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Mutation Analysis of the *Mycobacterium leprae folP1* Gene and Dapsone Resistance▽

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Diaminodiphenylsulfone (dapsone) has long been used as a first-line drug worldwide for the treatment of leprosy. Diagnosis for dapsone resistance of *Mycobacterium leprae* by DNA tests would be of great clinical value, but the relationship between the nucleotide substitutions and susceptibility to dapsone must be clarified before use. In this study, we constructed recombinant strains of cultivable *Mycobacterium smegmatis* carrying the *M. leprae folP1* gene with or without a point mutation, disrupting their own *folP* gene on the chromosome. Dapsone susceptibilities of the recombinant bacteria were measured to examine influence of the mutations. Dapsone MICs for most of the strains with mutations at codon 53 or 55 of *M. leprae folP1* were 2 to 16 times as high as the MIC for the strain with the wild-type *folP1* sequence, but mutations that changed Thr to Ser at codon 53 showed somewhat lower MIC values than the wild-type sequence. Strains with mutations at codon 48 or 54 showed levels of susceptibility to dapsone comparable to the susceptibility of the strain with the wild-type sequence. This study confirmed that point mutations at codon 53 or 55 of the *M. leprae folP1* gene result in dapsone resistance.

The massive use of dapsone for treatment of leprosy led to the isolation of resistant strains of *Mycobacterium leprae* as early as 1964 (11), only a few years after discovery of the drug. Dapsone is structurally related to the sulfonamides. The mechanism of dapsone resistance in *M. leprae* is thought to be associated with dihydropteroate synthase (DHPS) in a manner similar to the mechanism of resistance to sulfonamides developed in other bacteria. The sulfonamides are structural analogs of *p*-aminobenzoate (PABA) and act as antimetabolites by competing with PABA for the active site of DHPS (4). DHPS catalyzes the reaction between dihydropteridine pyrophosphate and PABA as a part of the biosynthetic pathway leading to tetrahydrofolate (5, 12), which acts as a cofactor in the biosynthesis of purines, pyrimidines, and amino acids. Resistance to the sulfonamides has been shown to be mediated by mutations of the chromosomal *folP* gene encoding DHPS (7, 14, 15). Point mutations in the *folP1* gene have been identified in dapsone-resistant strains of *M. leprae* (9, 10, 16). Because *M. leprae* cannot be cultivated on any artificial medium and requires 13 days to double in experimentally infected mice, DNA diagnoses to detect dapsone-resistant bacteria would be highly useful. However, not all nucleotide substitutions in the *folP1* gene give rise to drug resistance. Therefore, the relationship between drug susceptibility and each nucleotide substitution observed in clinical isolates requires clarification. Dapsone-resistant *M. leprae* isolates have shown mutation at codon 53 or 55 in the *folP1* gene (6, 10, 16). Mutation at codon 48 has also been detected in our clinical specimens (unpublished data). Williams et al. have analyzed two types of mutations at codons 53 and 55 of the *M. leprae folP1* gene using a *folP*-deficient *Escherichia coli* (16). However, their analysis is as yet insufficient for direct application as molecular diagnosis for dapsone resistance.

In this study, site-directed mutagenesis techniques were used to alter the wild-type *M. leprae folP1* gene at codons shown to be mutated in clinical isolates for testing the effects of these mutations on dapsone susceptibility in a *folP*-disrupted *Mycobacterium smegmatis* host.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α cells were grown in Luria-Bertani (LB) medium. *M. smegmatis* mc²155 and its transformants were grown in Middlebrook 7H9 medium (Difco, 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 *M. leprae folP1* gene was amplified by PCR from *M. leprae* Thai-53 and cloned into pMV261. Site-directed mutagenesis was performed using PCR with KOD DNA polymerase (Toyobo, Osaka, Japan) and the primers listed in Table 2. PCR products were purified and phosphorylated with T4 kinase and ATP and then ligated to become circular. The ligation mixture was used to transform *E. coli* DH5α, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants, and the mutated sequences were confirmed by sequencing. Mutations introduced in the *M. leprae folP1* gene are shown in Fig. 1A.

Disruption of the *folP* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc²155 cells were transformed with plasmids carrying the *M. leprae folP1* with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allelic exchange mutants were constructed by the temperature-sensitive mycobacteriophage method (3). Using the *M. smegmatis* mc²155 genome sequence (accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *folP* gene (MSMEG_6103). In order to disrupt the *folP* gene, DNA segments from 736 bp upstream through 286 bp downstream of the initiation codon of *M. smegmatis folP* and from 198 bp upstream through 832 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. Plasmids thus produced were digested with PacI and ligated to the PH101 genomic DNA excised from the phasmid phAE87 by PacI digestion. The ligated DNA was packaged using GigaPackIII Gold Packaging Extract (Stratagene, La Jolla, CA), and the resultant mixture was used for transduction of *E. coli* STBL2 (Life Technologies, Carlsbad, CA) to yield cosmid DNA. After *E.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
DH5α	Cloning host	
STBL2	Cloning host	
C600 Δ*folP*::Km[r]	*folP* mutant	7
M. smegmatis mc^{2}155		
Plasmids		
pYUB854	Cosmid vector	3
phAE87	Phasmid vector carrying full length DNA of mycobacteriophage PH101	3
pMV261	*E. coli*-mycobacteria shuttle plasmid vector (multicopy in mycobacteria)	13
pNN301[a]	pMV361-type integrative vector (single copy in mycobacteria)	13; this study

[a] pNN301 has an *int-attP* fragment of mycobacteriophage L5 instead of *oriM*.

coli was transduced and the transductants were plated on 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. smegmatis* transformant carrying the *M. leprae folP1* gene was infected by the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin- and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

Dapsone 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 dapsone (0.25 to 64 μg/ml). The MIC value for each strain was defined as the lowest concentration of dapsone needed to inhibit bacterial growth.

RESULTS

Construction of recombinant *M. smegmatis* strains. We prepared plasmids with point mutations in the *M. leprae folP1* gene. Each plasmid has 1 of 21 single point mutations at codon 48, 53, 54, or 55 (Fig. 1A). The first or second nucleotide at each codon was replaced by another nucleotide to change the amino acid residue. Mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae folP1* with or without a point mutation were individually introduced into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *folP* gene on their own chromosome (Fig. 1B). PCR analysis confirmed that the *folP* sequences in the recombinant strains were replaced by hygromycin resistance gene sequences (Fig. 2). Isolation of a *folP*-disrupted *M. smegmatis* strain carrying the *M. leprae folP1* with mutation 48-4 (mutation 4 at codon 48) was unsuccessful. All the strains except for the strains with mutation 48-5 or 53-4 showed comparable growth rates. The strains with mutation 48-5 or 53-4 grew a little more slowly than the strain with the wild-type sequence. These two mutations may reduce DHPS activity.

Dapsone susceptibility. Dapsone susceptibilities of the recombinant *M. smegmatis* strains were tested. As shown in Fig. 3, the MIC of dapsone for recombinant *M. smegmatis* carrying the wild-type *M. leprae folP1* gene was 0.5 μg/ml. MIC values for most of the strains with mutations at codon 53 or 55 were 2 to 16 times as high as the MIC for the strain with the wild-type sequence. Interestingly, two strains with alterations

TABLE 2. Primers used in this study

Primer	Sequence[a]	Application
MLFPWTF	GCGAATTCGTGAGTTTGGCGCCAGTGCA	Cloning of *M. leprae folP1*, forward
MLFPWTR	GCAAGCTTTCAGCCATCACATCTAACCT	Cloning of *M. leprae folP1*, reverse
MSFPUF	GCAGGCCTGTATCCTCATCCCGGACAGC	*folP* disruption, upstream forward
MSFPUR	GCTCTAGATGGTGTCGATGCTGATCGTG	*folP* disruption, upstream reverse
MSFPDF	GCAGATCTCGCAAACGTTTCCTCGGTAC	*folP* disruption, downstream forward
MSFPDR	GCACTAGTACTGGTCGATCTCCGACAGC	*folP* disruption, downstream reverse
MSFPF	TCACCGAGTACGGCATGAGC	Detection of *folP* disruption, forward
MSFPR	TAGAGCGCATGGATCAGCAG	Detection of *folP* disruption, reverse
MLFPR1	CGATTCGCCACCGACGTCGAC	Introduction of point mutations for codons 53, 54, and 55
MLFPR2	GTCGACAATCGCCGCGCCTT	Introduction of point mutations for codon 48
MLFP48-1	ATCGGTGGCGAATCGACCCG	Introduction of point mutation 48-1
MLFP48-2	CTCGGTGGCGAATCGACCCG	Introduction of point mutation 48-2
MLFP48-3	TTCGGTGGCGAATCGACCCG	Introduction of point mutation 48-3
MLFP48-4	GACGGTGGCGAATCGACCCG	Introduction of point mutation 48-4
MLFP48-5	GCCGGTGGCGAATCGACCCG	Introduction of point mutation 48-5
MLFP48-6	GGCGGTGGCGAATCGACCCG	Introduction of point mutation 48-6
MLFP53-1	GCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-1
MLFP53-2	ATCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-2
MLFP53-3	TCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-3
MLFP53-4	CCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-4
MLFP53-5	AACCGGCCCGGTGCCATTAG	Introduction of point mutation 53-5
MLFP53-6	AGCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-6
MLFP54-1	ACCAGGCCCGGTGCCATTAG	Introduction of point mutation 54-1
MLFP54-2	ACCCGGCCCGGTGCCATTAG	Introduction of point mutation 54-2
MLFP54-3	ACCTGGCCCGGTGCCATTAG	Introduction of point mutation 54-3
MLFP55-1	ACCCGGACCGGTGCCATTAG	Introduction of point mutation 55-1
MLFP55-2	ACCCGGGCCGGTGCCATTAG	Introduction of point mutation 55-2
MLFP55-3	ACCCGGTCCGGTGCCATTAG	Introduction of point mutation 55-3
MLFP55-4	ACCCGGCACGGTGCCATTAG	Introduction of point mutation 55-4
MLFP55-5	ACCCGGCGCGGTGCCATTAG	Introduction of point mutation 55-5
MLFP55-6	ACCCGGCTCGGTGCCATTAG	Introduction of point mutation 55-6

