of the bacilli/pathogens. Thus, a phenotypic resistance is defined as drug resistance in stationary-phase, persistent, and dormant bacilli. Antimicrobials usually act against actively growing bacteria but not against non-growing forms. The lack of susceptibility of the non-growing bacteria to antimicrobials is due to changes in bacterial metabolism or physiological state and is therefore called as a phenotypic resistance. The phenotypic resistance plays an important role in subclinical/asymptomatic latent TB infection in which dormant bacilli are existed.

Drug resistance in *M. tuberculosis* can be either intrinsic or acquired. Intrinsic resistance refers to nonsusceptibility due to unique features of *M. tuberculosis*, such as its natural resistance to penicillin or clarithromycin. Acquired resistance refers to susceptible *M. tuberculosis* becoming resistant to drugs as a result of genetic mutations. The genetic basis of antimicrobial resistance in *M. tuberculosis* to date indicates that the MDR phenotype is the result of accumulative mutations of target genes and proteins rather than the acquisition of an MDR transfer factor. **Table 1** summarizes mechanisms of drug action and resistance of mycobacteria [12, 13].

Since the time when antimicrobial drugs were first introduced to treat infectious diseases, drug resistance has emerged. Thus, the development of resistance is an inevitable consequence of the clinical use of antimicrobial drugs. This is because the large number of bacterial cells in populations allows for the selection of mutants that are resistant to the drugs. Genetic resistance is due to a change in the genotype resulting in a drug-resistant phenotype of bacteria that can be passed on to subsequent generations. A better understanding the mutations participating in drug resistance not only clarifies the mechanism of drug resistance and action of drugs but also facilitates

Table 2. Technologies for detection of drug-resistant *Mycobacterium tuberculosis*.

Technology	Description	Product	Skill/Training
Liquid culture	Broth-based culture systems detect MTB and can be used for drug susceptibility testing	BacT/ALERT 3D, MGIT	Extensive
Microscopic observation drug susceptibility (MODS)	Manual liquid culture technique uses an inverted light microscope and microscopy skills to detect MTB be used for drug susceptibility testing	Non-commercial	Extensive
Molecular line probe assay	Strip test detects MTB and genetic mutations indicating IHN and/or RIF resistance	GeoType MTBDR, MTBDRPlus, INNO-LiPA Rif.TB	Moderate
Automated detection and screening	Automated sample processing: DNA amplification and detection of MTB and RIF resistance	Cepheid Gene Xpert	Minimal

MTB: *Mycobacterium tuberculosis*
MGIT: Mycobacteria Growth Indicator Tube

simple and rapid detection of drug-resistant *M. tuberculosis* by techniques of molecular biology [14].

5. Detection of Drug-Resistant *M. tuberculosis*

The methods to test the susceptibility of mycobacteria generally accepted are based on the growth of mycobacteria on solid or in liquid media containing a specified concentration of a single drug (**Table 2**) ("gold standard"). Classical cultivation methods in the addition of drug to detect inhibition of growth show advantages, including 1) less expensive, affordable in resource-limited setting, 2) the clinical relevance of results predicting treatment success/failure for decades, 3) detection of resistance regardless of the precise mechanism and/or molecular basis, and 4) unaffected by certain mutations that may not cause resistance [15].

The microscopic-observation drug-susceptibility (MODS) assay [15] is considered to be an inexpensive tool for the bacteriologic diagnosis of TB and the detection of drug resistance. This method is based on direct inoculation of the selective Middlebrook 7H9 liquid culture medium in 24-well plates with a sputum specimen. Detection of the typical cord formation ("microcolonics") of *M. tuberculosis* in the wells on microscopic examination (under an inverted light microscope) constitutes the basis of diagnosis. Growth (or the lack) in drug-containing wells, as compared with growth in drug-free wells, is the basis for reporting the results as "susceptible" or "resistant" to anti-TB agent. The reported sensitivity of this method in the recovery of mycobacteria from sputum specimens was higher than the sensitivity of either the MB/BacT automated mycobacterial system or traditional culture. The major difficulty in the implementation of the MODS assay or any other new cultivation method is that of biosafety and requirement for extensive training/skill.

Recently, molecular biology-based detection of genetic mutations linked to drug resistance has been developed, and it has several advantages over the conventional phenotypic culture method, including molecular line probe assay and automated detection of *M. tuberculosis* and screening for drug-resistant *M. tuberculosis* [14, 16, 17]. Advantages include 1) speed (results may be available within hours or days vs weeks and/or months by cultivation), 2) better reproducibility, especially in low-level resistance, and 3) ability to work with poorly growing cultures.

It has been reported that a new automated nucleic acid-amplification test that may allow a relatively unskilled health care worker to diagnose TB and detect resistance to a key anti-TB drug, rifampin (RIF), within 90 minutes. A large, well-conducted, multicountry study evaluated an automated tuberculosis assay (Xpert MTB/RIF) for the presence of *M. tuberculosis* and resistance to RIF. With a single test, this assay identified 98% of patients with smear-positive and culture-positive TB (including more than 70% of patients with smear-negative and culture-positive disease) and correctly identified 98% of bacteria that were resistant to RIF. This test and others that are likely to follow have the potential to revolutionize the diagnosis of TB and detection of RIF resistance [17]. The assay has several critical advantages over conventional nucleic acid-amplification tests. The Xpert MTB/RIF assay is simple to perform with minimal training, is not prone to cross-contamination, requires minimal biosafety facilities, and has a high sensitivity in smear-negative TB. However promising these findings, issues involving the MTB/RIF assay may limit its utility. These issues include its high cost and limitations in testing only for resistance to RIF but not INH, PZA, and EMB of *M. tuberculosis*, a platform that detects a relatively small number of mutations.

6. Chemotherapeutic Intervention for TB and Development of New Anti-TB Drugs

Treatment of TB due to drug-susceptible disease requires at least 6 months of a cocktail therapy by standard first-line anti-TB agents, including isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA), based on a strategy of directly observed treatment, short course (DOTS). To control TB, the World Health Organization (WHO) and the International Union against Tuberculosis and Lung Disease (IUATLD) recommend the DOTS strategy, which has five elements: political

Table 3. Goals of anti-tuberculosis therapy.

1. Safety and simplify the treatment of active, drug-susceptible TB
2. Improve efficacy and safety and shorten duration of therapy for drug-resistant TB
3. Develop drugs for TB coinfected with HIV to avoid the induction by RIF of CYP450 that metabolizes anti-HIV drugs
4. Shorten therapy of latent TB infection

TB: tuberculosis
HIV: human immunodeficiency virus

Table 4. Anti-tuberculosis drug candidates in clinical development.

Drug	Developers	Mechanisms	Stage	Active against MDR-TB
Diamine SQ-109	Sequella	Inhibits cell wall biosynthesis	Phase 1	Yes
Diarylquinoline TMC207	Johnson & Johnson	ATP synthase inhibitor	Phase 3	Yes
Nitroimidazoles		Inhibit cell wall lipid synthesis		
OPC-67683	Otsuka		Phase 2	Yes
PA-824	Global TB Alliance		Phase 2	Yes
Fluoroquinolones		Inhibit DNA replication and transcription		
Gatifloxacin	Bayer, Global TB Alliance, CDC		Phase 3	Yes
Moxifloxacin	WHO-TDR, Lupin		Phase 3	Yes

MDR-TB: multidrug-resistant tuberculosis
ATP: adenosine triphosphate
CDC: Centers for Disease Control and Prevention
WHO: World Health Organization
TDR: Tropical Disease Research
DNA: deoxyribonucleic acid

commitment, diagnosis primarily by sputum-smear microscopy among patients attending health facilities, short-course treatment with effective case management (i.e., direct observation), regular drug supply, and systematic monitoring to assess outcomes of every patient started on treatment. Standard short-course regimens, which involve an initial phase of INH, RIF, PZA and EMB for the first 2 months followed by a continuation phase of INH and RIF for the last 4 months, can cure more than 95% of cases of new, drug-susceptible TB. Between 1995 and 2008, a total of 36 million people were treated successfully with the use of the DOTS approach, and 6 million lives were saved [4]. By contrast, treatment of MDR-TB can require more than 18 months of therapy. Identification of drugs that shorten the duration of treatment and thereby improve adherence is key to improving active TB treatment, decreasing demands on national TB control programs, and preventing further emergence of drug-resistant bacilli [1, 4]. Goals of TB chemotherapy are shown (**Table 3**).

