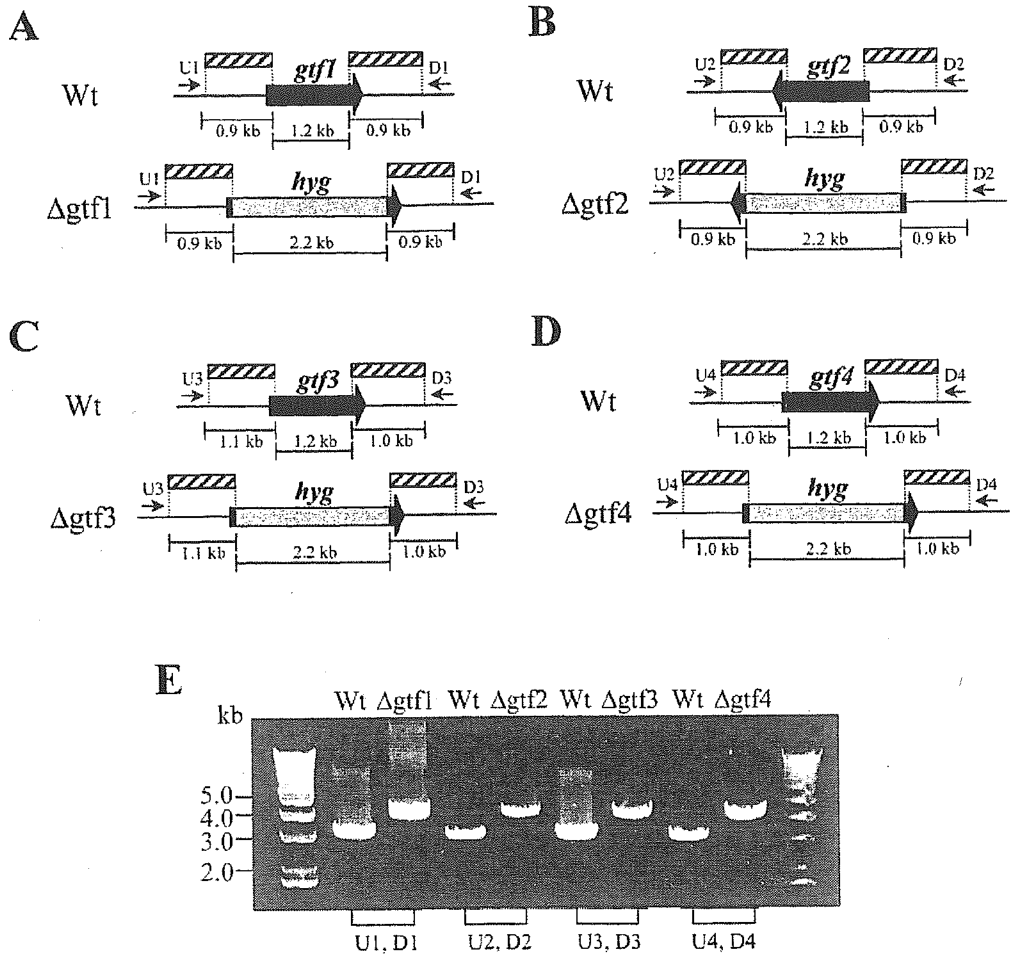

FIG. 1. Generation of *gtf* gene disruptants. (A to D) Schematic diagram of each *gtf* region on the chromosome of the wild-type *M. smegmatis* mc^{2}155 strain (Wt) and its gene disruptants Δgtf1, Δgtf2, Δgtf3, and Δgtf4. The shaded boxes indicate the regions included in recombinant phage for gene disruption. The black arrows represent the coding region of each *gtf* gene. The gray boxes represent the hygromycin resistance cassette (*hyg*). The primers used for PCR analysis are indicated by small arrows. (E) PCR analyses of the wild type and each disruptant using the primers indicated above.

alditol acetates were separated and analyzed by gas chromatography–mass spectrometry (GC/MS) on TRACE DSQ (Thermo electron) instrument equipped with an SP-2380 column (SUPELCO) using helium gas. The temperature program was from 52 to 172°C at 40°C/min and then 172 to 250°C at 3°C/min.

MALDI-TOF/MS analysis. To determine the total mass of the purified deacylated GPLs, matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectra (in the positive mode) were acquired on a QSTAR XL (Applied Biosystems) with a pulse laser emitting at 337 nm. Samples mixed with 2,5-dihydroxybenzoic acid as the matrix were analyzed in the reflectron mode with an accelerating voltage operating in positive ion mode of 20 kV.

RESULTS

Disruption of *gtf1*, *gtf2*, *gtf3*, and *gtf4* by allelic exchange. Four genes showing high similarity to the *rtfA* gene, involved in GPL biosynthesis of *M. avium*, were identified for the *M. smegmatis* mc^{2}155 strain (12). The homologies of their corresponding amino acid sequences with that of RtfA were around 60%. Three genes were found in the GPL biosynthetic gene cluster, namely, *gtf1*, *gtf2*, and *gtf3* (GenBank accession no. AY138899.1) (16), whereas one gene, designated *gtf4* (TIGR database no. 4839918 to 4841162), was located far from the other three genes. To examine whether these genes are responsible for GPL biosynthesis, we generated four gene disruptants, designated Δgtf1, Δgtf2, Δgtf3, and Δgtf4, using the specialized transducing mycobacteriophage containing the entire open reading frame, replacing with the hygromycin resistance cassette (2). For confirmation of the gene disruption, PCR analysis was performed on chromosomal DNA from each disruptant. To avoid the amplification of disrupted sequences derived from residual mycobacteriophage, we designed and used the primers located outside the sequences included in each mycobacteriophage as shown in Fig. 1A to D. As expected, around 3.0-kb fragments were amplified from mc^{2}155 (wild type), whereas around 4.0-kb fragments were amplified from each disruptant, because most of the *gtf* coding region (1.2 kb) was replaced by the hygromycin resistance cassette (2.2 kb) (Fig. 1E). These results demonstrated that allelic exchanges involving replacement of the *gtf* genes with the disrupted constructs have been successful.

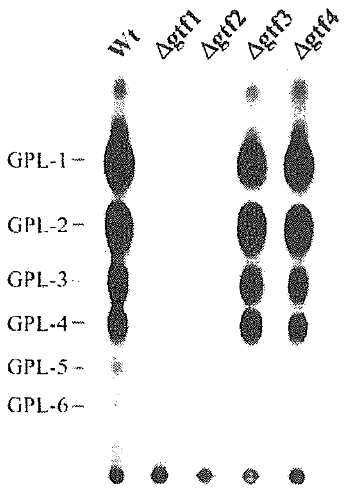

FIG. 2. TLC analyses of crude GPL extracts from the *M. smegmatis* mc^{2}155 strain (Wt) and its gene disruptants. The total lipid fraction after mild alkaline hydrolysis was spotted on plates and developed in $CHCl_3$-CH_3OH (9:1 [vol/vol]). GPLs were visualized by spraying with 10% H_2SO_4 and charring. Each total lipid fraction was extracted from an equal weight of harvested cells.

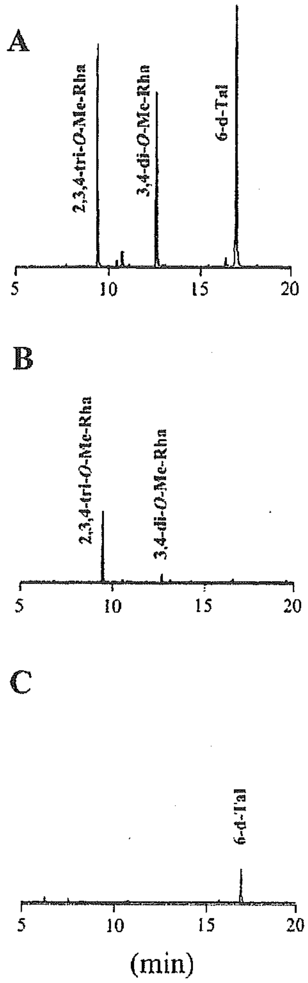

FIG. 3. GC/MS analyses of alditol acetates of sugars released from crude GPLs. GPLs were extracted from *M. smegmatis* strains: (A) mc^{2}155 strain, (B) Δgtf1, and (C) Δgtf2. Alditol acetate derivatives were prepared from the total lipid fraction after mild alkaline hydrolysis, which was extracted from an equal weight of harvested cells.

TLC analysis of gene disruptants. To investigate the effects of the mutation in each *gtf* gene, we examined GPL production of four gene disruptants. TLC analyses of total lipid fraction after mild alkaline hydrolysis revealed that wild-type mc^{2}155 mainly produced six components, designated GPL-1 to -6, whereas Δgtf1 and Δgtf2 lacked all six components and Δgtf3 lacked two minor ones (GPL-5 and GPL-6) found in the wild type (Fig. 2). In contrast, no differences in TLC profile were observed between Δgtf4 and the wild type (Fig. 2).

Characterization of Δgtf1 and Δgtf2. In Δgtf1 and Δgtf2, the TLC analyses showed that six GPL components contained in the wild type had disappeared. On the other hand, there is the possibility that both disruptants contained GPL derivatives which are structurally incomplete and hard to be detected by TLC analyses. To characterize the sugars included in GPL derivatives from both disruptants and to compare with the wild type, each total lipid fraction after mild alkaline hydrolysis was hydrolyzed, and the released monosaccharides as their alditol acetates were examined by GC/MS. Figure 3 shows that the profiles of the wild type gave three peaks corresponding to 2,3,4-tri-*O*-Me-Rha, 3,4-di-*O*-Me-Rha, and 6-d-Tal (Fig. 3A), whereas Δgtf1 lacked 6-d-Tal (Fig. 3B) and Δgtf2 lacked 3,4-di-*O*-Me-Rha and 2,3,4-tri-*O*-Me-Rha (Fig. 3C). Complementation of both disruptants with each respective gene restored the TLC profile of GPLs to that observed for the wild type (not shown). Therefore, the *gtf1* and *gtf2* genes are found to be responsible for transferring the 6-d-Tal and Rha residues, respectively.

