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# Alterations in the Superoxide Dismutase Gene of an Isoniazid-Resistant Strain of *Mycobacterium tuberculosis*

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**Genetic analysis of a set of six *Mycobacterium tuberculosis* strains differing in virulence for the guinea pig revealed an altered restriction enzyme fragmentation pattern associated with the superoxide dismutase (SOD) gene in a low-virulence, isoniazid-resistant strain. In addition, it was found that the SOD enzyme produced by the isoniazid-resistant strain differed in its electrophoretic mobility from the SOD of other *M. tuberculosis* strains. Detailed analysis of these strain-specific differences showed that the restriction fragment length polymorphism resulted from the presence of a copy of a repetitive element 552 bp upstream of the SOD gene and that the anomalous electrophoretic mobility arose from a single nucleotide change, resulting in replacement of an aspartic acid residue by histidine in the SOD enzyme of the isoniazid-resistant strain. Possible relationships between genetic changes and strain-dependent differences in virulence are discussed.**

Individual strains of *Mycobacterium tuberculosis* isolated from infected patients display marked differences in their abilities to cause disseminated disease in animal models of infection (10, 11), and several studies have tried to relate these differences in virulence to differences in biochemical properties of the strains. An ability to resist killing by hydrogen peroxide has been shown to correlate with high virulence, for example (7, 11), and alterations in lipid profile provide a marker for reduced virulence (3). In the present study, we have analyzed a panel of *M. tuberculosis* strains with the aim of identifying genetic differences which might correlate with reported differences in their virulence for the guinea pig.

Recent interest in genetic differences among *M. tuberculosis* strains has focused on the observation that Southern blot analysis using probes based on a repetitive genetic element specific to the *M. tuberculosis* complex generates distinctive genomic "fingerprints" which can be used to identify and monitor the spread of individual strains in infectious outbreaks (1, 5, 8, 15, 17). Three such probes (IS*6110*, IS*986*, and IS*987*) have been isolated (2, 4, 20), with sequence analysis showing them to be virtually identical, differing only in a few nucleotides and in their 3-bp terminal sequences (4, 9, 16). Sequence analysis of the repetitive element reveals a close similarity to insertion sequences identified in other bacteria, specifically, with the IS*3* family (9), suggesting that this element may have the ability to "hop" to different positions within the *M. tuberculosis* genome. The highly variable patterns observed in different isolates provide strong support for this suggestion, although the stability of the pattern during the subculture of individual strains for several months indicates that such transposition events are very infrequent (5, 8, 17).

The attenuated-vaccine strain *Mycobacterium bovis* BCG contains only a single copy of the repetitive element in all isolates tested, prompting the speculation that the absence of multiple mobile copies of the insertion sequence may bear some relation to the lack of virulence in this strain (4). Those authors went on to demonstrate that in both *M. tuberculosis* and *M. bovis* BCG, at least one copy of the insertion sequence is located within a region of the chromosome characterized by the presence of multiple short direct repeat sequences and described as a "hot spot" for insertion (4). Precise chromosomal locations of other copies of the insertion sequence have not been reported.

In this report, we describe the presence of a copy of the repetitive element adjacent to the superoxide dismutase (SOD) gene in an isoniazid-resistant isolate of *M. tuberculosis* which has low virulence in the guinea pig model.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and genomic DNA.** The bacterial strains and plasmids used in this study are listed in Table 1. The *M. tuberculosis* strains have previously been characterized with regard to their virulence in the guinea pig model, their sensitivity to hydrogen peroxide, and their catalase and SOD activities (7, 11). *M. tuberculosis* strains were grown with stirring at 37°C in Middlebrook 7H9 medium containing albumin-dextrose-catalase enrichment (Difco Laboratories; code 0713). To isolate genomic DNA, glycine was added to 4-week-old cultures at a final concentration of 1% (wt/vol), and the incubation was continued for another 24 h before the cultures were harvested. The *M. tuberculosis* cultures were centrifuged at 1,500 × *g* for 15 min at room temperature, and the cell pellets were resuspended in lysis solution (50 mM Tris HCl [pH 8.0], 10% sucrose, 2 mg of lysozyme per ml) in a 1/20 volume of the original culture. The mixture was incubated at 37°C for 1 h, and then EDTA (pH 8.0) and proteinase K were added to 100 mM and 200 μg/ml, respectively. The incubation was continued at 37°C for another hour, and then sodium dodecyl sulfate (SDS) was added to a final concentration of 2% (wt/vol) and the mixture was