MDR-TB is a form of TB that is difficult and expensive to treat and fails to respond to standard first line drugs. While drug-resistant TB is generally treatable, it requires extensive chemotherapy (up to two years of treatment) with second-line anti-TB drugs, which are more costly than first-line drugs and may produce adverse drug events that are more severe. Drug-resistant TB has required the most effective use of existing second-line anti-TB drugs, such as aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine/terizidone, and para-aminosalicylic acid, and other antimicrobials available to treat drug-resistant TB and develop new chemotherapeutic agents, particularly against MDR/XDR-TB. These situations demand research to improve therapeutic outcomes and optimize current treatments. Fortunately, over the past five years, TB drug development has shown remarkable progress [18–20]. For the first time in many years, there is now a coordinated portfolio of promising new compounds at every level of drug development, and several new drugs for TB therapy are being assessed in clinical trials (**Table 4**). Most of them can be effective against drug-resistant strains and shorten the duration of treatment.

Diamines:

The most promising diamine candidate is SQ-109 [21], EMB analogues with 1,2-diamine pharmacophere. SQ-109 inhibits the cell wall synthesis of drug-susceptible and -resistant mycobacteria. Because resistance rates of SQ-109 are low, it is considered that two gene changes are required for the resistance, and SQ-109 may have more than one target in *M. tuberculosis*. No serious adverse effects were reported in a phase 1 study.

Diarylquinolines:

The most active diarylquinolone is TMC207 [22], which is being assessed in phase 3 clinical trials. TMC207 inhibits adenosine triphosphate (ATP) synthase of mycobacteria and is effective against both drug-susceptible and -resistant *M. tuberculosis*. No cross-resistance to current anti-TB agents is thought, because the drug target differs from the available agents. No serious adverse events were reported.

Nitroimidazoles:

Nitroimidazoles have derived from the bicyclic nitroimidazofurans that were originally developed as

Table 5. Vaccine candidates for tuberculosis.

Candidate	Developers	Composition	Mode of action
Viral vectors			
MVA85A	University of Oxford	Ag85, mycolyl transferase, expressed by vaccinia virus vector, modified vaccinia Ankara	Boost response to BCG
Ad35 TB-S	Aeras, Crucell	Ag85 and Tb10.4, a subfamily of ESAT-6, expressed by adenovirus vector	Boost response to BCG
Protein			
Mtb72F	GlaxoSmithKline, Corixa, Aeras	Recombinant fusion protein in adjuvant	Boost response to BCG
Hybrid-1	Statens Serum Institut	Ag85-ESAT-6 fusion protein in adjuvant	Boost response to BCG
HyVac4-IC31	Statens Serum Institut, Aeras, Intercell	Ag85-Tb10.4 fusion protein in adjuvant	Boost response to BCG
Modified BCG			
rBCG30	UCLA, Aeras	Recombinant BCG overexpressing Ag85	Augmented immunogenicity of BCG
rBCGUre:Chly-	Max Planck Institute	Recombinant BCG for enhanced MHC class I presentation, induction of cytotoxic T lymphocytes	Augmented immunogenicity of BCG
HyVac4-IC31	Statens Serum Institut, Aeras, Intercell	Ag85-Tb10.4 fusion protein in adjuvant	Boost response to BCG
Inactivated whole cells			
Mycobacterium vaccae	AnHui Longcom Biologic Pharmacy	Environmental nonpathogenic mycobacteria	Adjunct to chemotherapy, varying results
Mycobacterium w	Immuvac by Cadila Pharmaceuticals	Environmental nonpathogenic mycobacteria	Adjunct to chemotherapy

UCLA: University of California, Los Angeles
ESAT: early secreted antigen target
BCG: bacillus Calmette-Guérin
MHC: major histocompatibility complex

chemotherapeutic agents for cancers. These are effective for actively growing and dormant *M. tuberculosis* (latent TB infection) and includes OPC-67683 and PA-824, and for drug-susceptible and -resistant *M. tuberculosis*. These are now in phase 2 clinical trials.

OPC-67683 [23]; OPC-67683 inhibits biosynthesis of mycobacterial lipids, such as mycolic acid. The anti-mycobacterial activity of OPC-67683 in vitro was better than INH and PA-824. No cross-resistance and serious adverse events were observed.

PA-824 [24]; PA-824 inhibits the synthesis of proteins and cell wall lipids and also produces nitric oxide that is needed to control growth of *M. tuberculosis*. This agent is active against both drug-susceptible and -resistant organisms and limited to *M. tuberculosis* complex. No cross-resistance was observed. No serious adverse events were found, although mild toxicity to kidneys was seen.

Fluoroquinolones:

Fluoroquinolones [25] are known as second-line drugs for TB. Among them, moxifloxacin and gatifloxacin are candidates for shortening the duration of treatment, because they have low minimum inhibitory concentrations (MICs) and strongest bactericidal activity. Both moxifloxacin and gatifloxacin are currently in phase 3 trials. These inhibit bacterial DNA gyrase that is essential for DNA supercoiling and necessary for chromosomal replication. No cross-resistance to first-line agents was observed, and thus these were effective for MDR-TB. These show certain adverse events, such as nausea, vomiting, myalgia, tremor, insomnia, and dizziness, but not irreversible and life-threatening events. In addition, prolongation of QT intervals found by electrocardiogram may be associated with ventricular tachycardia and dysglycemias, including hyperglycemia and hypoglycemia. Fluoroquinolones are contraindicated for general use in patients with <18 years old and pregnant women.

7. Prevention of TB: Vaccine Research and Development

Prevention is better than cure. Ultimately, global eradication of TB will require a new effective vaccine. Although vaccination with bacillus Calmette-Guérin (BCG), a live attenuated *M. bovis*, has been shown to decrease the risk of severe forms of childhood TB, but its efficacy against pulmonary TB of adults is questionable, which remains the main source of transmission. One of the major obstacles to develop effective vaccines combating TB is that *M. tuberculosis* establishes intracellular infection. Vaccines that merely induce antibodies cannot prevent the disease. Thus, a critical part of TB vaccine development is directed toward the induction of cell-mediated immunity mediated by protective type 1 helper T (Th1) cell responses. New TB vaccines better than the current BCG vaccines are greatly needed to control the disease, which kills approximately 2 million persons in the world today.

There are two potential vaccination strategies against TB: 1) vaccines can be given before infection (pre-exposure, prophylactic) to prevent infection (or more probably, disease) or 2) post-exposure (therapeutic) to eliminate or contain latent TB and prevent reactivation. Therapeutic vaccines can also be used in combination with drug therapy. The development of a more effective,

better standardized, affordable vaccine with durable activity and fewer adverse effects is a major priority.

There are now more than 10 vaccine candidates in development, and they use different antigens and delivery strategies. Vaccines and strategies under development and in clinical trials include 1) improved/recombinant BCG, 2) attenuated auxotroph *M. tuberculosis*, 3) subunit/component vaccines, and 4) DNA vaccines. These are based on a prime by BCG-boost system, a two-stage immunization approach (prime-boost). Candidate vaccines [12, 26, 27] have demonstrated activity in animal models that is equal to or superior to that of BCG, and human trials are underway (**Table 5**).

Among them, a leading vaccine candidate, modified vaccinia Ankara (MVA85) expressing antigen 85A encoding an important enzyme of lipid metabolism, mycolyl transferase, has been studied in BCG-unvaccinated and -vaccinated subjects. MVA 85A in a clinical trial induced a robust Th1 cell response (interferon-γ and interleukin 2) lasting more than 50 weeks. Th1 responses can lead to protection against *M. tuberculosis* infection. MVA 85A was shown to be safe and highly immunogenic in phase 1 trial, leading the way to larger trails in endemic areas [28].

