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Nucleotide Sequence of the *Mycobacterium leprae katG* Region

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Synthetic oligonucleotide primers based on the DNA sequence data of the *Escherichia coli*, *Mycobacterium tuberculosis*, and *Mycobacterium intracellulare katG* genes encoding the heme-containing enzyme catalase-peroxidase were used to amplify and analyze the *Mycobacterium leprae katG* region by PCR. A 1.6-kb DNA fragment, which hybridized to an *M. tuberculosis katG* probe, was obtained from an *M. leprae* DNA template. Southern hybridization analysis with a probe derived from the PCR-amplified fragment showed that the *M. leprae* chromosome contains only one copy of the putative *katG* sequence in a 3.4-kb *Eco*RI-*Bam*HI DNA segment. Although the nucleotide sequence of the *katG* region of *M. leprae* was approximately 70% identical to that of the *M. tuberculosis katG* gene, no open reading frame encoding a catalase-peroxidase was detectable in the whole sequence. Moreover, two DNA deletions of approximately 100 and 110 bp were found in the *M. leprae katG* region, and they seemed to be present in all seven *M. leprae* isolates tested. These results strongly suggest that *M. leprae* lacks a functional *katG* gene and catalase-peroxidase activity.

Two catalases have been extensively characterized in *Escherichia coli*. HPI (encoded by *katG*) is a prokaryotic broad-spectrum bifunctional peroxidase-catalase inducible by hydrogen peroxide (7). The *E. coli* catalase HPII (encoded by *katE*) is a monofunctional enzyme that has sequence similarities to eukaryotic catalase (17). Biochemical and serological characterizations of mycobacterial lysates have also identified two mycobacterial catalases (20). The mycobacterial T-catalase, which has been identified in most mycobacterial species, has substrate specificities similar to those of the *E. coli* HPI peroxidase-catalases. The mycobacterial M-catalase is a monofunctional HPII-like catalase which has a limited distribution within the mycobacterial genus. A few species, notably those in the *Mycobacterium terrae* complex, produce only M-catalase. *Mycobacterium tuberculosis* and *Mycobacterium bovis* produce only T-catalase. *Mycobacterium avium* and *Mycobacterium intracellulare* produce both classes of catalase (8, 9, 19, 21). Since mycobacteria proliferate inside macrophages, it has been speculated that catalases may protect acid-fast bacilli from the deleterious effects of peroxide and, therefore, may play a crucial role in the in vivo survival of mycobacteria. The virulence of two other intracellular pathogens, *Nocardia asteroides* and *Leishmania donovani*, has been related to their catalase content (2). Catalases probably enhance the pathogenicity of these microorganisms by metabolizing hydrogen peroxide, a toxic oxygen metabolite which is released by phagocytes in response to bacterial challenge. There is extensive evidence which suggests that peroxide and its associated toxic oxygen metabolites are responsible, in part, for the antimycobacterial activity of macrophages. The most direct evidence implicating catalases as mycobacterial virulence factors is derived from studies demonstrating the protective effect of exogenous catalase. In these experiments, exogenous catalase protected against the killing of *Mycobacterium microti* by lymphokine-activated murine macrophages (18). More recent studies have shown, however, that the resistance of *M. intracellulare* strains to peroxide does not correlate with their catalase content and that the susceptibility of *M. tuberculosis* to killing by activated macrophages is not related to peroxide susceptibility (14). Therefore, the role of catalase in mycobacterial virulence may be very complex and has not been clearly delineated.