Structural determination of GPL-5 and GPL-6 for characterization of Δgtf3. The TLC profile of Δgtf3 showed that two spots (GPL-5 and GPL-6) disappeared (Fig. 2). To reveal the biosynthetic role of the *gtf3* gene, GPL-5 and GPL-6 were purified from mc^{2}155 and their structures were determined. GC/MS analyses showed that GPL-5 and GPL-6 contained 6-d-Tal and 3,4-di-*O*-Me-Rha, which were identified as sugar moieties of GPL-3 and GPL-4 (Fig. 4A). However, an extra sugar, 3-*O*-Me-Rha, was also detected (Fig. 4A). MALDI-TOF/MS analyses revealed that the main molecular ions of GPL-5 (*m/z* 1,333.8) and GPL-6 (*m/z* 1,319.8) were 160 mass units higher than those of GPL-3 (*m/z* 1,173.9) and GPL-4 (*m/z* 1,159.9), respectively (Fig. 4B). These results confirmed the presence of 3-*O*-Me-Rha in GPL-5 and GPL-6 and also suggested that 3-*O*-Me-Rha was further added to GPL-3 and GPL-4. Although GPL-5 and GPL-6 contained same three sugars, the spectra showed that the main molecular ion of GPL-5 (*m/z* 1,333.8) was 14 mass units higher than that of GPL-6 (*m/z* 1,319.8) (Fig. 4Ba and 4Bb). These differences in total mass may be due to O methylation of fatty acid as observed in structures of GPL-1 and GPL-3, suggesting that fatty acid of GPL-5 was O methylated like GPL-1 and GPL-3 (16). To investigate the sugar linked to D-*allo*-Thr of the fatty acyl-tetrapeptide core, GPL-5 and GPL-6 were subjected to β-elimination treatment. The main ion peaks of treated GPL-5 and

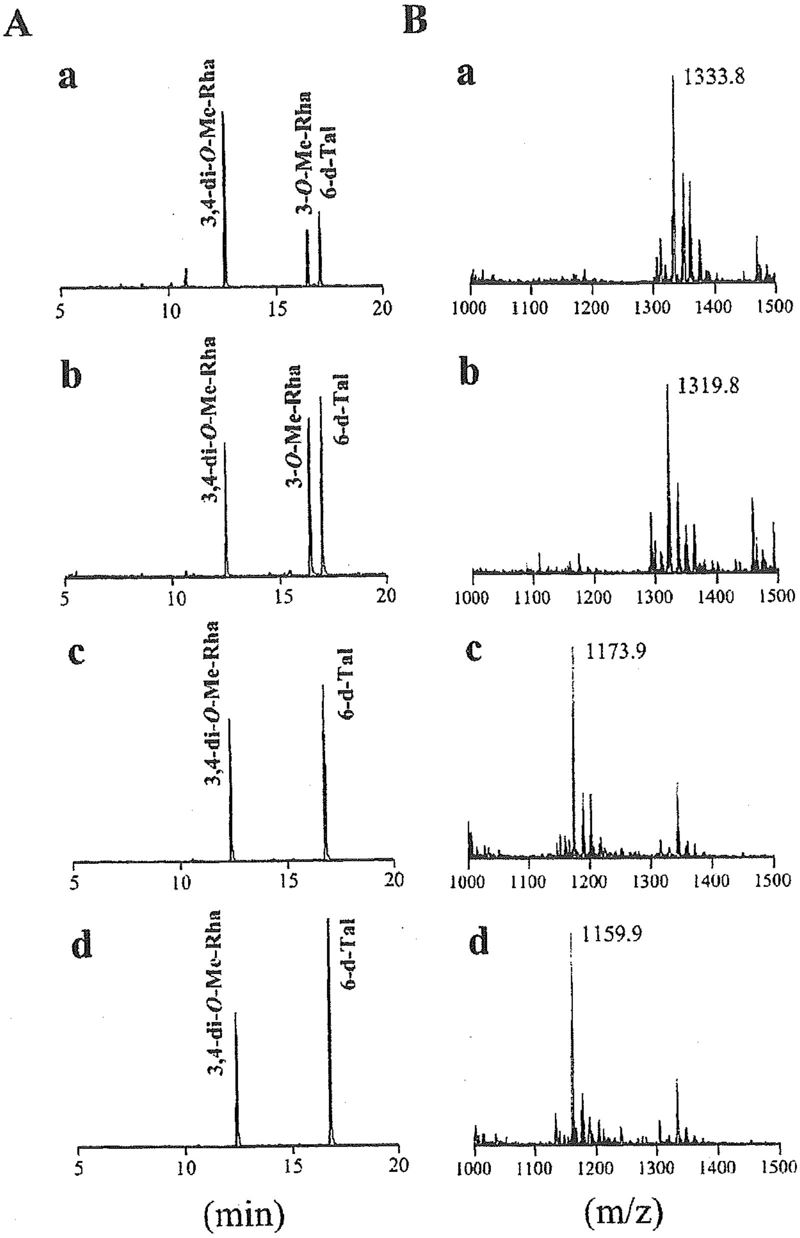

FIG. 4. Biochemical characterization of GPL-5 (a), GPL-6 (b), GPL-3 (c), and GPL-4 (d). (A) GC/MS analysis of alditol acetates of sugars released from each purified GPL. (B) MALDI-TOF/MS analysis of total molecular mass of each purified GPLs. (C) MALDI-TOF/MS analysis of total molecular mass of purified GPL-5 (a) and GPL-6 (b), which were subjected to β-elimination.

GPL-6 were *m/z* 1,171.7 and 1,157.7, respectively, which resulted in the loss of total mass of 162, suggesting that 6-d-Tal was linked to the position of D-*allo*-Thr (Fig. 4C). The linkage position of the sugars linked to the L-alaninol site of GPL-5 and GPL-6 was then determined by GC/MS analyses followed by perdeuteriomethylation. As shown in Fig. 5A, the GC profiles of alditol acetates from perdeuteriomethylated GPL-5 gave three peaks corresponding to 6-d-Tal, 3-*O*-Me-Rha, and 3,4-di-*O*-Me-Rha. The characteristic spectra of 3-*O*-Me-Rha and 3,4-di-*O*-Me-Rha, which are predicted to be linked to L-alaninol, are illustrated in Fig. 5B and C, respectively. The spectrum of 3-*O*-Me-Rha gave fragment ions at *m/z* 121, 134, and 165, which represent the presence of a deuteriomethyl group at positions C-2 and C-4. In contrast, no deuteriomethyl group was observed in 3,4-di-*O*-Me-Rha, whose C-2 position was acetylated, supported by the detection of fragment ions at *m/z* 131 and 190. The results from GC/MS analyses of perdeuteriomethylated GPL-6 were the same as those for GPL-5 (not shown). These observations demonstrated that GPL-5 and GPL-6 have the same sugar moieties, which are 6-d-Tal at

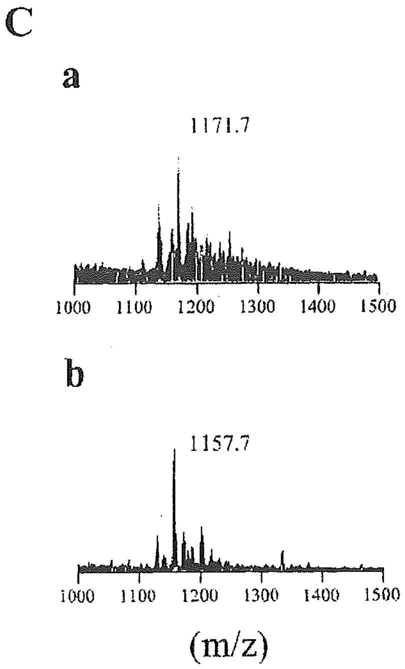

FIG. 4—*Continued.*

D-*allo*-Thr and 3-*O*-Me-rhamnosyl-(1→2)-3,4-di-*O*-Me-Rha at L-alaninol, indicating that 3-*O*-Me-Rha was linked to GPL-3 and GPL-4 (Fig. 6).

Overexpression of *gtf1*, *gtf2*, *gtf3*, and *gtf4* in *M. smegmatis* mc^{2}155. To investigate the effects of overexpression of each gene on GPL biosynthesis, we constructed four *gtf*-overexpressed strains in wild-type mc^{2}155 and compared the profile of total GPLs by TLC analyses. The results showed that the profiles of Wt/pMVgtf1, Wt/pMVgtf2, and Wt/pMVgtf4 were the same as that of Wt/pMV261, whereas Wt/pMVgtf3 produced two major compounds whose biochemical data corresponded to those of GPL-5 and GPL-6 (Fig. 7).

Characterization of *M. avium gtfA* and *gtfB*. We showed that both *M. smegmatis gtf1* and *gtf2* were responsible for glycosylation of the fatty acyl-tetrapeptide core. Comparison of the genome sequences encompassing the GPL biosynthetic gene cluster among several species of *M. avium* have shown that *gtfA* and *gtfB* (GenBank accession no. AF125999.1) are very similar to *M. smegmatis gtf1* and *gtf2*, respectively, in the corresponding putative amino acid sequences and might contribute to the glycosylation of the fatty acyl-tetrapeptide core (13). However, the function of each gene has not been thoroughly analyzed (13). Therefore, to confirm the role of *gtfA* and *gtfB*, we complemented Δgtf1 and Δgtf2 with the *gtf* expression vectors carrying *gtfA* (pMVgtfA) and *gtfB* (pMVgtfB). As shown in Fig. 8, TLC analyses revealed that *gtfA* and *gtfB* restored the production of wild-type GPLs in Δgtf1 and Δgtf2, respectively, whereas transformants with reverse vectors (Δgtf1/pMVgtfB and Δgtf2/pMVgtfA) did not produce wild-type GPLs. These results suggested that the function of *M. avium gtfA* and *gtfB* is the same as that of *M. smegmatis gtf1* and *gtf2*, respectively.

DISCUSSION

It has been shown that the *rtfA* gene of *M. avium* encodes a rhamnosyltransferase which synthesizes ssGPLs, while other genes involved in the glycosylation of the fatty acyl-tetrapeptide core remain unknown (12). In this study, we focused on the four genes of *M. smegmatis*, which show high similarity to *rtfA*, and generated their disruptants to characterize the role in the GPL biosynthesis.

In the early glycosylation steps of the fatty acyl-tetrapeptide core, we observed that the disruption of *gtf1* abolished the whole GPLs and led to the accumulation of *O*-Me-Rha derivatives without 6-d-Tal in Δgtf1 (Fig. 3B). Thus, we propose that the *gtf1* gene product catalyzes the transfer of 6-d-Tal to fatty acyl-tetrapeptide core. It is reported that the *M. avium* 104Rg strain, which has a spontaneous deletion in the genome region including *gtfA*, also accumulated *O*-methylated and nonmethylated Rha without 6-d-Tal (13, 30). This property is directly supported by our result that the *gtfA* could complement Δgtf1 (Fig. 8). However, *M. avium* 104Rg mainly contained nonmethylated Rha, whereas Δgtf1 derived from *M. smegmatis* mc^{2}155 contained only *O*-Me-Rha. These different observations may be due to differences in the substrate specificity of methyltransferase, because 2,3,4-tri-*O*-Me-Rha was present in *M. smegmatis* mc^{2}155 but was not identified in *M. avium* species (8, 25).

