Pleiotropic Antibiotic Resistance Mutations Associated with Ribosomes and Ribosomal Subunits in *Mycobacterium smegmatis*

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Viomycin-resistant strains isolated from *Mycobacterium smegmatis* demonstrated pleiotropic resistance to tuberactinomycin-*N*, capreomycin, streptomycin, and kanamycin as a result of mutational alteration of ribosomes, even though they were selected for resistance to a single antibiotic. The pleiotropic drug resistance of three mutants isolated by stepwise selection for resistance to viomycin was due to alteration of the 30S ribosomal subunit. One mutant, strain A, isolated independently by multiple-step selection to viomycin resistance, was resistant to viomycin, tuberactinomycin-*N*, and capreomycin through an alteration of the 50S ribosomal subunit, whereas it was sensitive to kanamycin but resistant to streptomycin through an alteration of the 30S ribosomal subunit. Three streptomycin-resistant strains, which were isolated by one-step selection at a high concentration of streptomycin, did not show significant co-resistance to any other antibiotics tested in culture and cell-free systems; streptomycin resistance in these mutants was localized on the 30S ribosomal subunit.

Pleiotropic drug resistance associated with viomycin (VM) resistance in mycobacteria has been reported (6, 9, 12, 13, 14, 15, 25, 26, 31, 32, 33, 34, 37, 38, 39). Three possible mechanisms for this drug resistance might be considered: (i) enzymatic inactivation of the drugs (17), (ii) alteration of the membrane leading to a change in permeability, and (iii) alterations at the site of action of the antibiotics, e.g., ribosomes (44).

As a continuation of our work, pleiotropic drug resistance was studied in culture and in cell-free systems. The results indicate that mutational alteration of the VM binding site on ribosomes led to pleiotropic drug resistance in *Mycobacterium smegmatis*.

The results were presented in abstract form at the XXIInd International Tuberculosis Conference (T. Yamada et al., XXIInd Int. Tuberc. Conf., Tokyo, abstract, p. 25, 1973).

MATERIALS AND METHODS

Antibiotics and reagents. Viomycin was a product of Pfizer Ltd. (Japan), and streptomycin (SM) sulfate was a product of Kaken Co., Ltd. (Japan). Kanamycin (KM) sulfate was obtained from Takeda Chemical Industries, Ltd. (Japan) and capreomycin (CPRM) from Shionogi Co., Ltd. (Japan). Tuberactinomycin-

N (TUM) was the gift of K. Hayano, The Research Laboratories, Toyo Jozo Co., Ltd., Japan. Transfer ribonucleic acid (*Escherichia coli* B) and poly(U) were obtained from Miles Laboratories, Inc. and pyruvate kinase and phosphoenolpyruvate monopotassium salt from Sigma Chemical Co. [¹⁴C]phenylalanine was purchased from Daiichi Pure Chemical Co., Ltd. (Japan).

Strains and culture medium. *M. smegmatis* ATCC 14468 and *E. coli* A 19 (ribonuclease I⁻), as described in a previous paper (44), were used. All culture media have been described previously (44).

Isolation of VM-resistant strains by one-step selection and replica-plate method. *M. smegmatis* was grown on the surface of glycerol agar medium at 37 C for 4 days. The cells were collected with a sterile needle, and aggregated cells were dispersed by gentle shaking in a flask containing glass beads. Sterilized distilled water was added to adjust optical density at 590 nm to 0.3, and 0.3 ml of bacterial suspension was streaked on the glycerol agar plates containing 2.5 µg of VM per ml. Spontaneous mutants were isolated after incubation at 37 C for 4 days. Each colony was passed through cellulose acetate filters (pore size, 3 µm) to obtain single cells. The cells thus obtained were grown on glycerol agar plates. The colonies were inoculated onto glycerol agar slants and stored. One of the three mutants was designated as strain O. An alternative method was also used to isolate mutants which had never been in contact with VM, as described below. The cells, grown on glycerol agar plates, were replicated onto plates containing 2.5 µg of

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VM per ml. Those colonies on the glycerol agar plate without VM, which were resistant to VM on replicate plates, were picked up with a sterile needle and streaked on the plates. The replica method was repeated three times. One of the resistant mutants thus obtained was designated as strain N.