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Characteristics[a] | Reference or source |
|---|---|---|
| Strain | | |
| *M. tuberculosis* 79112 | Clinical isolate from Indian patient, low virulence | 11 |
| *M. tuberculosis* 79157 | Clinical isolate from Indian patient, low virulence | 11 |
| *M. tuberculosis* 79500 | Clinical isolate from Indian patient, intermediate virulence | 11 |
| *M. tuberculosis* 79665 | Clinical isolate from Indian patient, low virulence | 11 |
| *M. tuberculosis* B1453 | Clinical isolate from British patient, low virulence, isoniazid resistant, catalase negative | 7 |
| *M. tuberculosis* I2646 | Clinical isolate from British patient, high virulence | 11 |
| *M. tuberculosis* H37Rv | Laboratory-maintained virulent strain | B. Allen, RPMS, London, England |
| *E. coli* TG1 | Standard host strain used for cloning; *supE hsd-5 thi* Δ(*lac-proAB*) [*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15] | 14 |
| Plasmid | | |
| pCR1000 | TA cloning vector for PCR, Kan$^r$ | Invitrogen Corp. |
| pUC18 | Cloning vector, Ap$^r$ | 19 |
| pUC19 | Cloning vector, Ap$^r$ | 19 |
| pYZ13 | pUC19 construct containing 4.4-kb *Kpn*I fragment including *M. tuberculosis* SOD gene | This work |
| pYZ33 | pUC18 construct containing 3.7-kb *Pst*I-*Eco*RV fragment containing partial SOD gene and upstream region including complete insertion sequence from strain B1453 | This work |
| pYZ43 | pCR1000 construct containing 700-bp PCR fragment of B1453 spanning 3′ insertion junction region | This work |

[a] Virulence of *M. tuberculosis* strains is based on the root index of virulence, which describes their abilities to cause disseminated infection in the guinea pig (11). Strains with a root index of virulence of less than 1 are listed as being of low virulence, and those with an index of greater than 1 are listed as high virulence (the root index of virulence for strain 79500 was reported as 0.98 [11]).

incubated at 55°C for 1 h. The lysate was then extracted with phenol saturated with buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and centrifuged at 2,000 × *g* for 10 min at room temperature. The supernatant was subsequently extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). Sodium chloride was added to the supernatant at a final concentration of 0.3 M, and the genomic DNA was precipitated with 2 volumes of 95% ethanol. After centrifugation in an MSE Micro Centaur at 13,000 rpm for 15 min, the DNA pellet was washed with 70% ethanol and dissolved in sterile Milli-Q-Ultrapure water.

**DNA manipulations and sequencing.** Standard DNA manipulations were performed essentially as described elsewhere (14). DNA probes used for Southern and colony hybridization were labeled with [α-$^{32}$P]dCTP by using a random primed labeling kit supplied by Boehringer Mannheim, Lewes United Kingdom. Partial genomic DNA libraries of *M. tuberculosis* B1453 and H37Rv were constructed on the basis of Southern blot analysis by cutting out and extracting restricted DNA fragments of a certain size range from low-melting-point agarose gels by using a GeneClean Kit and then cloning the fragments into pUC18 or pUC19 vectors. Oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were synthesized at the Department of Virology, Royal Postgraduate Medical School (RPMS), London, England, and at the Centre of Molecular Biology, Cantoblanco, Madrid, Spain. PCR was performed with genomic DNA from strain B1453 as described elsewhere (13) with oligonucleotide primers IS*1* (5′-TACTGCG GCGACGTCCCGCCG-3′; nucleotide residues 1242 to 1263 of the IS*6110* sequence described in reference 16) and S1 (5′-GGTCTGGCAAGGTGTATTCG-3′; complementary strand of nucleotide residues 136 to 117 of the SOD sequence described in reference 21). The amplified fragment was subcloned into pCR1000 vector by using the TA cloning kit (Invitrogen Corp.). Double-stranded plasmid DNA was sequenced by using both universal primer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and synthetic oligonucleotide primers (as indicated in Fig. 3) with a Sequenase kit (US Biochemical Corp., Cincinnati, Ohio) according to the manufacturer's protocol.