Mtb 72F represents a fusion protein comprising antigens Rv0125 and Rv1196 formulated in an oil-in-water emulsion with an adjuvant comprising lipopolysaccharide and a triterpene glycoside (AS02) produced by GlaxoSmithKline [29]. It triggers a strong memory response in the immune system. When properly activated, memory cells confer long-lasting immunity. The fused protein and a new kind of adjuvant, AS02, have previously shown to be safe and effective in clinical trials.

However, there is no identified surrogate marker for protection; identification of an improved vaccine will require long-term efficacy trials in humans. Thus, we are facing the difficulty in the research and development of novel vaccines for TB. An effective vaccine would be the most powerful tool for preventing TB and drug resistance, but a vaccine is not anticipated anytime soon. We must invest in research and development for better tools while maintaining the efficacy of the tools we have available today.

8. Concluding Remarks

The current strategy for TB control is based on reducing the spread of infection through the effective treatment of individuals with active disease and the vaccination of children. The WHO has initiated the DOTS campaign in many regions; however, this program has not been able to control the global TB epidemic or prevent the incidence of MDR strains of *M. tuberculosis* [1]. To control MDR-TB, intensified research on new diagnostic tests, drugs, and vaccines are needed, as described in this review. Scientists and industries need to develop radically improved tools for TB elimination, including new diagnostics, drugs, and vaccines.

Acknowledgements

This work is financially supported by Ministry of Health, Labour and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Research on Regulatory Science of Pharmaceuticals and Medical Devices), Health Sciences Research Grants, and Ministry of Education, Culture, Sports, Science and Technology.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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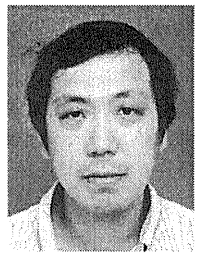

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 51, pp. 44153–44161, December 23, 2011

Lipid Phenotype of Two Distinct Subpopulations of *Mycobacterium bovis* Bacillus Calmette-Guérin Tokyo 172 Substrain*[S]

Received for publication, October 5, 2011, and in revised form, October 25, 2011 Published, JBC Papers in Press, October 26, 2011, DOI 10.1074/jbc.M111.310037

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Background: The heterogeneity of substrains of Bacillus Calmette-Guérin (BCG), the only available tuberculosis vaccine, was studied.
Results: BCG Tokyo 172 types I and II were different in morphology and lipid composition.
Conclusion: Type II lacks phenolic glycolipid/phthiocerol dimycocerosate due to a *ppsA* mutation, and its phenotype was implicated in host responses.
Significance: The lipid phenotype is important to evaluate the efficacy of BCG vaccines.

Bacillus Calmette-Guérin (BCG) Tokyo 172 is a predominant World Health Organization Reference Reagent for the BCG vaccine. Recently, the BCG Tokyo 172 substrain was reported to consist of two subpopulations with different colony morphologies, smooth and rough. Smooth colonies had a characteristic 22-bp deletion in *Rv3405c* of the region of difference (RD) 16 (type I), and rough colonies were complete in this region (type II). We hypothesized that the morphological difference is related to lipid phenotype and affects their antigenicity. We determined the lipid compositions and biosynthesis of types I and II. Scanning electron microscopy showed that type I was 1.5 times longer than type II. Phenolic glycolipid (PGL) and phthiocerol dimycocerosate (PDIM) were found only in type I. Although it has been reported that the RD16 is involved in the expression of PGL, type II did not possess PGL/PDIM. We examined the *ppsA-E* gene responsible for PGL/PDIM biosynthesis and found that the existence of PGL/PDIM in types I and II is caused by a *ppsA* gene mutation not regulated by the RD16. PGL suppressed the host recognition of total lipids via Toll-like receptor 2, and this suggests that PGL is antigenic and involved in host responses, acting as a cell wall component. This is the first report to show the difference between lipid phenotypes of types I and II. It is important to clarify the heterogeneity of BCG vaccine substrains to discuss and evaluate the quality, safety, and efficacy of the BCG vaccine.

Tuberculosis (TB)[2] is a major public health problem in the world. The World Health Organization (WHO) estimates that one-third of the world's population is infected with *Mycobacterium tuberculosis* and annually reports the global burden of the disease caused by TB (1). Moreover, the global spread of drug-resistant mycobacteria and the number of immunocompromised hosts, including victims of the human immunodeficiency virus (HIV) epidemic, are important problems. The *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine is the only available TB vaccine licensed under the WHO Expanded Program on Immunization. The BCG vaccine was first introduced in 1921 and has been used to prevent TB for nearly a century. The BCG strain was disseminated and maintained in different laboratories until the introduction of archival seed lots in the 1960s.

Currently, more than 14 BCG substrains are used throughout the world, and the heterogeneity of their genomics has been studied. These strains are different from each other and the original BCG strain and possibly affect the efficacy of the vaccine (2–5). All BCG substrains lack the region of difference (RD) 1. The BCG substrains are divided into early (represented by BCG Tokyo, Birkhaug, Sweden, and Russia) and late (including BCG Pasteur, Danish, Glaxo, and Prague) strains by the deletions and insertions of genes plus some single nucleotide

* This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan; and the Japan Health Sciences Foundation (Research on Publicly Essential Drugs and Medical Devices), the Ministry of Health, Labour and Welfare in Japan.
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB665170.
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[2] The abbreviations used are: TB, tuberculosis; BCG, Bacillus Calmette-Guérin; PGL, phenolic glycolipid; PDIM, phthiocerol dimycocerosate; RD, region of difference; MA, mycolic acid; FA, fatty acid; BMM, bone marrow-derived macrophage; TLR, Toll-like receptor; 2-*O*-Me-Rha, 2-*O*-methylrhamnose; PGL-tb, PGL of *M. tuberculosis*; WHO, World Health Organization.

polymorphisms (SNPs) of RDs (6). The first WHO International Reference Preparation for the BCG vaccine was established in 1965, and this International Reference Preparation was produced using BCG Tokyo 172. Based on collective international expert opinions from three WHO consultation meetings on the BCG vaccine in 2005, there is a demand for replacement of the first WHO International Reference Preparation for the BCG vaccine with substrain-specific BCG Reference Reagents. Three different substrains of BCG, Tokyo 172-1, Russian BCG-I, and Danish 1331, were identified and represent the predominant substrains used for BCG vaccine production and distribution worldwide (7). BCG Tokyo 172 strain has kept the position of International Reference Preparation or Reference Reagent for over 40 years, and the quality of this vaccine preparation is the best estimation of the viability and stability in the three Reference Reagent substrains (8–10).

Bedwell *et al.* (11) showed two genotypes in the BCG Tokyo 172 preparation that either have or do not have a characteristic 22-bp deletion in RD16. Honda *et al.* (12) identified two types of colony morphology, smooth and rough. In most cases, the smooth colonies showed a 22-bp deletion in *Rv3405c* of RD16 (type I), and rough colonies were complete in this region (type II). Both genotypes and colony morphologies have been found in common BCG Tokyo 172 vaccine preparations, but the major population was always type I.

For global BCG vaccination policy and practice, it is important to understand the heterogeneity in the morphology and genetics of BCG substrains (4, 13). The most common characteristic of acid-fast bacteria is that they are rich in lipids, and this may be reflected in the morphology (14, 15). The differences in the nature of the BCG Tokyo 172 subpopulation types I and II and vaccine efficacy were not fully understood except for the mutation in RD16. In this study, we demonstrate the heterogeneity of lipid components in types I and II and clarify the lipid phenotypes, their biosynthesis, and their functions.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—BCG Tokyo 172 subpopulation types I and II were isolated on Middlebrook 7H10 agar medium (Difco) with 0.5% glycerol and 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment (Difco) at 37 °C for 2 weeks (12). BCG Moreau, Connaught (ATCC 35745), and Pasteur (ATCC 35734) substrains and parent *M. bovis* Ushi 10 were used as reference strains. The identities and history were described previously (3).

