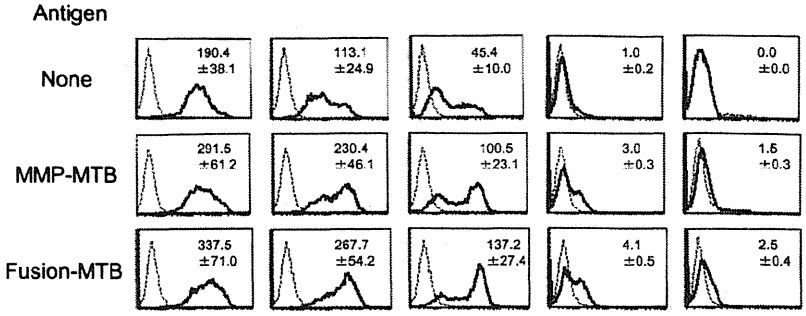


(p value)	HLA-ABC	HLA-DR	CD86	CD83	MMP
None vs MMP	p<0.05	p<0.05	p<0.05	p<0.001	p<0.05
None vs Fusion	p<0.05	p<0.05	p<0.05	p<0.005	p<0.01
MMP vs Fusion	p<0.05	p<0.05	p<0.005	p<0.05	p<0.005

FIG. 1. Expression of APC-associated molecules and MMP on DC by stimulation with recombinant proteins. Immature DC obtained from monocytes in the presence of rGM-CSF and rIL-4 were pulsed with either MMP-MTB or Fusion-MTB at 10 μg/ml on day 4 of culture. The DC were gated and analyzed on day 6 after the start of culture. Dotted lines, isotype-matched control IgG or IgM (for MMP); solid lines, the indicated test MAb. Representative results of three separate experiments are shown. The value in the top right corner of each graph is the mean fluorescence intensity of three independent experiments with a control Ig or the test MAb ± the standard deviation. Titers were statistically compared using Student's *t* test.

chromatography (TALON Metal Affinity Resins; Clontech Laboratories). Fusion-ML and Fusion-MTB were purified by two purification steps. Passage through a metal affinity column (TALON) was also used in the first step. The eluted crude proteins were applied to a HiLoad Superdex 200 pg column (GE Healthcare, Buckinghamshire, England) for further purification by gel filtration. Three major fractions were detected after the second step; one of them contained the target protein. The purified proteins (MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining (see Fig. S1 in the supplemental material).

Analysis of cell surface Ag. The expression of cell surface Ag on DC and lymphocytes was analyzed using a FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich), and 1×10^4 live cells were analyzed. For cell surface Ag analysis, fluorescein isothiocyanate (FITC)-conjugated MAbs against HLA-ABC (G46-2.6; BD Biosciences), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), and CD83 (HB15a; Immunotech, Marseille, France) were used.

The expression of MMP on Ag-pulsed DC or DC infected with *M. tuberculosis* at an indicated multiplicity of infection (MOI) was determined using the MAb against MMP-ML (M270-13, IgM, kappa), which probably detects MMP complexed with MHC molecules on the surface of DC (26), followed by FITC-conjugated anti-mouse Ig Ab (Tago Immunologicals, Camarillo, CA). The intracellular production of perforin was assessed as follows. Memory-type $CD8^+$ T cells were stimulated with Ag-pulsed DC for 5 days in the presence of memory-type $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with phycoerythrin-labeled MAb to CD8 and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences) and stained with an FITC-conjugated MAb to perforin (δG9; BD Biosciences) or an FITC-labeled isotype control.

APC functions of DC. The ability of Ag-pulsed DC and macrophages to stimulate T cells was assessed using an autologous APC-T cell coculture as previously described (15, 28). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450; Dynal Biotech) (28). The purity of the $CD4^+$ and $CD8^+$ T cells was more than 95% as assessed by FACScalibur. Naïve $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with MAb to CD45RO, which was followed by beads coated with MAb to goat anti-mouse IgG (Dynal Biotech). The purity of both subsets of naïve T cells was more than 97%. However, there was no contamination of memory-type T cells in the naïve T cell preparations. More than 98% of the $CD45RA^+$ T cells were positive for expression of the CCR7 molecule. Memory-type T cells were similarly produced by the treatment of cells with a MAb to CD45RA Ag. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and DC or macrophages pulsed with Ag were added to give the indicated APC/T cell ratio. Supernatants of APC-T cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, Ag-pulsed DC were treated with MAbs to HLA-ABC (W6/32; mouse IgG2a, kappa), HLA-DR (L243; mouse IgG2a, kappa), CD86 (IT2.2; mouse IgG2b, kappa, BD Biosciences), or MMP (M270-13) or normal mouse IgG or IgM. The treatment of DC with these MAbs did not affect the viability of the DC (not shown). Also, in some cases, Ag-pulsed DC were costimulated with CD40 ligand (CD40L; 1 μg/ml; PeproTech). The optimal concentration was determined in advance.

Measurement of cytokine production. Levels of the following cytokines were measured: IFN-γ produced by $CD4^+$ and $CD8^+$ T cells and IL-12p40 produced by DC stimulated for 24 h with Ag. The concentrations of these cytokines were quantified with enzyme-linked immunosorbent assay (ELISA) kits (Opt EIA Human ELISA Set; BD Biosciences). The detection limit of the IFN-γ ELISA kit is 3.0 pg/ml.

Statistical analysis. Student's *t* test was used to determine statistically significant differences.

RESULTS

Activation of DC by *M. tuberculosis*-derived recombinant proteins. For a recombinant protein to activate T cells, it must have the ability to activate APCs. We assessed the phenotypic change induced in DC by stimulation with MMP-MTB (gene name, Rv1876 or *bfrA*) and a fusion protein composed of BCG-derived HSP70 and MMP-MTB (Fusion-MTB) (Fig. 1). Both recombinant proteins upregulated the surface expression of HLA-ABC, HLA-DR, CD86, and CD83. However, Fusion-MTB more efficiently enhanced the expression of all of these molecules. Further, MMP-MTB- or Fusion-MTB-pulsed DC expressed molecules which react with anti-MMP-ML MAbs. Again, Fusion-MTB was more efficient than MMP-MTB in the induction of expression of the molecules. These results indicated that both recombinant proteins may have the ability to activate DC. To confirm this point, we measured the IL-12p40 production of DC by stimulation with the recombinant proteins (Fig. 2). We comparatively analyzed MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. All of the recombinant proteins induced the production of IL-12p40, but the levels of IL-12p40 produced by stimulation were as follows: MMP-MTB > MMP-

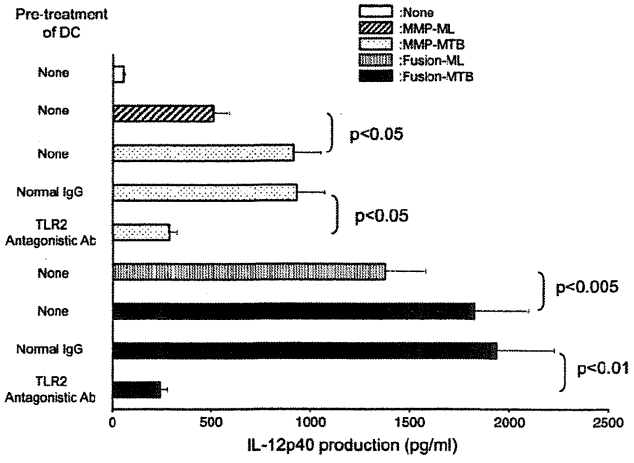


FIG. 2. IL-12p40 production by DC stimulated with recombinant proteins. Monocyte-derived DC from 5 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated recombinant protein at 10 μg/ml for 24 h. In some cases, immature DC were pretreated with normal murine IgG or TLR2-antagonistic Ab (10 μg/ml) and subsequently stimulated with recombinant protein for 24 h. The concentration of IL-12p40 was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± the standard deviation. Titers were statistically compared by Student's *t* test.

ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. In order to reveal the mechanisms leading to the activation of DC by MMP-MTB or Fusion-MTB, we pretreated immature DC with TLR2-antagonistic Ab and subsequently stimulated the treated DC with recombinant proteins, since both MMP-ML and Fusion-ML are reported to activate the NF-κB pathway through ligation with TLR2 (21, 31). While pretreatment of DC with normal murine IgG did not affect the production of IL-12 by stimulation with recombinant proteins, the pretreatment with TLR2-antagonistic Ab significantly inhibited the cytokine production caused by MMP-MTB and Fusion-MTB (Fig. 2). Also, we tested the effect of TLR4-antagonistic Ab on IL-12 production; however, the pretreatment of DC with TLR4-antagonistic Ab did not inhibit cytokine production (not shown).