When the *gtf2* gene was disrupted, we detected 6-d-Tal without Rha derivatives in GC/MS analysis, which demonstrates that the *gtf2* gene contributes to the transfer of Rha to the fatty acyl-tetrapeptide core (Fig. 3C). In addition, complementation revealed that the *gtfB* gene of *M. avium* had the same function as *gtf2* (Fig. 8). In the previous studies of GPL biosyntheses, the mutant accumulating 6-d-Tal-containing derivatives without the Rha residue have not been isolated from GPL-producing species so far. Our results directly indicated for the first time that 6-d-Tal-containing derivatives could be an intermediate for the biosynthetic pathways of GPLs.

As for the order of glycosylation steps regulated by *gtf1* and *gtf2*, we cannot determine which step takes place earlier, since both disruptants accumulated the intermediates having different component (Fig. 3B and C). For *M. avium* serovar 2, Eckstein et al. proposed a pathway in which the transfer of the Rha residue to the fatty acyl-tetrapeptide core occurred prior to that of 6-d-Tal, because a mutant strain, 104Rg, having the *gtfA* region deleted, accumulated the fatty acyl-tetrapeptide core with only the Rha residue (13). However, our results lead to the interesting possibility that there are two alternative glycosylation pathways for the formation of nsGPLs (Fig. 9). If the glycosylation should occur in a single pathway, we would expect the accumulation of a nonglycosylated intermediate in either of the disruptants, because one of the genes, *gtf1* or *gtf2*, would be responsible for the first step of glycosylation converting the fatty acyl-tetrapeptide core to a glycosylated intermediate. Thus, the detection of glycosylated intermediates from both Δgtf1 and Δgtf2 suggests that (i) the fatty acyl-tetrapeptide core could be the substrate for both Gtf1 and Gtf2 and (ii) the glycosylated intermediates could also be the substrates for both Gtf1 and Gtf2. We prove here that Gtf1 and Gtf2 have broad substrate specificity and propose that the fatty acyl-tetrapeptide core is glycosylated by Gtf1 and Gtf2 at the same

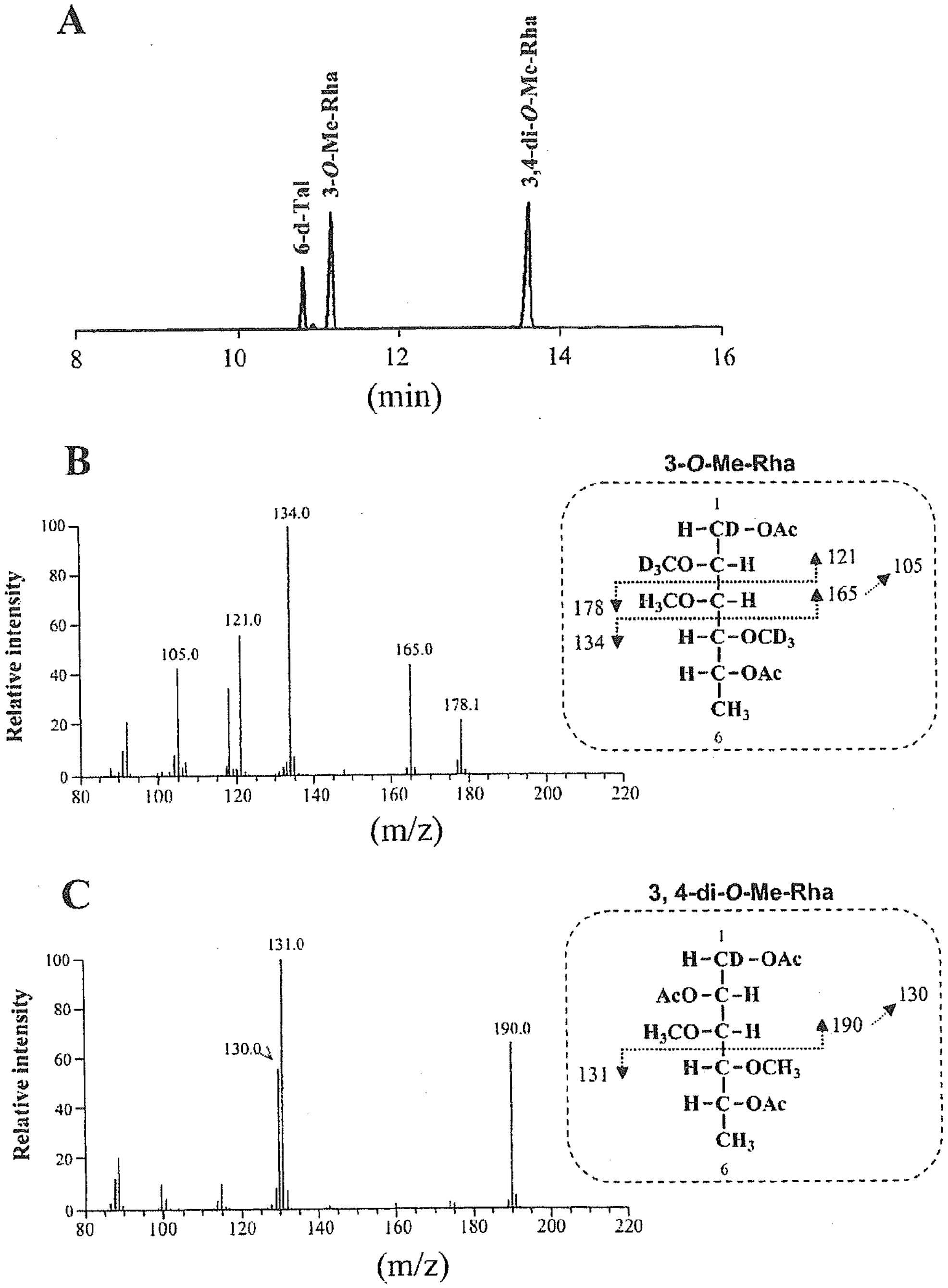

FIG. 5. GC/MS analysis of alditol acetates of sugars released from perdeuteriomethylated GPL-5. (A) GC profile. (B) Mass spectrum and fragment ion assignment corresponding to 3-*O*-Me-Rha. (C) Mass spectrum of fragment ion assignment corresponding to 3,4-di-*O*-Me-Rha.

time and then converted to the nsGPLs having both 6-d-Tal and *O*-Me-Rha via cross-glycosylations (Fig. 9).

Structural determination of GPL-5 and GPL-6 revealed that L-alaninol of the fatty acyl-tetrapeptide core was glycosylated with disaccharide (3-*O*-Me- and 3,4-di-*O*-Me-Rha), which was structurally different from GPLs including GPL-1 to -4 and ssGPLs (Fig. 6). However, it is reported that *M. fortuitum* complex produced GPLs which are glycosylated as in GPL-5 and GPL-6 as major components (19, 20). Therefore, these observations suggest that this type of glycosylation is not specific for *M. smegmatis*. GC/MS analyses of GPL-5 and GPL-6 indicated the presence of 3-*O*-Me-Rha in addition to 3,4-di-*O*-Me-Rha, and analyses of perdeuteriomethylated GPL-5 and GPL-6 showed that position C-1 of 3-*O*-Me-Rha is linked to position C-2 of 3,4-di-*O*-Me-Rha. Recent studies have shown that *M. smegmatis* mc^{2}155 newly produces two polar GPLs

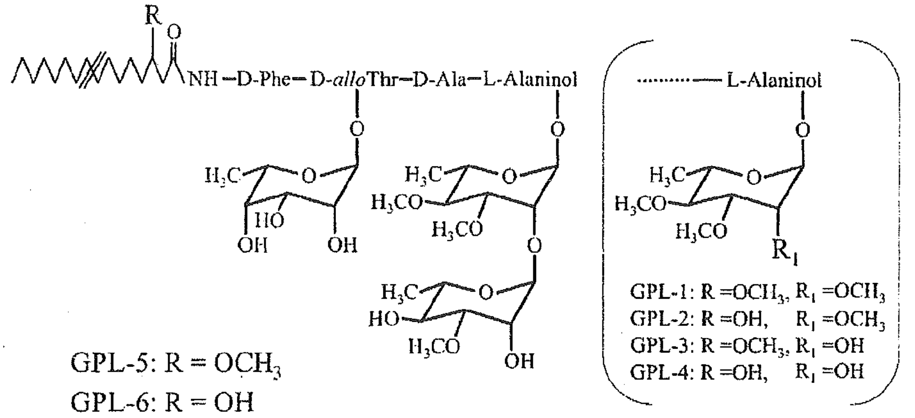
NH—D-Phe—D-alloThr—D-Ala—L-Alaninol
L-Alaninol
GPL-1: R =OCH$_3$, R$_1$ =OCH$_3$
GPL-2: R =OH, R$_1$ =OCH$_3$
GPL-3: R =OCH$_3$, R$_1$ =OH
GPL-4: R =OH, R$_1$ =OH
GPL-5: R = OCH$_3$
GPL-6: R = OH

FIG. 6. Proposed structures of GPL-5 and GPL-6. Figure in parentheses shows the structure of GPL-1, GPL-2, GPL-3, and GPL-4, which were characterized in previous studies (10, 16, 25).

which contained two units of 3,4-di-*O*-Me-Rha at L-alaninol of the fatty acyl-tetrapeptide core with no 3-*O*-Me-Rha at any other position when cultured in carbon-limited medium (23, 24). However, the reason for not being able to detect 3-*O*-Me-Rha remains unknown.

In the *gtf3*-overexpressed strain Wt/pMVgtf3, the productivities of GPL-5 and GPL-6 were much higher than those of other GPLs (Fig. 7). So, we can speculate that the expression level of *gtf3* is usually repressed and could be regulated by some environmental factors, such as the nutrient condition or the gene encoding sigma factor (23, 24). GC/MS analyses showed that GPL-5 and GPL-6 have the structures in which 3-*O*-Me-Rha is linked to GPL-3 and GPL-4. These results suggest that GPL-3 and GPL-4 could be the precursors of GPL-5 and GPL-6, respectively, and in Wt/pMVgtf3, overexpression of *gtf3* resulted in 2-*O*-rhamnosylation of 3,4-di-*O*-Me-Rha in GPL-3 and GPL-4 instead of 2-*O*-methylation for converting to GPL-1 and GPL-2, so that GPL-5 and GPL-6 were synthesized.

