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Molecular Analysis of Kanamycin and Viomycin Resistance in *Mycobacterium smegmatis* by Use of the Conjugation System

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We examined the molecular mechanisms of resistance to kanamycin and viomycin in *Mycobacterium smegmatis*. All of the *M. smegmatis* strains with high-level kanamycin resistance had a nucleotide substitution from A to G at position 1389 of the 16S rRNA gene (*rrs*). This position is equivalent to position 1408 of *Escherichia coli*, and mutation at this position is known to cause aminoglycoside resistance. Mutations from G to A or G to T at position 1473 of the *M. smegmatis rrs* gene were found in viomycin-resistant mutants which had been designated *vicB* mutants in our earlier studies. Using the *M. smegmatis* conjugation system, we confirmed that these mutations indeed contributed to kanamycin and viomycin resistance, and kanamycin susceptibility was dominant over resistance in a heterogenomic strain. Additional experiments showed that three of four *Mycobacterium tuberculosis* strains with high-level kanamycin resistance had a mutation from A to G at position 1400, which was equivalent to position 1389 of *M. smegmatis*.

Recent advances in mycobacterial genetics have made it possible to clarify molecular changes in drug-resistant *Mycobacterium tuberculosis* strains. About 95% of rifampin-resistant strains have one or two mutations in the RNA polymerase β-subunit gene (23, 24). Resistance to streptomycin is due to a mutation in either the ribosomal S12 protein gene (*rpsL*) or the 16S rRNA gene (*rrs*) (6). It is also known that resistance to isoniazid is due to changes in the catalase-peroxidase gene (*katG*) (8) or the *inhA* gene (2).

Kanamycin has been used as an important second-line antituberculosis drug for more than 30 years in Japan. This drug inhibits protein synthesis and interacts directly with ribosomes, similar to streptomycin and other aminoglycoside antibiotics (11). We have previously shown with in vitro polyphenylalanine-synthesizing systems that kanamycin-resistant *Mycobacterium smegmatis* had altered ribosomes which showed resistance to kanamycin (13).

In *Escherichia coli*, methylation of position 1405 or 1408 or a nucleotide change at position 1491 of the *rrs* gene causes resistance to kanamycin and some aminoglycoside antibiotics (3, 5). It appears possible that in mycobacteria, resistance to kanamycin is also conferred by the mutation in the *rrs* gene.

Viomycin and capreomycin, basic peptide antibiotics, also inhibit procaryotic protein synthesis and have been used as second-line antituberculosis drugs. Our earlier studies have shown that resistance to viomycin and capreomycin in *M. smegmatis* was caused by an altered RNA molecule in the 30S or 50S ribosomal subunit (29).

In this study, we analyzed the molecular mechanism of resistance to kanamycin and viomycin in *M. smegmatis* and found that one resistant mutant possessed one mutation in the *rrs* gene. We then confirmed the results by use of the conjugation system which had been described previously (25). Furthermore, we examined mutational sites of drug-resistant clinical isolates of *M. tuberculosis.*

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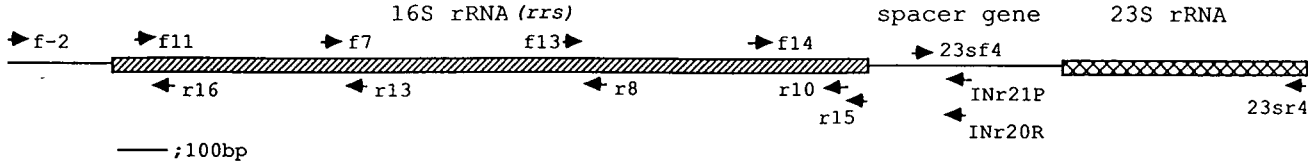
MATERIALS AND METHODS

Bacterial strains and media. For descriptions of *M. smegmatis* strains derived from strains Rabinowitchi (R), PM5 (P), and ATCC 14468 and conjugants obtained from conjugations between R and P strains, see Tables 1, 2, and 4. Methods for isolation of drug-resistant mutants and their genotypic characteristics were described previously (21). Briefly, the mutants were isolated by inoculating large amounts of cells, with or without UV irradiation, on medium containing 10 or 100 μg of kanamycin per ml or 20 μg of viomycin per ml. Frequencies of drug resistant mutants were very low even in the case of UV-irradiated cells (approximately 10^{-9}), but mutants could be obtained by single-step isolation. Strains E, M, A, and O-1 were isolated from ATCC 14468 by Yamada et al. by resistance to viomycin, but they showed pleiotropic drug-resistant phenotypes (27). The mutational sites for the resistance to kanamycin, viomycin, and streptomycin of these mutants have been characterized as being on the ribosomal subunits (14, 15, 27). Strains AC21, AC22, and AC23 were isolated by resistance to kanamycin but showed divergent levels of resistance. For characteristics of the conjugants obtained from conjugations between R44 and P18, R36 and P18, and R17 and P31, see Table 4.