[a] Restriction sites are underlined

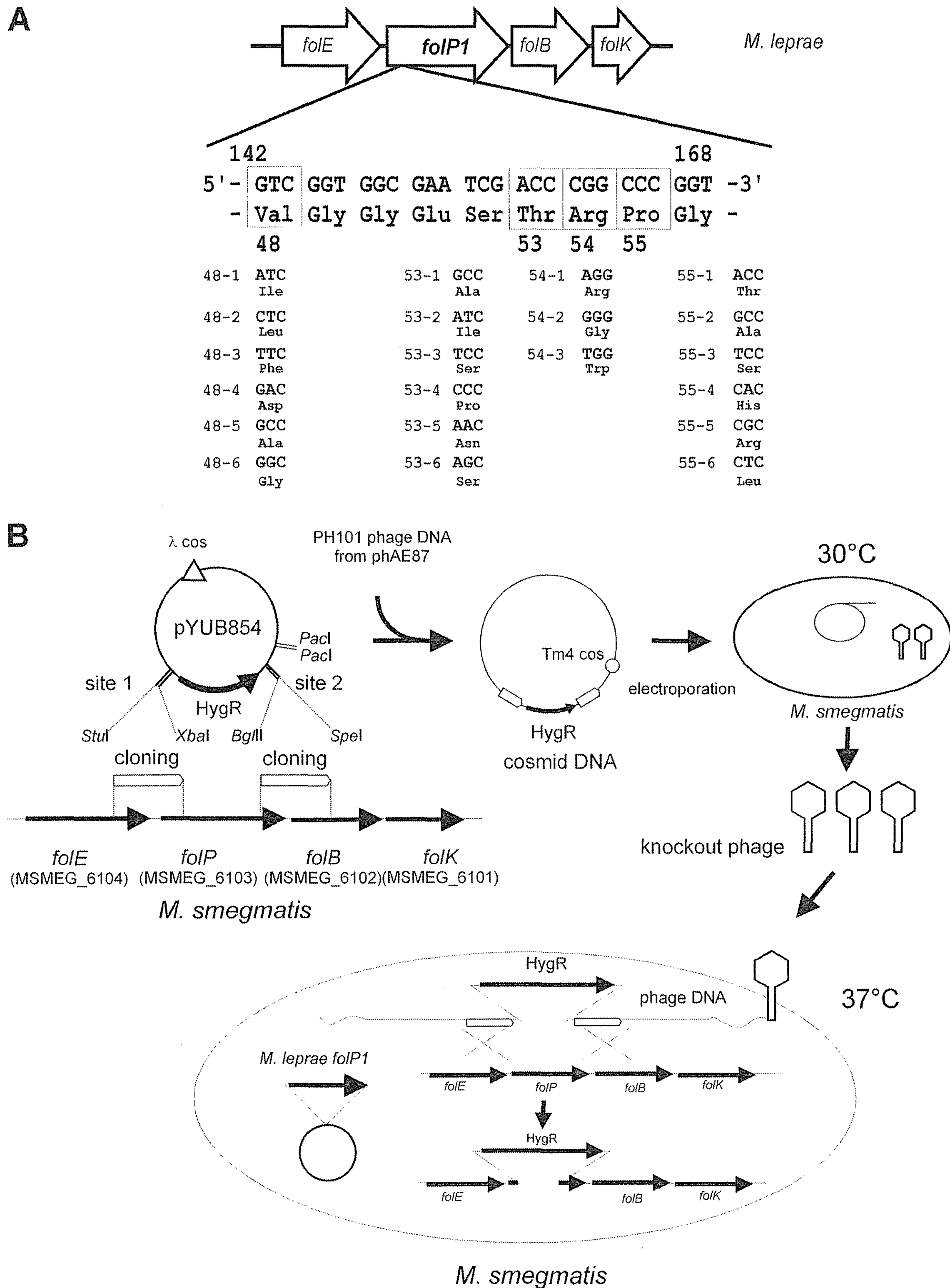

FIG. 1. Construction of recombinant *M. smegmatis* strains for dapsone susceptibility testing. (A) Point mutations introduced in the *M. leprae folP1* gene. Single nucleotide substitutions introduced in the *M. leprae folP1* at codons 48, 53, 54, and 55 are shown. Deduced amino acid residues are shown below the triplets. (B) Construction of *M. smegmatis* recombinants by allelic exchange.

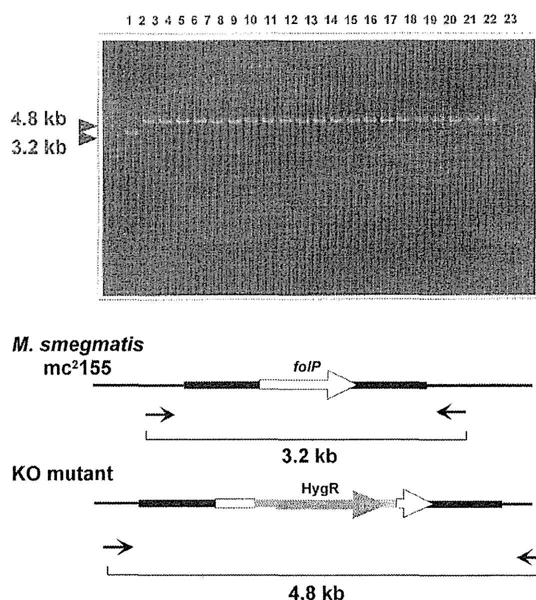

FIG. 2. PCR analysis to confirm the disruption of *folP*. Black arrows represent primers MSFPF and MSFPR for the PCR amplification. Lane 1, *M. smegmatis* mc^2155; lanes 2 to 22, *M. smegmatis* strains carrying the *M. leprae folP1* without mutation and *folP1* with mutations 48-1,48-2, 48-3, 48-5, 48-6, 53-1, 53-2, 53-3, 53-4, 53-5, 53-6, 54-1, 54-2, 54-3, 55-1, 55-2, 55-3, 55-4, 55-5, and 55-6, respectively; lane 23, negative control. KO, knockout.

in amino acids from threonine to serine (T53S) encoded by different nucleotide sequences (53-3 and 53-6) were more susceptible to dapsone than strains with the wild-type *folP1* sequence. MIC values for strains with mutations at codon 48 or 54 were comparable to MICs for strains with the wild-type sequence. MIC values of dapsone for the recombinant *M. smegmatis* strains are listed in Table 3. Using a multicopy plasmid may affect the expression levels of the *M. leprae folP1* and MIC values. Therefore, we tested all the mutations using

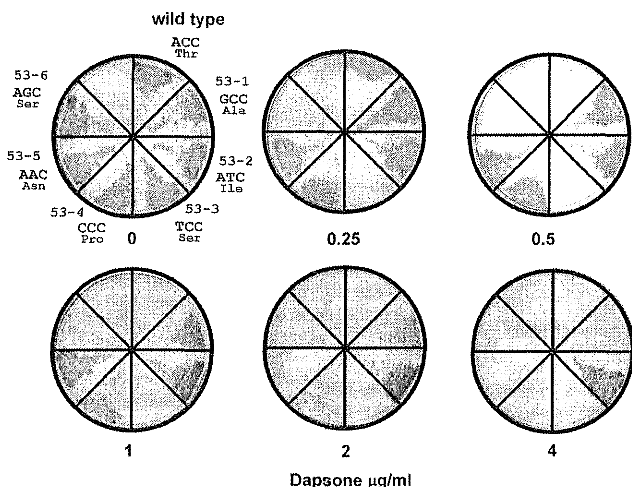

FIG. 3. Dapsone susceptibility of recombinant *M. smegmatis*. Results for *M. smegmatis* strains with point mutations at codon 53 of the *M. leprae folP1* are shown. Dapsone concentration is depicted below each plate.