Isolation of VM-resistant mutants with high level resistance by multiple-step selection. Strain O-1 was isolated spontaneously from strain O on glycerol agar plates containing 10 μ g of VM per ml. Strains E, M, and A were independently isolated by multiple-step selection as described previously (44). The growing cultures of *M. smegmatis* were filtered through cellulose acetate filters (pore size, 3 μ m), and then the filtrates were inoculated on glycerol agar plates containing 5 μ g of VM per ml. One of the surviving colonies was transferred to liquid medium containing 10 μ g of VM per ml. The surviving cells thus selected were transferred again to media containing 20 μ g of VM per ml. The mutant thus obtained was purified and designated as strain E. Another mutant isolated independently by similar procedures (as described above) was designated as strain M.

Isolation of SM-resistant mutants. *M. smegmatis* was streaked on the surface of glycerol agar plates containing 100 μ g of SM per ml and incubated at 37 C for 4 days. Mutants isolated and purified were designated as K-1 and K-2. Strain KS was isolated on glycerol agar plates containing 1,000 μ g of SM per ml.

Minimal inhibitory concentrations (MIC). Minimal inhibitory concentrations were determined on glycerol agar plates. The concentrations of antibiotics were 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 50, 100, 200, 500, 1,000, and 2,000 μ g/ml. Inoculum size was adjusted so as to deposit 80 to 170 colony-forming units on the surface of the agar. Colonies were scored after 3 or 4 days of incubation at 37 C, which allowed the visible growth of bacteria to the same extent as

that on the plate without antibiotic. The concentration of antibiotics which inhibited 100% of the numbers of colonies was expressed.

Ribosomes and supernatant fluid. Ribosomes, ribosomal subunits, and supernatant fluid were prepared as described previously (42).

Cell-free system. Experimental conditions for poly (U)-directed polyphenylalanine synthesis were as described previously (16, 42). The standard reaction mixture (0.1 ml) contained the following: 95 mM tris(hydroxymethyl)aminomethane, pH 7.8; 5.8 mM 2-mercaptoethanol; 7.5 mM phosphoenolpyruvate monopotassium salt; 0.9 mM adenosine triphosphate; 0.028 mM guanosine triphosphate; 0.1 mM [^{14} C]phenylalanine (specific activity, 100 μ Ci/5.5 μ mol); 0.186 mM each of 19 other ^{12} C-amino acids; 48 mM NH₄Cl; 1.5 μ g of phosphoenolpyruvate kinase; 20 μ liters of supernatant fluid of *E. coli* A 19 (absorbance at 280 nm, 25); 60 μ g of 30S ribosomal subunits; 120 μ g of 50S ribosomal subunits; 20 μ g of poly(U); 50 μ g of *E. coli* transfer ribonucleic acid; and 15 mM of MgCl₂. After incubation at 37 C for 45 min, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid, and the mixture was incubated for an additional 30 min at 95 C. The resulting acid-insoluble material was collected on membrane filters (Millipore Corp.; pore size, 0.45 μ m). Radioactivity was counted in a liquid scintillation spectrometer.

RESULTS

Minimal inhibitory concentrations of various antibiotics against parent, VM-resistant O and N strains of *M. smegmatis*. Minimal inhibitory concentration values for VM, TUM, CPRM, SM, and KM against the parent strain and strains O and N are shown in Table 1. The

TABLE 1. *Minimal inhibitory concentrations of antibiotics in M. smegmatis strains*

Strain	Minimal inhibitory concentration (μ g/ml) ^a					Selection
	VM	TUM	CPRM	SM	KM	
Parental	1.25	1.25	1.25	0.31	0.31	None
N	5.0	2.5	5.0	0.63	0.63	One-step VM resistance (2.5 μ g/ml)
O	5.0	5.0	10	0.63	0.63	
O-1	2,000	2,000	2,000	2,000	2,000	Two-step VM resistance (10 μ g/ml) (One-step from strain O)
E	50	100	100	0.63	2,000	Multiple-step VM resistance (20 μ g/ml)
M	50	50	1,000	2,000	2,000	
A	2,000	2,000	2,000	2,000	2,000	
KS	1.25	1.25	2.5	2,000	0.31	SM resistance (1,000 μ g/ml)
K-1	1.25	1.25	2.5	2,000	0.31	SM resistance (100 μ g/ml)
K-2	1.25	1.25	2.5	2,000	0.31	SM resistance (100 μ g/ml)