Multiple drug-resistant clinical isolates of *M. tuberculosis* (see Table 5) were donated from Higashi Hospital and Nougata Central Hospital in Fukuoka, Japan. Nine of them were rifampin resistant and possessed a point mutation in the *rpoB* gene (23). Four of them showed a high level of resistance to kanamycin (MICs, >200 μg/ml). One strain also showed a high level of resistance to viomycin. MICs for *M. tuberculosis* or *M. smegmatis* were determined by a method described previously (21, 23). Media used for *M. tuberculosis* were 7H9 and 7H10 (Difco Laboratories, Detroit, Mich.) and Ogawa's egg medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan). For *M. smegmatis*, nutrient agar medium and Davis minimal medium were employed.

DNA extraction, PCR amplification, and nucleotide sequencing. The method of DNA extraction and conditions for PCR amplification have been described previously (23). PCR amplification was performed with the Program Temp Control System PC-700 (Astec Co. Ltd., Tokyo, Japan) and a Takara Ex Taq Kit (Takara Shuzo Co. Ltd., Kyoto, Japan). Fourteen different primers (Fig. 1) were prepared on the basis of the sequences reported by Ji et al. (9) and Rogall et al. (18) and GenBank accession number Z17212, and these were used for the amplification of the *rrs* gene of *M. smegmatis* and its flanking regions. In particular, primers f14 and r10 or r15 were used for the detection of a mutational site causing kanamycin or viomycin resistance in *M. smegmatis* and *M. tuberculosis*. The mutational sites in the *rpsL* genes of streptomycin-resistant *M. smegmatis* and *M. tuberculosis* strains were detected by using primers f1 (5′-CCAACCATCCATCCAGCAGCTGGT-3′) and r1 (5′-CTAGCGGGATCC TTCTCCTTCTTGGCGCCGTA-3′) (17). All of the streptomycin-resistant mutants of *M. smeg-*



Sequence of the primers:

f-2:ATTAGACTGCCAGGGTTG (-215 to -198)	r16:CACCCGTTCGCCACTCGAGT (109 to 90)
f11:GGCGGCGTGCTTAACACATG(40 to 59)	r13:CGTATTACCGCGGCTGCTGG (515 to 496)
f7:CCTCTTTCACCATCGACGA (431 to 450)	r8:GGTAAGGTTCTTCGCGTTG (965 to 947)
f13:CACAAGCGGCGGAGCATGTG(914 to 933)	r10:CGGCTACCTTGTTACGACTT (1493 to 1474)
f14:CGGATCGGGGTCTGCAACTC(1283 to 1302)	r15:GCTCCTTAGAAAGGAGGTGA (1533 to 1514)
23Sf4:CTGCCAGACACACTATTGGG(1656 to 1675)	INr20R:CCCCCAACGGGACGGGAACG (1714 to 1695)
	INr21P:GTCAACACGGAGCCACCC (1725 to 1707)
	23Sr4:CACGGGCCCGCTGCTACTCG (483 to 464 of 23SrRNA)

FIG. 1. Strategies and primers used for the determination of the nucleotide sequences of the *rrs* gene, its flanking regions, and the spacer gene of *M. smegmatis*. Fourteen primers were prepared on the basis of the sequences of the *rrs* gene, its flanking regions, and spacer gene reported by Ji et al. (9) and T. Rogall et al. (18) and the 23S rRNA gene (GenBank accession number Z17212). DNA fragments were amplified by using combinations of the primers listed and sequenced by the dideoxy method. Primer INr20R was specific for the spacer gene of the *M. smegmatis* R strain, and primer INr21P was specific for that of the P strain. Numbers in parentheses indicate the nucleotide positions of primers (Fig. 2). ▨, *rrs* gene; ▩, 23S rRNA gene; ——, flanking region and spacer gene.

matis R and P strains (*str-7*, *str-15*, *str-23*, and *str-24* mutants) had the altered *rpsL* gene (AAG changed to AGG at codon 43). Synthesized primers were purchased from Greiner Japan Co. Ltd. (Kyoto, Japan).

The nucleotide sequencing reaction was carried out by using Dye Terminator Cycle Sequencing FS Ready Reaction Kits with a CATALYST 800 Molecular Biology Labstation (Perkin-Elmer Japan Co. Ltd., Tokyo, Japan). The nucleotide sequence was analyzed with an ABI 373A DNA sequencer (Perkin-Elmer Japan Co. Ltd.).

Conjugation of *M. smegmatis*. A method described previously was employed for the conjugation of *M. smegmatis* (25). Briefly, cell suspensions of equal concentrations of the parental strains (R44, R36, or R17 as a donor strain and P18 or P31 as a recipient strain) were mixed, plated on nutrient agar plates, and incubated for 48 h at 37°C. Cells grown on the agar plates were harvested, suspended in phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4), and diluted appropriately. Conjugants were selected by resistance to antibiotics (nutrient agar medium supplemented with 25 μg of kanamycin per ml, 12.5 μg of viomycin per ml, and/or 100 μg of streptomycin per ml) or by nutritional requirements (Davis minimal medium supplemented with 10 μg of amino acids per ml) and were analyzed for their *rrs* genes.