**Southern blotting.** For Southern blot analysis, DNA fragments were transferred to Hybond-N nylon membranes (Amersham plc, Amersham, United Kingdom) by using a Trans Vac TE80 vacuum transfer system (Hoefer Scientific, San Francisco, Calif.) according to procedures recommended by the manufacturer. Blots were probed under stringent conditions with $^{32}$P-labeled DNA fragments (14). In order to reprobe the same blot, nylon membranes were stripped by washing them twice with boiling 0.1% SDS as recommended by the manufacturer (Amersham plc).

**Western blotting (immunoblotting) and SOD activity staining.** Procedures involving polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions or in the presence of SDS (SDS-PAGE), visualization of SOD activity, and immunoblotting with monoclonal antibody D2D were carried out as described previously (21). Manganese-containing SOD from *Escherichia coli* was obtained from Sigma Chemical Co.

## RESULTS

**Alterations in SOD of *M. tuberculosis* B1453.** Genomic DNA was isolated from six clinical isolates of *M. tuberculosis* which had previously been shown to have differing levels of virulence in the guinea pig model (Table 1). DNA was digested with *Kpn*I or with a combination of *Eco*RI and *Kpn*I and analyzed for restriction fragment length polymorphisms by probing Southern blots with genes encoding defined mycobacterial antigens. When the blot was probed with a 1.1-kb *Eco*RI-*Kpn*I fragment containing the structural