^a Italicized numbers represent a change in minimal inhibitory concentration from the parental strain of a factor 4 or less. Numbers in bold face represent a change in resistance greater than fourfold from the parental strain.

parent strain was inhibited by VM, TUM, and CPRM at concentrations of 1.25 µg/ml and by KM and SM at concentrations of 0.31 µg/ml. In comparison with the parent strain, strains O and N, which were isolated by one-step selection, showed low level co-resistance to all of these antibiotics. Attempts to isolate mutants with high level resistance by one-step selection were without success.

Antibiotic sensitivity of *M. smegmatis* strain O-1 in culture and cell-free systems. Because mutants which were isolated by one-step selection were only weakly resistant to antibiotics when tested in cell-free systems, strain O-1 was isolated from strain O by an additional one-step selection to a higher concentration of VM. It is clearly seen in Table 1 that strain O-1 is co-resistant to high levels of TUM, CPRM, SM, and KM. In cell-free systems, ribosomes of strain O-1 were also co-resistant to these antibiotics but the supernatant fractions were not (unpublished data). Ribosomal subunits were isolated from the parent and resistant strains and were reconstructed in vitro to give normal and hybrid ribosomes, and the antibiotic sensitivity of these were tested in the cell-free system.

From Tables 2-6 it is clear that co-resistance to these antibiotics was localized on 30S ribosomal subunits.

TABLE 2. Localization of VM resistance on ribosomal subunits from VM-resistant strain O-1 in poly(U) system^a

Constitution of hybrids		VM ^a (µg/ml)	Incorporation	
50S	30S		Counts/min	% ^c
Sensitive	Sensitive	0	13,263	100
Sensitive	Sensitive	1	1,462	11
Resistant	Resistant	0	4,345	100
Resistant	Resistant	1	2,521	58
Resistant	Sensitive	0	7,109	100
Resistant	Sensitive	1	991	14
Sensitive	Resistant	0	6,243	100
Sensitive	Resistant	1	4,210	67

^a Experimental procedures as described in the text.

^b Antibiotic was added at the onset of incubation. Similar results were obtained at a concentration of 5 µg of VM per ml in separate experiments.

^c Incorporation on sensitive reconstituted particles in the presence of 1 µg of VM per ml ranged between 6 to 24% of control in 17 experiments with independent preparations.

TABLE 3. Localization of TUM resistance on ribosomal subunits from VM-resistant strain O-1 in poly(U) system^a

Constitution of hybrids		TUM ^a (µg/ml)	Incorporation	
50S	30S		Counts/min	% ^c
Sensitive	Sensitive	0	8,403	100
Sensitive	Sensitive	1	2,352	28
Resistant	Resistant	0	4,026	100
Resistant	Resistant	1	4,358	108
Resistant	Sensitive	0	8,189	100
Resistant	Sensitive	1	1,098	13
Sensitive	Resistant	0	6,516	100
Sensitive	Resistant	1	4,914	75

^a Experimental procedures as described in the text.

^b Antibiotic was added at the onset of incubation. Similar results were obtained at a concentration of 0.5 µg of TUM per ml in separate experiments.

^c Incorporation on sensitive reconstituted particles in the presence of 1 µg of TUM per ml ranged between 11 to 42% of control with 10 independent preparations.

TABLE 4. Localization of CPRM resistance on ribosomal subunits from VM-resistant strain O-1 in poly(U) system^a

Constitution of hybrids		CPRM ^a (µg/ml)	Incorporation	
50S	30S		Counts/min	% ^c
Sensitive	Sensitive	0	11,435	100
Sensitive	Sensitive	1	2,189	19
Resistant	Resistant	0	5,139	100
Resistant	Resistant	1	4,687	91
Resistant	Sensitive	0	9,253	100
Resistant	Sensitive	1	1,103	12
Sensitive	Resistant	0	8,430	100
Sensitive	Resistant	1	6,390	76

^a Experimental procedures as described in the text.

^b Antibiotic was added at the onset of incubation. Similar results were obtained at a concentration of 0.5 µg of CPRM per ml in separate experiments.