Nucleotide sequence accession numbers. The nucleotide sequence data for the flanking regions and the spacer genes of R44 and P18 will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers AB003595, AB003596, AB003597, and AB003598.

RESULTS

Nucleotide sequence analysis of 16S rRNA genes of wild-type *M. smegmatis* R and P strains. First, we determined the complete nucleotide sequences of the 16S rRNA (*rrs*) genes, the flanking regions, and the spacer genes of the kanamycin- and viomycin-sensitive *M. smegmatis* strains R41 and P16 (Table 1 and Fig. 1). The nucleotide sequences of the *rrs* genes were compared to that of *M. smegmatis* ATCC 14468 (18). The flanking regions and the spacer genes were compared to those of *M. smegmatis* NCTC8159 (9). The results are shown in Fig. 2. The nucleotide sequence of the *rrs* gene of the R strain was identical to that of strain ATCC 14468. The difference between the nucleotide sequences of the *rrs* genes of the R and P strains was minimal; only one base at position 177 was different. In contrast, the nucleotide sequences of the flanking regions and the spacer genes were different for strain NCTC8159 and the R and P strains (Fig. 2). In particular, there was a great difference between the nucleotide sequences of the spacer genes. These differences allowed us to identify the donor of the *rrs* gene in kanamycin- or viomycin-resistant recombinants.

Mutational analysis of the *rrs* genes of kanamycin-resistant *M. smegmatis* strains. Next, we determined the complete nucleotide sequences of the *rrs* genes, the flanking regions, and the spacer genes of the kanamycin-resistant *M. smegmatis* strains R42 (*nek-1*), R44 (*nek-3*), P33 (*nek-14*), and P34 (*nek-14*) and compared them to those of the kanamycin-sensitive strains R41 and P16. As shown in Table 1, these strains had a mutation from A to G at position 1389, which is equivalent to position 1408 of *E. coli*. By using primers f14 and r10, we also analyzed the nucleotide sequences of the 3′ ends of the *rrs* genes of kanamycin-resistant strains P31, E, M, O-1, AC21, AC22, and AC23 (Tables 1 and 2). Strain P31 (*nek-12*) had the

TABLE 1. Characteristics of drug-resistant *M. smegmatis* strains

Strain[a]	Auxotrophy	Drug resistance genotype[b]	Mutation at the *rrs* gene[c]
R17	*met*		—
R41*	*argA*	*str-7*	—
R42*	*met*	*nek-1 str-15*	A→G (1389)
R44*	*met*	*nek-3*	A→G (1389)
R43*	*met*	*vicB3*	G→A (1473)
R36	*met argA*	*vicB4*	G→A (1473)
R48*	*met*	*vicA1 str-15*	—
P16*	*leu*		—
P18	*leu his*	*str-23*	—
P31	*leu his argB*	*nek-12 str-23*	A→G (1389)
P33*	*leu his argB*	*nek-14*	A→G (1389)
P34*	*leu his argB*	*nek-14 str-24*	A→G (1389)
P29	*leu his argB*	*vicB17*	G→T (1473)
P41*	*leu his argB*	*vicB11*	G→A (1473)
P43	*leu his argB*	*vicA12*	—

[a] An asterisk indicates strains for which the nucleotide sequence of the *rrs* gene was determined completely.

[b] The MICs of kanamycin and viomycin for the drug-sensitive parental strain R were 2.5 and 5 μg/ml, respectively, and those for strain P were 1.25 and 10 μg/ml, respectively. *str*, resistant to 1,000 μg of streptomycin per ml; *nek*, resistant to 1,000 μg of kanamycin and neomycin per ml; *vic*, resistant to 50 to 300 μg of viomycin and capreomycin per ml.

[c] —, no mutation; numbers in parentheses indicate the mutational site in the *rrs* gene.