Activation of T cells by recombinant protein. The enhanced activation of DC by *M. tuberculosis*-derived MMP and fusion proteins may enable autologous T cell activation. The T cell-activating ability of the recombinant proteins was assessed chiefly by using DC as APCs. Memory-type $CD4^+$ T cells were purified from healthy, BCG-vaccinated individuals. All of the recombinant proteins activated the $CD4^+$ T cells with a small dose (~2.0 μg/ml) of Ags and a small dose of recombinant protein-pulsed DC (T cell/DC ratio, 80:1) (Fig. 3A). However, MMP-MTB and Fusion-MTB more efficiently activated the T cells than *M. leprae*-derived proteins did, and Fusion-MTB induced a significantly larger amount of IFN-γ than MMP-MTB did. When we used a higher concentration of recombinant proteins, such as 5 or 10 μg/ml, and used a higher number of DC, such as a T cell/DC ratio of 10:1, as a stimulator, similar statistically significant differences were observed (not shown). Further, only Fusion-MTB successfully activated memory-type $CD4^+$ T cells through macrophages, although a large dose of Ag and a high number of Ag-pulsed macrophages were required (T cell/macrophage ratio, 5:1) (Fig. 3A). The $CD4^+$ T cell-stimulating activity of MMP-MTB and Fusion-MTB was confirmed by using CD45RO-negative naïve $CD4^+$ T cells. All of the recombinant proteins, including MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB, activated naïve $CD4^+$ T cells, and Fusion-MTB was the most effective (Fig. 3B). Compared to memory $CD4^+$ T cells, naïve $CD4^+$ T cells required a larger dose of Ag (~10 μg/ml) and a higher number of Ag-pulsed DC (T cell/DC ratio, 10:1) to be activated. To address the mechanisms leading to the activation of naïve $CD4^+$ T cells by Fusion-MTB, Fusion-MTB-pulsed DC were treated with MAbs against HLA-DR, CD86, and MMP-ML molecules and subsequently used to stimulate naïve $CD4^+$ T cells (Fig. 3C). IFN-γ production by these naïve $CD4^+$ T cells was significantly inhibited by the surface treatment of the DC with the MAbs, and similarly, IL-2 production by naïve $CD4^+$ T cells was inhibited (not shown). The ability of MMP-MTB and Fusion-MTB to activate memory-type $CD8^+$ T cells was then assessed (Fig. 4A). Although, in contrast to memory-type $CD4^+$ T cells, a large dose of recombinant proteins was required, both *M. tuberculosis*-derived recombinant proteins induced significant production of IFN-γ from memory-type $CD8^+$ T cells. Further, the additional treatment of Ag-pulsed DC with CD40L upregulated the production of IFN-γ by $CD8^+$ T cells. In both cases, i.e., without and with CD40L treatment, Fusion-MTB induced significantly greater IFN-γ production than MMP-MTB did. In order to confirm the $CD8^+$ T cell-stimulating abilities of both MMP-MTB and Fusion-MTB, naïve $CD8^+$ T cells were also examined as responders. In this case, purified proteins from *M. leprae* were used as a control (Fig. 4B). Both MMP-MTB and Fusion-MTB activated naïve $CD8^+$ T cells to produce IFN-γ; however, the concentration of IFN-γ released from naïve $CD8^+$ T cells was low and a cytokine concentration of less than 35 pg/ml was produced, and the concentration of IFN-γ produced from naïve $CD8^+$ T cells by stimulation with Fusion-MTB was significantly lower than that from memory $CD8^+$ T cells ($P < 0.005$). The naïve $CD8^+$ T cell-stimulating activities of the recombinant proteins were as follows: MMP-MTB > MMP-ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. The IFN-γ production by naïve $CD8^+$ T cells was enhanced by the additional treatment of Ag-pulsed DC with CD40L, and the highest production of IFN-γ was achieved by Fusion-MTB; in this case, Fusion-MTB could induce an IFN-γ concentration of more than 100 pg/ml. To elucidate the mechanisms of the activation of naïve $CD8^+$ T cells by Fusion-MTB, Fusion-MTB pulsed DC were treated with MAbs to HLA-ABC and CD86 and subsequently used as a stimulator (Fig. 4C). IFN-γ production by naïve $CD8^+$ T cells was significantly inhibited by the treatment of the DC. One of the aims of $CD8^+$ T cell activation in terms of the host defense against *M. tuberculosis* is to produce cytotoxic $CD8^+$ T cells. To measure the production of cytotoxic $CD8^+$ T cells, we assessed the intracellular production of perforin in $CD8^+$ T cells which were stimulated with MMP-MTB or Fusion-MTB in the presence of $CD4^+$ T cells (Fig. 4D). Both recombinant proteins produced perforin-producing $CD8^+$ T cells, and Fusion-MTB seemed to produce them more efficiently.

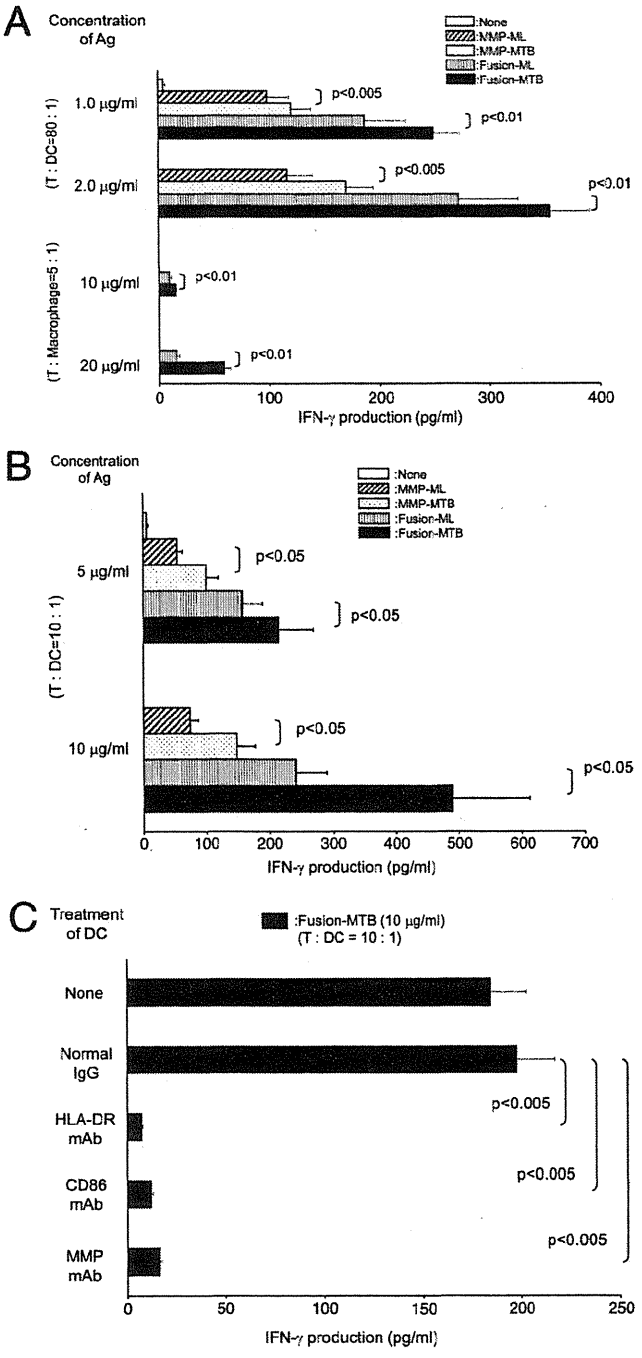


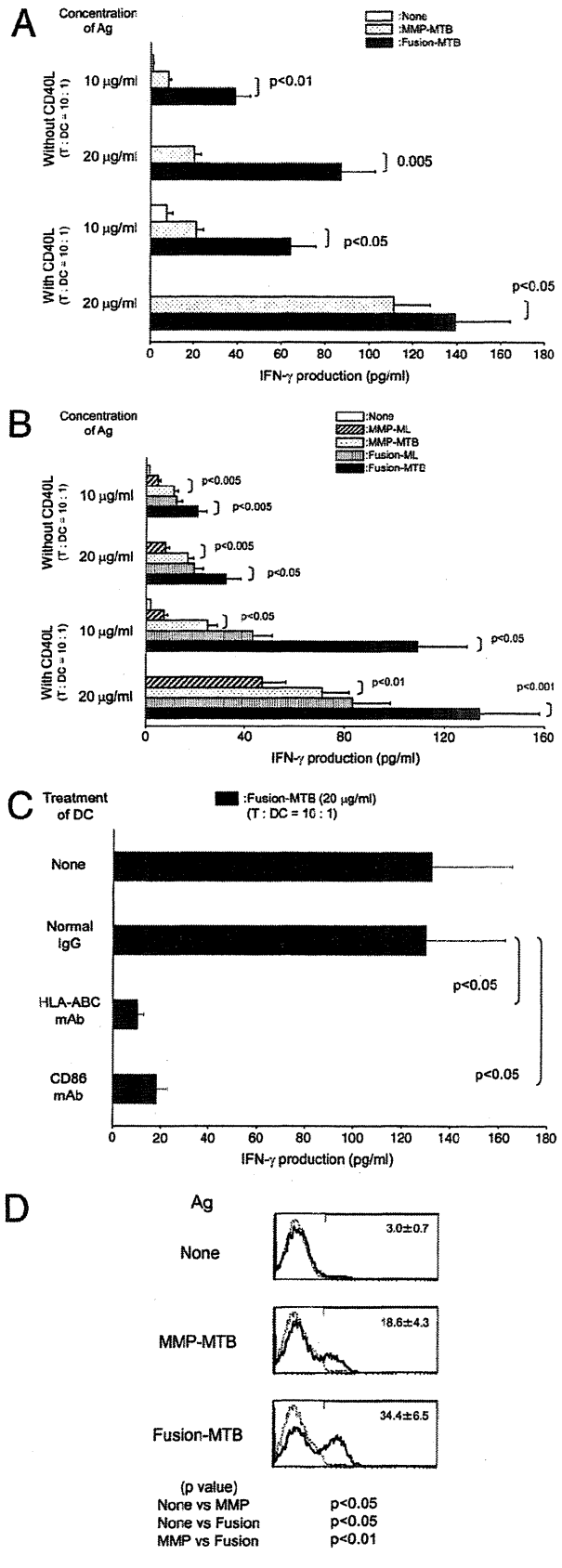
FIG. 3. (A) IFN-γ production by memory-type $CD4^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC or macrophages were pulsed with the indicated recombinant protein at the indicated concentration and used to stimulate memory-type $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ T cells (1×10^5) were stimulated with the indicated dose of Ag-pulsed DC or macrophages. (B) IFN-γ production by naïve $CD4^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with the indicated recombinant protein at 5 or 10 μg/ml and used to stimulate naïve $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naïve $CD4^+$ T cell activation by treatment of Ag-pulsed DC with MAb. Monocyte-derived DC were pulsed with Fusion-MTB at 10 μg/ml and subsequently treated at 10 μg/ml with MAb to HLA-DR, CD86, MMP, or normal murine IgG or IgM. These DC were used to stimulate naïve $CD4^+$ T cells (1×10^5) at a T cell/DC ratio of 10:1. IFN-γ produced from T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test.