TABLE 3. Dapsone susceptibility of the recombinant *M. smegmatis* strains

Strain or mutation	Dapsone MIC (μg/ml)	Reference of footpad test
Wild type	0.5	
48-1 (Val → Ile)	0.5	
48-2 (Val → Leu)	0.5	
48-3 (Val → Phe)	1.0	
48-4 (Val → Asp)	—[a]	
48-5 (Val → Ala)	1.0	
48-6 (Va → Gly)	1.0	
53-1 (Thr → Ala)	4.0	6
53-2 (Thr → Ile)	8.0	6, 10, 16
53-3 (Thr → Ser)	0.25	
53-4 (Thr → Pro)	2.0	
53-5 (Thr → Asn)	2.0	
53-6 (Thr → Ser)	0.25	
54-1 (Arg → Arg)	0.5	
54-2 (Arg → Gly)	1.0	
54-3 (Arg → Trp)	0.5	
55-1 (Pro → Thr)	1.0	
55-2 (Pro → Ala)	2.0	
55-3 (Pro → Ser)	2.0	
55-4 (Pro → His)	2.0	
55-5 (Pro → Arg)	8.0	6, 16
55-6 (Pro → Leu)	4.0	6, 10

[a] Isolation of an *folP*-disrupted *M. smegmatis* strain carrying the *M. leprae folP1* with mutation 48-4 was unsuccessful.

pNN301, a single-copy integrative vector, instead of pMV261 and obtained MIC values identical to those obtained with pMV261, suggesting that the expression levels did not influence the MIC values.

DISCUSSION

We first attempted using *E. coli* C600 Δ*folP*::Km^r transformants to determine the MIC of dapsone, but susceptibility of the recombinant *E. coli* strains to dapsone was not stable even in Mueller-Hinton medium. Subsequently, we tried to isolate a *folP*-deficient *M. smegmatis* strain by allelic exchange, given the closer association of *M. smegmatis* to *M. leprae* than *E. coli*. The selection held great promise as total-sequence comparison of *M. leprae* DHPS with *M. smegmatis* DHPS indicated 83% identity, whereas the identity between *M. leprae* DHPS and *E. coli* DHPS is only 41%, indicating the higher potential of *M. smegmatis* as a host for measuring MIC values of dapsone for *M. leprae* DHPS. However, isolation of *folP*-deficient *M. smegmatis* was unsuccessful. In *E. coli*, DHPS is not essential for bacterial growth when the cells are cultured with thymidine (7), but DHPS activity may be essential for the growth of *M. smegmatis* as it could not be replaced by any of the supplemented culture media tested. Hence, we then attempted to disrupt the *folP* gene on the *M. smegmatis* chromosome after introducing the *M. leprae folP1* gene into the cell to compensate for DHPS activity.

Comparison of the DHPS structures of *E. coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* has suggested that Ser53 and Pro55 of the *M. tuberculosis* DHPS, which correspond to Thr53 and Pro55 in *M. leprae*, may be the major sites of interaction with PABA, dapsone, and sulfonamides (1, 2, 8). In the present study, all mutations that cause amino acid sub-

stitutions at codon 55 resulted in dapsone resistance. Mutations at codon 53 also gave rise to dapsone resistance except for the T53S substitution, which resulted in less resistance to dapsone than the wild-type sequence (Fig. 3). The results for mutation 53-1, 53-2, 55-5, and 55-6 are consistent with the mouse footpad dapsone susceptibility testing of the *M. leprae* clinical isolates (6, 10, 16). Mutations at codon 48 or 54 showed comparable levels of susceptibility to dapsone as the wild-type sequence using dapsone susceptibility testing, but the MIC values for mutations 48-3, 48-5, 48-6, and 54-2 were slightly higher than the MIC for the wild-type sequence. Mutation 48-5 for V48A, which has been detected in our clinical samples (unpublished data), might give rise to low-level resistance to dapsone in *M. leprae*. This level of resistance should be very carefully examined by comparison with the results of footpad testing and clinical data. These data will help the molecular diagnosis of dapsone-resistant *M. leprae* with the goal of avoiding the wrong choice of drugs for chemotherapy.

Although these results should always be initially confirmed by clinical susceptibility testing as well, we believe that the method established in this study should have great utility in further attempts to determine the mutations responsible for giving rise to the dapsone resistance of *M. leprae.* The advantage of this method lies in the ability to functionally replace an essential gene of fast-growing mycobacteria with the *M. leprae* counterpart. The method may also be applicable to analysis of the rifampin resistance and quinolone resistance of *M. leprae*.

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Multiple Cases of Cutaneous *Mycobacterium massiliense* Infection in a "Hot Spa" in Japan[▽]

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Seven body polishers working in the same "hot spa" presented with multiple red nodules and papules on their hands and forearms. A causative agent was successfully isolated from two of the subjects and from a swab sample collected from the underside of a bed cover in the body-polishing facility. The two cutaneous isolates and the environmental isolate were rapidly growing mycobacteria that formed nonphotochromogenic smooth or smooth/rough colonies on Ogawa egg slants. They were identified as *Mycobacterium massiliense* by multigenotypic analysis using the 16S rRNA, *hsp65*, and *rpoB* genes and the 16S–23S rRNA internal transcribed spacer (ITS) region. However, the use of the 16S rRNA gene sequence and/or DNA-DNA hybridization (DDH Mycobacteria Kit) alone would not distinguish *M. massiliense* from mycobacteria in the *M. chelonae-M. abscessus* group. The three isolates were significantly more susceptible to clarithromycin, doxycycline, and minocycline than the *M. abscessus* and *M. bolletii* reference strains. One cutaneous isolate and the environmental isolate were in a related cluster by randomly amplified polymorphic DNA PCR (RAPD-PCR). Of the several mycobacterial species found in the day spa, only *M. massiliense* was isolated from biopsy specimens of the skin lesions, suggesting that this bacterium is a human skin pathogen. This is the first known report of cutaneous *M. massiliense* infections that could not be attributed to a prior invasive procedure. This is also the first report of *M. massiliense* infection in Japan.

Mycobacterium massiliense was initially isolated from the sputum of a patient with pneumonia in France in 2004 (1). Epidemiologically, *M. massiliense* has been recognized as an emerging pathogen in the United States (16, 24) and Brazil, where outbreaks have been associated with postsurgical and cosmetic procedures (2, 4, 22). In Korea, an outbreak was linked to intramuscular injections of an antimicrobial agent (9). This bacterium was also the source of a lethal case of sepsis in Italy and has been found in cystic fibrosis patients in France (15, 20). Among pulmonary *M. abscessus* group isolates, almost half of the isolates in Korea and 30% of those in the Netherlands are *M. massiliense* (8, 21). It has been suggested that *M. massiliense* should be reclassified taxonomically as a subspecies of *M. abscessus* (11). The clinical significance of differentiating these two species has also been explored (7). However, *M. massiliense* has not been fully characterized. Although mycobacteria are a frequent source of dermal infection, *M. massiliense* has never been reported as an etiological agent. This report describes the first case of an *M. massiliense* dermal infection in Japan.

Case Reports

In November 2007, a 49-year-old female who worked as a body polisher in a hot spa developed multiple red nodules and papules on her hands and forearms. The number of lesions gradually increased over several months, precipitating a visit to a local hospital in June 2008 (case 1). A skin biopsy specimen of a nodule stained with hematoxylin and eosin (H&E) revealed that the lesion was a structured form of granuloma that contained giant cells and infiltrating lymphocytes with necrosis. Acid-fast bacilli were identified by Ziehl-Neelsen staining.

In October 2008, multiple red nodules and papules appeared on the hands and forearms of a 26-year-old female who worked in the same body-polishing facility as the individual with case 1. She visited the same local hospital in December 2008 (case 2) and received similar biopsy results: acid-fast bacilli and granuloma formation with giant cells and infiltrating lymphocytes.

In addition to cases 1 and 2, in the same spa during the same period, there were five more puzzling cases of body polishers with similar symptoms. Three of these patients (with cases 3 to 5) visited the hospital. However, the presence of acid-fast bacilli was not confirmed, even after the observation of granulomas in the skin biopsy specimen of case 3. In April 2009, environmental sampling was conducted at this hot spa in order to discover the causative agent(s).