Mycobacterium leprae, the causative organism of leprosy, has not only defied all attempts at cultivation in vitro but also exhibits the longest generation time of all bacteria, requiring 13 days to double in experimentally infected mice (6). Catalase and peroxidase activities in *M. leprae* have been long discussed since they were first detected in human-derived *M. leprae* in 1967. Prabhakaran found low catalase and high peroxidase activities in *M. leprae* preparations (15), but there was no evidence to suggest that they were *M. leprae* (rather than host-derived) enzymes. More recently, superoxide dismutase and peroxidase were shown to be present in the organism, but studies with the inhibitor 3-amino-1, 2, 4-triazol and polyacrylamide gel electrophoresis indicate that the catalase activity in armadillo-derived *M. leprae* extracts is host derived (23). Lygren et al. also failed to detect catalase and peroxidase activities in *M. leprae* (8). Therefore, attempts to detect catalase in *M. leprae* have so far been unsuccessful. However, it has been shown by Southern hybridization techniques that a *katG* homolog is also present in *M. leprae* (5). We have examined whether or not the *katG* gene is present in *M. leprae*, and in this report, we describe nucleotide sequence analysis of the *M. leprae* DNA region homologous to those of other bacterial *katG* genes encoding a catalase-peroxidase.

E. coli AB1157, *M. tuberculosis* H37Ra, *M. bovis* BCG, and seven different isolates of *M. leprae* (Amami, Gushiken, Izumi, Kitazato, Kyoto, Thai-237, and Thai-53) were used in this study. *E. coli* AB1157, *M. tuberculosis* H37Ra, and *M. bovis* BCG were obtained from culture stocks maintained at the Leprosy Research Center, National Institute of Health, Tokyo, Japan. *E. coli* AB1157 was grown at 37°C in Luria-Bertani medium. *M. tuberculosis* H37Ra and *M. bovis* BCG were grown at 37°C in Middlebrook 7H9 medium with ADC enrichment (Difco Laboratories) to mid-exponential phase. The seven strains of *M. leprae* were originally isolated from biopsy materials of lepromatous leprosy (LL) patients. Two were obtained from LL patients in Thailand (Thai-237 and Thai-53), and five

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were obtained from LL patients from geographically distinct regions of Japan (Amami, Gushiken, Izumi, Kitazato, and Kyoto). The isolates were propagated by subinoculation in footpads of BALB/c *nu/nu* mice or in armadillos. The strain Thai-53 was obtained from laboratory-infected armadillo spleen by homogenization and centrifugation. The other strains of *M. leprae* used in this study were obtained from infected nude mouse footpads. *M. leprae* bacilli from host tissues were purified at 4°C as follows. Mouse footpads or armadillo spleens were minced and homogenized in phosphate-buffered saline and centrifuged at 3,500 × *g* for 10 min. The pellet was treated with 0.5 M NaOH, neutralized, and resuspended in phosphate-buffered saline containing 0.05% Tween 80. This was then centrifuged at 100 × *g* for 10 min to remove host cell components, and the supernatant from this step was then centrifuged at 3,500 × *g* for 20 min. The resulting pellet consisted of purified bacterial cells.

Genomic DNA from *E. coli*, *M. tuberculosis* H37Ra, and *M. bovis* BCG was prepared as follows. Cells were harvested by centrifugation and treated with lysozyme at 10 mg/ml for 1 h at 37°C. Sodium dodecyl sulfate (SDS) and proteinase K were then added to 1% (wt/vol) and 1 mg/ml, respectively, and the mixture was incubated for 2 h at 50°C. This was followed by phenol-chloroform extraction and RNase treatment (50 μg/ml, 30 min at 37°C). After phenol-chloroform extraction, DNA was precipitated with ethanol. Genomic DNA of *M. leprae* Thai-53 was purified by mechanical disruption as described previously (3). Briefly, the final bacterial pellet, obtained as described above, was resuspended in breaking buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 5% SDS. The bacterial suspension was then frozen in liquid nitrogen before being mechanically disrupted with a Polytron homogenizer, producing frozen powder. The resultant powder was then treated with phenol-chloroform and precipitated with ethanol. Genomic DNAs from the other strains of *M. leprae* were prepared by the freeze-thaw method. Bacterial suspensions were adjusted to 1.0×10^8 bacteria/ml and frozen at −80°C for more than 30 min, followed by being heated in boiling water for 10 min. This freeze-thaw cycle was repeated three times, and the resultant bacterial extracts were used for PCR.