Expression of MMP on APCs infected with *M. tuberculosis*. The molecule used as a vaccinating agent should be expressed on APCs infected with the pathogen. To reveal the expression of MMP on the surface of APCs, DC and macrophages were infected with *M. tuberculosis* H37Ra and H37Rv and analyzed by flow cytometry (Fig. 5). Both DC and macrophages expressed MMP molecules on their surface after infection with H37Ra and H37Rv. Expression levels seemed to be dependent on the dose of *M. tuberculosis* used for infection (not shown).

DISCUSSION

In vivo studies using various knockout mice indicate that adaptive immunities play an important role in inhibiting the multiplication of *M. tuberculosis* and that the activation of both $CD4^+$ T cells and $CD8^+$ T cells is an essential element of the control of *M. tuberculosis* infection (1, 12, 16). While $CD4^+$ T cells chiefly act in the initial phase of infection, $CD8^+$ T cells either producing IFN-γ or having cytotoxic killing activity contribute to the chronic or stationary phase of infection (7, 19, 46). Thus, the antigenic molecules which are used as an essential component of a vaccine should have the ability to activate not only naïve $CD4^+$ T cells and $CD8^+$ T cells but also APCs, including DC. So far, we have found MMP to be one of the immunodominant Ags of *M. leprae* (21) and found evidence that MMP-ML activated DC through ligation with TLR2, which resulted in the activation of the NF-κB pathway of host cells, and that DC pulsed with MMP-ML stimulated both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific manner (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by both T cell subsets of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

In addition, HSP70, one of the heat shock proteins, plays various roles in the upregulation of the ability of APCs to stimulate T cells (6, 10, 44, 45). Further, HSPs of both mammalian host cell and bacterial origins are reported to have chaperon activity (6, 44) and can effectively prime a cytolytic response (10, 45). In fact, we previously reported that HSP70 effectively induced the cross-priming of $CD8^+$ T cells through the cytosolic pathway when secreted from recombinant BCG in the phagosome of DC as part of a fusion protein (31). Also, others have reported that HSP65 activated naïve $CD8^+$ T cells and a DNA vaccine containing the *hsp65* gene inhibited the development of tuberculosis that is induced by the multiplication of subsequently challenged *M. tuberculosis* (50). Furthermore, vaccination of mice with recombinant BCG that secreted either MMP-ML or Fusion-ML, in which BCG was used as a vehicle, efficiently inhibited the multiplication of subsequently challenged *M. leprae*, although the fusion protein was more efficient in both activating naïve T cells and inhibiting *M. leprae* multiplication (22, 25, 31).



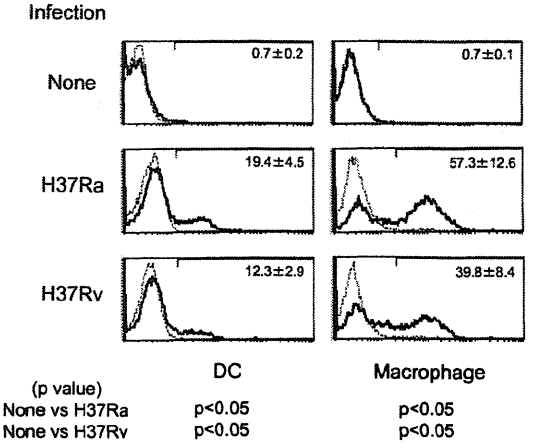


FIG. 5. Expression of MMP on DC and macrophage infected with *M. tuberculosis*. Monocyte-derived DC or macrophages were infected with either H37Ra or H37Rv at an MOI of 1.0 and cultured for another 2 days in the presence of rGM-CSF plus rIL-4 or rM-CSF, respectively. The DC and macrophages were gated and analyzed on day 5 after the start of culture. Dotted lines, control IgM; solid lines, MMP MAb. Results representative of three separate experiments are shown. The values are the mean percentages of major membrane protein II-positive DC or macrophages in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test.

MMP is commonly expressed in both pathological mycobacteria and BCG, so that it may be that MMP-MTB plays a substantial role in inhibiting the replication of *M. tuberculosis*; however, the homology of MMP between *M. leprae* (ML2038)

FIG. 4. (A) IFN-γ production by memory-type $CD8^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with MMP-MTB or Fusion-MTB at 10 or 20 μg/ml, costimulated with or without CD40L (1.0 μg/ml), and used to stimulate memory-type $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (B) IFN-γ production by naïve $CD8^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC were pulsed with the indicated recombinant protein at 10 or 20 μg/ml, further costimulated with or without CD40L (1.0 μg/ml), and used to stimulate naïve $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naïve $CD8^+$ T cell activation by treatment of Fusion-MTB-pulsed DC with MAb. Monocyte-derived DC were pulsed with MMP-MTB at 20 μg/ml, costimulated with CD40L (1.0 μg/ml), and subsequently treated at 10 μg/ml with MAb to HLA-ABC, CD86, or normal murine IgG. These DC were used to stimulate naïve $CD8^+$ T cells (1×10^5) at a T cell/DC ratio of 10:1. IFN-γ produced by T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test. (D) Intracellular production of perforin by $CD8^+$ T cells. Monocyte-derived DC were pulsed at 10 μg/ml with either MMP-MTB or Fusion-MTB and cultured with unseparated memory-type T cells (T cell/DC ratio, 40:1) for 5 days. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production. Values are the mean percentages of the $CD8^+$ T cell population that were perforin positive in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test. A representative of three separate experiments is shown.

and *M. tuberculosis* (Rv1876) is only 90.6% at the amino acid level. Therefore, we assessed the immunostimulatory activity of *M. tuberculosis*-derived MMP and its fusion with BCG-derived HSP70 by using MMP-ML and Fusion-ML as controls.

As expected, MMP derived from *M. tuberculosis* activated DC in terms of phenotypic change and cytokine production, and the cytokine production was associated with the ability of MMP-MTB to ligate with TLR2. MMP-MTB-pulsed DC activated both $CD4^+$ and $CD8^+$ T cells. In this respect, only a very small amount of MMP was required to induce vigorous activation of $CD4^+$ T cells, but not $CD8^+$ T cells, obtained from BCG-vaccinated healthy donors. These results may indicate that some subsets of $CD4^+$ T cells are primed with MMP by vaccination with BCG, whose MMP is 100% homologous to that of *M. tuberculosis*, as in the case of leprosy patients whose T cells were primed by *M. leprae* infection. However, in contrast to leprosy patients, only $CD4^+$ T cells are primed with MMP by BCG vaccination, which may be linked with the fact that the parent BCG less efficiently activates naïve $CD8^+$ T cells. Activation of T cells usually depends on APCs expressing Ags, so that successful production of MMP-reactive memory-type T cells could be achieved by administration of MMP since MMP could be expressed on the surface of DC after infection with *M. tuberculosis* H37Ra and H37Rv. This speculation might be supported by our preliminary experiments in which administration of MMP-MTB to C57BL/6 mice produced memory-type splenic T cells reactive to MMP-MTB *in vitro*, which produced IFN-γ because of this stimulation.

Fourteen amino acids of *M. leprae* MMP differ from those of *M. tuberculosis* MMP, and substitutions of amino acids between these mycobacteria are known to occur randomly. However, a MAb which recognizes the epitope expressed on DC pulsed with *M. leprae*-derived MMP could also detect a peptide expressed on the surface of DC pulsed with *M. tuberculosis*-derived MMP or infected with *M. tuberculosis*. The MAb against MMP-ML inhibited the activation of naïve $CD4^+$ T cells by stimulation with MMP-MTB-pulsed DC. These observations indicated that the regions common to the MMPs of *M. leprae* and *M. tuberculosis* were chiefly used as antigenic epitopes of $CD4^+$ T cells. However, the T cell activation by *M. tuberculosis*-derived MMP and Fusion-MTB is significantly stronger than that by *M. leprae*-derived proteins. The exact mechanism leading to the difference between the T cell-stimulating activities of the MMPs derived from these two pathological mycobacterial strains remains to be elucidated, but one possibility is that some parts of *M. tuberculosis*-derived MMP other than common regions have APC-immunomodulating activities that are associated with T cell activation. In fact, *M. tuberculosis*-derived MMP more efficiently activated DC than MMP-ML did, in terms of IL-12 production. However, both MMP-ML and MMP-MTB ligate TLR2; thus, MMP-MTB may have other unknown mechanisms that can induce the activation of DC more strongly. In this respect, we assessed the IL-1β-producing ability of MMP, but there was no apparent difference between the MMPs obtained from *M. tuberculosis* and *M. leprae* (not shown). It has been reported that the replacement of one amino acid of the T cell epitope of the antigenic determinant of Ag85B of *M. tuberculosis* strongly affects its T cell-stimulating activity, i.e., the ability to induce IFN-γ production (4). Therefore, a similar change may have occurred in the MMP system, although it has not been clearly defined.