Rv3405c of RD16—Bacterial cells were disrupted mechanically, and genomic DNA was extracted with phenol-chloroform followed by precipitation with ethanol. To examine *Rv3405c* of the RD16, PCR was performed with primers RD16-F (5′-GGCTGGTGTTTCGTCACTTC-3′) and RD16-R (5′-ACATTGGGAAATCGCTGTTG-3′).

Colony Morphology and Scanning Electron Microscopy—Colony morphology was observed using an optical microscope after incubation for 2 weeks on 7H10 agar plates. To prepare the bacteria for scanning electron microscopy, types I and II were grown in 7H9 broth with shaking to the midlog phase. The bacteria were collected, passed through a 5-μm membrane filter to remove aggregates, and then put on coverslips (Thermanox Plastic, 13-mm diameter; Thermo Fisher Scientific, Rochester, NY) coated with 0.1% (w/v) poly-L-lysine solution. The bacteria were fixed with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd., West Berkshire, UK) in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature, washed twice with 0.1 M phosphate buffer, and postfixed with 1% osmium tetroxide (TAAB Laboratories Equipment Ltd.) in 0.1 M phosphate buffer for 1 h at room temperature. The bacteria were then dehydrated in a graded ethanol series. After permutation with 3-methylbutyl acetate, critical point drying was performed using a Hitachi HCP-2 Critical Point Dryer (Hitachi High-Technologies Corp., Tokyo, Japan). Scanning electron microscopy was performed at ×5,000 in a Hitachi FE-SEM S-4700 instrument (Hitachi High-Technologies Corp.) using an accelerating voltage of 10 kV (16).

Analysis of Mycolic Acid Compositions—Mycolic acids (MAs) were analyzed as described previously (17). In brief, MAs of each subpopulation were liberated from the bacteria by alkaline hydrolysis using 10% potassium hydroxide in methanol at 90 °C for 2 h followed by extraction with *n*-hexane. After treatment with diazomethane, the methyl ester derivatives of total MAs were detected by thin-layer chromatography (TLC) on Silicagel G (Uniplate; 20 × 20 cm, 250 μm; Analtech, Inc., Newark, DE), which was developed with *n*-hexane-diethyl ether (90:15, v/v; three runs) and visualized by spraying with 20% sulfuric acid in ethanol and heating at 180 °C for 3 min. The ratio of each MA subclass was measured and calculated with ImageJ software. For each MA subclass, α-, methoxy-, and keto-MA was purified by preparative TLC until a single spot was obtained. The molecular species of the MA methyl esters were detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Comparisons of Fatty Acids and Carbohydrates—To determine the bacterial fatty acid (FA) and carbohydrate compositions, gas chromatography-mass spectrometry (GC/MS) of FA methyl ester, trimethylsilyl ester, and alditol acetate derivatives was performed (18, 19). The bacteria were sonicated, and total lipids were extracted with chloroform-methanol (2:1, v/v). The lipids were partitioned by a two-layer system with chloroform-methanol (2:1, v/v) and water. The organic phase was evaporated. Bacterial cells and total lipids were dried followed by the alkaline hydrolysis with 15% sodium hydroxide in 50% methanol at 100 °C for 72 h. After acidification by 6 M hydrochloric acid, FAs were extracted with *n*-hexane. FAs were methylated with diazomethane or trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA). Alditol acetate derivatives of carbohydrates were obtained by hydrolysis with 2 M trifluoroacetic acid at 120 °C for 2 h, reduction with 10 mg/ml sodium borohydride at 25 °C for 2 h, and acetylation with acetic anhydride at 100 °C for 1 h (20, 21).

Analysis and Conditions of Mass Spectrometry—MALDI-TOF MS was performed by using a SpiralTOF mass spectrometer (JMS-S3000; JEOL Ltd., Tokyo, Japan) for MA methyl esters and an Ultraflex II mass spectrometer (Bruker Daltonics, Billerica, MA) for glycolipids. The matrix was 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform-methanol (1:1, v/v), and it was analyzed in the reflectron mode with an accelerating

voltage operating in positive mode at 20 kV. GC/MS was performed by using a quadrupole mass spectrometer (GCMS-QP2010 Plus; Shimadzu Corp., Kyoto, Japan) equipped with a fused capillary column (SP-2380 and Equity-1; 30 m, 0.25-mm inner diameter; Supelco). Helium was used as the carrier gas. The temperature program for FA methyl esters was started at 60 °C, held for 1 min, increased 30 °C/min to 190 °C followed by an increase of 6 °C/min to 320 °C, and held for 3 min. The temperature program for FA trimethylsilyl esters was started at 60 °C, held for 1 min, increased 30 °C/min to 200 °C, increased 8 °C/min to 280 °C, and held for 16 min. The program for alditol acetate derivatives was started at 60 °C, increased 40 °C/min to 220 °C, held for 5 min followed by an increase of 10 °C/min to 260 °C, and held for 10 min. The molecular separator and ion source energies were 70 eV, and the accelerating voltage was 8 kV (20, 21).

Analysis of Total Lipids by Two-dimensional TLC—Two-dimensional TLC of total lipids was performed. The TLC plates were developed with three systems: first with chloroform-methanol-water (65:25:4, v/v/v) and second with chloroform-methanol-acetic acid-water (80:12:15:4, v/v/v/v) for the low hydrophobic fraction; first with chloroform-methanol-acetone-acetic acid (90:10:6:1, v/v/v/v) and second with chloroform-methanol-water (90:10:1, v/v/v) for the middle hydrophobic fraction; and first with *n*-hexane-ethyl acetate (98:2, v/v, three runs) and second with *n*-hexane-acetone (98:2, v/v) for the high hydrophobic fraction (22, 23). To detect phenolic glycolipids (PGLs), the TLC plates were developed first with chloroform-methanol (96:4, v/v) and second with toluene-acetone (80:20, v/v). The TLC plates were sprayed with 20% sulfuric acid in ethanol and heated at 180 °C for 3 min.

Sequencing of ppsA Region—The *ppsA* region of types I and II was amplified by PCR using primers *ppsA*-F (5′-CATATGACGGGAAGCATCAGTGGTGAAG-3′) and *ppsA*-R (5′-AAGCTTTCACACCGACCTCTCGGCCTCAG-3′). The amplified fragment was sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and a sequence analyzer (ABI3130xl; Applied Biosystems) (21).

Transformation of Type II with Type I ppsA—The *ppsA* fragments from type I (type I *ppsA*) and type II (type II *ppsA*) were amplified and cloned into pVV16, an expression plasmid vector for mycobacteria, downstream of the hsp60 promoter. Types I and II were transformed by incorporation of pVV16-type I *ppsA* and pVV16-type II *ppsA* by electroporation, and kanamycin-resistant colonies were isolated (21). Total lipids were prepared from heat-killed transformants, and the lipids produced were checked by TLC.

Host Responses to Total Lipids and Suppression by PGL—The host responses to total lipids were estimated by activations of murine bone marrow-derived macrophages (BMMs) of wild-type (C57BL/6), Toll-like receptor (TLR)2-KO, and TLR4-KO mice (Oriental Yeast Co., Ltd., Tokyo, Japan). The bone marrow cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 20% L929 cell supernatant at 37 °C in a 5% CO_2 atmosphere for 7 days, and adherent cells were harvested as BMMs. The BMMs were seeded at a concentration of 5×10^5 cells/ml on 96-well flat bottom tissue culture plates and stimulated with the total lipids (21). The lipid samples were stored at a concentration of 1 mg/ml in PBS containing 0.025% Tween 80 and diluted to the optimal concentration in each experiment. After 24-h incubation, the production of tumor necrosis factor (TNF)-α in the supernatants was measured using a DuoSet ELISA development kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. As positive controls, LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich) for TLR4 and Pam3CSK4 (InvivoGen, San Diego, CA) for TLR2 were used. Two independent experiments were performed and conducted in duplicate.