PCR was carried out in a 50-μl volume containing 100 ng of genomic DNA or 1 μl of bacterial extract with Takara ExTaq (Takara, Kyoto Japan) with reagents and protocols supplied by the manufacturer. Thermocycler reaction conditions were 1 min at 95°C, 2 min at 50°C, and 3 min at 72°C for 35 cycles. PCR products were analyzed by electrophoresis in a 0.7% agarose gel and ethidium bromide staining and were purified from the gel for direct nucleotide sequencing. The AmpliCycle Sequencing Kit (Perkin-Elmer) was used for nucleotide sequencing. The nucleotide sequences obtained were analyzed by the DNASIS computer program (Windows version; Hitachi Software Engineering).

For Southern hybridization analysis, PCR products or genomic DNA were electrophoresed in a 0.7% agarose gel and blotted onto nylon membranes. DNA probes were labelled with a Random Primer DNA Labelling Kit (Takara) with α-^{35}S-dCTP. Nylon membranes were prehybridized in 5 ml of a solution containing 50% formamide, 1 M NaCl, 1% SDS, and 100 μg of salmon sperm DNA per ml for 4 h at 37°C. After addition of the probe to the hybridization fluid, the membrane was incubated at 37°C for 18 h. Nonspecifically bound probe DNA was removed by washing of the membrane twice for 15 min in 0.1× SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature. Bound probe was detected with a BAS-1000 Mac System (Fuji Film, Tokyo, Japan).

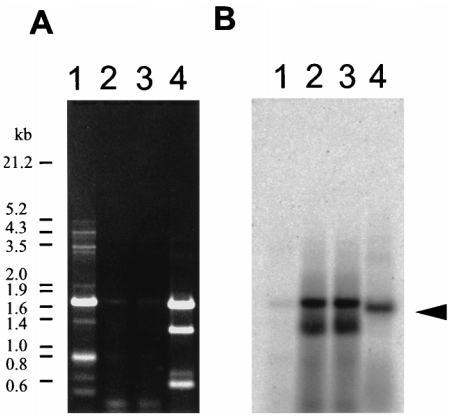

FIG. 1. Analysis of PCR amplification products from the *M. leprae katG* region with primers A and B. (A) Electrophoresis and ethidium bromide staining. (B) Southern hybridization. PCR products were blotted and hybridized with a 2.5-kb *katG* probe obtained from *M. tuberculosis* by PCR (nucleotides 1908 to 4446 [Fig. 4A]). The chromosomal DNAs used as templates were obtained from *E. coli* AB1157 (lane 1), *M. tuberculosis* H37Ra (lane 2), *M. bovis* BCG (lane 3), and *M. leprae* Thai-53 (lane 4). Small bands in lanes 2 and 3, which hybridized to the *M. tuberculosis katG* probe, are probably fragments amplified from the internal region of the *katG* sequence, because *M. tuberculosis* H37Ra has only one copy of the *katG* gene.

PCR amplification and nucleotide sequencing of *M. leprae katG* region. Two oligonucleotide DNA primers (primer A, 5′-ATAAAGCTTGCACGCCGCGGGCACCTACCG-3′; primer B, 5′-ATACTCGAGCAGGTTGACGAAGAAGTC-3′) binding to consensus sequences 1.7 kb apart within the *katG* open reading frame of *E. coli* (16), *M. intracellulare* (12), and *M. tuberculosis* (5) were synthesized for PCR amplification. A 1.6-kb DNA fragment was amplified when *M. leprae* Thai-53 DNA was used as a template, while a 1.7-kb fragment was produced when *E. coli*, *M. tuberculosis*, and *M. bovis* BCG genomic DNA templates were used (Fig. 1A). All of these bands hybridized to an *M. tuberculosis katG* probe under conditions of high stringency (Fig. 1B).