MATERIALS AND METHODS

Identification and characterization of isolates. Skin samples were decontaminated with *N*-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) (13). Briefly, an equal volume of NALC-NaOH solution (2% NaOH, 1.45% sodium citrate, 0.5% NALC) was added to as much as 10 ml of a skin specimen homogenized in normal saline. The mixture was vortexed and allowed to stand for 15 to 20 min before neutralization with sterile 0.067 M phosphate buffer (pH 6.8), to a final volume of 50 ml, and centrifugation at 3,000 rpm for 20 min. The supernatant was discarded, and the sediment was resuspended in 2 ml of phosphate-buffered saline. Half of the sediment was stored at −80°C, while the other half was used

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TABLE 1. Primers used in this study

Primer	Sequence (positions)	Target and/or purpose (amplified fragment size)	Reference
8F16S	5′-AGAGTTTGATCCTGGCTCAG-3′ (8–27)[a]	16S rRNA gene, PCR (ca. 1,500 bp), sequencing	17
1047R16S	5′-TGCACACAGGCCACAAGGGA-3′ (1047–1028)[a]		
830F16S	5′-GTGTGGGTTTCCTTCCTTGG-3′ (830–849)[a]		
1542R16S	5′-AAGGAGGTGATCCAGCCGCA-3′ (1542–1523)[a]		
TB11	5′-ACCAACGATGGTGTGTCCAT-3′	*hsp65*, PCR (441 bp), sequencing	19
TB12	5′-CTTGTCGAACCGCATACCCT-3′		
MabrpoF	5′-GAGGGTCAGACCACGATGAC-3′ (2112–2131)[b]	*rpoB*, PCR (449 bp), sequencing	This study
MabrpoR	5′-AGCCGATCAGACCGATGTT-3′ (2559–2541)[b]		
ITSF	5′-TTGTACACACCGCCCGTC-3′	16S–23S ITS region, PCR (ca. 340 bp), sequencing	14
ITSR	5′-TCTCGATGCCAAGGCATCCACC-3′		
OPA2	5′-TGCCGAGCTG-3′	RAPD-PCR	25
OPA18	5′-AGGTGACCGT-3′		
INS-2	5′-GCGTAGTGCGTCGGTGACAAA-3′		

[a] Nucleotide positions were assigned using the *Escherichia coli* 16S rRNA gene sequence as a reference.
[b] Primer design and nucleotide positions were based on the *M. tuberculosis rpoB* gene sequence (GenBank/EMBL/DDBJ accession no. L27989).

for acid-fast staining and inoculation into a 2% Ogawa egg slant (case 1) or Middlebrook 7H9 broth enriched with 10% oleic acid-albumin-dextrose-catalase (OADC; Nippon Becton Dickinson, Fukushima, Japan) (7H9 broth) (case 2). Mycobacterial isolates were subcultured on Middlebrook 7H11 agar plates enriched with 10% OADC (Nippon Becton Dickinson) for more than 3 days at 36.5°C.

A total of 15 environmental samples were collected from the body-polishing facility in sterile containers or bags. There were four water samples from different bathtubs, eight swab samples, and three scurf scrub equipment samples (two gloves and one brush). All samples were centrifuged at 3,000 rpm for 20 min to concentrate any organisms; the swab and equipment samples were stirred in sterile normal saline before centrifugation. Following centrifugation, precipitated samples were resuspended in normal saline and were added to 1.5 volume of 1 N hydrogen chloride. After incubation for 20 min, the samples were neutralized with 1 N NaOH. The mixture was centrifuged at 3,000 rpm for 20 min, and the sediment was resuspended in 1 ml of phosphate-buffered saline (5). Suspensions were inoculated onto 2% Ogawa egg slants or into 7H9 broth and were incubated at 36.5°C. Mycobacterial isolates were subcultured on Middlebrook 7H11 agar for more than 3 days at 36.5°C. The characteristics of the cultured isolates were determined as described previously (3).

DNA-DNA hybridization. DNA-DNA hybridization was performed with a DDH Mycobacteria Kit (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) to identify mycobacterial species (10). In brief, one-half loopful of a mycobacterial colony was used for the test. Biotin-labeled denatured DNA was extracted from a colony and was distributed into the wells of a microdilution plate where the single stranded DNA from 18 reference strains had been immobilized. After a 2-h hybridization at 55°C, hybridized DNA was detected with peroxidase-conjugated streptavidin and the substrate tetramethylbenzidine. The optical density at 630 nm was measured for each well within 30 min. The labeled strain was identified as one of the 18 species when the maximum color intensity was 1.9 times higher than the intensity of the negative control and the second strongest color intensity was lower than 70% of the maximum color intensity.

DNA extraction. One loopful of a mycobacterial colony on solid medium 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 mycobacterial sample was crushed in a bead-beating instrument (Magnalizer; Roche Diagnostics) at 3,000 rpm for 90 s with zirconia beads (diameter, 2 mm). Total genomic DNA was purified from the crushed suspension using the High Pure PCR template preparation kit according to the manufacturer's instructions (Roche Diagnostics) and was stored at −20°C.

Sequence and phylogenetic analysis. Sequences of clinical and environmental isolates, which had been preliminarily identified as *M. abscessus* by the DDH Mycobacteria Kit, were compared to those of the reference strains *M. massiliense* JCM 15300^T, *M. chelonae* JCM 6388^T, *M. abscessus* JCM 13569^T, and *M. bolletii* JCM 15297^T, obtained from the Japan Collection of Microorganisms of the Riken BioResource Center (BRC-JCM; Saitama, Japan). The majority of the 16S rRNA gene, the partial *hsp65* and *rpoB* genes, and the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) with the primers listed in Table 1. Both strands were sequenced with the BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems), and were run on the ABI Prism 310 genetic analyzer (Applied Biosystems) (13). Analyses were performed after removal of the primers from the sequences.

Similarity searches were performed in the DNA Data Bank of Japan (DDBJ) (6). Phylogenetic analyses were performed using the MEGA software package, version 4.0.2 (Build no. 4028) (18). The tree was constructed using the neighbor-joining method with Kimura's two-parameter distance correction model with 1,000 bootstrap replications.

RAPD-PCR. Randomly amplified polymorphic DNA PCR (RAPD-PCR) (25) was performed with three random primers in order to compare clinical and environmental isolates with the *M. massiliense* JCM 15300^T reference strain (Table 1). In brief, 50 μl of a mixture containing 60 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 250 μM each deoxynucleoside triphosphate (dNTP), 50 pmol of the primer, 1 U of *Taq* DNA polymerase (Takara Bio Inc., Japan), and 100 ng of total genomic DNA, which was freshly extracted or stored for as long as 30 days at −20°C, was used for the PCR. Amplification was performed in the Takara PCR thermal cycler SP using 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The PCR products were separated in the same run by 2% agarose gel electrophoresis and ethidium bromide staining. Strains were assigned to the same cluster when the same band patterns were observed with the three primers or one major band difference was observed in only one of the three primers.

Drug susceptibility assays. Drug susceptibility assays were performed with 7H9 broth microdilutions according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (23), with a modification in drug choice for rapidly growing mycobacteria. Amikacin (AMK), azithromycin (AZM), ciprofloxacin (CIP), clofazimine (CLF), clarithromycin (CLR), doxycycline (DOX), meropenem (MEM), minocycline (MIN), and panipenem (PAPM) were tested against the clinical and environmental isolates and the *M. abscessus*, *M. massiliense*, and *M. bolletii* reference strains. AZM was provided by Pfizer Japan Inc.; MEM and PAPM were provided by Dainippon Sumitomo Pharma Co. Ltd. and Daiichi Sankyo Co. Ltd., respectively; and the other drugs were purchased from Sigma-Aldrich Co. MIC testing was carried out in triplicate on different days, with two of three matching MICs used as the criteria for MIC determination. Susceptibility was evaluated according to the CLSI breakpoint recommendations.

Nucleotide sequence accession numbers. The DNA sequences of the 16S rRNA (1,468 bp), *hsp65* (401 bp), *rpoB* (409 bp), and ITS (298 bp) fragments from the reference strains (*M. massiliense* JCM 15300^T, *M. chelonae* JCM 6388^T, *M. abscessus* JCM 13569^T, and *M. bolletii* JCM 15297^T) and the clinical and environmental isolates have been deposited in the International Nucleotide Sequence Databases (INSD) through the DDBJ under accession numbers AB548592 to AB548611.

TABLE 2. Similarities of nucleotide sequences between case isolates and reference strains of closely related mycobacterial species

Isolate	Species for comparison[a]	% Identity			
		16S rRNA (1,468 bp)	*hsp65* (401 bp)	*rpoB* (409 bp)	ITS (298 bp)
Isolate 1	*M. abscessus*	99.9	98.8	97.6	99.0
	M. massiliense	99.9	100	100	100
	M. bolletii	99.9	99.3	98.3	99.0
	M. chelonae	99.8	92.5	96.1	89.9
Isolate 2	*M. abscessus*	99.9	98.8	97.6	99.0
	M. massiliense	99.9	100	100	100
	M. bolletii	99.9	99.3	98.3	99.0
	M. chelonae	99.8	92.5	96.1	89.9
Environmental isolate	*M. abscessus*	99.9	98.8	97.6	99.0
	M. massiliense	99.9	100	100	100
	M. bolletii	99.9	99.3	98.3	99.0
	M. chelonae	99.8	92.5	96.1	89.9

[a] Reference strains used for comparison were *M. abscessus* JCM 13569^T, *M. massiliense* JCM 15300^T, *M. bolletii* JCM 15297^T, and *M. chelonae* JCM 6388^T.

RESULTS

Isolation from skin and environmental samples. Bacteria isolated from the skin biopsy specimens of cases 1 and 2 were provisionally identified as *M. abscessus* by the DDH Mycobacteria Kit. None of the four environmental samples from the bathtubs yielded mycobacteria. However, mycobacteria grew from four swabs and two gloves used for the scurf scrub. The swab isolate from the underside of the bed cover in the body-polishing room was tentatively identified as *M. abscessus* by the DDH Mycobacteria Kit. The five remaining mycobacterial isolates included *M. nonchromogenicum*, from the stone wall of the body-polishing room; *M. terrae*, from a glove; and three *M. fortuitum* isolates (one from the spring spout, one from the wood wall of the body-polishing room, and one from a glove).