Figure 9 represents proposed glycosylation steps related to *M. smegmatis* and *M. avium*. We showed that the functions of *gtf1* and *gtf2* corresponded to those of *gtfA* and *gtfB*, respectively. This finding demonstrates that the biosynthetic pathway for nsGPLs, which is the glycosylation of the fatty acyl-tetrapeptide core with the 6-d-Tal and Rha residues, is common between *M. smegmatis* and *M. avium*. Moreover, the biochemical characterization of Δgtf2 and Δgtf1 suggested that the glycosylation pathways for nsGPLs might not be stringent. On the other hand, it has been shown that the *rtfA* gene of *M. avium* triggers the biosynthesis of ssGPLs by transfer of Rha to 6-d-Tal of nsGPLs (12). In *M. smegmatis*, our results indicated that the *gtf3* gene plays a role in synthesis of 3-*O*-Me-rhamno-

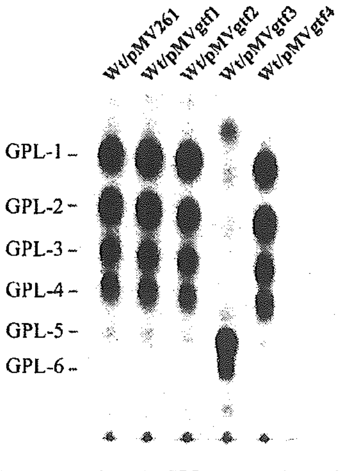
Wt/pMV261
Wt/pMVgtf1
Wt/pMVgtf2
Wt/pMVgtf3
Wt/pMVgtf4
GPL-1
GPL-2
GPL-3
GPL-4
GPL-5
GPL-6

FIG. 7. TLC analyses of crude GPL extracts from the *M. smegmatis* mc^{2}155 strain (Wt) transformed with *gtf* expression vectors. Total lipid fraction after mild alkaline hydrolysis was spotted on plates and developed in CHCl$_3$-CH$_3$OH (9:1 [vol/vol]). GPLs were visualized by spraying with 10% H$_2$SO$_4$ and charring. Each total lipid fraction was extracted from an equal weight of harvested cells.

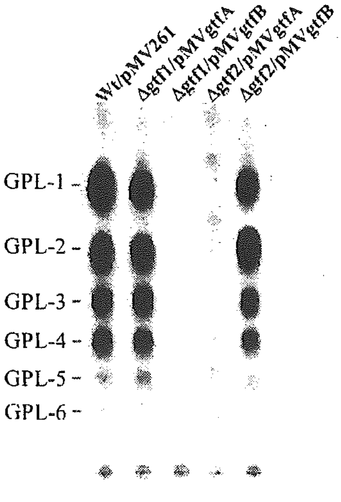
Wt/pMV261
Δgtf1/pMVgtfA
Δgtf1/pMVgtfB
Δgtf2/pMVgtfA
Δgtf2/pMVgtfB
GPL-1
GPL-2
GPL-3
GPL-4
GPL-5
GPL-6

FIG. 8. TLC analyses of crude GPL extracts from the *M. smegmatis* mc^{2}155 strain (Wt) and its gene disruptants transformed with *M. avium gtfA* and *gtfB*. Total lipid fraction after mild alkaline hydrolysis was spotted on plates and developed in CHCl$_3$-CH$_3$OH (9:1 [vol/vol]). GPLs were visualized by spraying with 10% H$_2$SO$_4$ and charring. Each total lipid fraction was extracted from an equal weight of harvested cells.

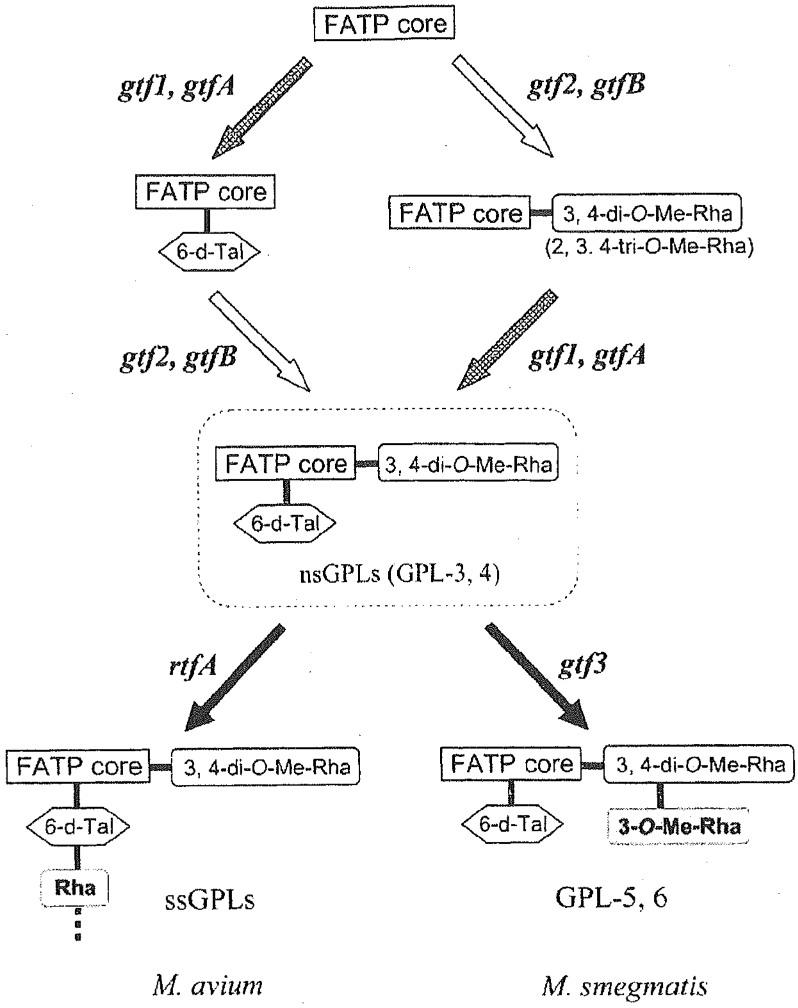

FIG. 9. Proposed biosynthetic pathways for GPLs of *M. smegmatis* and *M. avium*. FATP core, fatty acyl-tetrapeptide core.

syl-(1→2)-3,4-di-*O*-Me-Rha linked to L-alaninol of the fatty acyl-tetrapeptide core by transfer of an extra Rha residue to nsGPLs. Thus, the *rtfA* and *gtf3* genes have the ability to confer the biosynthetic differences between *M. avium* and *M. smegmatis*, suggesting that these genes may be responsible for the phylogenetic distinctions in the two species of mycobacteria.

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Identification of *Mycobacterium* species by comparative analysis of the *dnaA* gene

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Abstract

For the establishment of a diagnostic tool for mycobacterial species, a part of the *dnaA* gene was amplified and sequenced from clinically relevant 27 mycobacterial species as well as 49 clinical isolates. Sequence variability in the amplified segment of the *dnaA* gene allowed the differentiation of all species except for *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium microti*, which had identical sequences. Partial sequences of *dnaA* from clinical isolates belonging to three frequently isolated species revealed a very high intraspecies similarity, with a range of 96.0–100%. Based on the *dnaA* sequences, a species-specific primer set for *Mycobacterium kansasii* and *Mycobacterium gastri* was successfully designed for a simple loop-mediated isothermal amplification method. These results demonstrate that the variable sequences in the *dnaA* gene were species specific and were sufficient for the development of an accurate and rapid diagnosis of *Mycobacterium* species.

Introduction

Increasing reports of opportunistic infection by nontuberculous mycobacteria (NTM) in immunocompromised patients such as AIDS patients and elderly people are a matter of serious concern to public health (Horsburg, 1991; Montessori *et al.*, 1996; Primm *et al.*, 2004). The routine diagnosis of mycobacteriosis relies primarily on the detection of acid-fast-stained bacilli in the samples by microscopic observation, and the infecting mycobacterial species can be identified with conventional tests including observation of colony morphology and pigmentation, growth rate, and biochemical characteristics (Cernoch *et al.*, 1994; Metchock *et al.*, 1999). Disadvantages of this approach include the time taken to provide clinically relevant information. The clinician must initiate therapy for *Mycobacterium tuberculosis* against NTM infection several weeks before species identification (Montessori *et al.*, 1996), which may increase health care costs, and may reduce the social activity of the patients. Therefore rapid detection and identification of the species level of mycobacteria is required, both to decide whether measures are needed to prevent the spread of the disease and for adequate therapy (American Thoracic Society, 1997).

The mycobacterium species often implicated in NTM infection are *Mycobacterium avium–Mycobacterium intracellulare* complex (MAC), *Mycobacterium kansasii*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, and *Mycobacterium xenopi* (Wayne & Sramek, 1992; Metchock *et al.*, 1999; Primm *et al.*, 2004). *Mycobacterium gordonae*, *Mycobacterium gastri*, or most of the rapidly growing species are rarely pathogenic, but are often encountered as contaminant in clinical samples. Therefore, the discrimination of these species from pathogenic ones is an important diagnostic issue (Primm *et al.*, 2004).

Several studies have been conducted to develop rapid methods based on molecular technique for identifying mycobacterial species in recent years. The DNA sequences reported for such usage are those of 16S rRNA gene (Kirschner *et al.*, 1993; De Beenhouwer *et al.*, 1995; Cloud *et al.*, 2002), *recA* (Blackwood *et al.*, 2000), *rpoB* (Kim *et al.*, 1999), *gyrB* (Kasai *et al.*, 2000), *hsp65* (Plikaytis *et al.*, 1992; Brunello *et al.*, 2001), or 16S–23S internal transcribed spacer (ITS) (De Smet *et al.*, 1995; Roth *et al.*, 1998). The 16S rRNA gene and ITS-based methods are currently widely accepted as rapid and accurate for identifying mycobacteria (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Park *et al.*, 2000; Turenne *et al.*, 2001). However, some species have the same sequence or a very high similarity (Kim *et al.*, 1999; Kasai *et al.*, 2000). This fact indicates the need to develop more reliable and user-friendly molecule-based diagnostic tools.