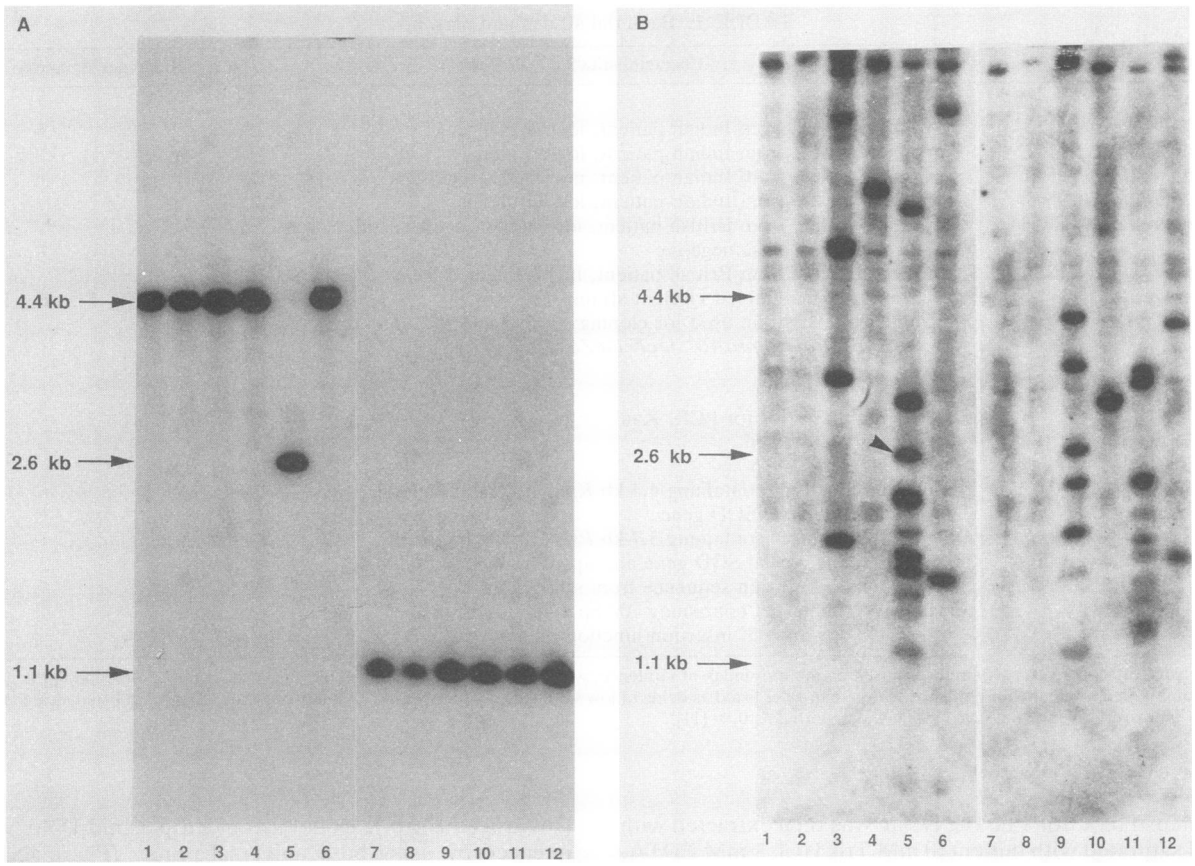


FIG. 1. Southern blot analysis of chromosomal DNAs from six *M. tuberculosis* isolates. Genomic DNAs from six *M. tuberculosis* isolates were digested with *Kpn*I (lanes 1 to 6) or *Kpn*I and *Eco*RI (lanes 7 to 12). After separation by agarose gel electrophoresis, DNA fragments were transferred to a nylon membrane and hybridized with a probe containing the SOD gene of *M. tuberculosis* (A) and with a PCR fragment from the *M. tuberculosis* repetitive element (B). The SOD probe hybridized to a 2.6-kb *Kpn*I fragment from strain B1453 (A, lane 5) in contrast to a 4.4-kb fragment in the other strains. The repetitive-element probe also hybridized to a 2.6-kb *Kpn*I fragment from B1453 (B, lane 5). In each case the 2.6-kb fragment could be digested by further incubation with *Eco*RI. Lanes: 1, *M. tuberculosis* 79112; 2, *M. tuberculosis* 79157; 3, *M. tuberculosis* 79500; 4, *M. tuberculosis* 79665; 5, *M. tuberculosis* B1453; 6, *M. tuberculosis* I2646. The same order was used for *Eco*RI-*Kpn*I-digested DNAs in lanes 7 to 12.

gene for *M. tuberculosis* SOD (the 23-kDa antigen [21]), strain B1453 showed a fragmentation pattern distinct from that of the other strains (Fig. 1A). The SOD probe hybridized with a 2.6-kb fragment of *Kpn*I-restricted DNA from B1453 in contrast to a 4.4-kb *Kpn*I fragment of DNA from the five other strains. After digestion with both *Kpn*I and *Eco*RI, the probe hybridized with a 1.1-kb fragment in all six strains, indicating the presence of an extra *Kpn*I site upstream of the SOD gene in the B1453 strain. The restriction pattern seen with the laboratory strain, *M. tuberculosis* H37Rv, was identical to those of the five other clinical isolates.

Strain B1453, an isoniazid-resistant isolate, lacks catalase activity but has previously been reported to express normal levels of SOD (7). Further analysis of the SOD activity expressed by strain B1453 confirmed this finding (data not shown) but revealed a difference in the electrophoretic mobility of the enzyme as detected by activity staining following nondenaturing PAGE (Fig. 2). Western blot analysis of the same extracts separated by SDS-PAGE showed no difference between the B1453 SOD and SOD from other strains (data not shown), indicating that the change in mobility of B1453 SOD on the nondenaturing gel is not due to an alteration in subunit molecular weight.

Thus, changes at the DNA and protein levels indicated that the SOD of strain B1453 differs from the "conventional" SOD found in other *M. tuberculosis* strains.

**Identification and precise localization of an insertion sequence adjacent to the SOD gene in strain B1453.** In view of the reported polymorphisms related to the *M. tuberculosis* repetitive element (4, 15, 17), we tested the possibility that the altered restriction pattern of the SOD gene in strain B1453 may have been generated by an insertion event. Further probing of the blot shown in Fig. 1A with a 245-bp PCR fragment from IS*986* (4) did indeed reveal the presence of a copy of the insertion sequence on a 2.6-kb *Kpn*I restriction fragment from strain B1453 which coincided exactly with the fragment identified by the SOD probe and which was absent from the other strains tested (Fig. 1B). After subsequent *Eco*RI digestion, we were unable to distinguish the expected 1.5-kb fragment containing the insertion sequence and conclude that it may overlap other similarly sized fragments (Fig. 1B, lane 11).

For precise localization of the insertion sequence in B1453, two strategies to determine the nucleotide sequences of the flanking 3′ and 5′ junction regions were adopted. For the 3′ junction region, a PCR reaction with oligonucleotide primers based on the 3′ end of the insertion sequence (IS*1*)