The clinical and environmental (bed cover) isolates were rapidly growing mycobacteria that formed nonphotochromogenic colonies at 25 to 37°C on 2% Ogawa egg slants and 7H11 agar plates but did not grow at 42°C. The isolates were negative for niacin, nitrate reduction, and Tween 80 hydrolysis and were positive for 5% NaCl tolerance, arylsulfatase (3 days), catalase, and urease. However, differences in colony morphology were observed: isolate 1 and the environmental isolate formed smooth colonies, while isolate 2 produced rough colonies.

Genotypic analysis. Nucleotide sequence analysis was performed with the three isolates and four reference strains (*M. abscessus*, *M. massiliense*, *M. bolletii*, and *M. chelonae*). The sequences of the 1,468-bp fragment of the 16S rRNA gene from the three isolates were identical. Only single or triple mismatches with *M. abscessus*, *M. massiliense*, and *M. bolletii*, or with *M. chelonae*, respectively, were found at nucleotide positions 1008 or 999, 1039, and 1265. The sequences of *hsp65*, *rpoB*, and the ITS region were also identical among the three isolates, showed complete identity with those of *M. massiliense*, and were 89.9 to 99.3% similar to those of *M. abscessus*, *M. bolletii*, and *M. chelonae* (Table 2). Phylogenetic trees, developed using sequences from the *hsp65* and *rpoB* genes, clustered the isolates with *M. massiliense* (Fig. 1), although the clustering was not as clear with trees developed using sequences from the 16S rRNA gene and the 16S-23S rRNA ITS region (data not shown). Confirmation of these three isolates as *M. massiliense* led to the supposition that *M. massiliense* might be the underlying cause of the cutaneous lesions and that the environment of the day spa led to the acquisition of the infections.

Randomly amplified polymorphic DNA PCR. Strain typing was performed by RAPD-PCR with three random primers to clarify the relatedness of the clinical and environmental *M. massiliense* isolates. A comparison of the OPA2 band patterns (Fig. 2, lanes 1 to 4) revealed distinct differences in the amplification patterns of the clinical isolates versus the *M. massiliense* reference strain. The patterns of isolate 1 and the environmental isolate differed by a minor band. The OPA18 and INS-2 band patterns of isolate 1 and the environmental isolate were identical or differed by only one minor band, though these band patterns were clearly different between the clinical isolates and the reference strain (Fig. 2, lanes 5 to 8 and 9 to

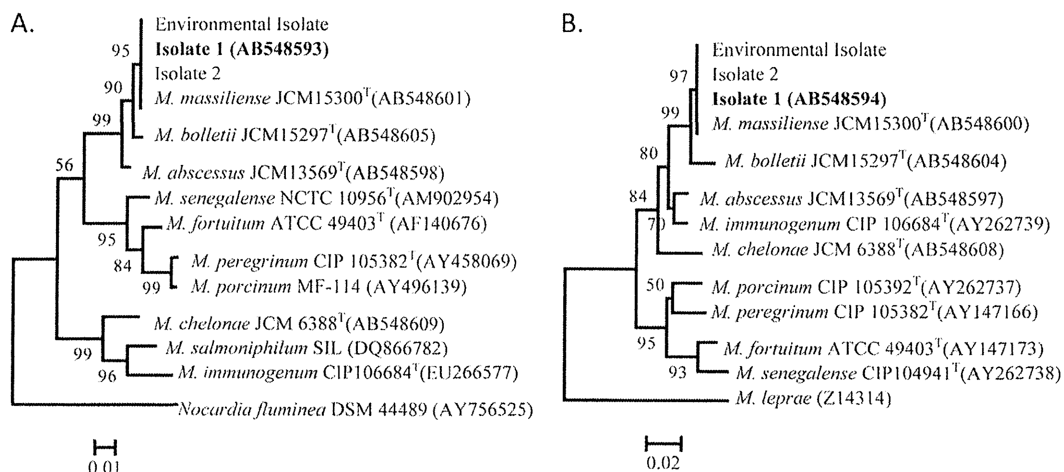

FIG. 1. Phylogenetic analysis based on the *hsp65* (A) and *rpoB* (B) genes of isolate 1 (boldface) and other rapidly growing mycobacteria. The numbers at the nodes are the percentages of bootstrap levels supported by 1,000 resampled data sets. Bootstrap values of <50% are not shown. *Nocardia fluminea* (A) and *M. leprae* (B) were used as outgroups.

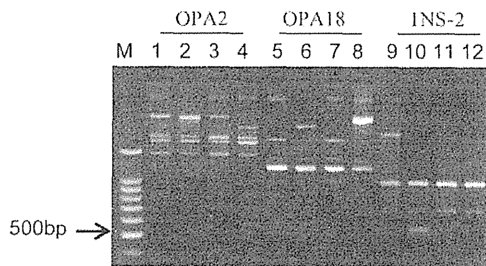

FIG. 2. Comparison of the RAPD-PCR patterns of the clinical isolates (isolates 1 and 2), the environmental isolate, and a reference strain (*M. massiliense* JCM 15300[T]) with three different primers. Lanes 1, 5, and 9, DNA from isolate 1; lanes 2, 6, and 10, DNA from isolate 2; lanes 3, 7, and 11, DNA from the environmental isolate; lanes 4, 8, and 12, DNA from the *M. massiliense* reference strain; lane M, DNA size marker (100-bp ladder). RAPD-PCR patterns produced with primers OPA2 (lanes 1 to 4), OPA18 (lanes 5 to 8), and INS-2 (lanes 9 to 12) are shown.

12). Therefore, isolate 1 and the environmental isolate were assigned to the same cluster by RAPD-PCR analysis but were different from isolate 2.

Assays for susceptibility to antimicrobial agents. The results of tests of the susceptibilities of the clinical and environmental isolates to antimicrobial agents are shown in Table 3. All three isolates exhibited susceptibility patterns similar to that of the *M. massiliense* reference strain (1, 11, 16), such as susceptibility to clarithromycin, minocycline, doxycycline, and amikacin and resistance to ciprofloxacin. The strains were also tested against azithromycin, clofazimine, meropenem, and panipenem, though these were not on the list of CLSI-recommended drugs (23). Notably, the MICs of azithromycin for the three isolates and the *M. massiliense* reference strain were lower than those for the *M. abscessus* and *M. bolletii* reference strains. No differences in the MIC were observed with clofazimine, meropenem, and panipenem.

DISCUSSION

In 2004, *M. massiliense* was proposed as a new species in the *M. chelonae-M. abscessus* group (1). Its 16S rRNA gene had complete identity with that of *M. abscessus* and more than 99.6% similarity with the *M. chelonae* and *M. immunogenum* genes. Therefore, genotypic analysis using single-target sequencing of the 16S rRNA gene would not distinguish *M. massiliense* from other mycobacteria in the *M. chelonae-M. abscessus* group. Two independent groups have reported on the inaccuracy of single-target sequencing for the diagnosis of *M. massiliense* (11, 12). Similarly, the DDH Mycobacteria Kit could not distinguish *M. massiliense* from *M. abscessus*, because the objective species of this kit were limited to 18 mycobacterial species: *M. tuberculosis*, *M. kansasii*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *M. szulgai*, *M. avium*, *M. intracellulare*, *M. gastri*, *M. xenopi*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, and *M. peregrinum*. However, with this kit, the one isolate provisionally identified as *M. abscessus* was easily distinguished from several environmental surveillance mycobacterial isolates.

The appearance of skin lesions among day spa workers led to the collection and analysis of workplace environmental samples. Although environmental surveillance was performed several months after case 1 first presented with symptoms, RAPD-PCR showed that isolate 1 and the environmental isolate were part of the same cluster (Fig. 2). In contrast, the same analysis revealed that isolate 2 belonged to a different cluster. The relationship between isolate 1 and the environmental isolate suggests that the unhygienic conditions in the day spa led to the acquisition of the infections, but the cause and effect could not be resolved, because the origin of isolate 2 was not specified. RAPD-PCR typing also showed that the *M. massiliense* reference strain isolated in France had a different amplification pattern, which was indicative of the geographical distinction between the Japanese and French isolates.

Based on published reports, this is the first presentation of cutaneous *M. massiliense* infections that were not preceded by an invasive procedure. *M. massiliense* may be more pathogenic to human skin than other species, since only *M. massiliense* was isolated from the skin biopsy specimens, though several species of mycobacteria were isolated from the day spa facility. The antimicrobial susceptibility profile of *M. massiliense* is shown in Table 3. Further studies are required to determine if the profile differs from those of other members of the *M. chelonae-M. abscessus* group and if any differences can be used as a typing tool. Interestingly, the MICs of azithromycin, clarithromycin,

TABLE 3. Results of drug susceptibility tests

Antimycobacterial drug[a]	MIC (μg/ml) for:					
	Isolate 1	Isolate 2	Environmental isolate	*M. massiliense* JCM 15300[T]	*M. abscessus* JCM 13569[T]	*M. bolletii* JCM 15297[T]
AMK	16	16	16	16	16	16
AZM	16	32	16	16	64	128
CIP	8	16	8	8	8	8
CLF	1	2	1	2	1	2
CLR	0.25	0.25	0.25	0.25	4	4
DOX	2	8	1	1	64	64
MEM	8	8	16	8	16	8
MIN	1	2	0.5	0.5	16	8
PAPM	64	32	64	64	64	64

[a] AMK, amikacin; AZM, azithromycin; CIP, ciprofloxacin; CLF, clofazimine; CLR, clarithromycin; DOX, doxycycline; MEM, meropenem; MIN, minocycline; PAPM, panipenem.

doxycycline, and minocycline for both clinical isolates, the environmental isolate, and the *M. massiliense* reference strain were much lower than those for the *M. abscessus* and *M. bolletii* reference strains. Reinvestigation of the genotypic and drug susceptibility characteristics of the *M. chelonae-M. abscessus* group is needed. However, some differences in drug susceptibilities have been described that may allow clinicians to differentiate *M. massiliense* from other mycobacteria in the *M. chelonae-M. abscessus* group and to design specific therapies targeting the organism (1, 11, 16). Further study is needed to document the clinical features of, and treatment options for, cutaneous *M. massiliense* infection.

