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Characterization of rifampicin-resistant Mycobacterium tuberculosis in Taiwan
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Sixty-three rifampicin-resistant (Rifr) isolates of Mycobacterium tuberculosis from Kaohsiung, Taiwan, were analysed for mutations in the core region (69 bp, codons 511–533) of the rpoB gene. Some 84·1 % (53/63) of the resistant isolates showed mutations in this region, especially in codons 531 (41·5 %), 526 (18·9 %), 516 (15·1 %) and 533 (7·5 %). Five novel alleles of a total of 16 different types of mutations were identified in Rifr isolates. Ten Rifr isolates (15·9 %) exhibited no mutations in the core region of rpoB. Also, they did not show mutations in another 365 bp fragment (codons 99–220) of rpoB. The agar proportion method was used to determine the relationship between the degree of rifampicin resistance and alterations in the core region of rpoB. The results revealed that the mean MIC was 92·38 μg ml⁻¹ for the 53 isolates with a mutation in the core region, whereas the mean MIC of the other 10 isolates without mutations was only 24·8 μg ml⁻¹. This indicates that the isolates with mutations in the core region had higher levels of resistance than those without mutations in this region. IS6110 restriction fragment length polymorphism (RFLP) was used for typing of 55 Rifr M. tuberculosis isolates. Isolates contained two to 19 copies of IS6110, with sizes ranging from 600 to 16 000 bp. The majority (85 %) contained six to 16 copies. No strains lacking IS6110 were found. A total of 54 of 55 RFLP types were defined at the 90 % similarity level. The observation of varied IS6110-associated banding patterns indicates that an outbreak of drug-resistant tuberculosis did not occur in this area.

INTRODUCTION
Among infectious diseases, tuberculosis (TB) is one of the most frequent causes of death in the world, with more than 2 million TB-related deaths reported each year (Dye et al., 1999). In 1997, an estimated 8 million new cases were reported and approximately 2 billion people, one third of the world’s population, were infected with Mycobacterium tuberculosis (Dye et al., 1999). TB is one of the most important communicable diseases in Taiwan. It was the twelfth most common cause of death in 1998. The rates of incidence and mortality of TB per 100 000 population were respectively 64·89 and 6·93 in the same year (Kan, 2000). In recent years, the control of TB has been impeded by the emergence of drug-resistant M. tuberculosis strains (Kan, 2000).

Rifampicin has proven to be an effective antituberculosis agent and its use has greatly shortened the duration of chemotherapy for the treatment of TB. Rifampicin resistance heralds higher rates of treatment failure and death for the patient and a poor outcome if the isolate is also resistant to isoniazid (Goble et al., 1993). The action of rifampicin is believed to interfere with transcription in bacteria by binding to the β subunit of RNA polymerase (the product of the rpoB gene) (Jin & Gross, 1988). Mutations in certain highly conserved codons of rpoB account for ‘single step’ high-level resistance to rifampicin in M. tuberculosis (Telenti et al., 1993). More than 90 % of rifampicin-resistant (Rifr) M. tuberculosis strains from different countries appear to harbour specific point mutations located in a 69-bp (core) region of rpoB (codons 511–533) (Mani et al., 2001; Matsiota-Bernard et al., 1998; Ohno et al., 1996; Williams et al., 1998).

Since Rifr strains are a matter of great concern in Taiwan (Chiang et al., 1998; Wang & Lin, 2001), it is of interest to study the molecular basis of rifampicin resistance in these local resistant isolates. One recent report concerned with the genotype of rpoB of Rifr M. tuberculosis isolates from northern Taiwan reported four substitutions and one insertion (Qian et al., 2002). The mutation patterns among large

Abbreviations: RFLP, restriction fragment length polymorphism; TBL, tuberculosis; UPGMA, unweighted-pair group method using average linkage.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the novel M. tuberculosis rpoB alleles found in this study are AF312232–AF312236.
numbers of isolates from Kaohsiung, in southern Taiwan, acquired in this work should allow a better understanding of any hot-spot regions on this gene for suitable rapid diagnosis and proper control of TB in Taiwan.