^c Incorporation on sensitive reconstituted particles in the presence of 1 µg of CPRM per ml ranged between 10 to 33% of control with nine independent preparations.

Comparison of the degree of pleiotropic drug resistance in two independently isolated mutants, E and M, of *M. smegmatis*. To check whether a VM-resistant mutant always associates pleiotropic drug resistance, two independ-

TABLE 5. Localization of SM resistance on ribosomal subunits from VM-resistant strain O-1 in poly(U) system^a

Constitution of hybrids		SM ^b (μg/ml)	Incorporation	
50S	30S		Counts/min	% ^c
Sensitive	Sensitive	0	8,403	100
Sensitive	Sensitive	10	3,149	37
Resistant	Resistant	0	4,026	100
Resistant	Resistant	10	3,035	75
Resistant	Sensitive	0	8,189	100
Resistant	Sensitive	10	3,602	44
Sensitive	Resistant	0	6,516	100
Sensitive	Resistant	10	5,472	84

^a Experimental procedures as described in the text.^b Antibiotic was added at the onset of incubation. Similar results were obtained at a concentration of 5 μg of SM per ml in separate experiments.^c Incorporation on sensitive reconstituted particles in the presence of 10 μg of SM per ml ranged between 37 to 69% of control with nine independent preparations.

ently isolated mutants, E and M, were tested for their degree of resistance to each antibiotic in culture. The degree of co-resistance of strain E to the aminoglycoside antibiotics did not parallel that of VM, whereas the level of co-resistance to VM, TUM, and CPRM was approximately similar (Table 1). Thus, strain E was resistant to SM at a low level and to KM at high levels. On the contrary, strain M was resistant to both KM and SM (more than 2,000 μg/ml) and to CPRM (1,000 μg/ml) at high levels.

As shown in Table 7, analysis of these strains in cell-free, polypeptide-synthesizing systems showed that co-resistance in both strains was localized on the 30S ribosomal subunits.

Characterization of strain A of *M. smegmatis*. It has been reported previously (44) that high-level VM resistance of *M. smegmatis* strain A was due to an alteration of the 50S ribosomal subunits (44). Therefore, it was of interest to examine the subunit localization of the co-resistance of these mutants in the cell-free system.

Strain A was sensitive to KM but resistant to high levels of VM, CPRM, TUM, and SM in culture (Table 1).

Resistance to TUM and CPRM in strain A was a property of the 50S ribosomal subunit which coincides with the localization of VM

resistance (Table 8). By contrast, resistance to SM was associated with the 30S ribosomal subunit as shown in Table 9. Since strain A was isolated by multiple-step selection, we consider that the localization of SM and VM resistance on different subunits arose through mutational alteration of the 30S ribosomal subunit, resulting in very low-level resistance to VM which was selected in one step. The degree of VM resistance, but not the associated SM resistance, might have been too low to detect in the cell-free system.

Analysis of streptomycin-resistant mutants of *M. smegmatis*. Three streptomycin-resistant mutants which were isolated independently by one-step selection were tested for sensitivity to VM, TUM, CPRM, and KM. None of these isolates gave significant increases of resistance to any other antibiotics tested (Table 1).

In a cell-free system, resistance of mutants KS, K-1, and K-2 to SM could be localized on the 30S ribosomal subunits (Table 10).

DISCUSSION

Antibiotic-resistant mutants of *M. smegmatis*, which by a single mutational event acquire pleiotropic low level drug resistance to VM, TUM, CPRM, SM, and KM, have been isolated. To isolate mutants with high level resist-

TABLE 6. Localization of KM resistance on ribosomal subunits from VM-resistant strain O-1 in poly(U) system^a

Constitution of hybrids		KM ^b (μg/ml)	Incorporation	
50S	30S		Counts/min	% ^c
Sensitive	Sensitive	0	11,435	100
Sensitive	Sensitive	10	5,551	49
Resistant	Resistant	0	5,139	100
Resistant	Resistant	10	5,124	100
Resistant	Sensitive	0	9,253	100
Resistant	Sensitive	10	3,678	40
Sensitive	Resistant	0	8,430	100
Sensitive	Resistant	10	6,866	81

^a Experimental procedures as described in the text.^b Antibiotic was added at the onset of incubation. Similar results were obtained at a concentration of 5 μg of KM per ml in separate experiments.^c Incorporation on sensitive reconstituted particles in the presence of 10 μg of KM per ml ranged between 24 to 50% of control with five independent preparations.