Recently, Notomi *et al.* (2000) have reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficacy, and rapidity under isothermal conditions. The LAMP reaction requires a *Bst* DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize six distinct sequences on the target DNA, the specificity of which should be extremely high. The amplification products are stem-loop DNA structures with several inverted repeats of the target. The advantage of the LAMP method is that the reaction is performed under isothermal conditions of between 60 and 65 °C. As a result, it requires only simple and cost-effective reaction equipment. The LAMP method has emerged as a powerful tool to facilitate genetic testing for various infectious diseases (Enosawa *et al.*, 2003; Iwamoto *et al.*, 2003; Kuboki *et al.*, 2003; Ihira *et al.*, 2004; Parida *et al.*, 2004; Thai *et al.*, 2004).

The purpose of our work is to identify a species-specific region of *Mycobacterium* sp., and to develop a LAMP assay that can differentiate clinically relevant species.

Materials and methods

Bacterial strains and preparation of genomic DNA

The bacteria used in this study comprised 27 strains and 49 clinical isolates as shown in Table 1. All strains except for *Mycobacterium leprae* were cultured on 1% Ogawa medium (Nissui, Tokyo, Japan) at 37 °C. *Mycobacterium leprae* was prepared from infected nude mouse food pad (Shepard, 1960). Genomic DNA was extracted from mycobacterial strains as follows. Mycobacterial cells were resuspended in 1.8 mL of sterile phosphate-buffered saline (PBS) containing 0.1 mm diameter zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK). The mixture was beaded for 20 s with a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo, Japan), transferred to a 1.5 mL microcentrifuge tube, and the genomic DNA was purified with proteinase K treatment and phenol/chloroform extraction followed by ethanol precipitation, then suspended in 100 μL distilled water.

Table 1. *Mycobacterium* species and strains used in this study and results of the loop-mediated isothermal amplification assay

			Primer set	
Species	Strains	Accession number	Kan32	Gas583
Mycobacterium abscessus	JATA 63-01 (ATCC 19977)	AB087684	–	–
Mycobacterium africanum	KK 13-02 (ATCC 25420)	AB087685	–	–
Mycobacterium avium	JATA 51-01 (ATCC 25291)	AB087686	–	–
	Clinical isolate 22 strains			
Mycobacterium bovis	JATA 12-01 (ATCC 19210)	AB087687	–	–
Mycobacterium chelonae	JATA 62-01 (ATCC 35752)	AB087688	–	–
Mycobacterium fortuitum	JATA 61-01 (ATCC 6841)	AB087689	–	–
Mycobacterium gastri	KK 44-02 (ATCC 15754)	AB087690	–	+
Mycobacterium gordonae	JATA 33-01 (ATCC 14470)	AB087691	–	–
Mycobacterium intracellulare	JATA 52-01 (ATCC 13950)	AB087692	–	–
	Clinical isolate 17 strains			
Mycobacterium kansasii	KK 21-01 (ATCC 12478)	AB087693	+	–
	Clinical isolate 10 strains		+	–
Mycobacterium leprae	Thai-53	AB087694	–	–
Mycobacterium malmoense	JATA 47-01 (ATCC 29571)	AB087695	–	–
Mycobacterium marinum	JATA 22-01 (ATCC 927)	AB087696	–	–
Mycobacterium microti	KK 14-01 (ATCC 19422)	AB087697	–	–
Mycobacterium nonchromogenicum	JATA 45-01 (ATCC 19530)	AB087698	–	–
Mycobacterium parafortuitum	ATCC 25807	AB087699	–	–
Mycobacterium phlei	ATCC 19249	AB087700	–	–
Mycobacterium scrofulaceum	JATA 31-01 (ATCC 19981)	AB087701	–	–
Mycobacterium simiae	KK 23-08 (ATCC 25275)	AB087702	–	–
Mycobacterium smegmatis	JATA 64-01	AB087703	–	–
Mycobacterium szulgai	JATA 32-01	AB087704	–	–
Mycobacterium terrae	KK 46-01 (ATCC 15755)	AB087705	–	–
Mycobacterium triviale	KK 50-02 (ATCC 23292)	AB087706	–	–
Mycobacterium tuberculosis	JATA 11-01 (H37Rv)	AB087707	–	–
Mycobacterium ulcerans	KK 43-01	AB087708	–	–
Mycobacterium vaccae	KK 66-01	AB087709	–	–
Mycobacterium xenopi	KK 42-01 (ATCC 19250)	AB087710	–	–

All strains were kindly donated by Dr Kashiwabara, NIID.

Clinical isolates were identified by Amplicore *Mycobacterium* kit (Roche Pharma, Basel, Switzerland) or conventional biochemical test (Jamal *et al.*, 2000).

Amplification of the region within *dnaA* gene

Highly polymorphic regions flanked by conserved regions were identified by aligning the *Mycobacterium* spp. *dnaA* sequences, which were available in GenBank at the time this study was initiated. These regions were used to design a pair of degenerate primers, U1F 5′-GTS CAR AAC GAR ATC GAR CG-3′ and U1R 5′-CCB GAY TCR CCC CAG ATG AA-3′. A schematic representation of the primer design is shown in Fig. 1a. PCR was performed in a TAKARA Thermal Cycler MP (TAKARA Biomedical, Otsu, Japan) with a reaction mixture consisting of 1 µL of genomic DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of 0.4 µM, 1 × PCR buffer with 1.5 mM $MgCl_2$ (TAKARA Biomedical), and 1.25 U of ExTaq (TAKARA Biomedical), with 10 µL PCRX Enhancer System solution (Gibco BRL, Rockville, MD) in a total volume of 50 µL. The PCR thermocycles were 3 min at 94 °C, followed by 30 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. PCR products were visualized by UV illumination of an ethidium bromide-stained 1.5% agarose gel and cut out to purify with EASYTRAP Ver.2 (TAKARA Biomedical) according to the manufacturer's instruction.

DNA sequencing and sequencing analysis

The ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems, Foster City, CA) was used for the sequencing of the PCR products. The same primers for amplification were used for sequencing. The sequencing reaction was performed in accordance with the instruction of the manufacturer. Sequencing products were purified with a Centrisep column (Princeton Separations, Adelphia, NJ).

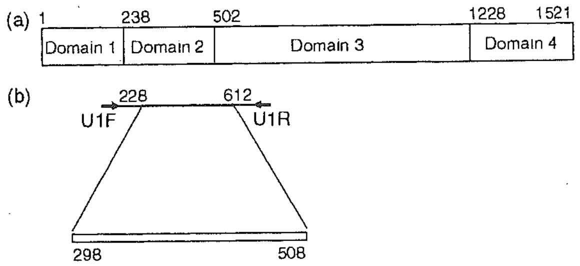


Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four domains. Domain 1 is involved in interaction with DnaB. Domain 2 constitutes a flexible loop. DNA unwinding required Domain 3. Domain 4 is sufficient for specific binding to DNA. Primers U1F and U1R were used to generate about 400 bp fragment from *dnaA* of 27 mycobacterial spp. (b) Analysis and comparison region used in this study are indicated by a bar (298–508 bp).

The sequencing output was analyzed by using the DNA Sequence Analyzer computer software (PE Biosystems). The partial *dnaA* sequences were aligned using the Clustal W algorithm (Thompson *et al.*, 1994) of the software DNASpace ver. 3.5 (Hitachi Software Engineering, Yokohama, Japan), and the alignment was manually corrected. A phylogenetic tree was generated by DNASpace ver. 3.5 (Hitachi Software Engineering) with a total of 1000 bootstraps. Pairwise similarity of the partial *dnaA* sequences was determined by using DNASIS package (Hitachi Software Engineering).

Species-specific LAMP assay for *Mycobacterium kansasii* and *Mycobacterium gastri*

A set of four primers comprising two inner primers and two outer primers that recognized six distinct regions on the target sequence were designed with PrimerExplorer Ver.3 (Fujitu, Tokyo, Japan). The detailed sequences of the primers are shown in Fig. 3. The two inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in late stages. FIP contains the sequence complementary F1 (F1c) and F2. BIP contains the complementary B1 (B1c) and B2. The two outer primers consist of F3 and B3.

The LAMP reaction was carried out in 25 µL of reaction mixture by using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tochigi, Japan) containing 2.4 µM (each) FIP and BIP, 0.2 µM (each) of the outer primers, F3 and B3, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 1.4 mM (each) of dNTP, 8 U of *Bst* DNA polymerase (New England BioLabs, Beverly, MA), and the template DNA. Amplification was undertaken in 0.5 µL microtubes in a heatblock under isothermal conditions of 63 °C for 60 min, followed by 80 °C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and precautions to prevent cross-contamination were observed. Two microliter aliquots of LAMP products were subjected to electrophoresis on a 4% agarose gel in Tris-borate-EDTA buffer followed by staining with ethidium bromide and were visualized on a UV transilluminator at 302 nm. The specificity of the LAMP-amplified products were further validated by restriction enzyme digestion with *Nae*I and *Hae*II for *M. kansasii* and *M. gastri*, respectively. The diluted genomic DNA was used for determining the sensitivity of the species-specific LAMP assay.

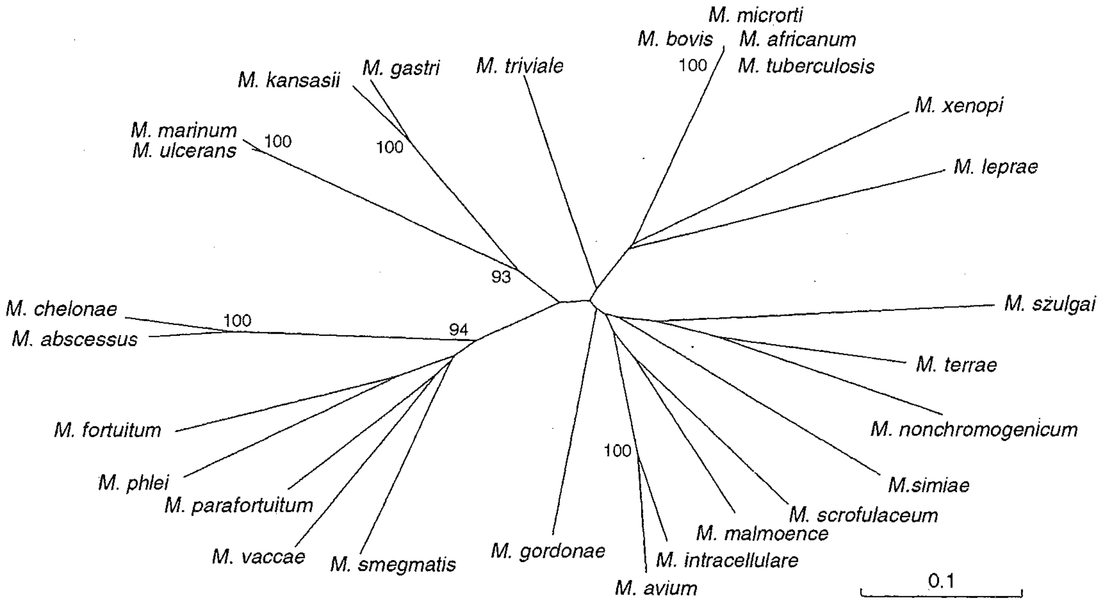


Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dna*A sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees; only values of > 90% are shown.