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Review Article

Innate Immune Effectors in Mycobacterial Infection

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Tuberculosis, which is caused by infection with *Mycobacterium tuberculosis* (Mtb), remains one of the major bacterial infections worldwide. Host defense against Mtb is mediated by a combination of innate and adaptive immune responses. In the last 15 years, the mechanisms for activation of innate immunity have been elucidated. Toll-like receptors (TLRs) have been revealed to be critical for the recognition of pathogenic microorganisms including mycobacteria. Subsequent studies further revealed that NOD-like receptors and C-type lectin receptors are responsible for the TLR-independent recognition of mycobacteria. Several molecules, such as active vitamin D_3, secretary leukocyte protease inhibitor, and lipocalin 2, all of which are induced by TLR stimulation, have been shown to direct innate immune responses to mycobacteria. In addition, Irgm1-dependent autophagy has recently been demonstrated to eliminate intracellular mycobacteria. Thus, our understanding of the mechanisms for the innate immune response to mycobacteria is developing.

1. Introduction

In humans, tuberculosis is one of deadly infectious diseases. Indeed, approximately 2 million tuberculosis patients die every year. The risk of disease is also increased by emergence of acquired immune deficiency syndrome and development of multidrug-resistant mycobacteria [1]. Therefore, it is important to understand the host defense mechanisms against mycobacteria. Inhalation of aerosols containing *Mycobacterium tuberculosis* (Mtb) causes tuberculosis. After inhalation, Mtb invades alveolar macrophages to enter into the host and establish the infection. The host, in turn, ignites defense responses through sequential activation of immunity, a combination of innate and adaptive immune systems. In the adaptive phase of immune responses, the importance of Th1/IFN-γ-mediated responses in mycobacterial infection has been well established [2]. In contrast, although macrophages are the major target of invasion by Mtb, how the innate arm of immunity mediates host defense against mycobacteria had long remained unknown. However, the mechanisms behind innate immune responses have been revealed in the past 15 years following the identification and characterization of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) [3]. Furthermore, it has been elucidated that TLR-dependent activation of innate immunity controls the development of adaptive immune responses [4]. The involvement of PRRs other than TLRs in the recognition of mycobacteria has also been revealed. In addition to the induction of adaptive immune responses, the PRR recognition of mycobacteria induces expression of several effector molecules participating in the innate host responses. The role of these innate effector molecules in mycobacterial infection is being elucidated. PRR-independent mechanisms for mycobacterial killing, such as autophagy, have also been revealed. In this paper, we will describe recent advances in our understanding of effectors that mediate innate immune responses against mycobacteria.

2. Toll-Like Receptors in Mycobacterial Infection

Innate immune responses after mycobacterial infection are initiated by recognition of mycobacterial components by PRRs, with mycobacterial components activating several

TLRs (Figure 1). Genomic DNA from a *Mycobacterium bovis* strain, bacillus Calmette–Guérin (BCG), have an ability to augment NK cell activity and induce type I IFNs from murine spleen cells and human peripheral blood lymphocytes. The immunostimulatory activity of mycobacterial DNA was ascribed to the presence of palindromic sequences including the 5′-CG-3′ motif, now called CpG motif [5], and now known to activate TLR9 [6]. The mycobacterial cell wall consists of several glycolipids. Among these, lipoarabinomannan (LAM) lacking mannose end capping, lipomannan (LM), and phosphatidyl-*myo*-inositol mannoside (PIM) are recognized by TLR2 [7, 8]. The 19-kDa lipoprotein of Mtb also activates macrophages via TLR2 [9, 10]. TLR4 is also presumed to recognize mycobacterial components.

The *in vivo* importance of the TLR-mediated signal in host defense to Mtb was highlighted in studies using mice lacking MyD88, a critical component of TLR signaling. MyD88-deficient mice are highly susceptible to airborne infection with Mtb [11–13]. In contrast to mice lacking MyD88, mice lacking individual TLRs are not dramatically susceptible to Mtb infection. Susceptibility of TLR2-deficient mice to Mtb infection varies between different studies [14, 15], while TLR4-deficient mice do not show high susceptibility to Mtb infection [16, 17]. A report demonstrates that TLR9-deficient mice are susceptible to Mtb infection and mice lacking both TLR2 and TLR9 are more susceptible [18]. These findings indicate that multiple TLRs might be involved in mycobacterial recognition. However, a recent report using mice lacking TLR2/TLR4/TLR9 indicated that these triple KO mice show a milder phenotype than MyD88-deficient mice [12]. Therefore, more intensive examination is required to reveal whether TLRs or molecules other than TLRs activating MyD88 mediate innate immune responses to mycobacterial infection. This study also demonstrated that Th1-like adaptive immune responses are induced even in Mtb-infected MyD88-deficient mice [12]. Therefore, the TLR/MyD88-independent component of innate immunity is involved in the induction of adaptive immune responses during mycobacterial infection. The TLR/MyD88-independent response might be induced by other PRRs described below.

3. Non-TLRs in Mycobacterial Infection

Several recent findings have indicated that PRRs other than TLRs evoke innate immune responses [19]. These include RIG-I-like receptors, NOD-like receptors (NLRs), and C-type lectin receptors. Among these PRRs, NOD-like receptors and C-type lectin receptors have been implicated in the innate recognition of mycobacteria (Figure 2).

NOD2 is a member of NLRs that recognize muramyl dipeptide (MDP), a core component of bacterial peptidoglycan, in the cytoplasmic compartment. Macrophages from NOD2-deficient mice show a defective cytokine production after Mtb infection [20]. Similarly, mononuclear cells of individuals homozygous for the *3020insC NOD2* mutation show a defective cytokine response after stimulation with Mtb [7]. Activation of the NOD2-mediated pathway is induced by stimulation with live Mtb, but not by heat-killed Mtb [8]. Live Mtb, which is localized in the phagosomal compartment within macrophages, stimulates the cytosolic NOD2 pathway by inducing phagosomal membrane damage [21]. The NOD2 ligand MDP is N-acetylated in most bacteria. However, MDP is N-glycolylated by N-acetyl muramic acid hydroxylase (NamH) in mycobacteria. Analyses using *M. smegmatis* namH mutant and NOD2-deficient mice showed that N-glycolyl MDP is recognized by NOD2. In addition, N-glycolyl MDP is the more potent NOD2 activator than N-acetyl MDP [22]. Thus, NOD2 contributed to the recognition of mycobacteria.

Several members of the NLR family, such as NLRP1, NLRP3, and IPAF, induce assembly of the inflammasome, which leads to caspase-1-dependent secretion of IL-1β and IL-18 [23]. The involvement of IL-1β and IL-18 in mycobacterial infection was demonstrated in studies using knockout mice [24–27]. A recent study demonstrated that mycobacteria inhibit the inflammasome-dependent casapase-1 activation leading to defective IL-1β production [28]. The inhibition of caspase-1 activation has further been shown to be mediated by an Mtb gene, *zmp1*, which encodes a putative Zn^{2+} metalloprotease. Thus, Mtb has a strategy that evades the inflammasome-mediated innate immune responses.

C-type lectin receptors, such as mannose receptor, were originally reported to mediate phagocytosis of mycobacteria [29]. Another C-type lectin receptor, DC-SIGN, has been shown to recognize mycobacteria, and thereby modulate the function of dendritic cells [30–32]. Recognition of mycobacteria by dectin-1 has been shown to induce gene expression such as TNF-α, IL-6, and IL-12 [33, 34]. In addition, macrophage inducible C-type lectin (Mincle) has recently been shown to recognize trehalose-6,6′-dimycolate (TDM: also called cord factor), a mycobacterial cell wall glycolipid that is the most studied immunostimulatory component of Mtb [35, 36], thereafter modulating macrophage activation. Thus, several C-type lectin receptors are involved in the recognition of mycobacteria.

CARD9 is involved in the signaling pathways of several PRRs including TLRs, NOD-like receptors, and FcRγ-associated C-type lectin receptors through association with Bcl-10 and MALT. Therefore, it is not surprising that CARD9-deficient mice are highly susceptible to Mtb infection. However, interestingly the high susceptibility of CARD9-deficient mice to the infection has been shown to be excessive inflammatory responses due to defective production of the immunosuppressive cytokine IL-10 [37]. Mincle is a member of C-type lectin receptors associated with FcRγ [38]. Accordingly, TDM-induced immune responses are mediated by the signaling pathway activating CARD9 [36, 39].