TABLE 7. Localization of co-resistance on ribosomal subunits from VM-resistant strains E and M in poly(U) system^a

Constitution of hybrids		Antibiotics ^b	Strain E		Strain M	
50S	30S		Concn (μg/ml)	Incorporation ^c (%)	Concn (μg/ml)	Incorporation ^c (%)
Sensitive	Sensitive			100		100
Sensitive	Sensitive	VM	0.5	14	1	11
Sensitive	Sensitive	TUM	1	28	0.5	11
Sensitive	Sensitive	CPRM	1	20	0.5	10
Sensitive	Sensitive	SM	1	54	10	69
Sensitive	Sensitive	KM	10	24	10	47
Resistant	Resistant			100		100
Resistant	Resistant	VM	0.5	85	1	59
Resistant	Resistant	TUM	1	63	0.5	48
Resistant	Resistant	CPRM	1	48	0.5	49
Resistant	Resistant	SM	1	51	10	95
Resistant	Resistant	KM	10	115	10	104
Resistant	Sensitive			100		100
Resistant	Sensitive	VM	0.5	32	1	13
Resistant	Sensitive	TUM	1	15	0.5	10
Resistant	Sensitive	CPRM	1	24	0.5	15
Resistant	Sensitive	SM	1	50	10	43
Resistant	Sensitive	KM	10	42	10	58
Sensitive	Resistant			100		100
Sensitive	Resistant	VM	0.5	85	1	40
Sensitive	Resistant	TUM	1	49	0.5	63
Sensitive	Resistant	CPRM	1	50	0.5	51
Sensitive	Resistant	SM	1	57	10	106
Sensitive	Resistant	KM	10	87	10	93

^a Experimental procedures as described in the text.^b Antibiotics were added at the onset of incubation.^c Results are expressed as percent polyphenylalanine synthesis. Incorporation of 100% corresponds to that observed on respective reconstituted or reconstructed ribosomes. The experiments were repeated at least three times with independent preparations and at various concentrations of antibiotics.

ance to VM, multiple-step selection on VM was necessary. The observations suggest that multiple alterations in or near the VM binding site on ribosomes would create high level resistance to various antibiotics.

Analysis of strains O-1, E, and M showed that pleiotropic drug resistance was due to alteration of the 30S ribosomal subunits. However, the comparison of levels of resistance showed that there was significant variation between different mutants in culture. The degree of resistance to VM and TUM was similar but different from that of resistance to SM, KM, and CPRM (Table 1). These observations suggest that the site of alteration of 30S ribosomal subunits in the VM-resistant mutants might be different from the binding site for CPRM (5) and aminoglycoside antibiotics, which involves the 30S ribosomal proteins (S12) specified by the *str A* gene in *E. coli* (19); the change in the VM

binding site might induce a concomitant conformational change in the CPRM and aminoglycoside binding site which varies depending on the particular mutant.

Strain A was also a multiple-step resistant mutant. Whereas resistance to VM, TUM, and CPRM in this strain was localized on the 50S ribosomal subunit, resistance to SM was due to alteration of the 30S ribosomal subunit. This suggests that the peptide antibiotics might act on both subunits. Therefore, mutational alteration of either the 30S or 50S ribosomal subunit might produce mutants resistant to these antibiotics, and also the response of either subunit to peptide antibiotics could be modified by reassociation with altered subunits to 70S ribosomes. The levels of resistance on 30S ribosomal subunits to the peptide antibiotics, which might be associated with resistance to SM, was too low to allow any definite conclusions.

The subunit localization and the levels of resistance to SM and VM led us to analyze SM-resistant mutants selected against high concentrations of SM. Three high level SM-resistant mutants were sensitive to VM, TUM, CPRM, and KM in culture and in cell-free systems. This observation suggests that the locus of VM resistance might be different from that of SM resistance on 30S ribosomal subunits, but alteration of the locus of VM resist-

ance might give some functional effect to the site of SM resistance.

The mode of action of VM and SM has been compared (7, 28). In cell-free systems of *E. coli*, SM caused misreading whereas VM did not. The different mode of action of these antibiotics would be expected from the results obtained above.