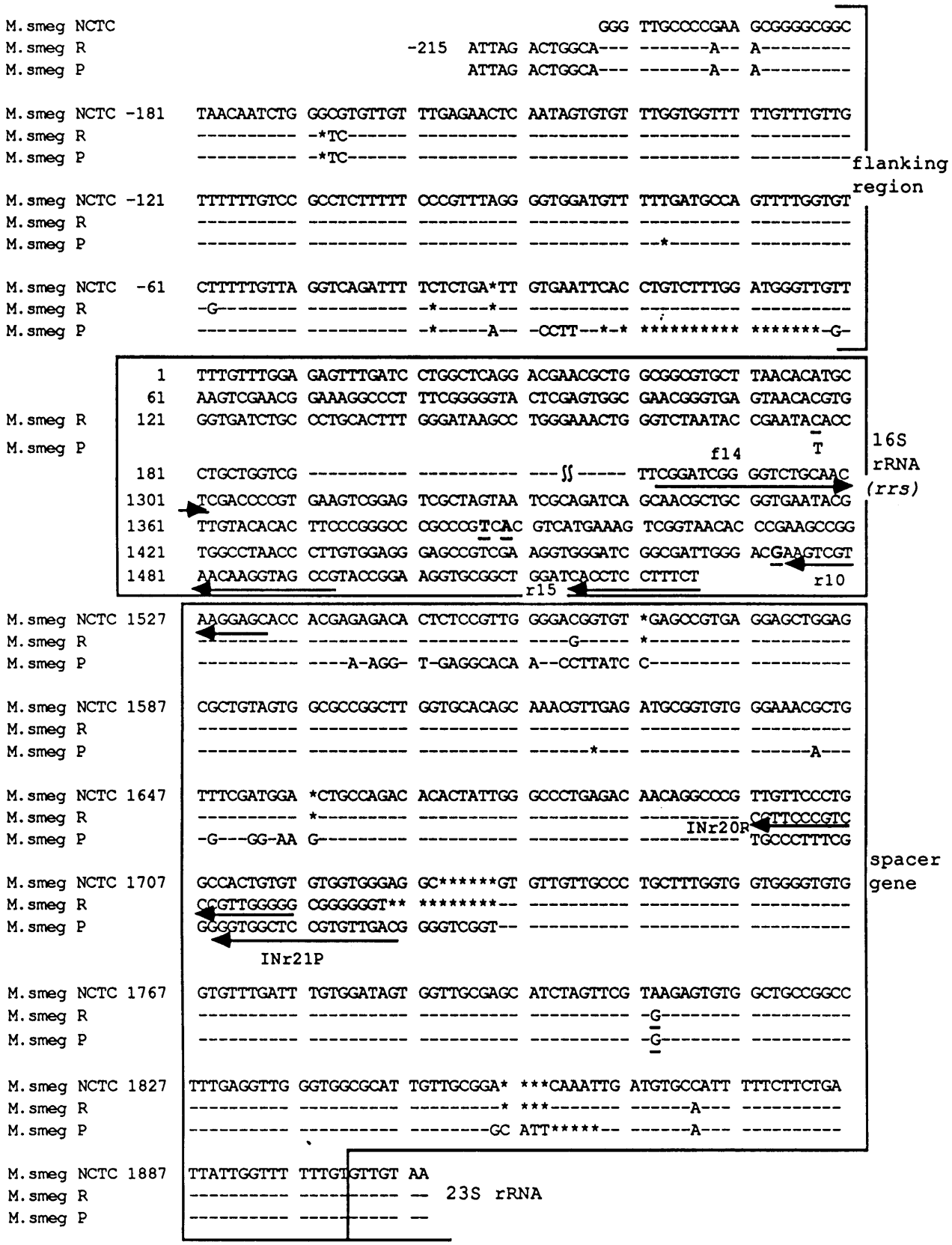


FIG. 2. Alignment of the sequences of the *rrs* genes, their flanking regions, and the spacer genes of *M. smegmatis* (M. smeg) R, P, and NCTC8159 (NCTC) (9). The mutation at position 1387 or 1389 (underlined and boldface T or A) causes kanamycin resistance (*nek*). A mutation at position 1473 (underlined and boldface G) confers viomycin resistance (*vicB*). The combination of the f14 and r10 primers was used to detect the *nek* mutation, and that of the f14 and r15 primers was used to detect the *vicB* mutation. The nucleotide sequences from position 191 to 1280 are omitted because there were no differences with the sequence of ATCC 14468. –, same nucleotide as in *M. smegmatis* NCTC8159; ∗, deletion of the nucleotide.

TABLE 2. Characteristics of *M. smegmatis* ATCC 14468 and its drug-resistant mutants

Strain	Resistance to aminoglycoside and peptide antibiotics[a]	Mutation(s) in the *rrs* gene[c]
ATCC 14468		—
E	VIC (50) (30S)[b], NEK (1,000) (30S)	A→G (1389)
M	VIC (50) (30S), NEK (1,000) (30S), STR (1,000) (30S)	A→G (1389)
A	VIC (1,000) (50S), STR (1,000) (30S)	—
O-1	VIC (1,000) (30S), NEK (1,000) (30S), STR (1,000) (30S)	A→G (1389), G→T (1473)
AC21	NEK (1,000)	A→G (1389)
AC22	NEK (200)	T→A (1387)
AC23	NEK (25)	T→C (1387)

[a] VIC, viomycin and capreomycin; NEK, kanamycin and neomycin; STR, streptomycin. Numbers in parentheses indicate MICs (in micrograms per milliliter).
[b] Mutated ribosomal subunit (14, 15, 27).
[c] Numbers in parentheses indicate the mutational sites. —, no mutation.

same mutation at the same site. In the case of the derivatives of strain ATCC 14468, mutants expressing a high level of resistance to kanamycin (MIC > 1,000 μg/ml) (strains E, M, O-1, and AC21) also had the same mutation at the same site (Table 2). Strains AC22 (MIC = 200 μg/ml) and AC23 (MIC = 25 μg/ml), however, had a different mutation at a different position (T to A or T to C at position 1387). Strain A, which is sensitive to kanamycin but resistant to viomycin, had no mutation in the *rrs* gene (Table 2).