The 1.6-kb fragment amplified from *M. leprae* Thai-53 was sequenced. The nucleotide sequence of the fragment showed significant homology to other bacterial *katG* genes. To further analyze the *katG* region of *M. leprae*, we amplified the flanking

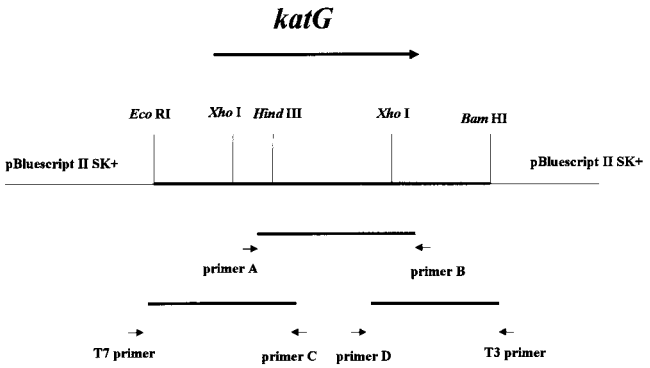

FIG. 2. Strategy for amplification of the *katG* region and flanking regions of *M. leprae*. Primers A and B were designed on the basis of sequence data from the *E. coli*, *M. intracellulare*, and *M. tuberculosis katG* genes. Primers C and D were designed on the basis of the nucleotide sequence of the DNA fragment obtained from *M. leprae* by PCR with primers A and B.