The correlation between the level of resistance to rifampicin and different mutational sites in the 69-bp core region of rpoB seemed to vary in different regions (Mani et al., 2001; Williams et al., 1998; Ohno et al., 1997). Thus, the MICS of rifampicin for Rifr strains of M. tuberculosis with known alterations in rpoB were determined.

Resistance to rifampicin has previously been associated with mutations in the early region of rpoB in addition to mutations in the middle or end regions of this gene in Escherichia coli (Jin & Gross, 1988; Liatsyn et al., 1984). Recently, Heep et al. (2000) also reported a single, novel amino acid mutation at codon 149 in a clinical isolate of Helicobacter pylori that developed rifabutin resistance during therapy. A homologous mutation was also found in some Helicobacter pylori recently, detection of laboratory contamination (Ramos et al., 1999; Dunlap et al., 2000). The rifampicin concentration was 1.0 μg/ml in the susceptibility of Mycobacterium tuberculosis strains. The correlation between the level of resistance to rifampicin for Rifr strains of M. tuberculosis was examined for potential mutations in the early part of rpoB.

The genetic polymorphism of Rifr M. tuberculosis isolates was evaluated by IS6110 DNA fingerprinting (van Embden et al., 1993). The 1355-bp insertion sequence IS6110, belonging to the IS3 family of insertion elements of enterobacteria, has been found exclusively in members of the M. tuberculosis complex (Thierry et al., 1990). It is widely used as a probe for strain differentiation (Cave et al., 1991), confirming suspected cases of transmission (Edlin et al., 1992; Zaa et al., 1995), detection of laboratory contamination (Ramos et al., 1999; Dunlap et al., 1995) and distinguishing between exogenous re-infection or endogenous reactivation (Shafer et al., 1995; Small et al., 1993). In this work, we compared the RFLP patterns of Rifr T. M. tuberculosis isolates from patients in Kaohsiung by using IS6110 as a probe in order to examine the epidemiological relatedness of the isolates.

METHODS

M. tuberculosis strains. Clinical Rifr isolates of M. tuberculosis were obtained from the Taiwan Provincial Kaohsiung Chronic Disease Prevention Center, the Kaohsiung Medical University Hospital and the Kaohsiung Chang Gung Memorial Hospital during 1996–1998, representing at least one-third of all cases in Kaohsiung. They were identified by conventional methods that included routine microscopy, culture and positive nitrator and niacin tests (Kent & Kubica, 1985). The standard M. tuberculosis strains H37Rv (ATCC 27294) and Mt.14323 (van Embden et al., 1993) and rifampicin-susceptible strains employed as controls.

Drug-susceptibility testing. The test followed the modified agar proportion method using Middlebrook 7H10 agar plates to determine the susceptibility of M. tuberculosis clinical isolates (Kent & Kubica, 1985; Inedfed & Nash, 1996). The rifampicin concentration was 1.0 μg/ml. If the number of colonies that grew on the rifampicin-containing plate was <1% of the number of colonies that grew on a drug-free medium, the isolate was defined as susceptible to rifampicin. The isolate was resistant if the number was >1%.

To determine the MIC of rifampicin of each isolate, serial twofold dilutions of rifampicin were incorporated in 7H10 agar at concentrations that ranged from 0 to 256 μg/ml. Sets of quadrant Petri dishes (one quadrant in each plate contained drug-free medium) were inoculated with each isolate. Plates were incubated at 37°C in the presence of 5% CO2. Each plate was checked weekly and results were recorded after weeks 3 and 4. The MIC was defined as the lowest concentration of drug that inhibited growth of the bacterial population by more than 99%.