Mutational analysis of the *rrs* genes of viomycin-resistant *M. smegmatis* strains. The complete nucleotide sequences of the *rrs* genes of the viomycin-resistant *M. smegmatis* strains R43 (*vicB3*), R48 (*vicA1*), and P41 (*vicB11*) were determined and compared to those of viomycin-sensitive strains R41 and P16 (Table 1). The *vicB* mutants R43 and P41 had a mutation from G to A at position 1473. However, the *vicA* mutant R48 had no mutations in the *rrs* gene. These results suggested that a mutation at position 1473 caused viomycin resistance in *vicB* strains. By using primers f14 and r15, we also analyzed the nucleotide sequences of the 3′ ends of the *rrs* genes of viomycin-resistant strains R36 (*vicB4*), P29 (*vicB17*), P43 (*vicA12*), E, M, A, and O-1. As expected, *vicB* mutants R36 and P29 had a mutation from G to A or G to T at position 1473. We have previously shown that *vicA* mutants had an altered 50S ribosomal subunit (28), and no mutation was observed in the *rrs* gene of *vicA* mutant P43 or R48. One derivative of ATCC 14468, strain O-1, expressing a high level of resistance to viomycin (MIC = 1,000 μg/ml) and in which the mutation was localized on a 30S ribosomal subunit (15), also had a mutation from G to T at position 1473 in addition to a mutation causing kanamycin resistance. In contrast, no mutation at position 1473 was found in strains E and M, which had low-level viomycin resistance (Table 2). Strain A, in which viomycin resistance is localized on a 50S ribosomal subunit (14) like a *vicA* mutation, had no mutation in the 3′ end of the *rrs* gene (Table 2).

The *vicB* mutations were of two types, either from G to A or from G to T at position 1473. The mutation from G to T (*vicB*) caused a higher level of cross-resistance to kanamycin than the mutation from G to A (Table 3) (13, 21).

Analysis of the association between the mutation in the *rrs* gene and kanamycin resistance by using the conjugation system. As reported previously, in conjugation between R and P strains, the chromosome was transferred from R to P (12). To elucidate whether the mutation at position 1389 was involved in the drug-resistant phenotype, eight conjugants in which kanamycin resistance was transferred from the donor to the recipient strain were selected from a conjugation between R44 (*met nek-3*) and P18 (*leu his str-23*) (Table 4). All of the conjugants were also resistant to streptomycin and required both histidine and leucine, which indicates that only a part of the chromosome was transferred from R44 to P18. The *rrs* genes of the conjugants were analyzed after PCR amplification with primers f-2 and r13 for the flanking region, primers f14 and INr20R for the spacer gene of the R strain, and primers f14 and INr21P for the spacer gene of the P strain (Fig. 2). We then determined their nucleotide sequences. The 3′ end, and the spacer gene of all conjugants were amplified by R-specific primer INr20R and not by P-specific primer INr21P (data not shown). All of them showed a mutation from A to G at position 1389 (Table 4). These results indicated that the *rrs* genes of kanamycin-resistant conjugants were transferred from the kanamycin-resistant donor strain and that the mutation at position 1389 was associated with the kanamycin resistance. The conjugants showed a change from AAG to AGG at codon 43 of the *rpsL* gene, the same as in the recipient strain P18 (data not shown).

TABLE 3. Levels of resistance to viomycin and cross-resistance to kanamycin of *vicB* mutants

Strain	Genotype	MIC (μg/ml)		Mutation in *rrs* at position 1473
		Viomycin	Kanamycin	
R36	*vicB4*	100	5	G→A
R43	*vicB3*	50	10	G→A
P29	*vicB17*	300	100	G→T
P41	*vicB11*	100	2.5	G→A

Analysis of the association between the mutation in the *rrs* gene and viomycin-capreomycin resistance by use of the conjugation system. Similar experiments were carried out with

TABLE 4. Analysis of conjugants between R and P strains

Strain	Auxotrophy	Drug resistance genotype	Amplification[a] with:	
			INr20R	INr21P
R44	*met*	*nek-3*	+ (1389)	−
P18	*leu his*	*str-23*	−	+ (w)
R44 × P18 (8)[b]	*leu his*	*str-23 nek-3*	+ (1389)	−
R36	*argA met*	*vicB4*	+ (1473)	−
P18	*leu his*	*str-23*	−	+ (w)
R36 × P18 (3)[b]	*leu his*	*str-23 vicB4*	+ (1473)	−
R17	*met*		+ (w)	−
P31	*leu his argB*	*str-23 nek-12*	−	+(1389)
R17 × P31 conjugants				
RP22	*leu his*	*str-23*	+ (w)	+ (1389)
RP86	*his*	*str-23*	+ (w)	+ (1389)
RP152	*leu argB*	*str-23 nek-12*	−	+ (1389)
RP165	*argB*	*str-23 nek-12*	−	+ (1389)
RP202	*argB his*	*str-23 nek-12*	−	+ (1389)
RP238	*argB*	*str-23*	+ (w)	+ (1389)