Nucleotide Sequence Accession Number—The nucleotide sequence of type II *ppsA* has been deposited in the NCBI GenBank™ database under accession number AB665170.

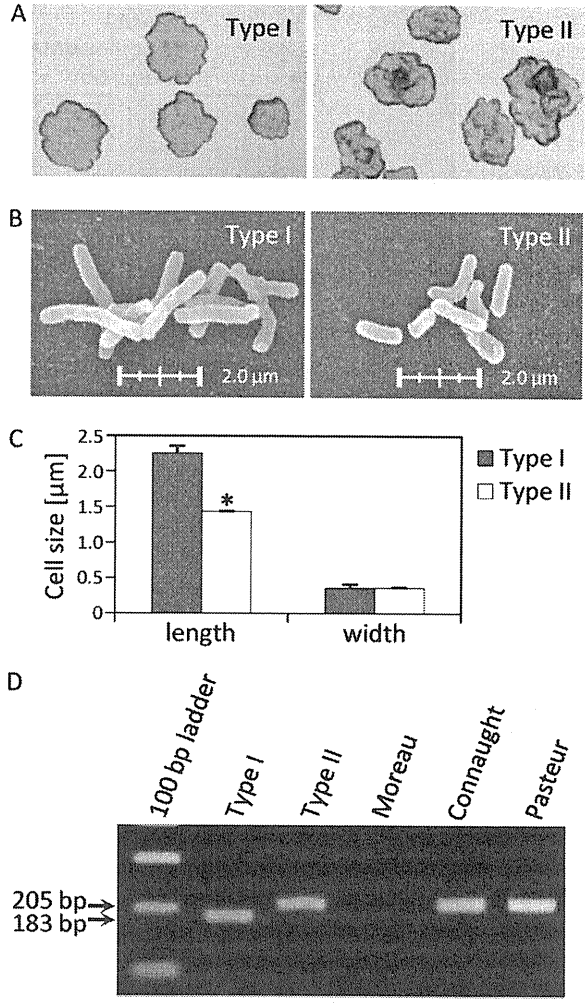

FIGURE 1. **Genotypes and morphologies of BCG Tokyo 172 type I and II subpopulations.** *A*, the colony morphology was observed by an optical microscope after incubation for 2 weeks on 7H10 agar plates. Type I was smooth, irregular, and flat, and type II was rough, raised, and convex. The *magnification* is ×4. Scanning electron microscopy (*B*) and a comparison of bacterial cell size (*C*) are shown. Types I and II were grown in 7H9 broth with shaking to the midlog phase. The bacteria were collected and filtered through a 5-μm membrane to remove aggregates. The average bacterial cell length was 2.25 ± 0.11 μm for type I and 1.44 ± 0.05 μm for type II. The *magnification* is ×5,000. The data are means ± standard deviations (SD) for 20 bacteria. *, $p < 0.001$. *D*, PCR of *Rv3405c* of the RD16. Type I showed a characteristic 22-bp deletion in *Rv3405c*, and type II was complete in this region. BCG Moreau, Connaught, and Pasteur substrains were used as reference strains.

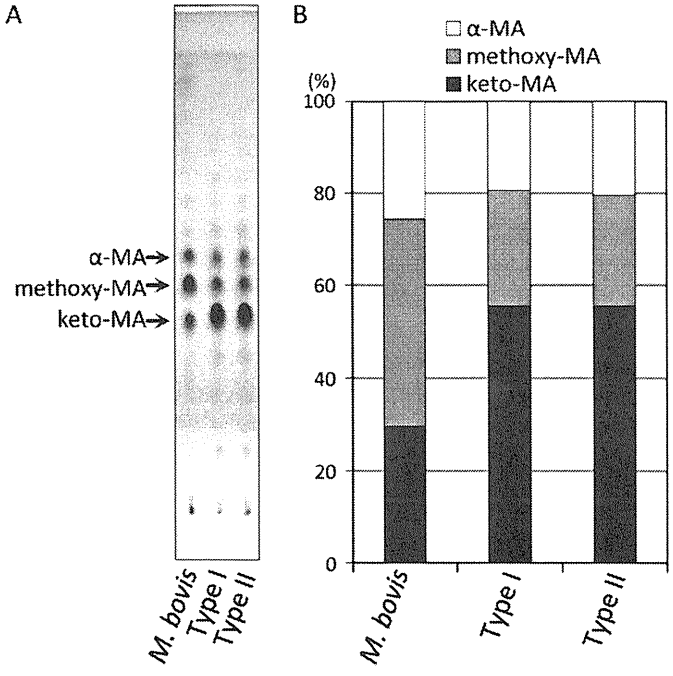

FIGURE 2. **Comparison of MA subclasses.** *A*, the TLC plate was developed with *n*-hexane-diethyl ether (90:15, v/v; three runs) and visualized by spraying with 20% sulfuric acid in ethanol and heating at 180 °C for 3 min. *B*, the ratio of each MA subclass was measured and calculated with ImageJ software. The keto-MA was over 50%, and the ratio of MA subclasses was almost the same in types I and II but significantly different from that of the BCG parent strain *M. bovis* Ushi 10.

TABLE 1
Fatty acid compositions in bacterial cells and total lipids from types I and II

Fatty acids[a]	Composition[b]			
	Bacterial cells		Total lipids	
	Type I	Type II	Type I	Type II
	%			
C14:0	1.5	2.3	1.4	1.9
C15:0	0.5	0.3	0.2	0.8
C16:1	0.5	0.8	0.8	1.3
C16:0	28.3	30.4	27.5	38.4
C17:0	0.9	1.7	1.0	2.6
C18:1	2.4	2.2	5.1	8.2
C18:0	17.3	18.0	12.1	15.8
C20:0	0.6	1.3	1.8	1.0
C22:0	1.1	0.8	0.8	0.4
C24:0	3.2	2.2	3.0	1.6
C25:0	ND	1.8	0.7	1.2
C26:0	29.7	27.9	13.7	9.8
C28:0	ND	ND	0.2	0.1
10-Methyl C16:0	1.2	ND	0.6	1.3
10-Methyl C17:0	0.2	0.5	0.1	0.9
10-Methyl C18:0	8.5	8.8	11.4	12.2
10-Methyl C19:0	ND	ND	0.1	ND
br C23:0	0.1	ND	0.6	ND
br C26:0	0.9	ND	4.4	0.4
br C27:0	0.5	ND	1.7	ND
br C29:0	1.3	0.2	8.7	0.1
br C30:0	0.4	ND	1.8	ND
br C32:0	0.6	0.2	1.4	0.1
br C33:0	ND	ND	0.2	0.1
Unidentified	0.4	0.8	0.7	1.9
Total	100	100	100	100

[a] br, multiple methyl-branched.
[b] Composition is given as the percentage of total integrated chromatographic areas. ND, not detected.

RESULTS

Colony Morphology and Genotypes of Types I and II

The colony morphologies of types I and II were different. The type I colonies were smooth, irregular, and flat, and those of type II were rough, raised, and convex (Fig. 1*A*). The scanning electron microscopy showed that the average bacterial length was 2.25 ± 0.11 μm for type I and 1.44 ± 0.05 μm for type II. Thus, the bacterial cell of type I was 1.5 times longer than that of type II, although there was no significant difference in their widths (Fig. 1, *B* and *C*).

Next, we examined the PCR products of the *Rv3405c* locus in RD16. Type I amplified a 183-bp band, whereas a 205-bp band was detected in type II that was identical to those of BCG Connaught and Pasteur. BCG Moreau showed the deletion of the specific band (Fig. 1*D*). These results clarified that type I had a 22-bp deletion in the *Rv3405c* locus and that of type II was complete.