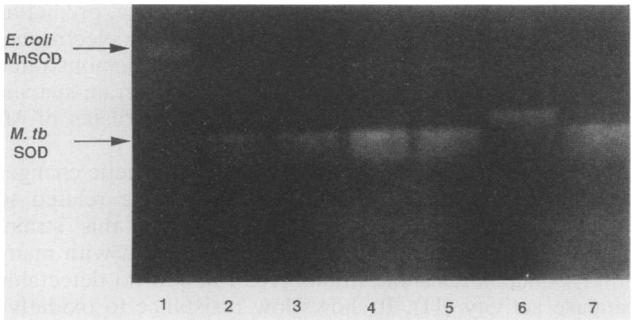


FIG. 2. PAGE analysis of SOD from six *M. tuberculosis* strains. Soluble extracts prepared from the six *M. tuberculosis* strains were stained for SOD activity following separation by PAGE. SOD from strain B1453 (lane 6) had a decreased electrophoretic mobility compared with mobilities of SOD from the other five strains. Lanes: 1, *E. coli* Mn-containing SOD; 2, *M. tuberculosis* 79112; 3, *M. tuberculosis* 79157; 4, *M. tuberculosis* 79500; 5, *M. tuberculosis* 79665; 6, *M. tuberculosis* B1453; 7, *M. tuberculosis* I2646.

and the 5′ end of the SOD gene (S1) was performed. A fragment of approximately 700 bp was amplified and sequenced with universal and oligonucleotide primers (as indicated in Fig. 3) after being cloned into the pCR1000 vector (pYZ43). The orientation of the insertion element could be determined from the size of the PCR fragment, and the direction of transcription of the insertion sequence was seen to be the same as that of the SOD gene (Fig. 3). To determine the sequences at the 5′ junction region, a 3.7-kb *Pst*I-*Eco*RV fragment was cloned from a partial pUC18 DNA library constructed by inserting 3- to 4-kb *Pst*I-*Eco*RV genomic DNA fragments from B1453 into pUC18 cut with *Pst*I-*Sma*I. A positive clone, pYZ33, was identified by colony hybridization, using as a probe a *Pst*I-*Eco*RI fragment which contains a portion of both the SOD gene and its upstream region (Fig. 3). The insert from pYZ33 was subjected to sequence analysis with an oligonucleotide primer corresponding to nucleotide residues 150 to 130 of the repetitive element (16), as indicated in Fig. 3. In order to

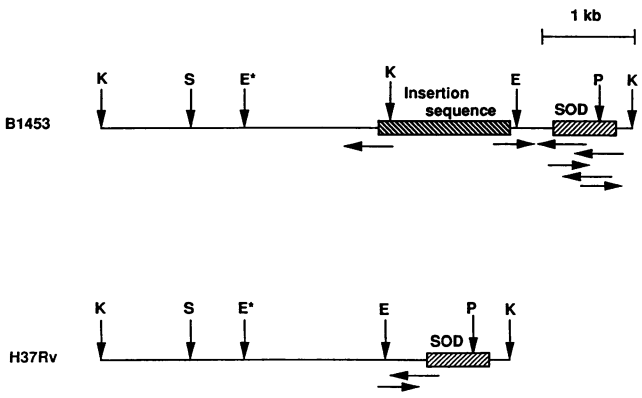


FIG. 3. Restriction map and sequencing strategy. Restriction maps for the SOD gene and upstream region in DNAs from *M. tuberculosis* B1453 and H37Rv are shown. The horizontal arrows below each map indicate the fragments sequenced by using synthetic oligonucleotide primers as described in the text. Transcription of both the SOD gene and the open reading frames within the repetitive element occurs from left to right. Abbreviations: E, *Eco*RI; E*, *Eco*RV; K, *Kpn*I; P, *Pst*I; S, *Stu*I.