[a] Results of the amplification with primers f14 and INr20R or primers f14 and INr21P (Fig. 2). The number in parentheses shows the mutational site determined by the nucleotide sequence analysis of PCR products: 1389, A→G mutation at position 1389, causing kanamycin resistance; w, wild type; 1473, G→A mutation at position 1473, causing viomycin resistance.
[b] Conjugants obtained from conjugation between the donor R strain and the recipient P strain. The number in parentheses shows the strain number tested.

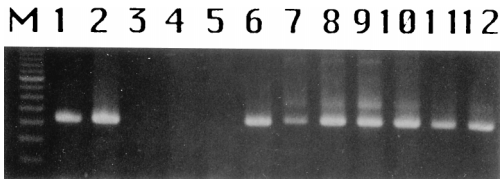


FIG. 3. Analysis of the origin of the *rrs* gene in the conjugants obtained from a conjugation between R17 (sensitive to kanamycin and neomycin) and P31 (*nek-12*). A DNA fragment containing the 3′ end of the *rrs* gene and the spacer gene was amplified by PCR with the primers f14 and INr20R for detection of the R-type gene (lanes 1 to 6) or with the primers f14 and INr21P for detection of the P-type gene (lanes 7 to 12). Amplified products were used to determine the nucleotide sequences (Table 4). Lanes 1 and 7, RP22 Km^s; lanes 2 and 8, RP86 Km^s; lanes 3 and 9, RP152 Km^r; lanes 4 and 10, RP165 Km^r; lanes 5 and 11, RP202 Km^r; lanes 6 and 12, RP238 Km^s; lane M, molecular weight markers.

strains R36 (*argA met vicB4*) and P18 (*leu his str-23*) to verify the mutation determining the viomycin resistance (Table 4). Three *leu his vicB str* conjugants were chosen, and their *rrs* genes were analyzed. All of them had a mutation from G to A at position 1473 (Table 4). Furthermore, all conjugants exhibited the nucleotide sequence of donor strain R in the flanking region, the 3′ end, and the spacer gene, like kanamycin-resistant conjugants. The results indicated that the mutation at position 1473 was involved in the viomycin resistance of *M. smegmatis.*

Determination of whether kanamycin susceptibility is dominant or recessive in heterogenomic strains. Bercovier et al. (4) demonstrated that *M. smegmatis* has two sets of rRNA operons and *M. tuberculosis* has only one set of rRNA operons. It is therefore necessary to determine whether *M. smegmatis* strains, which have one copy each of the wild-type and the resistant rRNA gene, show sensitive or resistant phenotypes, because in *E. coli*, resistance to kanamycin is recessive (1). We have demonstrated previously that the *nek*, *vicA*, and *vicB* loci are closely linked with each other and also linked to the *argA* and the *argB* loci (21). Stable heterogenomic conjugants which were phenotypically sensitive to antibiotics but produced resistant segregants at high frequencies were obtained when the *arg* locus was used as a selection marker in conjugations between drug-sensitive and -resistant strains (21). This strongly suggested that these conjugants possessed both the wild-type and the resistant *rrs* genes.

To prove this possibility, we isolated three kanamycin-sensitive conjugants (RP22, RP86, and RP238), which segregated resistant cells at a high frequency, from a conjugation between R17 (*met*; kanamycin and neomycin sensitive) and P31 (*leu his argB nek-12 str-23*) (Table 4). As a control, three kanamycin-resistant conjugants (RP152, RP165, and RP202) were also isolated by the selection of *met*$^+$ *argB* from the same conjugation. These conjugants were analyzed to determine the nucleotide sequences of the 3′ end and the spacer of the *rrs* gene by using primers f14 and either INr21P or INr20R. As shown in Fig. 3, three sensitive conjugants (RP22, RP86, and RP238) were amplified by both the INr20R and INr21P primers. However, the remaining three resistant conjugants, RP152, RP169, and RP202, were amplified only by primer INr21P. The nucleotide sequence analysis revealed that the R-type *rrs* gene amplified by primer INr20R had no mutation and that the P-type *rrs* gene amplified by primer INr21P had a mutation from A to G at position 1389. These results indicated that three sensitive conjugants possessed both one wild-type and one mutated *rrs* gene. It was also indicated that three resistant conjugants possessed only the mutated *rrs* genes.