When we compared the immunostimulating activities of MMP-MTB and Fusion-MTB in terms of the activation of APC and T cells, the latter showed higher activity in the activation of both DC and $CD4^+$ and $CD8^+$ T cells. The exact mechanism of the high immunostimulating activity of the fusion protein is not fully known, but it may be associated with previous reports indicating that HSPs play a varied role in enhancing the ability of APCs to stimulate T cells (6, 10, 44, 45). In fact, the fusion protein induced the expression of higher levels of APC-associated molecules on DC than MMP did. Further, Fusion-MTB may be useful to produce cytotoxic $CD8^+$ T cells because the fusion protein efficiently produced perforin-producing $CD8^+$ T cells, although both MMP-MTB and Fusion-MTB produced cytotoxic $CD8^+$ T cells. Moreover, the fusion protein upregulated the expression of CD40 on DC (not shown) and treatment of Fusion-MTB-pulsed DC with CD40L induced the production of a larger dose of IFN-γ from both naïve $CD4^+$ T cells (not shown) and naïve $CD8^+$ T cells (Fig. 4B). These results indicate that the use of HSP70 as part of a fusion protein may make APCs susceptible to various conditioning molecules, including CD40L. This observation is in the line with the fact that only Fusion-MTB-pulsed monocyte-derived macrophages successfully activated $CD4^+$ T cells, probably MMP primed, when conditioned with CD40L.

Taken together, the data present here suggest that MMP, alone or as part of fusion protein, is highly immunogenic and may be useful for developing new vaccine against tuberculosis, at least in combination with BCG, ESAT-6, or other molecules.

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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* mc^{2}155 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* mc^{2}155 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* mc^{2}155 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

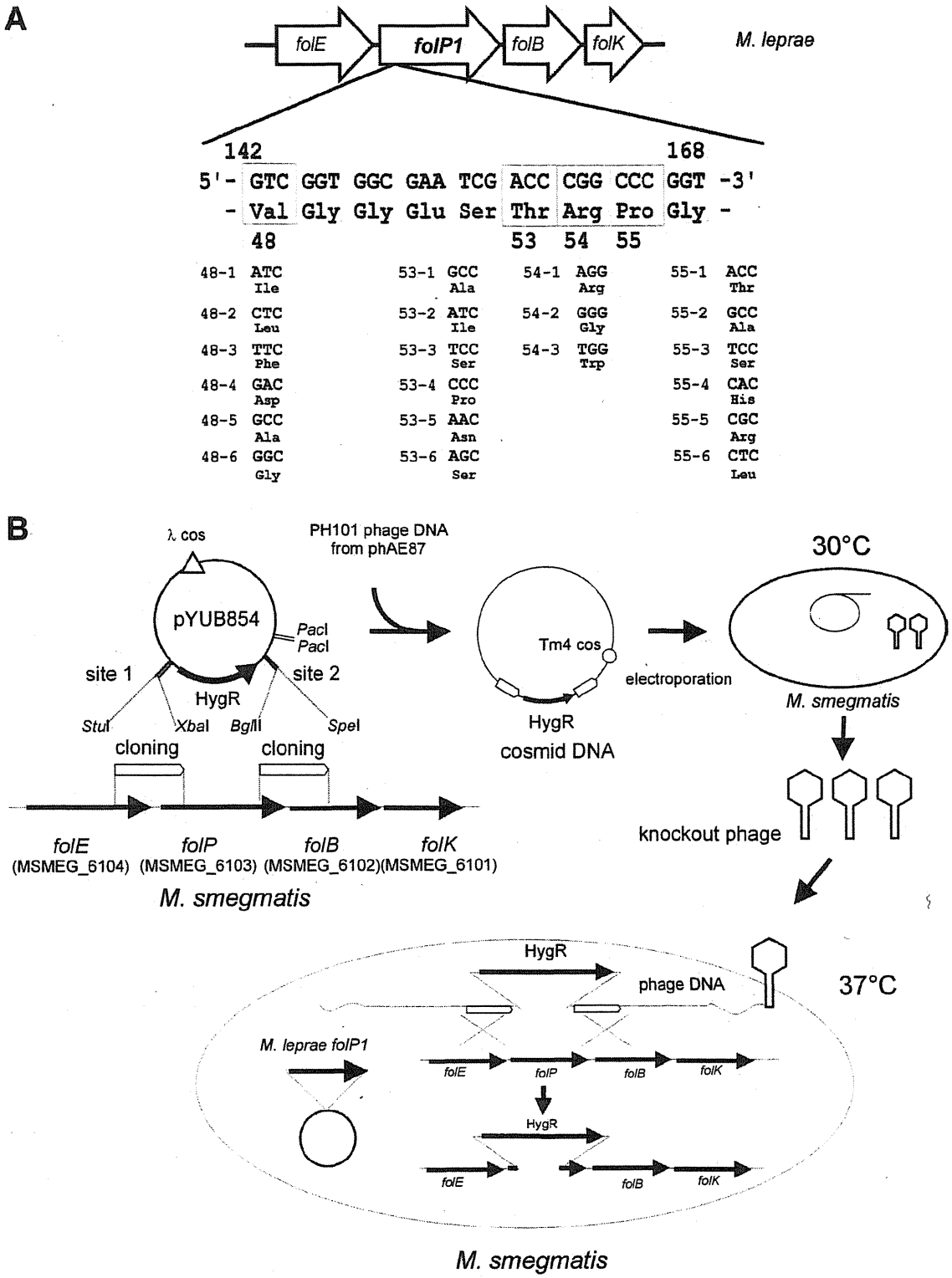

FIG. 1. Construction of recombinant *M. smegmatis* strains for dapsone susceptibility testing. (A) Point mutations introduced in the *M. leprae folP1* gene. Single nucleotide substitutions introduced in the *M. leprae folP1* at codons 48, 53, 54, and 55 are shown. Deduced amino acid residues are shown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

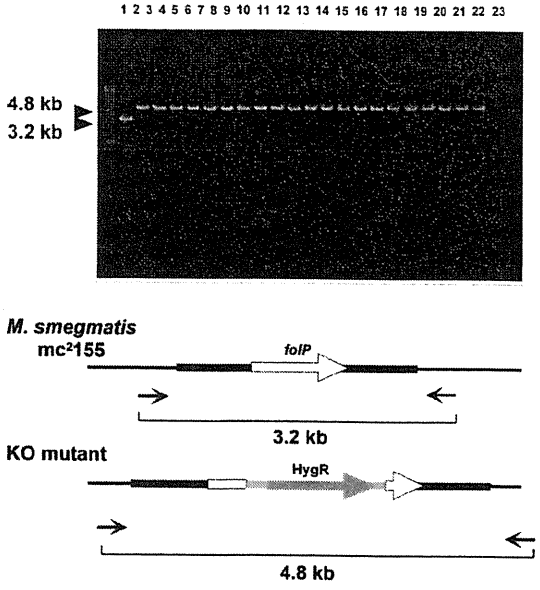

FIG. 2. PCR analysis to confirm the disruption of *folP*. Black arrows represent primers MSFPF and MSFPR for the PCR amplification. Lane 1, *M. smegmatis* mc²155; lanes 2 to 22, *M. smegmatis* strains carrying the *M. leprae folP1* without mutation and *folP1* with mutations 48-1,48-2, 48-3, 48-5, 48-6, 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

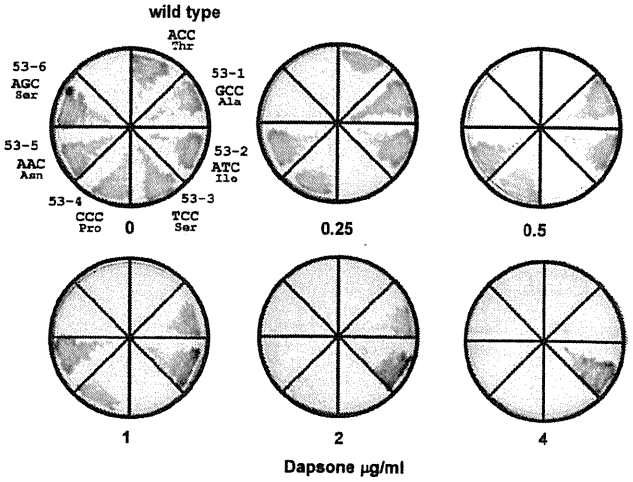

FIG. 3. Dapsone susceptibility of recombinant *M. smegmatis*. Results for *M. smegmatis* strains with point mutations at codon 53 of the *M. leprae folP1* are shown. Dapsone concentration is depicted below each plate.

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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MAJOR ARTICLE

Analysis of Drug-Resistant Strains of *Mycobacterium leprae* in an Endemic Area of Vietnam

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(See brief report by Ramien and Wong, e133–e135.)

Background. Multidrug therapy has effectively reduced the number of leprosy cases in the world. However, the rate of reduction has decelerated over the years, giving early detection of *Mycobacterium leprae* and epidemiological study of relapse renewed relevance in attempts to eliminate the disease.

Methods. A molecular epidemiological survey for drug-resistant *M. leprae* was conducted in the central and highland regions of Vietnam. A total of 423 samples taken from patients, including 83 patients with new cases, 321 patients receiving treatment, and 19 patients with relapse, were studied for detection of *M. leprae* with mutations relating to drug resistance by sequencing the drug resistance determining region of the *folP1*, *rpoB*, and *gyrA* genes, which are responsible for dapsone, rifampicin, and ofloxacin resistance, respectively.

Results. Nineteen mutations were found in the *folP1* gene samples, and no mutations relating to drug resistance were found in either the *rpoB* or *gyrA* genes. Samples from patients with relapse showed *folP1* mutation rates as high as 57%, and the mutation rates in samples from new and recent cases were <10%. Patients with relapse who had histories of treatment with dapsone monotherapy showed high mutation rates (78%), compared with patients with relapse who had previously only received multidrug therapy (33%).

Conclusions. Our study indicated high rates of dapsone resistance in patients with relapse, compared with patients with new and recent cases of leprosy. Moreover, it was observed that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy.