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-792 GTCGAGATCC GTCGCGACTA CACACACACC CAGCATCTCG ACCATCGCGA CTCCGGCCGA CGACGGCTAA
-722 CGAGCACGTT CGCCCCACCC GCCCCCGCAG CAACAACACA ACGGCACGGC AGCAGCTGAT CACTGCCCAA
-652 AACACGCACC CACATCAGAT GCAGAACCCC TTGACAACCA ATAGGGAATC TCTTCACGAA TGAGGGGGCA
                  GGT + insertion in strain B1453
-582 GTTGGGGTTT GAATCCGCCG GTTTCCAGTA GGTATCTGTC GGCTTAGTTG GTGAGATTGC GAAAGCCGAG
-512 GGTCGATCCC CGGAGGTGCT CGACGCGGCC GCTGATCGCT TCGGTCGGCG GGTTGACCGT GGTCACTGTT
-442 TTGGGCGTCG ATCCACTGCG GGAATTCCCA CTACCACGTC CGGCCGGATC ACCGGCGACT CGCGGTGCAC
-372 GGCCCGCTCC AGCACCTCCT TGGTCAATTC GTTAGCCGTC CCCGCCAACT GCCCAGCCGT CGACTTCTTC
-302 TTGCCCACCC ACCCCATAGA CCTTCGCCAC ACAGCGCCTT CCGTCCACCC AACAGCGGTC CGATGACGGA
-232 CCCCCGACGG GGACTTCAGC GACCAGGAAC GCGCCCATAG ACGTGGTATC AGCCTGGGGG CGTCCTGGTA
-162 GCCTATGCCG TCCGCCCTGG GGCATCGACC CCAAGGTCGT TGTTGCGACG CGAGCGGTCA TGGAGCAGGG
-92  TTGACTTGTC AAGCTAGAGC CAGCCCATCG CGTGGGAGGC ACCCGCGCGA AAAGAAACAT CGGACGATCA
-22  TTTCATCGAA GGAAGGAATG CC GTG → (SOD gene)
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FIG. 4. Nucleotide sequence of SOD upstream region, showing the position of the insertion in strain B1453. The nucleotide sequence of a 792-bp fragment preceding the SOD gene in *M. tuberculosis* H37Rv is shown, with residues numbered with respect to the first nucleotide of the structural gene. Analysis of regions flanking the insertion in strain B1453 revealed an identical nucleotide sequence, differing only in the presence of the repetitive element itself between residues −553 and −552 as shown. Duplication of the GGT triplet between nucleotides −552 and −550 at the other end of the insertion sequence is highlighted.

determine the nucleotide sequence of the corresponding SOD upstream region gene in *M. tuberculosis* H37Rv, the relevant 4.4-kb *Kpn*I fragment containing the SOD gene was cloned into pUC19 from a similarly constructed partial DNA library of H37Rv. The SOD upstream sequence in H37Rv was established from a positive clone (pYZ13) by using oligonucleotide primers as shown in Fig. 3.

Comparison of nucleotide sequences upstream of the SOD structural gene in *M. tuberculosis* H37Rv and in strain B1453 showed that the repetitive element was located 552 bp from the initiation codon of the SOD gene (Fig. 4). With the exception of the insertion itself, nucleotide sequences upstream of the SOD gene were identical in the two strains. The sequence was also determined for approximately 100 nucleotides from each end of the insertion element in B1453. Sequences of both ends were identical to those previously reported for the *M. tuberculosis* insertion element except that in this case, GGT replaced CGA (IS*6110*), GCG (IS*986*), or CCC (IS*987*) as the 3-bp terminal repeat. The short direct repeats characteristic of the insertional hot spot identified for IS*987* (4) were not present in the SOD upstream region. Inspection of the nucleotide sequence did not identify any open reading frame in either orientation disrupted by an insertion in this position.

**Alteration in the coding region of the SOD gene in strain B1453.** While the demonstration of an upstream insertion sequence accounted for the SOD-associated restriction fragment length polymorphism in strain B1453, it did not explain the altered electrophoretic pattern observed for the SOD enzyme in this strain. Further comparison of the nucleotide sequence of the structural gene for SOD in strain B1453 with that previously derived for the conventional *M. tuberculosis* SOD (21) indicated that this difference arose from an additional genetic alteration in strain B1453 (Fig. 5). A single base change of G to C at nucleotide residue 124 in the SOD gene of strain B1543 predicts the substitution of histidine for aspartic acid at amino acid residue 42 of the translated protein. The consequent decrease in overall negative charge of B1453 SOD would account for its decreased PAGE mobility.