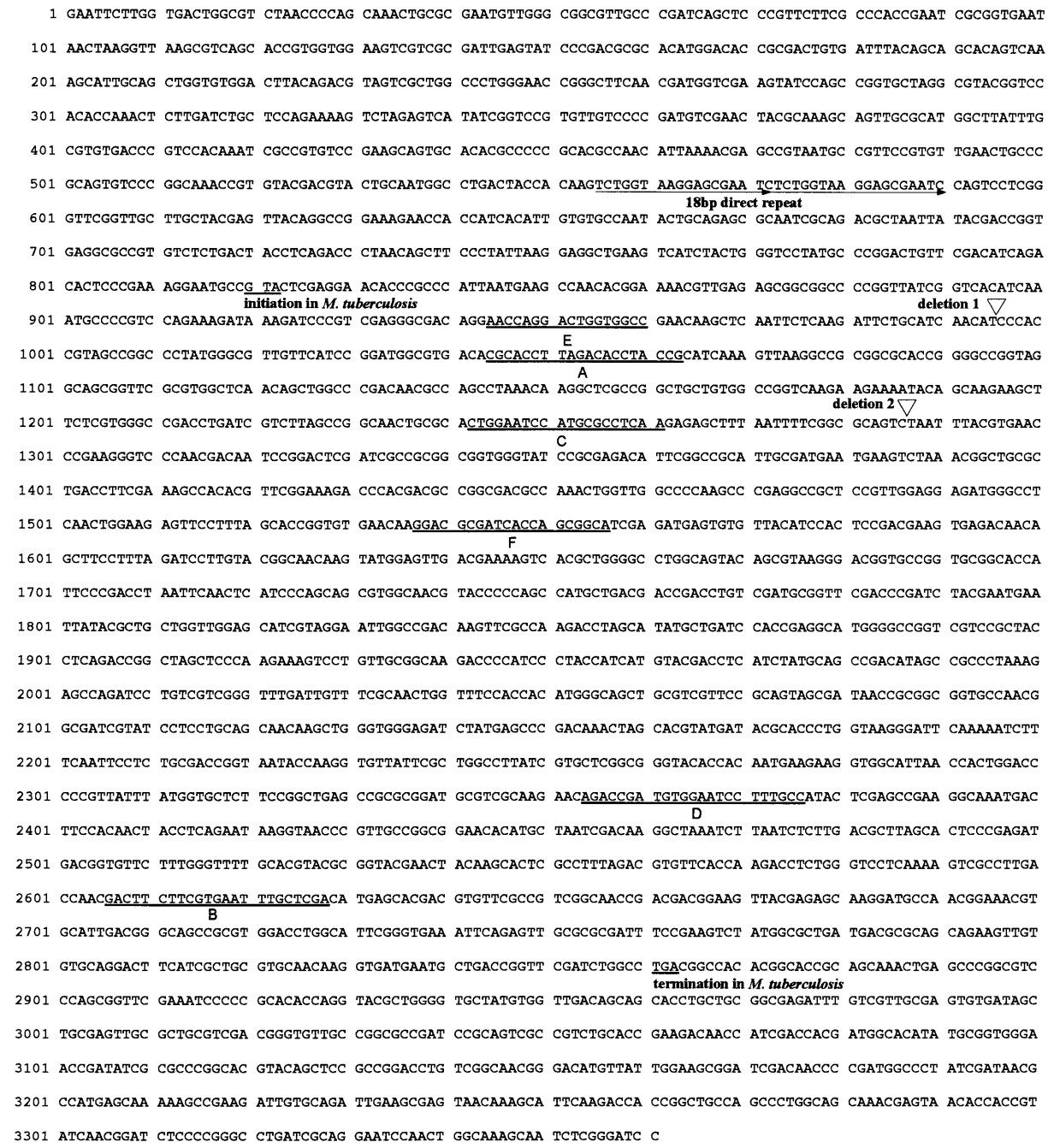

FIG. 3. Nucleotide sequence of the *M. leprae katG* region. Two major deletions (deletion 1 and deletion 2) are indicated by open triangles. GTA (nucleotides 819 to 821) corresponds to the initiation codon GTG in *M. tuberculosis*, and TGA (nucleotides 2861 to 2863) corresponds to the *M. tuberculosis* termination codon. The underlined sequences labeled A to F indicate the binding regions of primers A to F.

region of the *M. leprae* Thai-53 *katG* sequence by PCR. The PCR amplification strategy is summarized in Fig. 2. To amplify the whole region of *M. leprae katG* by PCR, we determined its flanking restriction sites by Southern hybridization analysis with a 1.1-kb *Hin*dIII-*Xho*I fragment excised from the PCR product. A single restriction fragment was detected in Southern blots of *M. leprae* DNA digested with the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Kpn*I (data not shown). This hybridization pattern confirmed the *M. leprae* derivation of the PCR-amplified fragment and strongly suggests a simple genetic organization with a singular chromosomal locus within the *M. leprae* genome. Further Southern hybridization analysis indicated that the *katG* homologous sequence was on a 3.4-kb *Eco*RI-*Bam*HI fragment (data not shown), so *M. leprae* genomic DNA was digested with *Eco*RI and *Bam*HI and ligated into pBluescript II SK+ plasmid. The ligated DNA was used as a template for PCR amplification of the *katG* region with two primer sets hybridizing to the vector plasmid and the *katG* sequence of *M. leprae* DNA, respectively (Fig. 2).