Leprosy is a chronic infectious disease caused by infection with *Mycobacterium leprae*. The present strategy for leprosy control is based on the multidrug therapy (MDT), recommended by the World Health Organization (WHO) [1], which has successfully reduced the number of leprosy cases in the world. However, transition in the number of registered cases and new cases amounting to ~210,000 and ~250,000, respectively, has almost come to a standstill [2]. Drug-resistant strains were first found in 1964, 1976, and 1997 [3–5]. MDT was designed to prevent the emergence and spread of drug-resistant strains. However, a strain showing resistance to both dapsone and rifampicin was reported in 1993 [6], and at present, there are further reports indicating the emergence of *M. leprae* strains resistant to multiple drugs [5, 7]. At present, the rapid detection and control of such drug-resistant strains is essential in countries approaching leprosy elimination levels, such as Vietnam.

MDT has been quite successful in Vietnam, and elimination of leprosy (prevalence rate, < 1/10,000 population) was achieved on the national level in 1995 [8]. The prevalence rate per 10,000 population in 2006

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was .07 [8, 9]. However, the majority of patients with leprosy are found in the central and highland regions of Vietnam [10], consisting of 11 provinces, including 4 provinces in the highland region and 7 provinces in the delta region. In 2005, the number of patients with leprosy was 236, spread through 4 provinces of the highland region; the prevalence rate of newly detected cases was 3.5 cases/10,000 population, although the overall prevalence rate was .25 cases/100,000 population on the national level. The rate of newly detected cases in the 7 delta region provinces was 1.38 cases/10,000 population [8, 9]. These cases not only present the danger of being possible infectious sources for leprosy but also harbor the risk of developing into relapse cases. However, little is known regarding the effects of drug-resistant *M. leprae* in patients with leprosy, especially in cases of relapse.

Therefore, in the present study, molecular epidemiological studies on drug-resistant strains were conducted in 11 provinces primarily in the central and highland regions that represent the areas where leprosy is endemic in Vietnam.

MATERIALS AND METHODS

Sensitivity of Polymerase Chain Reaction

The number of bacilli isolated from nude mice footpads was counted using the method described by Shepard et al [11]. Serial 10-fold dilutions of the enumerated *M. leprae* bacilli were used for polymerase chain reaction (PCR) in our study.

Clinical Specimens

Samples (from slit-skin smears or punch biopsies) were taken from patients with leprosy after receipt of informed consent in primarily the central and highland regions of Vietnam (including 11 provinces: Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Kontum, Gialai, Daknong, and Daklak), and the samples were classified as new (before starting MDT), recent (receiving MDT), and relapse cases. Relapse was defined as development of new skin lesions after completion of MDT and increase in bacterial index by >2 log units in any lesion.

The total of 423 samples included those from 83 patients with new cases, 321 patients with recent cases (receiving treatment), and 19 patients with relapse (collection period: March 2004–August 2009). Among 16 patients with relapse who had positive results of *M. leprae*–specific PCR, 9 cases were determined to be relapse after dapsone monotherapy (7–20 years), 3 as relapse after complete MDT, 2 as second relapse (the first after dapsone monotherapy and the second after MDT), and 2 as relapse after ofloxacin treatment. Samples were obtained from the skin lesions of patients (smear on blade or biopsy soaked in 1 mL of 70% ethanol at room temperature in the field, before being sent to Quyhoa National Leprosy & Dermato-Venereology Hospital laboratory).

DNA Extraction, Nested PCR, and Sequencing

M. leprae templates from both dilutions of *M. leprae* bacilli and slit-skin smears were prepared by treatment with lysis buffer at 60°C overnight, as described elsewhere [12]. Nested PCR amplification of the RLEP regions of *M. leprae* was performed under conditions described elsewhere with minor modifications, using the primers listed in Table 1 [13]. In brief, PCR amplification using special reagents (20 mM Tris-HCl [pH, 7.5], 8 mM magnesium chloride, 7.5 mM DTT, 2.5 mg BSA, 150 μM deoxynucleotides, 1.5 mM magnesium sulphate, and 2.5 units KOD-plus-Ver.2 DNA polymerase [Toyobo]) was performed using sample DNA as templates. Both first and second PCR conditions were as follows; strand separation at 94°C for 4 min, denaturing at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 20 s plus 1-s increment per cycle for 25 cycles. Products from the first PCR (0.5 uL) were used as templates in the second PCR. The nested PCR for DRDR was performed using the primer pairs listed in Table 1. Mutations were measured on the *folP1* gene for dapsone [14], the *rpoB* gene for rifampicin, and the *gyrA* gene for ofloxacin [15, 16]. Nested PCR conditions for drug resistance were different from that for RLEP-nested PCR. In brief, PCR amplification using standard reagents (10 mM Tris-HCl [pH, 8.3], 2 mM magnesium chloride, 250 μM dNTPs, and 2.5 units TaKaRa Ex Taq DNA polymerase [Takara shuzo]) was performed using sample genomic DNA as templates. The primer pairs used to amplify the specific drug-resistant genes are shown in Table 1. The reaction condition was 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 35 cycles.

The amplicons were visualized by agarose gel electrophoresis, and DNA was recovered from the gel using Mini-Elute gel extraction kits (Qiagen). The recovered DNA molecules were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems) and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The sequence data were analyzed by DNA analysis program Genetyx-MAC, version 15 (GENETYX), and were compared with those in the GenBank database.

RESULTS

PCR Sensitivity

Serial dilutions of the bacilli of 1×10^8–1×10^0 were prepared to determine PCR sensitivities. Genomic DNAs were extracted from the diluents with use of methods described under Materials and Methods [11]. The previously reported RLEP-nested PCR (named RLEP-L) was capable of detecting 1×10^2 bacilli in samples (Figure 1a) [13]. The newly designed RLEP-nested PCR, using K1 and K2 primers for the first PCR and LP1 and LP2 primers for the second PCR (named RLEP-K), is capable of detecting comparable counts of bacilli (Figure 1b), and RLEP-K

Table 1. Sequences of Primers Used in this Study

Name	Usage	Gene	Sequence, 5′→3′	Reference	Size, bp
K1	First PCR (F)	RLEP	CGTGGGTGTGAGGATAGTTGT-	Present study	268
K2	First PCR (R)	RLEP	GATCATCGATGCACTGTTCACT-	Present study	
LP1	First or second PCR (F)	RLEP	TGCATGTCATGGCCTTGAGG-	13	129
LP2	First or second PCR (R)	RLEP	CACCGATACCAGCGGCAGAA	13	
LP3	Second PCR (F)	RLEP	TGAGGTGTCGGCGTGGTC	13	99
LP4	Second PCR(R)	RLEP	CAGAAATGGTGCAAGGGA	13	
F1	Second PCR (F)	*folP1*	GCAGGTTATTGGGGTTTTGA	Present study	312
F2	First PCR(R)	*folP1*	CCACCAGACACATCGTTGAC	Present study	
F3	Second PCR (F)	*folP1*	CTTGATCCTGACGATGCTGT	Present study	245
F4	Second PCR(R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	
F5	Sequencing primer (F)	*folP1*	ATCCTGACGATGCTGTCCA	Present study	–
F4	Sequencing primer (R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	–
R1	First PCR (F)	*rpoB*	CAGACGCTGATCAATATCCGT	Present study	358
R2	First PCR (R)	*rpoB*	CAGCGGTCAAGTATTCGATC	Present study	
R3	Second PCR (F)	*rpoB*	CAATATCCGTCCGGTGGTC	Present study	337
R4	Second PCR (R)	*rpoB*	GTATTCGATCTCGTCGCTGA	Present study	
R5	Sequencing primer (F)	*rpoB*	ACGCTGATCAATATCCGTCC	Present study	–
R6	Sequencing primer (R)	*rpoB*	CGACAA TGAACCGATCAGAC	Present study	–
G1	First PCR (F)	*gyrA*	ACGCGATGAGTGTGATTGTGG	Present study	336
G2	First PCR (R)	*gyrA*	TCCCAAATAGCAACCTCACC	Present study	
G3	Second PCR(F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	291
G4	Second PCR (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	
G3	Sequencing primer (F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	–
G4	Sequencing primer (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	–

products are visualized more clearly with less smear bands. Therefore, the new RLEP-K system was used for detection in further experimentation with use of clinical samples.

Using DNAs extracted from the serial dilutions of *M. leprae*, we determined the sensitivity of the nested PCR for DRDRs. The limit of amplification by PCR was 1×10^3–1×10^4 bacilli (Figure 1 c–e).

RLEP-nested PCR for Clinical Samples

The PCR methods were applied on 423 clinical samples collected from areas of endemicity in Vietnam. First, we tested RLEP-K for detection of *M. leprae* after extraction of DNA from smear samples. Positive bands were obtained by gel electrophoresis using RLEP-K on 290 samples. The positivity rate was 69%. The patients supplying the 290 samples were divided into 3 categories: new, relapse, and recent cases. Positive rates of RLEP-K by category were 75%, 84%, and 66%, respectively (Table 2).

Mutations in Clinical Samples

Samples positive by RLEP-nested PCR were applied for mutation experiments on the DRDRs of *folP1*, *rpoB*, and the *gyrA* gene. Nineteen mutations were found in 187 *folP1* samples, but no mutations related to drug resistance were noted in 163 *rpoB* and 147 *gyrA* gene samples. The mutations detected on *folP1* were as follows: 6 cases of ACC to ATC in codon 53(threonine to isoleucine), 9 cases of CCC to CGC in codon 55 (proline to arginine), and 4 cases of CCC to CTC (proline to leucine). Two new cases, 8 relapse cases, and 9 recent cases had mutations on *folP1*. Mutation rates in the 3 categories were 6.1%, 57%, and 6.4%, respectively (Table 3).