Profiles of Bacterial Components in Types I and II

Mycolic Acid Subclasses—MAs are the most characteristic components of the acid-fast bacteria. The TLC of MA methyl esters showed the presence of three MA subclasses; α-, methoxy-, and keto-MA in both subpopulations (Fig. 2*A*). The composition of each subclass was keto- > methoxy- > α-MA. Keto-MA was over 50%, and the ratio of MA subclasses was significantly different from that of *M. bovis* Ushi 10 (Fig. 2*B*). The molecular species were assigned by MALDI-TOF MS, and the main species was C78:2 for α-MA, C84:1-OCH_3 for methoxy-MA, and C84:1 for keto-MA, respectively (supplemental Fig. 1). These results imply that types I and II had identical distributions of MAs.

Fatty Acids—The FA compositions of bacterial cells and total lipids are summarized in Table 1. The major FA species were C16:0, C18:0, 10-methyl C18:0 (tuberculostearic acid), and C26:0. The ratio of each FA from bacterial cells was almost the same in both subpopulations. The bacterial cells contained much C18:0 and C26:0 FAs and less 10-methyl C18:0 FAs compared with total lipids. The C24:0 and C26:0 FAs may be components of triglycerol (24). The multiple methyl-branched C26:0 and C29:0 FAs in total lipids were much higher in type I compare with type II.

Carbohydrates—The alditol acetate derivatives of carbohydrates in bacterial cells and total lipids were detected by GC/MS. The ratio of each carbohydrate in bacterial cells was identical in both subpopulations. In total lipids, type I contained 11.2% 2-*O*-methylrhamnose (2-*O*-Me-Rha), although only a trace was detected in type II (Table 2). This result strongly implied the deletion of glycolipids containing 2-*O*-Me-Rha in type II.

Lipids—The heterogeneity of lipid components of types I and II was examined by two-dimensional TLC (Fig. 3). Total lipids were developed with three different solvent systems to detect low, middle, and high hydrophobic lipid components. In the low hydrophobic fraction, the TLC patterns were similar in both subpopulations, and phosphatidylinositol mannosides and phosphatidylethanolamine were detected as major components. The middle hydrophobic fraction mainly contained several mycoloyl glycolipids, cord factor (trehalose 6,6′-dimycolate), and trehalose 6-monomycolate. Interestingly, an

TABLE 2
Carbohydrate compositions in bacterial cells and total lipids from types I and II

Carbohydrates	Composition[a]			
	Bacterial cells		Total lipids	
	Type I	Type II	Type I	Type II
	%			
2-*O*-Methyl Rhamnose	0.5	tr	11.2	0.4
Rhamnose	0.8	0.6	0.6	0.2
Ribose	1.6	1.2	ND	ND
Arabinose	15.7	14.8	0.8	1.0
Xylose	0.2	0.2	ND	ND
Mannose	12.8	13.3	42.7	47.5
Galactose	14.9	14.6	1.1	1.7
Glucose	45.8	47.2	10.6	12.9
myo-inositol	4.6	4.7	23.9	28.5
Unidentified	3.3	3.3	9.1	7.9
Total	100	100	100	100

[a] Composition is given as the percentage of total integrated chromatographic areas. tr, trace (less than 0.05%); ND, not detected.

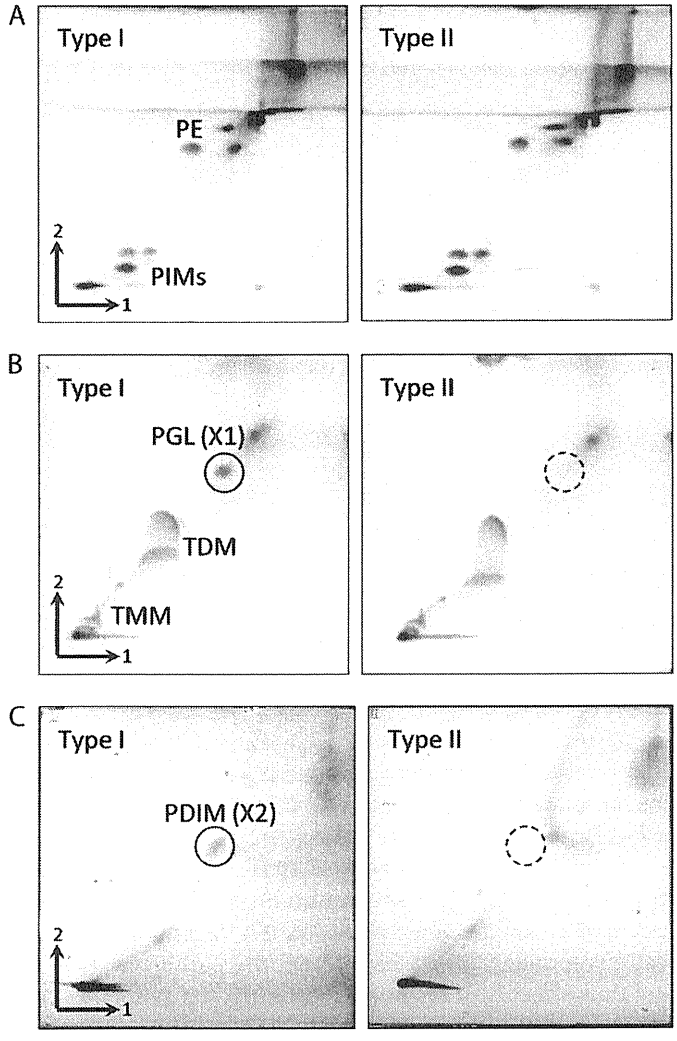

FIGURE 3. **Two-dimensional TLC of total lipids extracted from types I and II.** The TLC plates were developed with three systems: chloroform-methanol-water (65:25:4, v/v/v) followed by chloroform-methanol-acetic acid-water (80:12:15:4, v/v/v/v) for the low hydrophobic fraction (*A*), chloroform-methanol-acetone-acetic acid (90:10:6:1, v/v/v/v) followed by chloroform-methanol-water (90:10:1, v/v/v) for the middle hydrophobic fraction (*B*), and *n*-hexane-ethyl acetate (98:2, v/v; three runs) followed by *n*-hexane-acetone (98:2, v/v) for the high hydrophobic fraction (*C*). The TLC plates were sprayed with 20% sulfuric acid in ethanol and heated at 180 °C for 3 min. *PIMs*, phosphatidylinositol mannosides; *PE*, phosphatidylethanolamine; *TMM*, trehalose 6-monomycolate; *TDM*, cord factor (trehalose 6,6′-dimycolate).

unknown spot (X1) at the upper side of cord factor was detected as a yellow-brown spot only in type I. In the same manner, one more unknown spot (X2) expressed only in type I was detected in the high hydrophobic fraction.

We identified the precise structures of X1 and X2 by using mass spectrometry. The positions of X1 and X2 in TLCs developed with each solvent system suggested that X1 and X2 were PGL and phthiocerol dimycocerosate (PDIM), respectively (22). X1 and X2 were purified by preparative TLC. The MALDI-TOF MS spectrum of X1 showed *m/z* 1531 and other mass units at 14-Da intervals for $[M + Na]^+$ as molecule-related ions in positive mode (Fig. 4*A*). In addition, the MS/MS spectrum showed fragment ion peaks *m/z* 1371 based on the elimination of methyl deoxysugar, *m/z* 1135 and 1093 based on the elimination of the C26:0 and C29:0 FAs, *m/z* 696 based on the elimination of both C26:0 and C29:0 FAs, and *m/z* 535 for the phenolic phthiocerol that lacked methyl deoxysugar and C26:0 and C29:0 FAs (Fig. 4*B* and supplemental Fig. 2A). Alkaline hydrolysis of X1 was performed, and the FA was extracted. A trimethylsilyl ester of the FA fraction was analyzed by GC/MS. The main peaks of C26:0 and C29:0 were detected, and fragmented ions *m/z* 146, 187, and 229 were methyl-branched at every other branch from the α-position (supplemental Fig. 2B). This result was supported by the compositions of FA in total lipids from types I and II (Table 1). The GC/MS of the partially deuteromethylated alditol acetate derivatives of the X1 sugar moiety were detected as 2-*O*-methyl-3,4-di-*O*-deuteromethyl-1,5-di-*O*-acetylrhamnitol and assigned to 2-*O*-Me-Rha (supplemental Fig. 2C). The MALDI-TOF MS and MS/MS of X2 showed *m/z* 1349 for $[M + Na]^+$ as molecule-related ions (Fig. 4*C*) and *m/z* 952 and 910 based on the elimination of C26:0 and C29:0 FAs (Fig. 4*D*). As a result, X1 was identified as PGL, and X2 was identified as PDIM, which is composed of PGL. The proposed structure and fragment patterns of PGL/PDIM are shown in Fig. 4*E*. Analysis of the lipid distributions of types I and II clarified that type II did not produce PGL/PDIM, which contribute to the pathogenesis of *M. tuberculosis* and *M. bovis* (15).