DNA extraction. Genomic DNA was extracted as described by van Soolingen et al. (1994) with modifications. Bacteria were harvested from the Lowenstein–Jensen slopes, heat-killed and incubated with lysozyme (1 h, 37°C) followed by digestion with 50 μg proteinase K in 10% SDS for 30 min at 65°C. A further incubation with CTAB/NaCl for 10 min at 65°C was followed by partition using chloroform/isoamyl alcohol (24:1, v/v). Genomic DNA was extracted with phenol/chloroform and precipitated with 100% ethanol.

PCR amplification of mycobacterial strains. Aliquots of purified mycobacterial DNA (10–20 μg) were added to PCR reagents. The 157-bp rpoB fragment (nt 1846–2002) was amplified by using the primers Tr8 (5’-TGCAGGTCTCCGAACCTCCTCA-3’) and Tr9 (5’-TGGCC GGATCGAAGGATG-3’) as described previously (Telenti et al., 1993). To target a 365-bp fragment (early part) of rpoB in M. tuberculosis, primers Th176F (5’-CTTCTCCGGTGATGCTCGTGTG- 3’) and Thb176R (5’-CGGCGTTTGGCAGCGTCAAAGGTC-3’) were used (Heep et al., 2000).

Purification of PCR products and DNA sequencing. Template DNA was purified from the PCR products by using QUIAquick PCR purification kit (Quagen). Nucleotide sequence was performed with the ABI PRISM Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and the reactions were analysed on an ABI PRISM 373A DNA sequencer.

Restriction fragment length polymorphism (RFLP). Fifty-five Rifr M. tuberculosis isolates available for RFLP analysis. DNA fingerprinting was performed as described previously (van Embden et al., 1993; van Soolingen et al., 1994). Briefly, genomic DNA was digested with PvuII for 4–6 h before being separated electrophoretically in a 1% agarose gel in 1X TBE running buffer at a constant voltage of 32 V for 16 h. Next, the separated DNA fragments were transferred onto Hybond nylon membrane (Amersham). Hybridization was then performed using a digoxigenin-labelled 245-bp fragment of IS6110 (25 ng μl−1) as the DNA probe. Membranes were hybridized overnight under stringent conditions at 65°C. Hybridized digoxigenin-labelled probe was detected with the DIG luminescent detection kit (Boehringer Mannheim) following the manufacturer’s instructions.

As recommended previously (van Embden et al., 1993), PvuII-digested genomic DNA of M. tuberculosis reference strain Mt.14323 served as an external control of the hybridization conditions and a mixture of PvuII-digested supercoiled DNA ladder and HaeIII-digested φX174 DNA served as an internal marker.

Cluster analysis. DNA fingerprints were analysed by the GelCompar software (version 4.0; Applied Maths). A cluster was defined as two or more isolates with identical RFLP patterns when five or more copies of RFLP were present. Autoradiograms were digitized by using a scanner with an optical resolution of 300 d.p.i. The sizes of IS6110 RFLP fragments were calculated by comparison of their mobilities with those of a set of internal markers of known molecular sizes (van Soolingen et al., 1994). The accuracy of the normalization procedure was controlled by comparing the IS6110 fingerprint patterns of reference strain M. tuberculosis Mt.14323. The fingerprint patterns were analysed for similarity by using the Dice coefficient and a dendrogram was
calculated with the unweighted-pair group method using average linkage (UPGMA) according to the supplier's instructions. Band positions were determined by using the peak-finder function of the GelCompar software and were controlled manually by comparison with the original IS6110 autoradiogram.

Statistical analysis. The Mann–Whitney test was used for comparing mean MICs with the software package SPSS (SPSS Institute); \( P < 0.05 \) was considered significant.