Some missense mutations, of which the association with drug resistance is unknown, were detected in the *rpoB* gene from clinical samples. The mutations were detected in 7 patients at codons 517, 532, and 556. One patient with relapse showed a mutation from CAG (glutamine) to CAT (histidine) at codon 517. One new patient showed 2 mutations at codon 517 from CAG (glutamine) to CAT (histidine) and at codon 532 from GCG (alanine) to TCG (serine). Sequence electropherograms indicated double peaks of a second nucleotide at codon 556 in 3 patients categorized as having recent cases. One peak was G (identical to that of wild-type), and the other peak was T, which changed the amino acid from glycine (GGC) to valine (GTC; data not shown).

The Relation between Treatment and Drug-Resistant Mutations in Patients with Relapse

Patients with relapse were categorized into 4 groups, by treatment history (Table 4). Group 1 comprised those treated with dapsone monotherapy. Group 2 was treated with MDT for 24 months. Group 3 included patients who had received

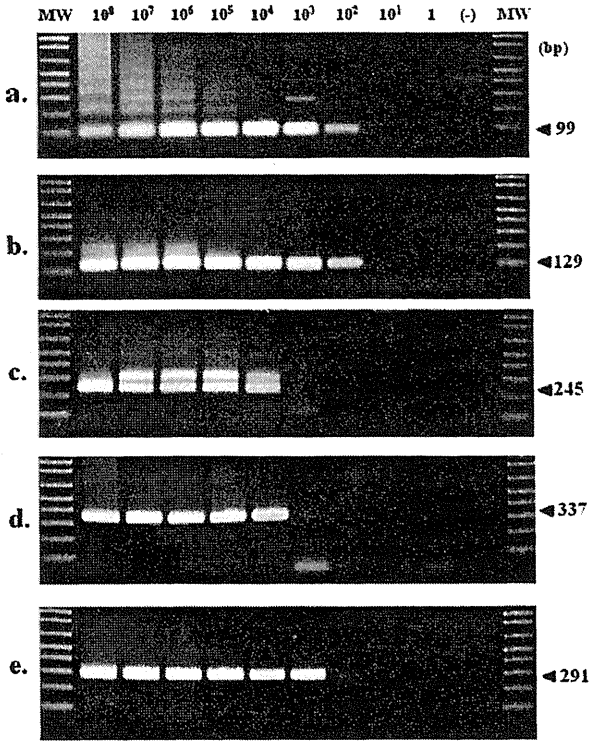

Figure 1. Sensitivity of nested polymerase chain reaction (PCR). The nested PCR products were visualized on 2 % agarose gel. A, RLEP-nested PCR (RLEP-L) using primers, LP1-LP4 (final products size, 99 bp). B, RLEP-nested PCR (RLEP-K) using primers, K1, K2, LP1, and LP2 (final products size, 129bp). C, *folP1*-nested PCR using F1-F4. D, *rpoB*-nested PCR using R1-R4. E, *gyrA*-nested PCR using G1-G4.

a diagnosis of second relapse—once after treatment with dapsone monotherapy and, subsequently, after MDT for 24 months. Group 4 was treated with ofloxacine monotherapy. Eight of the 14 patients with *folP1*-amplified relapse cases (57%) had mutations on the *folP1* gene. Seven (78%) of 9 patients with relapse who were categorized in groups 1 and 3 also had *folP1* mutations. However, 2 patients in group 4 had no mutations on any of the 3 genes.

Monitoring of Mutations in Patients

One hundred seven slit-skin smear samples from 43 patients were taken with consents at different times from each patient for monitoring mutations under treatment. Table 5 shows the difference in mutation results between 5 such patients. The other 38 patients showed no mutation during monitoring. Patients A, B, and C, who had new cases, showed a similar pattern, with no mutation at first testing and mutation in codon 53 on the *folP1* gene during MDT. However, double peaks of T and C in the second base were observed on *folP1* in the 3 patients. Patients D and E, who had relapse cases and finished dapsone monotherapy 20 years earlier, had a mutation on *folP1* in 2005 and no mutation after MDT.

Table 2. Polymerase Chain Reaction Positivity in New, Relapse, and Recent Cases

Case category	No.	RLEP	*folP1*	*rpoB*	*gyrA*
New	83	62 (75%)	33	39	43
Relapse	19	16 (84%)	14	15	13
Recent	321	212 (66%)	140	109	91
Total	423	290 (69%)	187 (64%)	163 (56%)	147 (51%)

DISCUSSION

The most popular PCR method for *M. leprae* detection with high sensitivity and specificity is probably the RLEP-nested PCR method, because the RLEP regions are specific for *M. leprae*, with >28 copies dispersed through the *M. leprae* genome [17]. New primers were designed for the RLEP-nested PCR in our study. This system using the new primers was termed RLEP-K. RLEP-K products appear to be a somewhat sharper and stronger band on agarose gel electrophoresis, compared with that that of previous RLEP-nested PCR (ie, RLEP-L). The RLEP-K detected *M. leprae* in 69% of the Vietnam samples. The remaining 31% of the samples were deduced as being cases either cleared of *M. leprae* by chemotherapy or those having <100 bacilli, which was below the detection limit of RLEP-K. We also designed new primers for amplification and sequencing of DRDR in the drug-resistance related genes *folP1*, *rpoB*, and *gyrA*, which were applied in examining the Vietnam samples. The mutation rates of *folP1* in new and recent cases were 6.1% and 6.4%, respectively. In contrast, the mutation rate in relapse cases was quite high, at 57%. The result indicated a strong correlation between mutation rate and relapse. Two possible reasons were conceived regarding the high positive rate of dapsone resistance in patients with relapse: (1) reinfection by the primary drug-resistant strain (7 of 8 samples indicating relapse were collected in the province in central Vietnam, which had the highest prevalence of leprosy and high rate of relapse (data not shown) and (2) reactivation of dapsone-resistant strains capable of

Table 3. Number of Mutations on *folP1*

Case category	No. of PCR-positive cases	No. of mutations (mutation ratio)	No. of mutation in mutation types
New	33	2 (6.1%)	2 (55th: CCC-CGC)
Relapse	14	8 (57%)	2 (53rd: ACC-ATC)
			3 (55th: CCC-CGC)
			3 (55th: CCC-CTC)
Recent	140	9 (6.4%)	4 (53rd: ACC-ATC)
			4 (55th: CCC-CGC)
			1 (55th: CCC-CTC)

Table 4. Mutations Noted in RLEP-Positive Relapse Cases, by Treatment Group

Group	Past treatment	No.	Mutation on *folP1*	Mutation on *rpoB*	Mutation on *gyrA*
1	DDS	7	5	0	0
2	MDT (24 months)	3	1	1[a]	0
3	DDS plus MDT (24 months)	2	2	0	0
4	OFX	2	0	0	0
All	...	14	8	1[a]	0

Abbreviations: DDS (diaminodiphenylsulfone), dapsone monotherapy; MDT,multidrug treatment; OFX, Ofloxacine monotherapy.

[a] Unknown DR mutation

persisting after chemotherapy, discussed below. Although it is still unclear whether the relapses are caused by reinfection by *M. leprae* or by reactivation of persistent *M. leprae*, close correlation between drug resistance and relapse have been recognized in several studies [18, 19].

The proportion of samples showing mutation on the *folP1* gene related to dapsone resistance was 10.2% (19 of 187) in samples from the central and highland regions of Vietnam (Table 3). Comparison with previous reports from South Korea (19.2%) indicates lower rates of relapse in these regions of Vietnam [20].

No mutation was found in the DRDR regions of *rpoB* in all samples. Mutation frequencies of the *rpoB* gene are also very low in other reports. Regarding other areas in Southeast Asia, no cases of rifampicin resistance have been detected in the Philippines, 1 (1.9%) of 54 cases in Myanmar, and 4 (3.3%) of 121 cases in Indonesia. However, in Japan, where the prevalence of leprosy is very low, the reported rate of rifampicin resistance is very high, at 29.5% (26 of 88 cases) [21]. The long-term use of drugs outside the standard MDT regimen in Japanese leprosy cases might have been instrumental in promoting this rifampicin resistance.

As such, no mutations have been found in the DRDR of the *M. leprae rpoB* gene derived from patients with leprosy, including relapse cases in Vietnam. A possible explanation for this could be the success of leprosy control in Vietnam and efficacy of properly administered MDT in which rifampicin—with its bactericidal properties—was effective in suppressing the occurrence of drug-resistant bacilli. In contrast, dapsone (not bactericidal in itself, although capable of suppressing growth), which had previously been used as monotherapy, may have enabled bacteria surviving in the patient receiving treatment to develop mutations, giving them resistance against the drug. Although occurrence of drug-resistant *M. leprae* was kept very low after application of MDT, 7 of 9 samples with drug-resistant mutations had previously been treated by dapsone monotherapy (Table 4). Jing et al [22] reported that patients with multibacillary leprosy who were retreated with MDT after dapsone monotherapy may have lower risk of early relapse while continuing to carry the risk of late relapse. Our observations suggest the possibility that efficacy of MDT may be hampered in some patients by the presence of surviving dapsone-resistant *M. leprae* in their bodies, which could develop into late relapse. Similar observations have been reported, suspecting involvement of the effects of dapsone monotherapy in patients with relapse [23].

There was no mutation in the major sites for drug resistance on the *rpoB* gene. However, we observed mutations at 3 positions, codons 517, 532, and 556, which have not been associated with rifampicin resistance. These mutations in the *rpoB* gene are a finding calling for further clarification.