Results

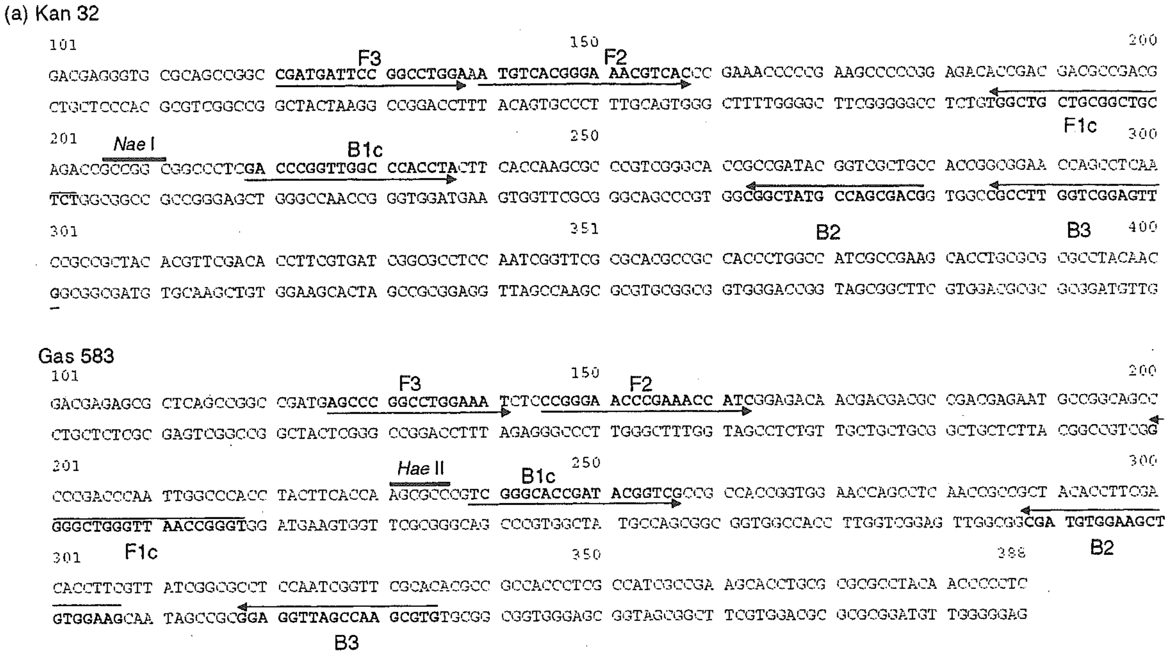
Comparison of partial *dnaA* sequence to identify the *Mycobacterium* species

For the species identification of mycobacterial species, we analyzed some possible variable regions of mycobacterial sequences deposited in the GenBank, and found the 5′ part of the *dnaA* gene as a candidate target for PCR amplification. The PCR products with U1F and U1R, from 27 mycobacterial species, showed the ragged pattern around 400 bp in size (data not shown). Therefore, we determined nucleotide sequences, corresponding to position 228–612 bp of *Mycobacterium tuberculosis*, of all 27 species (Fig. 1a). The alignment of the sequence shows that the region (298–508 bp) in the amplified products had the highest species-specific variability (Fig. 1b). The size of the variable fragment in *dnaA* ranged from 154 bp in *M. triviale* to 232 bp in *M. kansasii*. The variable region exhibits a reasonable number of nucleotide substitution and insertion or deletion sites, which is important for the development of a differential diagnostic tool. The lowest interspecies similarity was 28.2% in *M. leprae* versus *M. vaccae*. The similarity between *M. avium* and *M. intracellulare* was 78.3% and that between *M. marinum* and *M. ulcerans* was 97.7%. Pathogenic *M. kansasii* were easily differentiated from nonpathogenic *M. gastri* (83.6%). The sequences of *M. tuberculosis*, *M. microti*, *M. africanum*, and *M. bovis* were found to be identical, except for one nucleotide substitution that occurred in *M. bovis*. When clinical isolates from clinically relevant mycobacterial strains were analyzed, the following minor variation was found among each species: 97.7–100% (*M. avium*) and 96.0–100% (*M. intracellulare*). We did not find any intraspecies variation in 10 clinical isolates and the standard strain of *M. kansasii*. Because other reports using different systems revealed the existence of more than one sequevar (Yang *et al.*, 1993; Alcaide *et al.*, 1997), we may need to examine a bigger number of clinical isolates.

The unrooted phylogenetic tree showed that the 27 mycobacterial species were resolved by the variable region in the *dnaA* sequence (Fig. 2). All rapidly growing species, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. parafortuitum*, *M. phlei*, *M. vaccae*, and *M. smegmatis*, made a cluster that was clearly separated from those of the other species so far examined. On the other hand, *M. kansasii*, *M. gastri*, *M. avium*, and *M. intracellulare* are clinically relevant species; however, the branch of the former two species was obviously segregated from one of the later two species, which was supported by high bootstrap values. The results indicated that the partial *dnaA* sequence could be useful for the differentiation of NTM (Fig. 2).

Identification of mycobacteria by *dnaA* sequence-targeted species-specific LAMP assay

Several sets of primers designed from the *dnaA* sequence were evaluated for their specificity and sensitivity by the LAMP method. One set of primers named Kan-32 for *M. kansasii* and Gas-583 for *M. gastri* was selected (Fig. 3), and


(a) Kan 32
101
150
200
F3
F2
GACGAGGGTG CGCAGCCGGC CGATGATTCC GGCCTGGAAA TGTCACGGGA AACGTCACCC GAAACCCCCG AAGCCCCCGG AGACACCGAC GACGCCGACG
CTGCTCCCAC GCGTCGGCCG GCTACTAAGG CCGGACCTTT ACAGTGCCCT TTGCAGTGGG CTTTTGGGGC TTCGGGGGCC TCTGTGGCTG CTGCGGCTGC
F1c
201
Nae I
B1c
250
300
AGACCGCCGG CGGCCCTCGA CCCGGTTGGC CCACCTACTT CACCAAGCGC CCGTCGGGCA CCGCCGATAC GGTCGCTGCC ACCGGCGGAA CCAGCCTCAA
TCTGGCGGCC GCCGGGAGCT GGGCCAACCG GGTGGATGAA GTGGTTCGCG GGCAGCCCGT GGCGGCTATG CCAGCGACGG TGGCCGCCTT GGTCGGAGTT
301
351
B2
B3
400
CCGCCGCTAC ACGTTCGACA CCTTCGTGAT CGGCGCCTCC AATCGGTTCG CGCACGCCGC CACCCTGGCC ATCGCCGAAG CACCTGCGCG CGCCTACAAC
GGCGGCGATG TGCAAGCTGT GGAAGCACTA GCCGCGGAGG TTAGCCAAGC GCGTGCGGCG GTGGGACCGG TAGCGGCTTC GTGGACGCGC GCGGATGTTG
Gas 583
101
150
200
F3
F2
GACGAGAGCG CTCAGCCGGC CGATGAGCCC GGCCTGGAAA TCTCCCGGGA ACCCGAAACC ATCGGAGACA ACGACGACGC CGACGAGAAT GCCGGCAGCC
CTGCTCTCGC GAGTCGGCCG GCTACTCGGG CCGGACCTTT AGAGGGCCCT TGGGCTTTGG TAGCCTCTGT TGCTGCTGCG GCTGCTCTTA CGGCCGTCGG
201
Hae II
B1c
250
300
CCCGACCCAA TTGGCCCACC TACTTCACCA AGCGCCCGTC GGGCACCGAT ACGGTCGCCG CCACCGGTGG AACCAGCCTC AACCGCCGCT ACACCTTCGA
GGGCTGGGTT AACCGGGTGG ATGAAGTGGT TCGCGGGCAG CCCGTGGCTA TGCCAGCGGC GGTGGCCACC TTGGTCGGAG TTGGCGGCGA TGTGGAAGCT
301
F1c
350
388
B2
CACCTTCGTT ATCGGCGCCT CCAATCGGTT CGCACACGCC GCCACCCTCG CCATCGCCGA AGCACCTGCG CGCGCCTACA ACCCCCTC
GTGGAAGCAA TAGCCGCGGA GGTTAGCCAA GCGTGTGCGG CGGTGGGAGC GGTAGCGGCT TCGTGGACGC GCGCGGATGT TGGGGGAG
B3

(b)

Kan 32	
F3	CGATGATTCCGGCCTGGA
B3	GTTGAGGCTGGTTCCGC
FIP	TCTCGTCGGCGTCGTCGGTATGTCACGGGAAACGTCAC
BIP	GACCCGGTTGGCCCACCTAGCAGCGACCGTATCGGC

Gas 583	
F3	AGCCCGGCCTGGAAAT
B3	GTGCGAACCGATTGGAGG
FIP	TGGGCCAATTGGGTCGGGGCCGGGAACCCGAAACCATC
BIP	TCGGGCACCGATACGGTCGGAAGGTGTCGAAGGTGTAGC

Fig. 3. Location of oligonucleotide primer sets Kan 32 and Gas 583, used for the loop-mediated isothermal amplification method. For *Mycobacterium kansasii* partial *dnaA* gene (GenBank accession number AB087693) and for *Mycobacterium gastri* partial *dnaA* gene (GenBank accession number AB087690). A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer. The unique restriction enzyme recognition sites in the amplified product are shown with a bold bar. (b) List of each primer sequence.

by using these primer sets, a successful LAMP product appeared as a ladder of multiple bands (Fig. 3a).