**RESULTS**

In this study, 63 Rif\(\text{r} \) isolates and 12 rifampicin-sensitive (Rifs) clinical isolates were first examined for mutations in a 157-bp fragment of \( rpoB \). Of 63 Rif\(\text{r} \) isolates, 84·1 % (53/63) of the isolates showed mutations in this region. When compared with the published sequence, 15·9 % (10/63) of the Rif\(\text{r} \) isolates and 12 Rifs strains exhibited no mutations in this region. The most prevalent mutation sites were in codons 531 (41·5 %), 526 (18·9 %), 516 (15·1 %) and 533 (7·5 %) (Table 1). The mutations found within the 69-bp core region of \( rpoB \) were highly resistant to rifampicin, with MICs ranging from 8 to 256 \( \mu \text{g ml}^{-1} \), and the mean MIC was 92·38 \( \mu \text{g ml}^{-1} \) (Table 1). The MICs for the remaining 10 resistant isolates were between 2 and 128 \( \mu \text{g ml}^{-1} \) and the mean MIC was 24·8 \( \mu \text{g ml}^{-1} \). Our results revealed that strains with mutations in the 69-bp core region of \( rpoB \) in 53 of the resistant isolates showed mutations in two separate codons: codons 511 (CTG\(\rightarrow\)CGG; Leu\(\rightarrow\)Pro) and 516 (GAC\(\rightarrow\)GCC; Asp\(\rightarrow\)Gly); codons 526 (CAC\(\rightarrow\)TAC; His\(\rightarrow\)Tyr) and 521 (CTG\(\rightarrow\)CTA; Leu\(\rightarrow\)Leu); codons 526 (CAC\(\rightarrow\)CGG; His\(\rightarrow\)Arg) and 529 (CGA\(\rightarrow\)CAA; Arg\(\rightarrow\)Gln); and codons 516 (GAC\(\rightarrow\)AAC; Asp\(\rightarrow\)Asn) and 533 (CTG\(\rightarrow\)CGG; Leu\(\rightarrow\)Pro) (Table 1).

The agar proportion method was used to understand the relationship between the degree of resistance to rifampicin and the mutation site. Growth of the 12 sensitive strains was inhibited at rifampicin concentrations \( \leq 0·25 \text{ \mu g ml}^{-1} \), indicating the susceptibility of these strains. Isolates with mutations in the 69-bp core region of \( rpoB \) were highly resistant to rifampicin, with MICs ranging from 8 to 256 \( \mu \text{g ml}^{-1} \), and the mean MIC was 92·38 \( \mu \text{g ml}^{-1} \) (Table 1). The MICs for the remaining 10 resistant isolates were between 2 and 128 \( \mu \text{g ml}^{-1} \) and the mean MIC was 24·8 \( \mu \text{g ml}^{-1} \). Our results revealed that strains with mutations in the 69-bp core region of \( rpoB \) had significantly higher MICs than those without mutations in this region (\( P < 0.05 \)).

For the 10 Rif\(\text{r} \) isolates without mutations in the 157-bp fragment, the possibility of mutations occurring in the early region (365-bp fragment) of \( rpoB \) was then examined. PCR-single-strand conformation polymorphism analysis and DNA sequencing exhibited patterns the same as that of \( M. \) tuberculosis isolates and their levels of rifampicin susceptibility