Next, we isolated two kanamycin-resistant segregants from each of the kanamycin-sensitive conjugants RP22, RP86, and RP238. The six resistant segregants were analyzed for the nucleotide sequences of the 3′ end and the spacer of the *rrs* gene, using primers f14 and either INr21P or INr20R. Two of six segregants had only the P-type *rrs* gene. This indicated that the R-type *rrs* gene of the parent strain was replaced by the P-type *rrs* gene. However, the remaining four segregants had both the R-type and the P-type *rrs* genes (data not shown). The nucleotide sequence analysis revealed that not only the P-type *rrs* gene but also the R-type *rrs* gene possessed the point mutation from A to G at position 1389. This indicated that the R-type *rrs* gene transferred from the donor strain had acquired the point mutaion spontaneously. These results indicated that two mechanisms were present to produce drug-resistant segregants. One is an intrachromosomal recombination between the resistant and the sensitive loci which resulted in the substitution of the sensitive locus for the resistant one. The other is a mutation in the sensitive locus. These results indicated that kanamycin resistance is recessive in *M. smegmatis* in the same way as it is in *E. coli.*

Analysis of multiple drug-resistant *M. tuberculosis* clinical isolates. Ten drug-resistant strains of *M. tuberculosis* clinical isolates were analyzed for partial nucleotide sequences of the *rrs* gene (from primer f7 to the 3′ end) and the *rpsL* gene. The nucleotide sequences of the *rrs* genes were compared with those reported by Suzuki et al. (22) and Kempsell et al. (10). As shown in Table 5, three strains (HM7, HR1, and HR5) with

TABLE 5. Characteristics of drug-resistant *M. tuberculosis* strains

Strain	Resistance to aminoglycoside and peptide antibiotics[a]	Mutation[b] in:		Remarks[c]
		S12 protein	*rrs* gene	
HM26	STR (>200)	ND	A→C (513)	R, E
HM7	STR (>200), KM (>200), VM (>200)	Lys→Arg (43)	A→G (705)	R, E, P
HR1	STR (>200), KM (>200), VM (12.5)	Lys→Arg (43)	A→G (1400)	R
HM22	STR (>200), KM (>200), VM (12.5)		A→C (513), A→G (1400)	R, E, P
HR5	STR (100), KM (>200), VM (25)	Lys→Gln (88)	A→G (1400)	R, E
HR4	STR (50)			R, I, E, P
HM6	STR (12.5), KM (25)			R, I
HR2	STR (12.5), KM (12.5)			R, E
NM53	KM (6.25), VM (6.25)			
HR8	VM (6.25)			R

[a] Numbers in parentheses indicate MICs (in micrograms per milliliter). STR, streptomycin; KM, kanamycin; VM, viomycin.
[b] ND, not determined. Numbers in parentheses indicate the mutational site.
[c] R, resistant to rifampin; I, resistant to isoniazid; E, resistant to ethambutol; P, resistant to *para*-aminosalicylic acid.

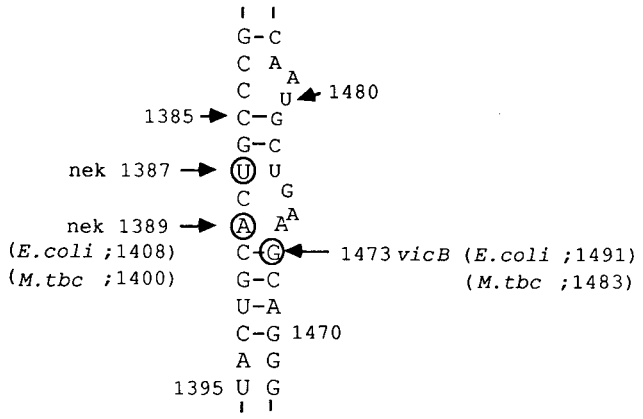


FIG. 4. Locations of mutations on the *rrs* gene which cause kanamycin or viomycin resistance. A portion of the region including positions 1387, 1389, and 1473 of *M. smegmatis* is enlarged from the secondary structure based on the data of Fourmy et al. (7). Nucleotide numbering for *M. tuberculosis* (*M. tbc*) and *E. coli* is based on the data of Kempsell et al. (10) and Moazed et al. (16), respectively.

high-level streptomycin resistance exhibited a mutation in the *rpsL* gene from lysine to arginine at codon 43 or from lysine to glutamine at codon 88. Both mutations are known to cause streptomycin resistance (6, 17). Two mutants (HM26 and HM22) with high-level streptomycin resistance had a mutation in the *rrs* gene at position 513, which is also known to be involved in the resistance (6, 17). Three mutants (HR1, HM22, and HR5) of four with high-level kanamycin resistance showed an alteration in the *rrs* gene from A to G at position 1400. This position is equivalent to position 1389 of *M. smegmatis* and position 1408 of *E. coli* (Fig. 4). One mutant (HM7) which showed high-level resistance to viomycin, kanamycin, and streptomycin did not exhibit mutations at positions 1400 and 1483 (equivalent to position 1473 of *M. smegmatis*). Instead, it had a mutation at position 705. The streptomycin resistance of this strain was due to the change in the *rpsL* gene. It is not known whether the mutation at position 705 is involved in the resistance to kanamycin and viomycin. Further analysis is necessary to determine the association between the resistance phenotype and the mutation. Strains (HR4, HM6, HR2, NM53, and HR8) with low-level resistance against streptomycin, kanamycin, and/or viomycin (MIC $<$ 50 μg/ml) had no mutation in either the *rpsL* or the *rrs* gene.