The species specificity and intraspecies stability of each primer set were examined with purified DNA from 27 mycobacterial species and 10 clinical isolates of *M. kansasii.* We subjected each sample to amplification using Kan-32 or Gas-583 primer set. The results obtained by electrophoretic examination are summarized in Table 1. Although 200 pg of nontargeted species DNA were not amplified, significant amplification of targeted respective isolates was observed after a 60 min incubation at 63 °C. To confirm that the amplification products had corresponding DNA structures, the amplified products were digested with restriction enzymes and the size of the fragments was analyzed by electrophoresis. *Nae*I cuts between F1 and B1c for the *M. kansasii* amplicon; *Hae*II was used for the *M. gastri* amplicons. The sizes of the fragments generated after digestion were in good agreement with sizes predicted theoretically from the expected DNA structure: 100 and 93 bp by *Nae*I digestion, and 123 and 98 bp by *Hae*II digestion (Fig. 4a). Thus, we concluded that each primer set was species specific.

We next assessed the sensitivity of the assay. Serially diluted *M. kansasii* or *M. gastri* genomic DNA was used. The results of a typical experiment are shown in Fig. 4b. Amplified DNA was readily visible when 500 copies of genomic DNA were present in a 60 min incubation assay. The detection limit did not change with a longer incubation period (data not shown).

Discussion and conclusions

For the identification of species, a target gene must be conserved among strains and species. As the DnaA protein is generally conserved among microbial organisms (Mizrahi *et al.*, 2000), this coding region could be used for the target analysis. Four functional domains of the DnaA protein have been defined (Messer *et al.*, 1998). Domain 1 is involved in oligomerization and interaction with DnaB, Domain 2 constitutes a flexible loop, Domain 3 has ATPase function, and Domain 4 is sufficient for specific binding to DNA. The variable region that we identified in the *dnaA* sequence was equivalent to the Domain 2 coding nucleotide sequence

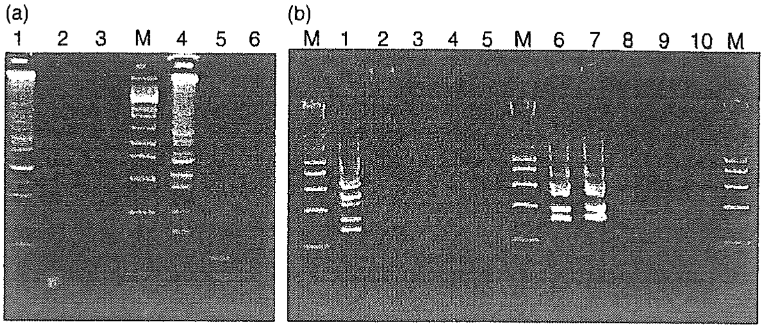


Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer, Kan 32, in the presence of genomic DNA from *M. kansasii* (lanes 1 and 2) and *M. gastri* (lane 3); lane 2, LAMP product from lane 1 after digestion with *Nae* I; lanes 4–6, LAMP carried out with *M. gastri* primer, Gas 583, in the presence of genomic DNA from *M. gastri* (lanes 4 and 5) and *M. kansasii* (lane 6). lane 5, LAMP product from lane 4 after digestion with *Hae* II. (b) Serial dilution of purified *M. kansasii* or *M. gastri* genomic DNA was amplified to determine the sensitivities by LAMP. Lanes: M, 100 bp DNA ladder; lanes 1–5 LAMP carried out with Kan 32 primer set in the presence of genomic DNA of *M. kansasii*, lane 1, 1000 copies; lane 2, 500 copies; lane 3, 100 copies; lane 4, 10 copy; lane 5, distilled water. lanes 6–10 LAMP carried out with gas 583 primer set in the presence of genomic DNA of *M. gastri*, lane 6, 1000 copies; lane 7, 300 copies; lane 8, 100 copies; lanes 9, 10 copy; lane 10, distilled water.

(Fig. 1). This domain is the least conserved region in the *dna A* gene with respect to sequence and length among *M. smegmatis*, *M. tuberculosis*, and *M. leprae* (Fsihi *et al.*, 1996). However, comparative studies of this region using 27 mycobacteria have not been reported and, as far as we know, this is the first report indicating the usefulness of the *dnaA* Domain 2 sequence as a differential diagnostic tool.

An accurate and rapid bacterial identification greatly contributes to this field of medication. Several methods based on molecular biological techniques have been reported. The sequences that have been reported include *hsp65*, 16S rRNA gene, and ITS (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Springer *et al.*, 1996; Messer & Weigel, 1997; Roth *et al.*, 1998; Brunello *et al.*, 2001). Each gene has several advantages and disadvantages. An excessive degree of variability is found in the *hsp65* gene (Telenti *et al.*, 1993), which may hinder the development of reliable probes. While 16s rRNA gene sequence is identical in *M. kansasii* and *M. gastri* and shows narrow divergenicity within species (Taylor *et al.*, 1997), ITS sequence can be used to distinguish between *M. kansasii* and *M. gastri* (Roth *et al.*, 1998). While *M. kansasii* is a representative pathogenic mycobacteria, *M. gastri* does not induce an apparent disease. The discrimination between these mycobacteria provides useful information to select the appropriate therapy. The percent similarity of ITS between two species was 93% (Roth *et al.*, 1998), and that of the *dnaA* variable region was found to be 83.6%. These observations may indicate the usefulness of the *dnaA* gene for discrimination of these species, at least in complement with ITS.

The recent trend in genetic testing is to make systems fully automatic with high-throughput analysis. Although this may be an ideal approach, it requires expensive equipment as well as a well-trained person in diagnostic laboratories. The LAMP method could be conducted under isothermal conditions ranging from 60 to 65 °C by a single enzyme. The only equipment needed for LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature around 63 °C. LAMP does not require a thermal cycling step, and an isothermal reaction for a short time (60 min) is enough to amplify the target DNA to a detectable level. As PCR and other molecular biological techniques are conducted in well-equipped laboratories, these methodologies are often impracticable under a field diagnosis.

In this paper, we demonstrated that the *dnaA* region could be an effective new nucleotide region for the diagnosis of NTM infection and that the LAMP method could be applied for a *dnaA* gene-based differential diagnostic tool.

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ORIGINAL ARTICLE

Polymorphism of the 5′ flanking region of the IL-12 receptor β2 gene partially determines the clinical types of leprosy through impaired transcriptional activity

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Background: Individual differences in T cell responsiveness to interleukin 12 (IL-12), resulting from inherited factors, may be responsible for differences in the intensity of cell mediated immune (CMI) responses in patients with leprosy, a disease with a wide clinical spectrum.
Aim: Polymorphisms in the 5′ flanking region of the IL12RB2 gene were analysed to determine potential immunogenetic factors affecting CMI responses, using leprosy as a model.
Methods: Polymorphisms in the 5′ flanking region of IL12RB2 were examined using direct sequencing techniques, and allele frequencies between patients with lepromatous leprosy and patients with tuberculoid leprosy were compared. The effect of these single nucleotide polymorphisms (SNPs) on IL12RB2 expression was estimated using the dual luciferase reporter gene assay in Jurkat T cells.
Results: Several SNPs, including −1035A>G, −1023A>G, −650delG, and −465A>G, were detected within the 5′ flanking region of IL12RB2. The frequency of haplotype 1 (−1035A, −1023A, −650G, −464A) was high in the general Japanese population, but was significantly lower in lepromatous patients compared with tuberculoid patients and healthy controls. Reporter gene assays using Jurkat T cells revealed that all haplotypes carrying one or more SNP exhibited a lower transcriptional activity compared with haplotype 1.
Conclusion: SNPs within the 5′ flanking region of IL12RB2 affect the degree of expression of this gene and may be implicated in individual differences in CMI responsiveness to mycobacterial antigens, leading to lepromatous or tuberculoid leprosy.

The importance of the cell mediated immune (CMI) response is well established in the host defence to mycobacterial pathogens.[1 2] Leprosy, a chronic disease caused by infection with *Mycobacterium leprae*, shows a wide spectrum of clinical features.[3] Patients with tuberculoid type leprosy (T-lep) show a high CMI response to *M leprae*, with resistance to infection, whereas patients with lepromatous leprosy (L-lep) show a poor CMI response to the pathogen and have a progressive form of the disease. Although a leishmaniasis model using BALB/c and C57BL6 mice has improved our understanding of cellular and genetic control mechanisms for infectious diseases and allergy, no such models have yet been established in humans. We propose leprosy as an alternative model in humans.

> "Polymorphisms in the 5′ flanking region of IL12RB2 may affect the expression of the interleukin 12 receptor β2 chain, resulting in individual differences in the intensity of cell mediated immune responses to mycobacteria"

Interleukin 12 (IL-12) is secreted from macrophages and dendritic cells and is a potent inducer of interferon γ production by T helper type 1 (Th1) cells, which is in part dependent upon the degree of expression of the IL-12 receptor (IL-12R) on the cell surface.[4–6] IL-12R is composed of two protein subunits, referred to as the β1 and β2 chains, and expression of the β2 chain is a crucial determinant of Th1/Th2 balance, because STAT4 is activated through interaction with a tyrosine residue on the cytoplasmic domain of the IL-12R β2 subunit.[7–10] It has been shown that the expression of IL-12Rβ2 is greater in tuberculoid lesions than in lepromatous lesions, whereas the expression of IL-12Rβ1 is similar in both.[11] We hypothesise that the susceptibility to several diseases related to mycobacterial pathogens could be determined by the degree of expression of IL-12Rβ2, which might be regulated by genetic factors, including IL12RB2 polymorphism.

One hypothesis is that polymorphisms in the 5′ flanking region of IL12RB2 may affect the expression of IL-12Rβ2, resulting in individual differences in the intensity of CMI responses to mycobacteria. We examined single nucleotide polymorphisms (SNPs) within the 5′ flanking region of IL12RB2 as feasible markers to determine susceptibility to the disease and the effect of these SNPs on the transcription of IL-12Rβ2 molecules.

MATERIALS AND METHODS

Study population

Genomic DNA samples were collected from 176 Japanese patients with leprosy—130 with L-lep and 46 with T-lep—and 68 healthy Japanese donors. Patients were clinically diagnosed, according to the description of Ridley and Jopling, referring to results of the Mitsuda test and their sequelae.[3] Donors were recruited into our study under informed consent guidelines approved by the human ethical committee of Saitama Medical School, Japan.

Abbreviations: CMI, cell mediated immunity; IL-12, interleukin 12; IL-12R, interleukin 12 receptor; L-lep, lepromatous leprosy; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; Th, T helper; T-lep, tuberculoid type leprosy

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Table 1 Allelic distribution in patients with leprosy

SNPs	Clinical form	Allele frequency	OR	RR	p Value
−1035A>G	L-lep	24.6%	3.97	3.24	<0.001
	T-lep	7.6%			
−1023A>G	L-lep	24.2%	2.95	2.48	<0.01
	T-lep	9.8%			
−650delG	L-lep	28.8%	3.74	2.95	<0.001
	T-lep	9.8%			
−464A>G	L-lep	23.1%	3.64	3.03	<0.01
	T-lep	7.6%			

Frequencies of four single nucleotide polymorphisms (SNPs) were compared between lepromatous (L-lep) and tuberculoid (T-lep) patients. The χ^2 test was used to compare differences in the distribution of clinical phenotypes and allele frequencies. The odds ratio (OR) and relative risk (RR) were also calculated by comparing the frequency of variant alleles between two clinical types of leprosy.