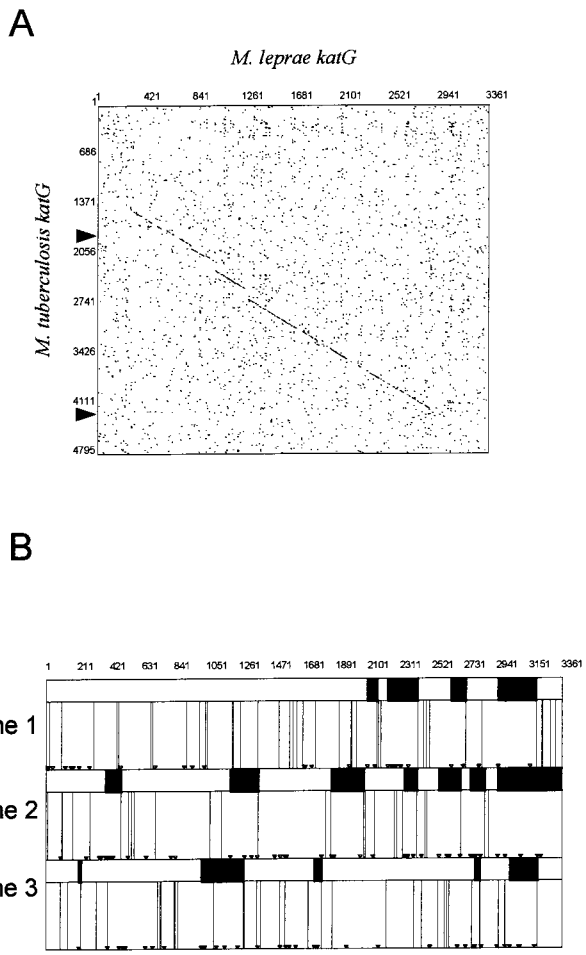

FIG. 4. Computer-aided nucleotide sequence analyses of the *M. leprae katG* region. (A) Homology plot analysis of homology between the *M. leprae katG* region and the *M. tuberculosis katG* gene. Arrowheads indicate the positions of the initiation codon (nucleotide 1979) and the termination codon (nucleotide 4184). (B) Open reading frames found in the sequenced *M. leprae katG* region. Open arrowheads and vertical lines in the lower column of each frame represent initiation and termination codons, respectively. Black bars in the upper column represent open reading frames predicted by the Fickett method (4).

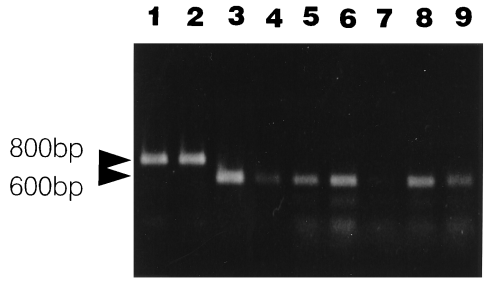

FIG. 5. PCR amplification analysis of mycobacterial *katG* regions. Amplification was carried out with primers E and F. The chromosomal DNA used as the template was obtained from *M. tuberculosis* H37Ra (lane 1), *M. bovis* BCG (lane 2), *M. leprae* Thai-53 (lane 3), *M. leprae* Izumi (lane 4), *M. leprae* Kitazato (lane 5), *M. leprae* Amami (lane 6), *M. leprae* Kyoto (lane 7), *M. leprae* Gushiken (lane 8), and *M. leprae* Thai-237 (lane 9).

The amplified DNA fragments were sequenced directly by thermal cycle sequencing, and the complete nucleotide sequence was determined (Fig. 3). Upon inspection of the resultant sequence, the 3.4-kb fragment was found to contain 3,361 nucleotides with an overall dG+dC content of 56.5%. Although the homology plot analysis indicated that the *M. leprae katG* region has significant homology to the *M. tuberculosis katG* gene (approximately 70% identical [Fig. 4A]), several lesions in the *M. leprae katG* sequence were found. The sequence GTA was found in place of the initiation codon (sequence GTG) in *M. tuberculosis* (Fig. 3). Moreover, two major deletions of approximately 100 and 110 bp located at the 5′-terminal region of the *katG* sequence were found together with several minor deletions and insertions. Furthermore, many termination codons were detected in all three frames of the sequence, and upon analysis for the presence of open reading frames with high coding probability values, no candidate *katG* gene was detected (Fig. 4B). The presence of the multiple lesions described above strongly suggests that the *katG* gene has been inactivated in *M. leprae* Thai-53.