| Mutated codon(s) | Specific mutation(s) | \( n \) (%)\(|^*\) | MIC (\( \mu \text{g ml}^{-1} \))\(|^+\)| |
|----------------|----------------------|---------------------|------------------|
| **Resistant** | | | |
| 531 | TCG(Ser)\(\rightarrow\)TTG(Leu) | 22 (41·5) | 32 (1), 64 (9), 128 (11), 256 (1) |
| 526 | CAC(His)\(\rightarrow\)GAC(Asp) | 3 (5·7) | 128 |
| | CAC(His)\(\rightarrow\)GCC(Asp) | 2 (3·8) | 32 |
| | CAC(His)\(\rightarrow\)TAC(Tyr) | 5 (9·4) | 32 (1), 128 (3), 256 (1) |
| 516 | GAC(Asp)\(\rightarrow\)GTC(Val) | 3 (5·7) | 32 (1), 64 (2) |
| | GAC(Asp)\(\rightarrow\)TAC(Tyr) | 3 (5·7) | 16 (1), 64 (2) |
| 533 | CTC(Leu)\(\rightarrow\)CCG(Pro) | 4 (7·5) | 8 (1), 64 (2), 128 (1) |
| 511 | CTC(Leu)\(\rightarrow\)CCG(Pro) | 1 (1·9) | 32 |
| 522 | TCG(Ser)\(\rightarrow\)TTG(Leu) | 1 (1·9) | 8 |
| 511, 516 | CTC(Leu)\(\rightarrow\)CCG(Pro), GAC(Asp)\(\rightarrow\)GCC(Gly)\(|^\ddagger\)| | 2 (3·8) | 64 |
| | CTC(Leu)\(\rightarrow\)CCG(Pro), GAC(Asp)\(\rightarrow\)TAC(Tyr) | 1 (1·9) | 128 |
| 526, 521 | CAC(His)\(\rightarrow\)GCC(Asp), GCA(Arg)\(\rightarrow\)GAA(Gln)\(|^\ddagger\)| | 1 (1·9) | 64 |
| 526, 529 | CAC(His)\(\rightarrow\)GCC(Asp), GCA(Arg)\(\rightarrow\)GAA(Gln)\(|^\ddagger\)| | 1 (1·9) | 128 |
| 516, 526 | GAC(Asp)\(\rightarrow\)GCC(Ala), CAC(His)\(\rightarrow\)GAC(Asp) | 1 (1·9) | 64 |
| 516, 533 | GAC(Asp)\(\rightarrow\)AAC(Ass), CTC(Leu)\(\rightarrow\)CCG(Pro)\(|^\ddagger\)| | 1 (1·9) | 64 |
| **Susceptible** | | | |
| None | | 10 | 2 (2), 4 (1), 8 (2), 16 (2), 32 (2), 128 (1) |

\( ^*\)Percentages of isolates carrying mutations in the core region are given in parentheses.  
\( ^+\)Where strains with the same mutation(s) gave different MIC values, the number of strains exhibiting each MIC is given in parentheses.  
\( ^\ddagger\)Novel pattern of substitution.
tuberculosis Mt.14323 (data not shown). These results demonstrated that these 10 Rif r isolates did not show mutations in the 157-bp or 365-bp fragment of rpoB.

In this study, 55 Rifr M. tuberculosis isolates were analysed by the standardized DNA fingerprinting method with IS6110 as a genetic marker. The IS6110 fingerprint patterns generated were highly variable (Fig. 2). The number of copies of IS6110 per isolate varied from two to 19, with sizes ranging from 600 to 16 000 bp. The majority of the 55 isolates (85 %), contained six to 16 copies, with a median of 10 bands (Fig. 3). No isolates lacking IS6110 were found.

The similarities of all 55 IS6110 fingerprint patterns were then analysed. A total of 54 of 55 RFLP types were defined at the 90 % similarity level (Fig. 4). Two (C14 and C389) of 17 isolates with mutations in codon 531 had 95·5 % similarity, with different drug-resistance patterns. Isolate C14 was susceptible to kanamycin and ethambutol, while C389 was resistant to both of these drugs. Close relatedness was not observed between other resistant isolates carrying mutations in either of the two other highly mutated sites, codons 526 and 516 (data not shown).

DISCUSSION

It was observed that the majority (75·5 %) of Rif r M. tuberculosis isolates contained missense mutations that led to substitutions of amino acids at Ser-531 (41·5 %), His-526 (18·9 %) or Asp-516 (15·1 %) in the core region of rpoB. While codon 531 is the most common site of nucleotide substitutions worldwide, mutations at the other two prevalent sites (codons 526 and 516) in our strains occur at different frequencies in strains from other geographical regions (Qian et al., 2002; Pozzi et al., 1999; Taniguchi et al., 1996). Distinctly, about 8 % of Rif r M. tuberculosis isolates displayed point mutations in codon 533 in this study. However, this missense mutation in codon 533 only occurred at low frequency (< 3·3 %) or no mutations at all were reported in other geographical regions (Mani et al., 2001; Pozzi et al., 1999; Bartfai et al., 2001; Valim et al., 2000). These differences may be attributed to geographical genetic differences in Rifr M. tuberculosis strains and the transmission of these strains among patients in different countries.