DISCUSSION

In this study, we found that mutations at specific sites in the 16S rRNA gene (*rrs*) generated resistance to kanamycin in mycobacteria. Viomycin-capreomycin resistance was also due to a change in the *rrs* gene. Our previous studies with *M. smegmatis* showed that kanamycin resistance is due to a change in a 30S ribosomal subunit (28) and that viomycin-capreomycin resistance is conferred by at least two different genes, *vicA* and *vicB* (13, 28). By experiments involving reconstitution of a 30S ribosomal subunit, we showed that a *vicB* mutant has an altered 16S rRNA (29). We have also demonstrated that the *vicA*, *vicB*, and *nek* genes are closely linked with each other (13, 21). We now could determine that mutants with high-level kanamycin resistance had an *rrs* gene with a mutation at position 1389. Position 1389 is equivalent to position 1408 of the *E. coli rrs* gene, which is known to be associated with resistance to aminoglycosides. Most recently, Sander et al. reported the same mutation at position 1408 of amikacin-, gentamicin-, and tobramycin-resistant *M. smegmatis* strains (20). The *vicB* mutants exhibited a point mutation at position 1473 of the *rrs* gene. From conjugation experiments, we could confirm that these mutations were indeed associated with the drug resistance.

It is interesting that A-1389 and G-1473 are located close to each other in the secondary structure of 16S rRNA, as shown in Fig. 4. As has been reported, viomycin and kanamycin are structurally different, but these drugs show cross-resistance in *M. smegmatis* and also in *M. tuberculosis* (13, 19, 21, 26). De Stasio et al. (5) reported that in *E. coli*, a mutation at position 1491 (equivalent to position 1473 of *M. smegmatis*) caused resistance to kanamycin, paromomycin, and other aminoglycoside antibiotics. It is also interesting that the mutation from G to T at position 1473 appeared to cause a higher level of resistance to viomycin and kanamycin than the G-to-A mutation (Table 3). Fourmy et al. reported that aminoglycoside antibiotics bind to the A site of 30S rRNA, which includes positions 1387, 1389, and 1473, and cause misreading of the genetic code in *E. coli* (7).

We found that two strains with an intermediate level of resistance to kanamycin (AC22 and AC23) possessed a mutation at position 1387 of the *rrs* gene. It seems plausible that the mutation at this position is also involved in the resistance to kanamycin. Further analysis is necessary to confirm the association between the mutation and the resistance.

Among viomycin-resistant mutants of ATCC 14468, strains O-1, E, and M showed a high level of resistance to kanamycin (Table 2). Our present analysis showed that only strain O-1 had the mutation at position 1473 in addition to that at position 1389 of the *rrs* gene. Probably, both mutations, at positions 1389 and 1473, are necessary for expression of a high level of resistance to viomycin. The mutation at position 1473 alone did not cause such a high level of resistance to viomycin, as shown in Table 3. The kanamycin-resistant phenotypes of strains E and M were also due to the changes in the *rrs* gene. No mutations which determine viomycin resistance were observed in the *rrs* genes of strains E and M. These two strains were isolated by serial transfers of the culture to medium containing increasing amounts of viomycin (27). It seems plausible that these mutants possess another change(s) in a phenotype such as permeability, in addition to the mutation at position 1389 of the *rrs* gene.

In previous studies, we have noted that certain conjugant classes become heterogenomic in kanamycin and viomycin resistance (21). It is now clear that coexistence of the resistant and the sensitive *rrs* genes in *M. smegmatis* results in phenotypically sensitive heterogenomic conjugants. This also affects frequencies of the appearance of resistant mutants, since both genes must be mutated simultaneously or sequentially to express resistance against kanamycin or viomycin. In fact, frequencies of resistant mutants are very low, especially in the case of viomycin-resistant mutants (21).

In *M. tuberculosis*, three (75%) of four mutants with high-level kanamycin resistance showed a mutation at position 1400 of the *rrs* gene (Table 5). This position is equivalent to position 1389 in *M. smegmatis*. Strain HM7 of *M. tuberculosis* showed a high level of resistance to streptomycin, kanamycin, and viomycin. This strain had an alteration in the *rpsL* gene which causes the resistance to streptomycin. However, no mutation was found in the *rrs* gene except for an A-to-G mutation at position 705. It is not known whether this alteration is associated with kanamycin and/or viomycin resistance. Further analysis is necessary to confirm the association between the resistance and the mutation at position 705.

We have shown that 50S rRNA was also involved in viomy-

cin resistance in *M. smegmatis* (29). It seems possible that *vicA* mutants possess an altered 23S rRNA gene. We are now analyzing the nucleotide sequences of *vicA* mutants.

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