Genetic Analysis of PGL/PDIM Biosynthesis

The *ppsA-E*, *drrA-C*, *papA5*, and *mas* genes are reported to be responsible for PGL/PDIM biosynthesis (25). We compared the sequences of the *ppsA* gene of types I and II. Type II *ppsA* had a single base insertion at nucleotide 379 and two single base substitutions at nucleotides 275 and 2415 compared with type I *ppsA* (Fig. 5*A*). As a result, a frameshift due to the insertion of this adenine relocated the stop codon from codon 1877 to 129, and an abnormal truncated protein was synthesized. To check the activity of the type II *ppsA*, we constructed type I and II transformants by inserting pVV16-type I *ppsA* and pVV16-type II *ppsA*. The type II transformant with type I *ppsA* restored the production of PGL (Fig. 5, *B* and *C*). These results imply that the type II *ppsA* is not functional and that the PGL/PDIM deletion in type II was caused by mutation of the *ppsA* gene.

Suppression of Host Responses by PGL

To explore the antigenicity of the lipids, BMMs derived from wild-type mice were stimulated with total lipids of types I and

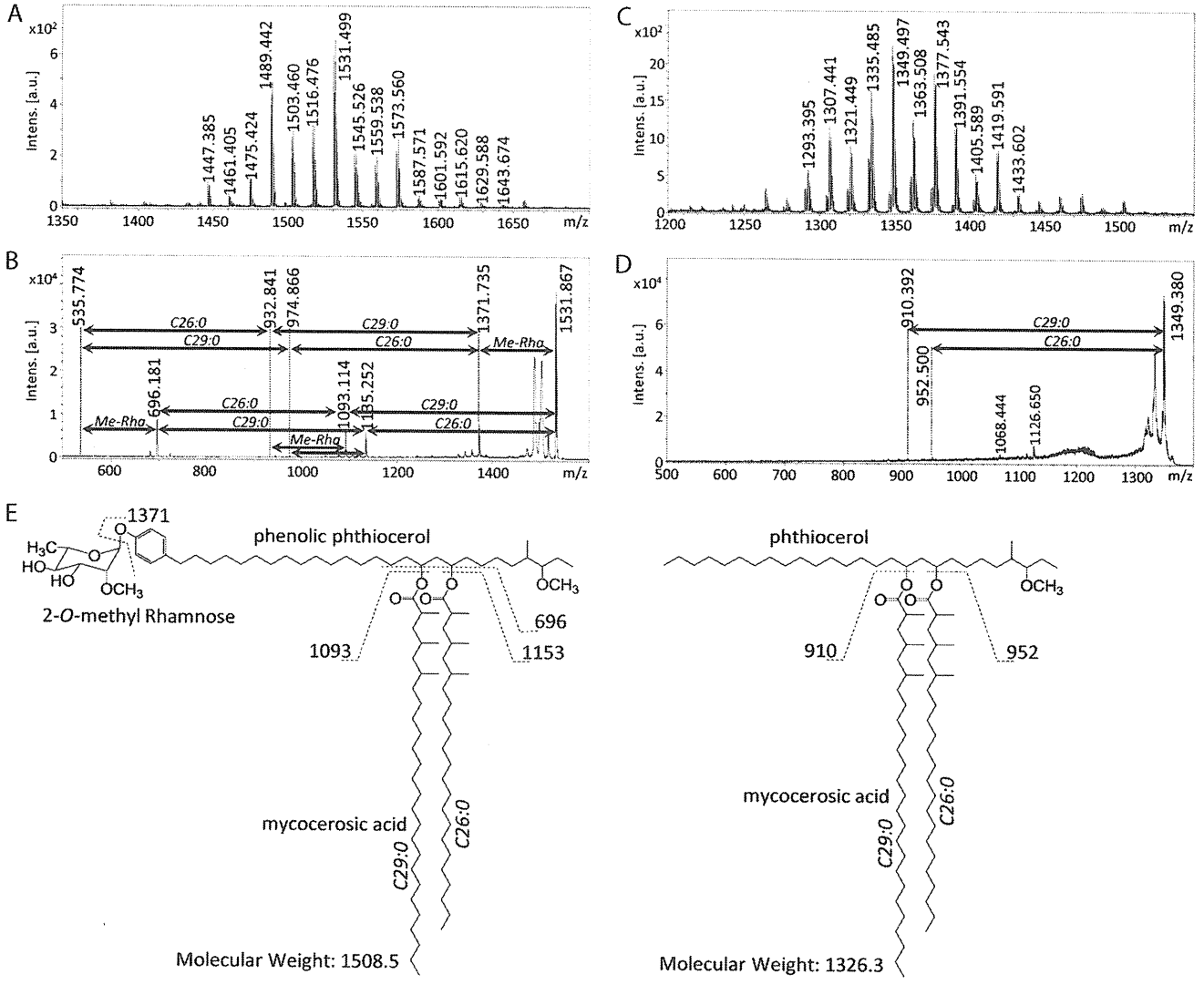

FIGURE 4. **Structural analyses of PGL/PDIM by mass spectrometry.** *A*, MALDI-TOF MS of PGL from type I showed the sodium adduct ion $[M + Na]^+$ with a major peak at *m/z* 1531 as a molecule-related ion. *B*, MALDI-TOF MS/MS of *m/z* 1531 showed four major fragment ions (*m/z* 696, 1093, 1135, and 1371) based on elimination of FAs and/or monomethylrhamnose from PGL. *C*, MALDI-TOF MS of PDIM from type I showed the sodium adduct ion $[M + Na]^+$ with a major peak at *m/z* 1349. *D*, MALDI-TOF MS/MS of *m/z* 1349 showed two minor fragment ions (*m/z* 910 and 952) based on elimination of FAs from PDIM. *E*, proposed structures and fragment patterns of PGL/PDIM from type I. *Intens.*, intensity; *a.u.*, arbitrary units.

II. The BMMs were activated by the total lipids and released TNF-α into the supernatant in a dose-dependent manner but did not respond to the PGL purified from type I. BMMs derived from TLR4-KO mice responded to the total lipids, but BMMs from TLR2-KO mice did not (Fig. 6*A*). These results imply that components of the total lipids stimulated BMMs via TLR2. Next, stimulations were performed using a mixture of total lipids and PGL at ratios of 10:1, 10:5, and 10:10. The TNF-α release was decreased in a concentration-dependent manner by the addition of PGL. This result implies that activation of the TLR2 pathway via total lipids was suppressed by PGL (Fig. 6, *B* and *C*).

DISCUSSION

BCG was introduced as a live attenuated vaccine for TB using the original BCG in 1921. Since then, the BCG substrains have undergone spontaneous mutations in the laboratory. To produce a stable and safe vaccine, it is important to clarify the differences of each substrain and estimate whether BCG substrains have similar protective potency. Of the representative phenotypes, BCG substrains obtained from the Pasteur Institute before 1927 produced methoxy-MA, but those obtained after 1931 failed to synthesize methoxy-MA (2). A point mutation in the *mma3* gene is responsible for this phenotype (26, 27). MAs, the major cell wall component, play a key role in the pathogenesis of TB and thus affect host responses. Recently, the genomes of the BCG Pasteur, Tokyo 172, and Moreau substrains were sequenced, and the genome of BCG Tokyo 172 had 20 insertion or deletion mutations of less than 20 bp and 68 SNPs compared with that of BCG Pasteur (4, 28, 29). The heterogeneity of BCG substrains is being clarified. BCG Tokyo 172 is important as a standard substrain of the BCG vaccine used by the WHO (8). It has been noted that BCG Tokyo 172 is composed of two subpopulations with different morphologies (12). Therefore, the relationships between bacterial phe-

notypes and genotypes should be evaluated in detail to validate the quality and protective effects of the BCG vaccine.