Analysis of polymorphism in the 5′ flanking region of IL12RB2

Genomic DNA was sequenced for polymorphisms in the 5′ flanking region of IL12RB2 using direct sequencing. Briefly, a fragment spanning −1247 to +55 of IL12RB2 was polymerase chain reaction (PCR) amplified and subsequently sequenced using the ABI 3730 DNA sequencer (PerkinElmer Life Sciences, Wellesley, Massachusetts, USA). The sequence data obtained were compared with the GenBank database (GenBank accession number AL389925) to determine the SNPs on IL12RB2. Numbers of base positions were defined as the distance from the start point of the reported cDNA sequence.[12] The haplotypes were determined by cloning the PCR products into the pGEM-T Easy plasmid (Promega, Madison, Wisconsin, USA) and sequencing.

Evaluation of transcriptional activity of the 5′ flanking region of IL12RB2

For transfection studies, the dual luciferase reporter gene assay system (Promega) was used with the IL12RB2–pGL3 plasmid constructs. The plasmids comprised a 1.3 kb NheI/HindIII digested PCR fragment of IL12RB2 ligated to the pGL3 basic vector (Promega). Jurkat T cells (1×10^7) were electroporated with 25 μg of plasmid DNA (IL12RB2–pGL3 constructs) and a control *Renilla* luciferase reporter plasmid (pRL-TK, 25 ng; Promega) using a Gene Pulser (Bio-Rad Laboratories, Hercules, California, USA), as described previously.[13] Cells were then cultured for 48 hours in the presence of antihuman CD3 monoclonal antibody (PharMingen, San Diego, California, USA), and antihuman CD28 monoclonal antibody (PharMingen) for the final 24 hour period. Cell lysates were prepared, and both firefly and *Renilla* luciferase activities were evaluated using a dual luminometer Fluoroskan Ascent FL (Thermo Electron Oy, Vantaa, Finland).

Table 2 Haplotypes of the 5′ flanking region of IL12RB2

Haplotype	−1035	−1023	−650	−464	Frequency (%)
1	A	A	G	A	41.2
2	G	G	del	G	32.4
3	A	A	del	A	11.8
4	G	G	G	A	8.3
5	A	A	del	G	5.9

Haplotypes were determined by sequencing 34 subcloned polymerase chain reaction amplified DNA fragments from 17 donors with heterozygous alleles in at least in one of the positions −1035, −1023, −650, and −464, but not from donors carrying homozygous alleles at all positions.

RESULTS

Analysis of polymorphism in the 5′ flanking region of IL12RB2

Twelve SNPs located within the 5′ flanking region of IL12RB2 (−1247 to +55), comprising −1047delT, −1035A>G, −1033T>C, −1023A>G, −650delG, −568A>C, −557T>C, −550T>C, −464A>G, −464A>C, −202T>C, and −188A>C, were identified. The surveillance study of 176 patients (130 with L-lep and 46 with T-lep) revealed significant differences in frequencies of SNPs between these patients groups; in particular: −1035A>G, −1023A>G, −650delG, and −464A>G (table 1).

We randomly selected 17 healthy donors heterozygous in at least one of the positions −1035, −1023, −650, and −464 to determine linkage disequilibrium. Five haplotypes were determined by sequencing 34 subcloned PCR amplified DNA fragments from the 17 heterozygous donors. Haplotype 1 consisted of −1035A, −1023A, −650G, and −464A, and haplotype 2 consisted of −1035G, −1023G, −650del, and −464G, and accounted for 73.6% of the haplotypes detected in our study (table 2).

Haplotype frequency was calculated based on the assumption that each group would be in accordance with the Hardy–Weinberg equilibrium because subjects were selected from the Japanese Wajin population of mainland Japan (Honshu). p Values were calculated using the StatView (SAS Institute, Cary, North Carolina, USA) statistical software program, by comparing the frequency of haplotype 1 in patients with L-lep leprosy, those with T-lep leprosy, and healthy controls. The frequency of haplotype 1 was significantly lower in patients with L-lep compared with those with T-lep and healthy donors (fig 1), suggesting that haplotype 1 might contribute to the intensity of CMI responses to mycobacteria.

Evaluation of transcriptional activity of the 5′ flanking region of IL12RB2

The transcriptional activity of each reporter construct was determined using the dual luciferase reporter gene assay. The transcriptional activity of haplotype 1 was significantly higher than haplotypes 2, 3, 4, and 5 (fig 2). Each experiment was performed using triplicate wells and was repeated four times. Because each SNP is a genetic factor potentially able to reduce the expression of IL-12Rβ2 molecules, a poor CMI response to mycobacteria may occur.

DISCUSSION

It was recently reported that the lack of IL-12Rβ1 expression caused by mutations in IL12RB1 resulted in human immunodeficiency, thereby demonstrating the essential role

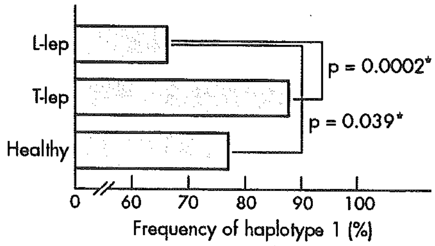

Figure 1 Frequency (%) of haplotype 1 in the three groups: patients with lepromatous leprosy (L-lep), patients with tuberculoid type leprosy (T-lep), and healthy controls. The haplotype frequency was calculated based on the assumption that each group would be in accordance with the Hardy–Weinberg equilibrium because subjects were selected from the Japanese Wajin population of mainland Japan (Honshu). p Values were calculated using the StatView statistical software program, by comparing the frequency of haplotype 1 in the L-lep leprosy, T-lep leprosy, and healthy control groups.

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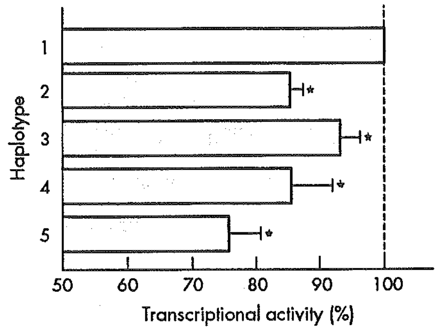

Figure 2 Basal promoter activity of reporter constructs containing −1247 to +55 of IL12RB2 harbouring each haplotype. The transcriptional activity of each haplotype is indicated by the percentage of relative luciferase units compared with the haplotype 1 construct. Each experiment was assayed using triplicate wells and was repeated four times and similar results were obtained. $^*p < 0.05$; χ^2 test.

of IL-12 in resistance to infections caused by intracellular bacteria.[14–16] However, no differences in the expression of IL-12Rβ1 on T cells were detected between donors, including patients and healthy subjects (data not shown). It has been reported that IL-12Rβ2 is absent in freshly isolated peripheral blood mononuclear cells, whereas up to 72% of resting peripheral blood mononuclear cells from normal volunteers express IL-12Rβ1 molecules, and that IL-12Rβ2 is expressed selectively in Th1 cells but not in Th2 cells.[9 17] Moreover, because IL-12Rβ2 has tyrosine residues in the cytoplasmic domain that play a role in signal transduction, we hypothesised that IL-12Rβ2, but not IL-12Rβ1, could be important in explaining the low CMI responses induced by IL-12 in T cells from patients with leprosy.

> "Individual differences in the intensity of the cell mediated immune response to mycobacteria are probably regulated primarily by the degree of expression of IL-12Rβ2, rather than possible conformational changes"

Alternatively spliced mRNA with the absence of IL12RB2 exon 15 leads to the loss of induction of interferon γ production.[18] Similarly, we have previously found several coding SNPs of IL12RB2, but could not determine an effect on CMI response intensity or on alternatively spliced mRNA (data not shown). Epidemiological studies recently performed by two independent groups demonstrated that there was no influence of IL12RB2 coding SNPs on susceptibility to mycobacterial infection.[19 20] Therefore, individual differences in the intensity of the CMI response to mycobacteria are probably regulated primarily by the degree of expression of IL-12Rβ2, rather than possible conformational changes caused by the coding SNPs detected in the Japanese population. Bleharski *et al* showed differences in gene expression profiles according to the clinical type of leprosy,[21] and the IL-12R gene was included among those differentially expressed between patients with L-lep and T-lep.

The differences in transcriptional activity between haplotype 1 and other haplotypes are marginal, except for that between haplotypes 1 and 5. These findings suggest that haplotype 5 might be more closely associated with susceptibility to lepromatous leprosy than haplotypes 2, 3, and 4. To investigate this issue, it will be necessary to analyse the frequency of all haplotypes in the patient population. Moreover, the functional effects of SNPs −650 and −464 on the transcriptional mechanism should also be elucidated.

Take home messages

- Single nucleotide polymorphisms (SNPs) within the 5′ flanking region of the IL12RB2 gene could affect the expression of the interleukin 12 receptor β2 chain (IL-12Rβ2) and result in the individual differences in the intensity of cell mediated immune responses that lead to the lepromatous and tuberculoid types of leprosy
- Haplotype 5 appears to be more closely associated with susceptibility to lepromatous leprosy than haplotypes 2, 3, and 4, but further investigations are necessary
- These SNPs may also affect susceptibility to allergy because IL-12Rβ2 is involved in the allergic response

An SNP at position −464 of IL12RB2 was found to have high transcriptional activity compared with the wild-type allele, possibly because of disruption of a GATA site.[22] This appears to contradict our present data, although the effects of SNPs on the binding affinity with GATA-3 were not directly tested in this report. It was determined that the main target of GATA-3 is not IL-12Rβ2, but rather STAT4.[23] Further studies are needed to determine the precise molecular mechanism.

Taken together, we conclude that SNPs within the 5′ flanking region of IL12RB2 could affect the expression of IL-12Rβ2, thus causing individual differences in the intensity of CMI responses leading to the lepromatous and tuberculoid types of leprosy. It is probable that these SNPs also affect susceptibility to allergy, because IL-12Rβ2 is implicated in the Th1/Th2 balance in the allergic response. A study investigating this hypothesis is currently under way.

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