TLRs and C-type lectin receptors are expressed on the plasma membrane or the endosomal/phagosomal membrane, whereas NOD-like receptors are expressed within the cytoplasm. Indeed, distinct patterns of TLR- and NOD-like receptor-mediated gene expression profiles have been demonstrated in infection with intracellular bacteria [40]. Thus, several PRRs recognize mycobacteria in distinct sites within the host cells (macrophages) to synergistically induce effective host defense responses.

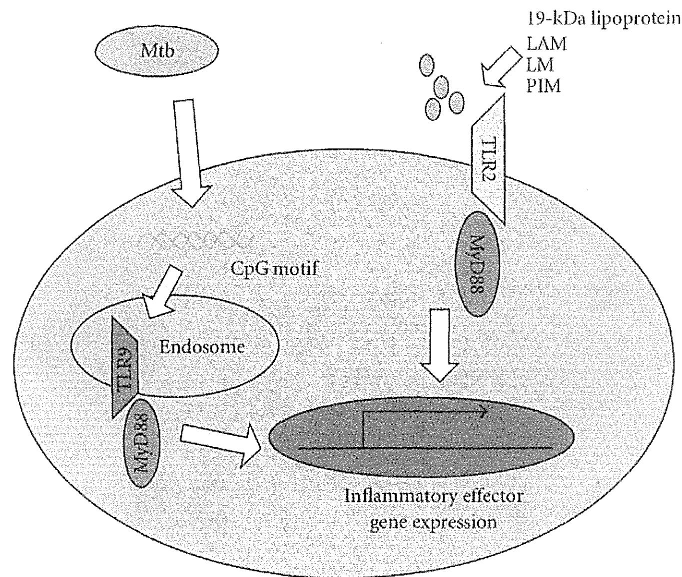

Figure 1: Recognition of mycobacteria by Toll-like receptors. TLR2 recognizes several mycobacterial-derived components. TLR9 recognizes mycobacterial DNA including the CpG motif within endosomal compartments. TLR-dependent recognition of mycobacteria induces activation of signaling pathways via the adaptor molecule MyD88, leading to activation of gene expression.

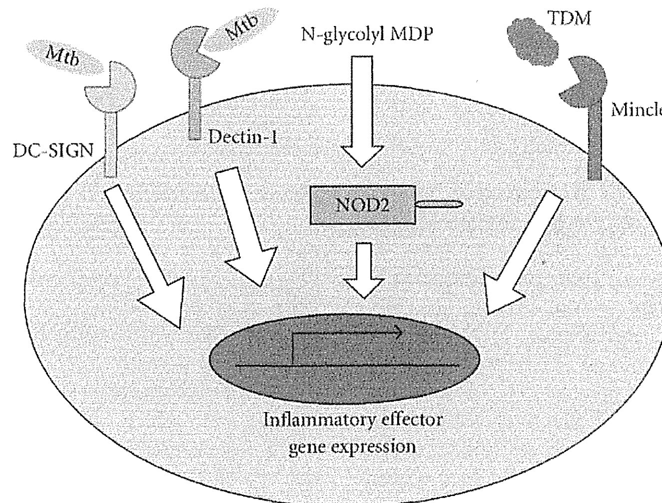

Figure 2: Recognition of mycobacteria by pattern recognition receptors. Several pattern recognition receptors, such as NOD-like receptors and C-type lectin receptors, mediate the TLR-independent recognition of mycobacteria. NOD2, a member of NOD-like receptors, recognizes mycobacterial N-glycolyl MDP within the cytoplasm. DC-SIGN and dectin-1 are members of C-type lectin receptors, which are implicated in the recognition of mycobacteria. In addition, Mincle has been shown to recognize TDM (a mycobacterial cell wall glycolipid).

4. Effectors for Mycobacterial Killing

The recognition of mycobacteria by several PRRs induces the expression of several genes that mediate host defense (Figure 3). Among these gene products, vitamin D receptor (VDR) and Cyp27b1, a 25-hydroxyvitamin D_3 1-α-hydroxylase that catalyzes inactive provitamin D into the bioactive form of vitamin D (1, 25 $(OH)_2D_3$), have been shown to be induced by TLR2 ligands in human macrophages [41]. Stimulation of macrophages with 1, 25 $(OH)_2D_3$ induces the expression of the antimicrobial peptide cathelicidin, and thereby enhances the antimycobacterial killing activity [42]. In addition to cathelicidin, the small cationic antimicrobial peptide defensin mediates innate immune responses to Mtb [43, 44]. Experimental infection of the lung epithelial cell line A549 with Mtb strongly induces production of human β-defensin HBD-2, which leads to Mtb killing [43]. HBD-2 expression has also been shown to be induced by TLR2 [45].

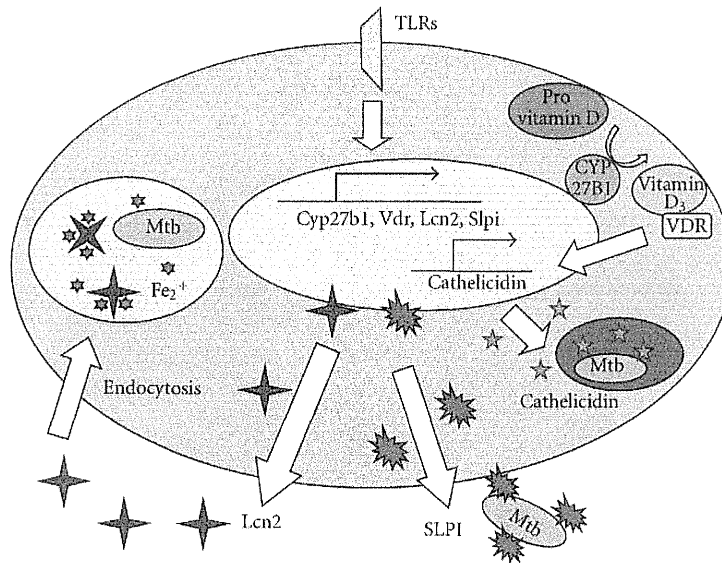

FIGURE 3: TLR-dependent innate response to mycobacteria. Several TLR-dependent gene products mediate innate immune responses to mycobacteria. Mycobacterial stimulation of TLR2 induces expression of Cyp27b1 and vitamin D receptor (VDR), both of which are involved in vitamin D_3-dependent induction of cathelicidin which directly kills mycobacteria. TLR-dependent induction of SLPI mediates disruption of the mycobacterial cell wall. Lcn2, which is also induced by TLR stimulation, is internalized into the alveolar epithelial cells and inhibits mycobacterial growth by sequestering iron uptake.

Gene expression analyses of the lung of mycobacteria-infected mice have identified several TLR-dependent genes that are involved in innate immune responses during mycobacterial infection. These genes include *Slpi*, encoding secretory leukocyte protease inhibitor (SLPI), and *Lcn2*, encoding lipocalin 2 (Lcn2). SLPI is a secreted protein composed of two cysteine-rich whey acidic protein (WAP) domains [46–48]. SLPI was named after its presence in secretions and its function as a serine protease inhibitor. SLPI was originally shown to mediate wound healing [49, 50]. SLPI is produced by bronchial and alveolar epithelial cells as well as alveolar macrophages and is secreted into the alveolar space at the early phase of mycobacterial respiratory infections. Recombinant mouse SLPI effectively inhibits the *in vitro* growth of BCG and Mtb through disruption of the mycobacterial cell wall structure. Cationic residues within the WAP domains of SLPI are essential for the disruption of mycobacterial cell walls. Moreover, SLPI-deficient mice are highly susceptible to mycobacterial infection [51]. The mechanism by which SLPI attaches to the membrane of mycobacteria has been elucidated. SLPI recognizes mannan-capped lipoarabinomannans and phosphatidylinositol mannoside, which are conserved in mycobacteria. Thus, SLPI might act as a PRR in order to bind to the mycobacterial membrane [52].

Lcn2 (also known as neutrophil gelatinase-associated lipocalin, 24p3, or siderocalin) was originally identified in the granules of human neutrophils. Lcn2 is a member of the lipocalin protein family and able to bind to small hydrophobic molecules, siderophore. It is a bacterial molecule made in iron-limited environment and facilitates iron uptake by bacteria [53–58]. The expression of Lcn2 is increased in macrophages of LPS-treated mice [59]. In addition, it is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 inhibits *in vitro* growth of Mtb by binding the mycobacterial siderophore carboxymycobactin, thereby sequestering iron uptake. Moreover, Lcn2-deficient mice are highly susceptible to intratracheal infection with Mtb. Lcn2 is internalized into alveolar epithelial cells by endocytosis and colocalized with mycobacteria within the cells. Therefore, Lcn2 presumably sequesters iron uptake of mycobacteria within epithelial cells and thereby inhibits their intracellular growth. Within macrophages, the endocytosed Lcn2 and mycobacteria show distinct patterns of subcellular localization, which might allow growth of mycobacteria within macrophages [60]. Thus, Lcn2, which is secreted into the alveolar space during the early phase of mycobacterial infection, is endocytosed into alveolar epithelial cells, thereby inhibiting mycobacterial growth [61].