First, we characterized the lipids in types I and II biochemically because we suppose that mycobacterial lipids may affect colony morphology (smooth and rough; Refs. 30–32). The type I bacterial cells were significantly longer than those of type II (Fig. 1*C*). Scherman *et al.* (33) have reported that the mutant cells of *M. tuberculosis* with H37Rv disrupting the *Rv3779* gene are significantly shorter than the wild-type parent strain. *Rv3779* is responsible for the direct synthesis of polyprenoyl-phosphomannopyranose and indirectly for lipoarabinomannan, lipomannan, and phosphatidylinositol mannosides. It is considered that disruption of the *Rv3779* gene induces decreases of lipomannan, lipoarabinomannan, and phosphatidylinositol mannosides and affects the cell growth and shape (33). Based on that report, we examined the *Rv3779* gene of types I and II. The *Rv3779* sequences were completely identical in both types I and II (data not shown), and thus, this gene cluster is not associated with cell length. The shorter cells of type II may be linked with slow growth, disruption of PGL/PDIM, and a change of morphology.

Next, we found that only type II lacked PGL/PDIM. The distribution of various species of glycolipids and phospholipids in the cell envelope determines the cell surface properties, cell wall permeability, and bacterial phenotype. For example, the glycopeptidolipids produced by several non-tuberculous mycobacteria are associated with smooth/rough colony morphology, biofilm formation, and sliding motility (30–32). PGL/PDIM play critical roles in the cell wall permeability and in the killing activity of host macrophages (15, 34, 35).

Chen *et al.* (22) compared the PGL/PDIM production in 12 BCG substrains and showed a deficiency of PGL/PDIM in three substrains, BCG Japan, Moreau, and Glaxo. They have suggested that *Rv3405c* encodes a transcription factor of the TetR family and is responsible for the biosynthesis of PGL/PDIM in BCG Japan and Moreau strains but not in BCG Glaxo. They also reported that insertion of the transformant of pRv3405c into BCG Japan partially restored the synthesis of PGLs but not PDIM (22). Our results are inconsistent with the report by Chen *et al.* (22). Although the *Rv3405c* gene was intact in type II, the production of PGL/PDIM was inhibited (Fig. 3). The type II *ppsA* had a single base insertion and two single base substitutions compared with type I *ppsA*. Moreover, the type II transformant with type I *ppsA* inserted recovered PGL/PDIM production. These results suggest that mutation of the *ppsA* gene is

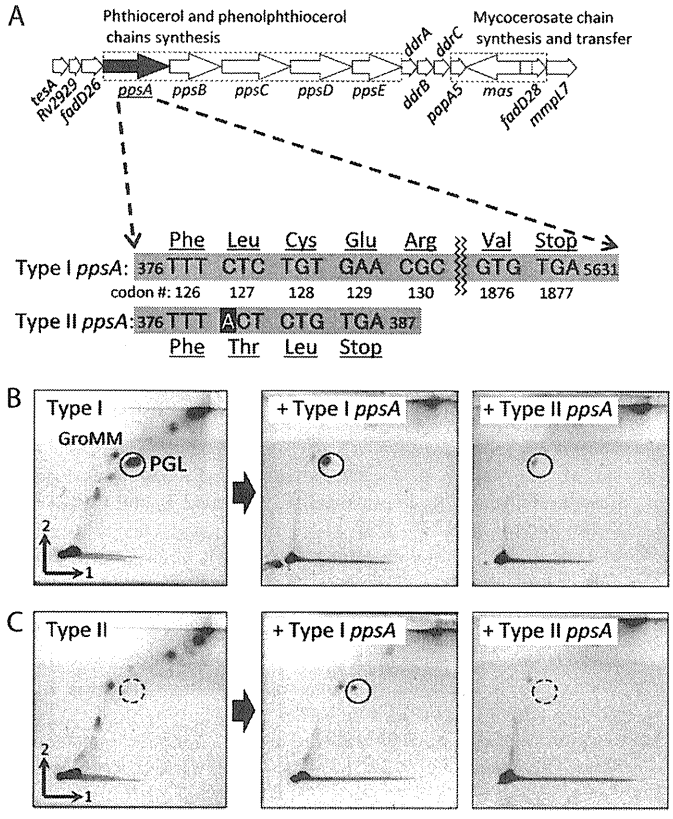

FIGURE 5. **Genetic organization of PGL/PDIM locus (25) and PGL expression.** *A*, the nucleotide sequence of *ppsA* genes showed that the type II *ppsA* had a single base insertion and two single base substitutions. The frameshift due to the insertion of this adenine relocated the stop codon from codon 1877 to 129, and an abnormal truncated protein was synthesized. *B* and *C*, total lipids extracted from type I (*B*) and II (*C*) transformants with type I *ppsA* or type II *ppsA* inserted. The manifestation of PGL was confirmed by insertion of the type I *ppsA* into the type II transformant. The TLC plates were developed with chloroform-methanol (96:4, v/v) first and then toluene-acetone (80:20, v/v). *GroMM*, glycerol monomycolate.

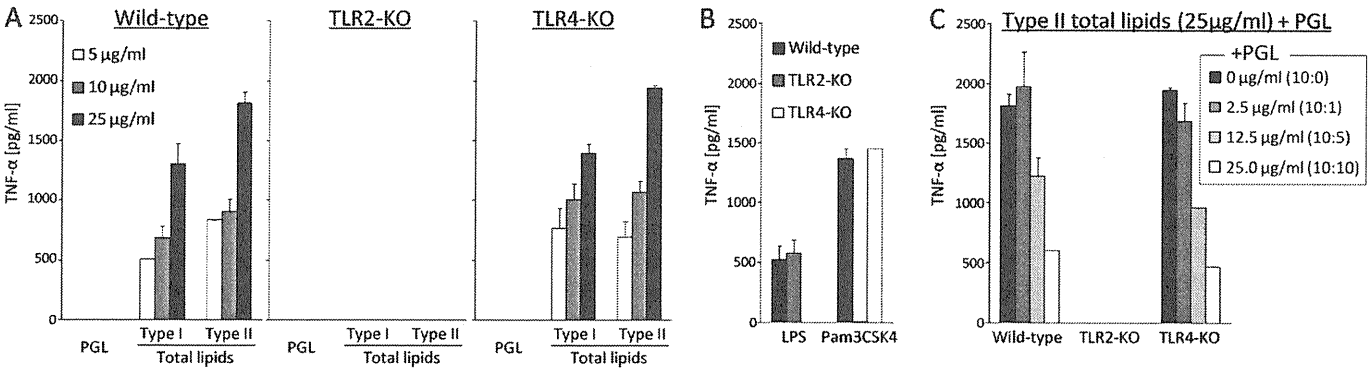

FIGURE 6. **Inhibition of proinflammatory cytokine release from murine macrophages by PGL.** *A*, BMMs were seeded at a concentration of 5×10^5 cells/ml on 96-well flat bottom tissue culture plates and stimulated with the purified PGL and total lipids. After 24-h incubation, the TNF-α production in the supernatants was measured. *B*, as positive controls, LPS (0.5 μg/ml) from *E. coli* 055:B5 was used for TLR4, and Pam3CSK4 (0.5 μg/ml) was used for TLR2. *C*, the BMMs were stimulated with type II total lipids (25 μg/ml) in the presence of increasing amounts of type I PGL (0, 2.5, 12.5, and 25 μg/ml). The TNF-α release was decreased in a PGL ratio-dependent manner. The data are means ± standard deviations (SD) for two independent experiments done in duplicate.