To further determine whether *katG* inactivation was specific for strain Thai-53 or was a more general property of *M. leprae*, we examined a collection of clinical *M. leprae* isolates (seven strains in total). Two oligonucleotide primers (primer E, 5′-GGAAACCAGGACTGGTGGCC-3′; primer F, 5′-TGCCGCTGGTGATCGCGTCC-3′) which can hybridize to both the *M. leprae* and the *M. tuberculosis katG* sequences were designed to amplify the *katG* internal fragment through the two deletions. As shown in Fig. 5, while an approximately 800-bp fragment was amplified from *M. tuberculosis* and *M. bovis* BCG, a 600-bp fragment was amplified from all *M. leprae* strains tested. All strains showed the same size of PCR product as strain Thai-53, suggesting that these deletions were present in all *M. leprae* strains examined. Moreover, we also amplified the 3.4-kb DNA segment from strain Kyoto by PCR and determined the complete sequence of the *katG* region. The nucleotide sequence of the 3.4-kb *katG* region of strain Kyoto was 100% identical to that of strain Thai-53 (data not shown).

Conclusions. We have determined the nucleotide sequences of PCR-amplified DNA fragments from two strains of *M. leprae*, which show significant homology to other bacterial *katG* genes. However, nucleotide sequence analysis suggests that this region is not functional as a *katG* gene for the following reasons. (i) The initiation codon GTG in the sequence of the *M. tuberculosis katG* gene is replaced by the sequence GTA in *M. leprae*. (ii) Many termination codons are present in all three frames of the 3.4-kb *Eco*RI-*Bam*HI *katG* sequence of *M. leprae*, resulting in no open reading frame which can encode a catalase-peroxidase enzyme. (iii) The *M. leprae katG* sequence contains two deletions of approximately 100 and 110 bp at the 5′-terminal region. Bacterial catalase-peroxidases consist of two related domains, both of which show strong similarity to yeast cytochrome *c* peroxidase protein, suggesting that they have evolved by means of a gene duplication event. Only the N-terminal domain of these enzymes, however, is predicted to convey the catalase-peroxidase activity (22). The two deletions found in the *M. leprae katG* sequence are located near the codons corresponding to Arg-104, Trp-107, and His-108 of other bacterial catalase-peroxidase proteins, essential amino acids for active-site modulation and H_2O_2 binding (5, 22). Since the deletions were also detected in the other strains of *M. leprae* tested and since *M. leprae* contains no genetic polymorphism (24, 25), *M. leprae* species probably lack T-catalase encoded by the *katG* gene. Zhang et al. have shown that deletions of the *katG* gene can confer resistance to isoniazid (INH) in *M. tuberculosis* (26, 27). Moreover, transfer and expression of the wild-type *M. tuberculosis katG* gene into INH-resistant *katG* mutant strains of *M. tuberculosis* and *Mycobacterium smegmatis* can increase their sensitivity to INH. So far, mechanisms for

resistance and susceptibility to INH have not been completely revealed, but the absence of a functional *katG* gene may explain the natural resistance of *M. leprae* to INH.

The immunopathogenic mechanisms of mycobacteria are not well understood, and mycobacterial virulence has not yet been clearly defined. Because it is thought that mycobacteria proliferate in an environment with high concentrations of toxic oxygen molecules, it has been suggested that catalases may play a role in intracellular proliferation of these acid-fast bacilli. Many investigators have demonstrated that INH-resistant strains of *M. tuberculosis* with defects in catalase-peroxidase activity have reduced virulence for guinea pigs (10, 13). Recently, the *katE* gene of *M. avium* encoding mycobacterial M-catalase was cloned and sequenced (11). We examined whether or not *M. leprae* has the *katE* gene by Southern hybridization with a *katE* gene probe obtained from *M. avium* by PCR. The results suggested that *M. leprae* does not have the *katE* gene either (data not shown). Deficiencies in enzymatic defense against oxygen-free radicals may be a factor in the slow growth of *M. leprae* in vivo.

It has not yet been revealed why *M. leprae* cannot be cultivated outside an animal, but it has been speculated that *M. leprae* lacks some genes essential for growth in vitro. Peroxides form in many culture media (1), and it is possible that one of the reasons *M. leprae* is difficult to grow in vitro is that it may not be able to remove this peroxide effectively.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D89336.

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