Table 5. Monitoring of 5 Patients with Multibacillary Leprosy for *folP1* Mutation

Patient	Case category	Date of sample obtainment	Sample site (method of obtainment)	*folP1* mutation
A	New	2006 April 3	Abdomen (biopsy)	None[a]
		2007 January 30	Earlobe (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Abdomen (smear)	53rd (ACC→ATC/ACC)
B	New	2005 May 31	Earlobe (smear)	None
		2006 March 24	Skin (smear)	None
		2007 November 2	Skin (smear)	53rd (ACC→ATC/ACC)
C	New	2006 July 20	Skin (smear)	None
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
D	Relapse	2005 November	Earlobe (smear)	55th (CCC→CGC)
		2007 January	Skin (smear)	None
E	Relapse	2007 Jannuary 17	Arm (smear)	None
		2007 January 30	Earlobe (smear)	55th (CCC→CGC)
		2007 January 30	Arm (smear)	None

[a] ACC ATC/ACC indicates double peaks in second base at codon 53.

To reveal the possible relation between treatment and gene mutation, some patients with leprosy were monitored for gene mutations in light of drug treatments. The results showed incidence of dapsone-resistant *M. leprae* in patients receiving MDT, suggesting that some of the patients with relapse who were previously treated with dapsone monotherapy might have persistent infections with dapsone-resistant *M. leprae*. Furthermore, samples derived from different sites of lesions in the same patient sometimes showed different results (Table 5). The results suggest that we need to know the relation between the situation of patients with leprosy and drug resistance.

Overall, our study indicated a high ratio of dapsone resistance in patients with relapse, compared with the other patients with leprosy. In contrast, an unexpected outcome of our study was that we were unable to find mutations on the *rpoB* gene in patients with relapse. Moreover, it was shown that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy. To clarify the relationship between relapse, drug resistance, and dapsone monotherapy, it might be necessary to investigate persistence of drug-resistant *M. leprae* through large-scale surveillance.

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Potential conflicts of interest. All authors: no conflicts.

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

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Glycopeptidolipids (GPLs) are one of the major glycolipid components present on the surface of *Mycobacterium avium* complex (MAC) that belong to opportunistic pathogens distributed in the natural environment. The serovars of MAC, up to around 30 types, are defined by the variable oligosaccharide portions of the GPLs. Epidemiological studies show that serovar 4 is the most prevalent type, and the prognosis of pulmonary disease caused by serovar 4 is significantly worse than that caused by other serovars. However, little is known about the biosynthesis of serovar 4-specific GPL, particularly the formation of the oligosaccharide portion that determines the properties of serovar 4. To investigate the biosynthesis of serovar 4-specific GPL, we focused on one segment that included functionally unknown genes in the GPL biosynthetic gene cluster of a serovar 4 strain. In this segment, a putative hemolytic protein gene, *hlpA*, and its downstream gene were found to be responsible for the formation of the 4-*O*-methyl-rhamnose residue, which is unique to serovar 4-specific GPL. Moreover, functional characterization of the *hlpA* gene revealed that it encodes a rhamnosyltransferase that transfers a rhamnose residue via 1→4 linkage to a fucose residue of serovar 2-specific GPL, which is a key pathway leading to the synthesis of oligosaccharide of serovar 4-specific GPL. These findings may provide clues to understanding the biological role of serovar 4-specific GPL in MAC pathogenicity and may also provide new insights into glycosyltransferase, which generates structural and functional diversity of GPLs.

The genus *Mycobacterium* has a unique feature in the cell envelope that contains a multilayered structure consisting of peptidoglycan, mycolyl-arabinogalactan complex, and surface glycolipids (8, 12). It is known that these components play a role in protection from environmental stresses, such as antimicrobial agents and host immune responses (8, 12). Some of them are recognized as pathogenic factors related to mycobacterial diseases, such as tuberculosis and leprosy (8, 12). In case of nontuberculous mycobacteria that are widely distributed in the natural environment as opportunistic pathogens, glycopeptidolipids (GPLs) are abundantly present on the cell envelope as surface glycolipids (34). GPLs have a core structure in which a fatty acyl-tetrapeptide is glycosylated with 6-deoxy-talose (6-d-Tal) and *O*-methyl-rhamnose (*O*-Me-Rha) (2, 5, 13). This structure is common to all types of GPLs, and GPLs with this structure that have not undergone further glycosylation are termed non-serovar-specific GPLs (nsGPLs) (2, 5, 13). Structural diversity generated by further glycosylations, such as rhamnosylation, fucosylation, and glucosylation, is observed for the oligosaccharide portion linked to the 6-d-Tal residue of nsGPLs from *Mycobacterium avium* complex (MAC), a member of the nontuberculous mycobacteria consisting of two species, *M. avium* and *M. intracellulare* (2, 5, 34). Consequently, these nsGPLs with varied oligosaccharides lead to the formation of the serovar-specific GPLs (ssGPLs) that define around 30 types of MAC serovars (10).

The properties of MAC serovars are known to be notably different from each other and also to be closely associated with the pathogenicity of MAC (3, 6, 18, 30, 31, 32). Various epidemiological studies indicate that serovar 4 is the most prevalent type and is also one of the serovars frequently isolated from AIDS patients (1, 20, 33, 36). Additionally, pulmonary MAC disease caused by serovar 4 is shown to exhibit a poorer prognosis than that caused by other serovars (23). With respect to host immune responses to MAC infection, serovar 4-specific GPL is reported to have characteristic features that are in contrast to those of other ssGPLs (21, 30). Structurally, serovar 4-specific GPL contains a unique oligosaccharide in which the oligosaccharide of serovar 2-specific GPL is further glycosylated with 4-*O*-methyl-rhamnose (4-*O*-Me-Rha) residue through a 1→4 linkage (Table 1) (24). Therefore, it is thought that the presence of 4-*O*-Me-Rha and its linkage position are important in exhibiting the specificity of biological activities. The biosynthesis of the oligosaccharide portion in several ssGPLs is currently being clarified (15, 16, 17, 25, 26), while that of serovar 4-specific GPL is still unresolved. In this study, we have focused on the genomic region predicted to be associated with GPL biosynthesis in the serovar 4 strain and explored the key genes responsible

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TABLE 1. Oligosaccharide structures of serovar 2- and 4-specific GPLs

Serovar	Oligosaccharide	Reference
2	2,3-di-*O*-Me-α-L-Fuc-(1→3)-α-L-Rha-(1→2)-L-6-d-Tal	9
4	4-*O*-Me-α-L-Rha-(1→4)-2-*O*-Me-α-L-Fuc-(1→3)-α-L-Rha-(1→2)-L-6-d-Tal	24

for the formation of 4-*O*-Me-Rha that might determine the specific properties of MAC serovar 4.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation. Table 2 indicates the bacterial strains and vectors used in this study. MAC strains were grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 supplemented with 10% Middlebrook ADC enrichment (BBL). For GPL production, *Mycobacterium smegmatis* strains were cultured in 2× YT broth (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) with 0.2% Tween 80. DNA manipulation of *M. smegmatis* strains was conducted as previously described (27). PCR amplification was done by two-step PCR using TaKaRa LA *Taq* with GC buffer, with the following program: denaturation at 98°C for 20 s and annealing-extension at 68°C for an appropriate time depending on the length of the targeted region. *Escherichia coli* strain DH5α was used for the routine manipulation and propagation of plasmid DNA. Antibiotics were added as required: kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for *M. smegmatis*; hygromycin B, 150 μg/ml for *E. coli* and 75 μg/ml for *M. smegmatis*. Oligonucleotide primers used in this study are listed in Table 3.

Construction of expression vectors. For generation of the serovar 2-specific GPL (GPL-S2)-producing strain, the vector possessing *rtfA*, *mdhtA*, *merA*, and *gtfD* genes was constructed. The *rtfA* gene was amplified from genomic DNA of *M. avium* strain JATA51-01 using primers RTFA-S and RTFA-A. The *mdhtA*, *merA*, and *gtfD* genes were amplified as one operon from the previously constructed vector pMVΔmtfF using primers MDHTA-S2 and GTFD-A2 (26). After construction of pMV261a, in which an AflII site was introduced into pMV261, the above two PCR products were digested with each restriction enzyme and cloned into the BamHI-PstI and PstI-AflII sites of pMV261a to give pMV-rtfA-mdhtA-merA-gtfD (Table 2). The fragments for construction of expression vectors were amplified from genomic DNA of MAC serovar 4 strain (ATCC 35767) using the following primers: HLPA-S and HLPA-A for *hlpA*, HLPA-S and ORF2-A for *hlpA-orf2*, and ORF3-S and ORF5-A for *orf3-orf4-orf5*. These PCR products were digested with each restriction enzyme and cloned into the EcoRI-ClaI, EcoRI-HindIII, and PstI-EcoRI sites of pYM301a to give pYM-hlpA, pYM-hlpA-orf2, and pYM-orf3-orf4-orf5, respectively (Table 2).

Isolation and purification of GPLs. To isolate whole-lipid extracts, harvested bacterial cells were mixed with $CHCl_3/CH_3OH$ (2:1 [vol/vol[) for several hours at room temperature. The extracts in organic phase were separated by adding water and evaporated until dry. To remove the lipid components except for GPLs, the whole-lipid extracts were subjected to mild alkaline hydrolysis to prepare the crude GPLs as previously described (27, 28). For analytical thin-layer chromatography (TLC), crude GPLs on silica gel 60 plates (Merck) were developed with $CHCl_3/CH_3OH/H_2O$ (30:8:1 [vol/vol/vol]), followed by spraying with 10% H_2SO_4 and charring. Purified GPLs were prepared from crude GPLs by preparative TLC on the same plates and extracted from the bands corresponding to each GPL. To determine the linkage position of sugar moieties, perdeuteriomethylation was performed for purified GPLs as previously described (7, 11, 15).