Unexpectedly, 10 (15·9 %) of the 63 Rif r clinical isolates in this study showed no mutations in the sequenced 157-bp region of rpoB, despite the fact that these isolates were resistant to rifampicin. A comparable high frequency (20 %; 4/20) of Rif r isolates with no mutations in this core region was also reported recently from northern Taiwan (Qian et al.,...
The frequency is comparatively higher than those that have been reported for Rifr isolates from other geographical areas. More than 90% of Rifr strains from other regions had mutations located in the 69-bp core region (Telenti et al., 1993; Mani et al., 2001; Matsiota-Bernard et al., 1998; Ohno et al., 1996; Williams et al., 1994). DNA sequencing of the early part of rpoB in these 10 Rifr isolates showed no mutations in a 365-bp region. This indicated the possible occurrence of an alteration outside the two regions of rpoB examined, such as mutations in V176F (Heep et al., 2001) and codon 381 (Taniguchi et al., 1996). Among other explanations, several additional genes may be involved in rifampicin resistance. A change in the antibiotic permeability of a membrane or in metabolism could also give rise to the rifampicin-resistant phenotype (Hui et al., 1977; Konno et al., 1973).

In general, amino acid substitution in the 69-bp core region led to higher levels of resistance to rifampicin than those isolates without mutations in this region ($P < 0.05$, Table 1). Some investigators have even demonstrated that different levels of resistance are associated with different mutational
sites in this region (Ohno et al., 1996, 1997; Taniguchi et al., 1996). Thus, strains with mutations in either codon 531 or 526 were usually highly rifampicin-resistant, as revealed in this report and others (Ohno et al., 1996; Williams et al., 1998; Taniguchi et al., 1996). However, in contrast to previous findings on mutations in codon 533 (Ohno et al., 1996), our isolates with amino acid substitution in this codon (Leu → Pro) did not show a consistently low level of resistance (MIC < 2 μg ml⁻¹). Likewise, high MICs (>128 μg ml⁻¹) for isolates with mutations in codon 516 or 533 were also observed in India (Mani et al., 2001). Thus, the association between particular mutational sites on rpoB and the drug susceptibility of multidrug-resistant M. tuberculosis strains is not apparent in some areas, including Taiwan. Noticeably, the same mutation in the same codon in the 69-bp region did not reflect the same level of drug resistance in these resistant isolates (Table 1; Ohno et al., 1996). Some other additional factor(s) might contribute to the variation in drug resistance (Hui et al., 1997; Konno et al., 1973). Thus, sequencing of rpoB is not completely able to replace traditional methods of susceptibility testing to detect the level of rifampicin resistance of M. tuberculosis.

The results showed that Rif M. tuberculosis strains from this area were highly polymorphic. The majority of the TB cases were therefore presumed to be the result of re-activation of previously contracted M. tuberculosis infections. Furthermore, our results showed that most isolates, even with the same mutated codon, did not have similar patterns or locations of IS6110 copies (Fig. 5). This suggests that acquisition of rifampicin resistance followed the infection of a rifampicin-susceptible strain in this work. In contrast to a report from Cape Town, where most drug-resistant TB cases represented new infections (van Rie et al., 2000), our results did not reveal an outbreak of Rif M. tuberculosis strains in this area.

In conclusion, five novel alleles, as well as some universal prevailing mutations, in the core region of rpoB were observed in our Rif M. tuberculosis isolates. The mutated codon might affect the MIC for rifampicin, although other additional factors might contribute to variation in drug resistance. Analysis of RFLP patterns of our Rif M. tuberculosis isolates suggested that latent re-activation rather than active transmission accounted for most TB cases. Nevertheless, we consider that the establishment of a DNA fingerprinting bank will be extremely helpful for tracing recent sources of infection, for the control of a possible spread of multidrug-resistant organisms and for the surveillance of TB in general.

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