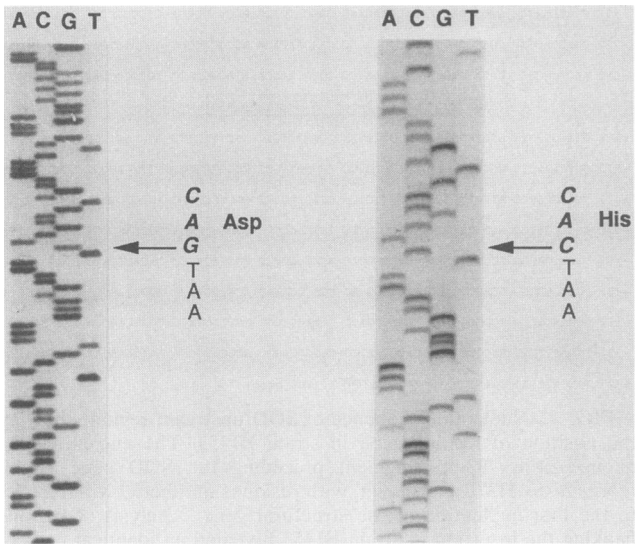


FIG. 5. Alteration of a single nucleotide residue within the coding sequence of the SOD gene in *M. tuberculosis* B1453. Substitution of C for G at nucleotide residue 124 of the SOD gene is illustrated by comparison of the appropriate region of a sequencing gel for the conventional SOD gene (left-hand panel) with the corresponding region of the altered B1453 SOD gene (right-hand panel). The nucleotide substitution predicts an alteration in amino acid sequence which is consistent with the relative decrease in the overall negative charge of B1453 SOD as judged by electrophoretic mobility.

## DISCUSSION

Screening a panel of six clinical isolates of *M. tuberculosis* allowed us to identify two distinctive features related to the SOD gene in one of the isolates, strain B1453. Southern blot analysis of genomic DNA from this strain revealed an anomalous restriction enzyme fragmentation pattern when the SOD gene was used as a probe, and the SOD enzyme from B1453 had an altered mobility during polyacrylamide gel electrophoresis. Since it has previously been demonstrated that there is a correlation between the abilities of different strains of *M. tuberculosis* to withstand oxidative killing and their abilities to cause disseminated disease in guinea pigs (11), we reasoned that detailed characterization of the abnormal features of SOD in B1453 might provide some insight into molecular mechanisms of strain variation in *M. tuberculosis* and also perhaps into the genetic basis of mycobacterial virulence.

The restriction fragment length polymorphism revealed by the SOD probe was found to result from the presence of a copy of a repetitive DNA element approximately 500 bp upstream from the SOD structural gene in strain B1453. Localization of a copy of the repetitive element adjacent to the SOD gene in a clinical isolate lends further support to the idea that it may function as an insertion sequence in *M. tuberculosis*. In particular, the observed terminal duplication of the 3-bp target site (GGT) is a feature characteristically associated with insertion events (6, 12). The presence of the insertion sequence at this point is unlikely to have a direct effect on the structure of the SOD enzyme, and a second genetic change is responsible for the altered PAGE mobility of SOD in strain B1453. In this case, a single nucleotide change was identified in the structural gene for the enzyme, with a corresponding substitution of a histidine residue for aspartic acid in the derived amino acid sequence, predicting a charge alteration consistent with the differing electrophoretic properties of the protein. Thus, we have demonstrated two distinct genetic mechanisms by which strain-specific biological features are generated in clinical isolates of *M. tuberculosis*.

It is interesting to consider whether these genetic changes associated with the SOD gene in B1453 are related to previously reported biological properties of this strain. B1453 is resistant to isoniazid and, in common with many other isoniazid-resistant strains (10, 18), has no detectable catalase activity (11). It shows low resistance to oxidative killing in vitro and is of low virulence in the guinea pig model (11). Since catalase is required to remove hydrogen peroxide, the toxic product of the SOD reaction, it can be proposed that an alteration in SOD activity may be required for survival of catalase-negative strains. However, although we have shown that SOD from strain B1453 has altered electrophoretic mobility, we have found no difference in overall levels of SOD activity between different strains, in confirmation of previously published results (7). It can still be argued that the presence of the insertion sequence may modify regulation of SOD expression in some specific circumstances, but it does not appear to alter the constitutive level of gene expression under standard laboratory conditions. Screening of a further panel of five catalase-negative, isoniazid-resistant isolates of *M. tuberculosis* has failed to identify additional examples of strains with SOD-related insertions or with altered SOD enzymes.

In conclusion, this study has defined the genetic basis of two strain-specific features of an *M. tuberculosis* isolate, but our present evidence does not allow us to determine whether either of these genetic changes plays a role in the low-virulence or drug resistance properties of this strain. Progress in the genetic analysis of mycobacterial virulence will depend on development of techniques for generation of defined stable mutations in *M. tuberculosis* and for transfer of normal and mutated genes between strains. Identification and characterization of natural isolates carrying defined mutations in individual genes may assist in development of such genetic systems for *M. tuberculosis* and may ultimately provide insights into the way in which strain-specific genetic differences can influence interactions between the pathogen and the infected host.

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