GC-MS and MALDI-TOF MS analysis. Purified and perdeuteriomethylated GPLs were hydrolyzed in 2 M trifluoroacetic acid (2 h, 120°C), and the released sugars were reduced with $NaBD_4$ and then acetylated with pyridine/acetic anhydride (1:1 [vol/vol]) at room temperature overnight. The resulting alditol acetates were analyzed by gas chromatography-mass spectrometry (GC-MS) with a GCMS-QP2010 (Shimadzu) equipped with a SP-2380 column (Supelco) using helium gas. The temperature program was from 52 to 172°C with an increase in temperature of 40°C/min, 172 to 223°C at 3°C/min, and then 223 to 270°C at 40°C/min. To determine the total mass of the purified GPLs, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were acquired with an Ultraflex II (Bruker Daltonics). Samples were dissolved in chloroform-methanol (2:1 [vol/vol]) at a concentration of 1 mg/ml, 1 μl was applied directly to the sample plate, and then 1 μl of 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform/methanol (1:1 [vol/vol]) was added as a matrix. The purified GPL was analyzed in the reflectron mode with an accelerating voltage operating in a positive mode of 20 kV (17).

Nucleotide sequence accession number. The 6.8-kb genomic region amplified from the MAC serovar 4 strain (ATCC 35767) by using primers GTFB-S1 and MDHTA-A2 has been deposited in the DDBJ nucleotide sequence database under accession no. AB550236.

RESULTS

Previously, the A5 strain, one of the MAC serovar 4 strains, was reported to contain a genomic region similar to the GPL biosynthetic gene cluster identified in other serovars (22). However, to date, there are no studies clarifying the biosynthetic pathways involved in the formation of 4-*O*-Me-Rha, which is unique to serovar 4-specific GPL. To explore this glycosylation pathway, we focused on one segment interposed with the *gtfB* and *mdhtA* genes whose organization was shown to vary in strains of other serovars (14, 22). In this study, using another serovar 4 strain, ATCC 35767, whose genomic information is unknown, we designed various primers for PCR amplification of a focused segment based on the sequences from other serovar strains. After the testing of primer pairs, a

TABLE 2. Bacterial strains and vectors used in this study

Strain or vector	Characteristic(s)	Source or reference
Bacterial strains		
E. coli DH5α	Cloning host	TaKaRa
M. smegmatis mc^{2}155	Expression host	29
M. intracellulare ATCC 35767	MAC serovar 4 strain	35
M. avium JATA51-01	Source of the *rtfA* gene	26
Vectors		
pYM301a	Site-specific integrating mycobacterial vector carrying an *hsp60* promoter cassette	25
pMV261a	*E. coli-Mycobacterium* shuttle vector carrying an *hsp60* promoter cassette with an AflII site	This study
pMVΔmtfF	Source of *mdhtA*, *merA*, and *gtfD* genes	26
pMV-rtfA-mdhtA-merA-gtfD	pMV261a carrying *rtfA*, *mdhtA*, *merA*, and *gtfD* genes	This study
pYM-hlpA	pYM301a carrying the *hlpA* gene	This study
pYM-hlpA-orf2	pYM301a carrying the *hlpA* gene and ORF2	This study
pYM-orf3-orf4-orf5	pYM301a carrying ORF3, ORF4, and ORF5	This study

TABLE 3. Oligonucleotide primers used in this study

Primer	Sequence[a]	Restriction site
RTFA-S	5′-CGGGATCCCATGAAATTTGCTGTGGCAAG-3′	BamHI
RTFA-A	5′-AACTGCAGCTCAGCGACTTCGCTGCGCTTC-3′	PstI
MDHTA-S2	5′-GCTCTAGACTGCAGAAAAACCAACTTCTACTGCCTGACCTG-3′	PstI
GTFD-A2	5′-GGAATTCTTAAGTCTACGGTTCTGCGCTTCGTTCTTTG-3′	AflII
HLPA-S	5′-GGAATTCGTGACAACGACGCCACCAGT-3′	EcoRI
HLPA-A	5′-CCATCGATACTACGCTGCCGCGCTAGGCG-3′	ClaI
ORF2-A	5′-CCCAAGCTTCTCAGACTCTAACGTACAGTTC-3′	HindIII
ORF3-S	5′-CACCTGCAGAAATGACCGCCACAACCAGGGC-3′	PstI
ORF5-A	5′-GCAGAATTCCTACGGCGCCAATTCGATGAG-3′	EcoRI
GTFB-S1	5′-GGAACTCCTGCACCTTGGGGCCGT-3′	
MDHTA-A2	5′-GGTGCGGGTCAACGTAGAGGTG-3′	

[a] Underlining indicates the restriction site.

6.8-kb fragment was amplified with primers GTFB-S1 and MDHTA-A2 (Fig. 1). Nucleotide sequences of the amplified fragments were similar to that of the GPL biosynthetic gene cluster from the A5 strain (94% identity in nucleotide sequences) (GenBank accession no. AY130970.1). This segment contains five complete open reading frame (ORF) genes (Fig. 1): the ORF1 gene, similar to a putative hemolytic protein gene (*hlpA*) previously found in the GPL biosynthetic gene cluster of the serovar 2 strain (69% identity in amino acid sequences) (GenBank accession no. AF125999.1) (14); the ORF2 gene, an undefined gene showing low similarity to some *O*-methyltransferases; and the ORF3, ORF4, and ORF5 genes, with amino acid sequences almost identical to those of three proteins, including GtfTB, which were previously identified as biosynthetic enzymes for serovar 8-specific GPL (GenBank accession no. AB437139.1) (25).

Prior to functional analysis of each ORF, it was necessary to prepare a strain producing the substrate for the enzymes participating in the biosynthesis of serovar 4-specific GPL. Since serovar 4-specific GPL has a structure in which the terminal Fuc residue of serovar 2-specific GPL is further glycosylated with 4-*O*-Me-Rha, we created a recombinant *M. smegmatis* strain (termed MS-S2) by introducing the plasmid vector pMV-rtfA-mdhtA-merA-gtfD possessing *M. avium rtfA*, *mdhtA*, *merA*, and *gtfD* genes, which were previously shown to convert nsGPLs to serovar 2-specific GPL with a terminal Fuc residue (termed GPL-S2) (26). For five ORFs, we first examined the function of the ORF1 (termed *hlpA*) and its downstream ORF2 gene by TLC analysis of recombinant strains, because these have not been functionally defined and it is difficult to predict the role of each gene. In comparison with the profile of the control strain (MS-S2/pYM301a) (Fig. 2, lane A), the new products (GPL-S4) were observed for the strain with the *hlpA* gene introduced (MS-S2/pYM-hlpA) (Fig. 2, lane B). Moreover, when the expression vector covering both

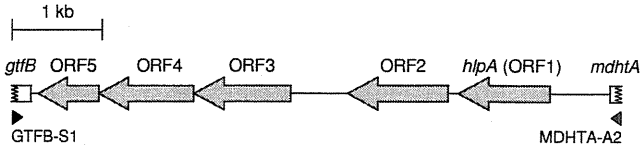

FIG. 1. Organization of the 6.8-kb genomic segment isolated from MAC serovar 4 strain (ATCC 35767). Filled triangles indicate the primers used for PCR amplification.

hlpA and ORF2 was introduced into MS-S2 (MS-S2/pYM-hlpA-orf2), another new product (GPL-S4M) appeared (Fig. 2, lane C). These observations indicated that GPL-S2 was converted to structurally different compounds by the expression of *hlpA* and that the compounds generated by *hlpA* were further modified by ORF2. As for the ORF3, ORF4, and ORF5 genes, which show a high similarity to the biosynthetic genes for serovar 8-specific GPL, we further generated a strain having three ORFs (MS-S2/pYM-orf3-orf4-orf5) and examined the GPL production by TLC analysis (Fig. 2, lane D). The results indicated the appearance of known product GPL-S8, previously shown to have a sugar residue of serovar 8-specific GPL, with no GPL-S4 and GPL-S4M (25), demonstrating that the enzymes encoded by three ORFs might act on the serovar 1-specific GPL which was produced as a precursor of GPL-S2 and subsequently yielded GPL-S8.

Because the compounds produced by *hlpA* and ORF2 were structurally unidentified, we performed a GC-MS analysis of the products GPL-S2, GPL-S4, and GPL-S4M, which were purified from recombinant strain MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively. Although two spots were seen for each product, this might be due to a different methylation pattern for the fatty acid portion, which is often observed with GPL biosynthesis of *M. smegmatis* and does not affect oligosaccharide structure (19, 25). In GC-MS profiles of GPL-S2 and GPL-S4, the classes of the detected sugar residues, Fuc, 6-d-Tal, Rha, and 2,3,4-tri-*O*-Me-Rha, were found to be identical to each other (Fig. 3A and B). However, it was observed that the intensity of the Rha residue in GPL-S4 was higher than that of the other sugars, while in GPL-S2, the intensity of the Rha residue was lower than that of Fuc, indicating that the proportion of Rha content in GPL-S4 was relatively large compared to that in GPL-S2. These results implied that the *hlpA* gene mediates the transfer of an additional Rha residue to GPL-S2. In contrast, the profiles of GPL-S4M showed the presence of 4-*O*-Me-Rha that is specifically observed for serovar 4-specific GPL (Fig. 3C), demonstrating that ORF2 encodes a rhamnosyl 4-*O*-methyltransferase and that both genes are responsible for the formation of the unique sugar residue of serovar 4-specific GPL. Furthermore, we confirmed the molecular masses of products GPL-S2, GPL-S4, and GPL-S4M by MALDI-TOF MS analysis (Fig. 4). Each product contained two main pseudomolecular ions ($[M + Na]^+$) with 14 mass unit differences, indicating the