5. Autophagy in Mycobacterial Infection

Phagocytosis of myobacteria and PRR-dependent recognition of mycobacteria activate several effector functions in macrophages (Figure 4). Maturation of phagosomes is a crucial step in the elimination of intracellular bacteria. The natural-resistance-associated macrophage protein (Nramp1), which is encoded by Slc11a1, is thought to mediate transportation of divalent cations in the phagosomal membrane and thereby sequesters iron (Fe^{2+}) from mycobacteria to enhance bacterial killing by macrophages [62]. Polymorphisms of the *SLC11A1* gene have been associated with susceptibility to several infectious diseases,

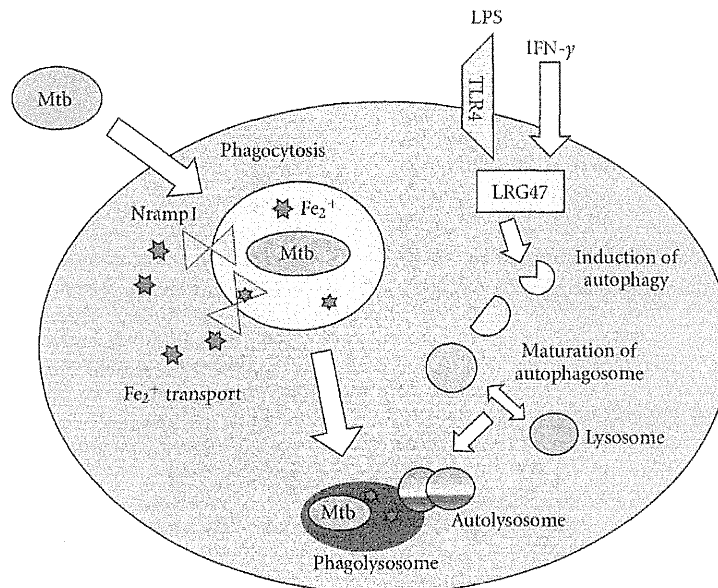

FIGURE 4: Effectors that mediate mycobacterial killing in macrophages. Macrophages eliminate invading mycobacteria by activating several effector functions, such as phagosomes and autophagy. Nramp1 is expressed in the phagosomal membrane and presumably mediates mycobacterial killing by sequestering iron uptake. IFN-γ and the TLR4 ligand induce expression of LRG47, which in turn stimulates autophagy in macrophages. Autophagy is responsible for mycobacterial killing by promoting fusion of mycobacterial phagosomes to lysosomes.

including tuberculosis [63, 64]. However, *in vivo* studies have shown that Nramp1-deficient mice are not more susceptible than wild-type mice to infection with virulent Mtb [65]. Thus, the role of Nramp1 in mycobacterial infection is still controversial. This might be due to the presence of other killing mechanisms for mycobacteria in macrophages. Indeed, autophagy has recently been shown to be involved in host defense against several intracellular pathogens that reside within phagosomes [66]. Autophagy was originally identified as a homeostatic mechanism for the catabolic reaction of cellular constitutes [67, 68]. It has been demonstrated that autophagy mediates innate immune responses against mycobacteria by promoting phagolysosomal maturation within macrophages [69, 70]. Autophagy is induced by IFN-γ-dependent induction of a member of the immunity-related p47 guanosine triphosphatases (IRG) family, LRG47 (also known as Irgm1) in murine macrophages [69]. The importance of LRG47 in resistance to Mtb infection was demonstrated in LRG47-deficient mice, which show high susceptibility to infection [71]. A subsequent study demonstrated that stimulation of macrophages with the TLR4 ligand LPS leads to the MyD88-independent induction of autophagy, which enhances mycobacterial colocalization with the autophagosomes. Since LPS stimulation induces expression of LRG47, the TLR signaling establishes a close relationship between innate immunity and autophagy in mycobacterial infection [72]. In humans, the most equivalent gene to murine Irgm1 is IRGM. IRGM has also been implicated in the induction of autophagy in mycobacteria-infected human macrophages [73]. Irgm1 has been shown to associate with the mycobacterial phagosome by interacting with phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P(2)) and PtdIns(3,4,5)P(3) [74]. The connection of the IRG family of proteins with autophagy has been further demonstrated in an alternative intracellular infection model. In this study, Irgm3 (also known as IGTP) has been implicated in autophagy induction in macrophages infected with *Toxoplasma gondii* [75].

p62 (also called A170 or SQSTM1) directly binds to cytosolic polyubiquitinated proteins and thereby induces their autophagic clearance [76, 77]. It has also been shown that p62 targets intracellular *Salmonella typhimurium* decorated by ubiquitinated proteins to induce autophagy [78]. In the case of mycobacteria residing in the phagosome, p62 delivers cytosolic ubiquitinated proteins to autophagolysosomes where they are proteolytically processed to products that are able to kill mycobacteria [79]. In accordance with this finding, it has been shown that mycobacterial killing by ubiquitin-derived peptides is enhanced by autophagy [80].

As described above, 1, 25 $(OH)_2D_3$ mediates antimycobacterial activity via induction of cathelicidin. A recent report demonstrated that 1, 25 $(OH)_2D_3$-mediated expression of cathelicidin induces autophagy [81]. Thus, several innate immune effectors are closely interacted.

6. Human Genetics in Tuberculosis

In addition to the intensive studies using murine models, considerable advances have been made in our understanding of the susceptibility to Mtb infection in humans through the identification of mutations and polymorphisms of

innate immunity-related genes in tuberculosis patients. As described above, polymorphisms of the *SLC11A1* gene are associated with tuberculosis. Subsequent studies identified a significant distinction between tuberculosis patients and healthy controls in *TLR2* Arg753Gln polymorphism genotype, indicating that the *TLR2* polymorphism influences the susceptibility of Mtb infection [82]. *VDR* polymorphisms have also been implicated in the susceptibility of Mtb infection [83]. These studies suggest that several genes, which have been revealed to be critical in innate responses in mouse models of Mtb infection, regulate Mtb infection in humans.

7. Conclusion

Since the discovery of TLRs at the end of the 20th century, rapid advances have been made in our understanding of the mechanisms for activation of innate immunity. Accordingly, innate immunity has been revealed to have a pivotal role in host defense against mycobacteria. The TLR-independent mechanisms for the innate immune response to mycobacteria have also been elucidated. The emergence of multidrug-resistant Mtb is now a major public health problem all over the world. In this context, it is highly critical to develop a new strategy for the treatment of Mtb-infected patients that supplements the conventional antimycobacterial chemotherapeutic drugs. More precise understanding of the innate immune response to Mtb will pave the way for the development of an effective drug that targets the host innate immunity for the treatment of tuberculosis.

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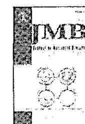

Structural Insights into the Novel Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate Phosphorylase from *Mycobacterium tuberculosis* H37Rv

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Rv2613c is a diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) phosphorylase from *Mycobacterium tuberculosis* H37Rv. Sequence analysis suggests that Rv2613c belongs to the histidine triad (HIT) motif superfamily, which includes HIT family diadenosine polyphosphate (Ap_nA) hydrolases and Ap_4A phosphorylases. However, the amino acid sequence of Rv2613c is more similar to that of HIT family Ap_nA hydrolases than to that of typical Ap_4A phosphorylases. Here, we report the crystal structure of Rv2613c, which is the first structure of a protein with Ap_nA phosphorylase activity, and characterized the structural basis of its catalytic activity. Our results showed that the structure of Rv2613c is similar to those of other HIT superfamily proteins. However, Asn139, Gly146, and Ser147 in the active site of Rv2613c replace the corresponding Gln, Gln, and Thr residues that are normally found in HIT family Ap_nA hydrolases. Furthermore, analyses of Rv2613c mutants revealed that Asn139, Gly146, and Ser147 are important active-site residues and that Asn139 has a critical role in catalysis. The position of Gly146 might influence the phosphorylase activity. In addition, the tetrameric structure of Rv2613c and the presence of Trp160 might be essential for the formation of the Ap_4A binding site. These structural insights into Rv2613c may facilitate the development of novel structure-based inhibitors for treating tuberculosis.

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Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). Every year, approximately 1.7 million people die from TB worldwide, and more than 8.9 million people are newly infected with *M. tuberculosis*.[1] Furthermore, multidrug-resistant TB and extensively drug-resistant TB have become serious problems recently.[2] As a result, novel anti-TB drugs are needed urgently. To facilitate the structure-based design of new anti-TB drugs, our group has investigated the structure–function relationships of *M. tuberculosis* proteins such as the NAD kinase–NAD complex[3] and Rv2613c, which is a novel diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) phosphorylase (EC 2.7.7.53).[4] The *Rv2613c* gene was previously shown to be an essential gene in *M. tuberculosis* H37Rv,[5] and Rv2613c was recognized as a target for new anti-TB drugs by *in silico* analysis.[6] Therefore, we have determined the

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Abbreviations used: Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; HIT, histidine triad; Ap_nA, diadenosine polyphosphate; TB, tuberculosis; SAD, single-wavelength anomalous dispersion; PDB, Protein Data Bank; AMW, adenosine monotungstate; IB2, P^1,P^2-methylene-P^3-thio-diadenosine triphosphate; SeMet, selenomethionine.

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