Similar phenomena which suggest certain functional relationships between chloramphenicol and erythromycin binding sites has been reported (18, 27, 29, 30, 36, 40). It has been reported and summarized in several studies that ribosomes often show alteration in their response to antibiotics when selection has been carried out by unrelated antibiotics (1, 2, 4, 21).

TABLE 8. Localization of TUM and CPRM resistance on ribosomal subunits from VM-resistant strain A in poly(U) system^a

Constitution of hybrids		Concentration of antibiotics ^b (μg/ml)		Incorporation	
50S	30S	TUM	CPRM	Counts/min	%
Sensitive	Sensitive	0	0	2,543	100
Sensitive	Sensitive	1	0	1,003	42
Sensitive	Sensitive	0	1	792	33
Resistant	Resistant	0	0	1,467	100
Resistant	Resistant	1	0	1,600	110
Resistant	Resistant	0	1	1,272	87
Resistant	Sensitive	0	0	2,137	100
Resistant	Sensitive	1	0	2,027	95
Resistant	Sensitive	0	1	2,100	100
Sensitive	Resistant	0	0	1,896	100
Sensitive	Resistant	1	0	861	45
Sensitive	Resistant	0	1	881	47

^a Experimental procedures as described in the text.

^b Antibiotic was added at the onset of incubation.

TABLE 9. Localization of SM resistance on ribosomal subunits from VM-resistant strain A in poly(U) system^a

Constitution of hybrids		SM ^b (μg/ml)	Incorporation	
50S	30S		Counts/min	%
Sensitive	Sensitive	0	2,623	100
Sensitive	Sensitive	5	940	36
Resistant	Resistant	0	1,478	100
Resistant	Resistant	5	1,627	110
Resistant	Sensitive	0	2,584	100
Resistant	Sensitive	5	772	29
Sensitive	Resistant	0	2,226	100
Sensitive	Resistant	5	1,544	70

^a Experimental procedures as described in the text.

^b Antibiotic was added at the onset of incubation.

TABLE 10. Localization of SM resistance on ribosomal subunits from SM-resistant strains KS, K-1, and K-2 in poly(U) system^a

Constitution of hybrids		SM ^b (μg/ml)	KS		K-1		K-2	
50S	30S		counts/min	%	counts/min	%	counts/min	%
Sensitive	Sensitive	0	8,227	100	11,750	100	1,418	100
Sensitive	Sensitive	5	3,385	41	4,635	39	450	32
Resistant	Resistant	0	2,016	100	3,740	100	1,035	100
Resistant	Resistant	5	1,525	76	2,757	74	945	91
Resistant	Sensitive	0	1,220	100	10,011	100	1,483	100
Resistant	Sensitive	5	546	36	4,356	44	547	37
Sensitive	Resistant	0	2,502	100	4,260	100	640	100
Sensitive	Resistant	5	1,498	60	2,795	66	634	99

^a Experimental procedures as described in the text.

^b Antibiotic was added at the onset of incubation. Similar results were obtained at a concentration of 10 μg of SM per ml.

Resistance to SM has been studied in various organisms. In *E. coli*, three possible mechanisms have been proposed: (i) an alteration of the 30S ribosomal protein specified by the *str A* gene (19), (ii) an alteration in permeability to SM specified by the *str B* gene (22), and (iii) enzymatic inactivation of SM by adenylation or phosphorylation (3, 20, 35, 43, 45). In *Salmonella typhimurium*, two possible mechanisms have been shown: (i) an alteration of ribosomes specified by the *str A* gene (23, 41) and (ii) an alteration in permeability to SM specified by the *str B* gene (23, 41). In *Bacillus subtilis*, alteration of ribosomes appears to be the only mechanism of resistance to SM thus far reported (8, 10, 11, 24).

In *M. smegmatis*, two possible mechanisms can now be proposed: (i) an alteration of 30S ribosomal subunits which might be analogous to the ribosomal alteration specified by the *str A* gene in *E. coli*, and (ii) an alteration of 30S ribosomal subunits which might be conferred by the VM-resistance locus.

Some genetic analysis of mutant A of *M. smegmatis* has been carried out. Preliminary results suggested that genetic loci for VM were multiple and some of them were linked to arg B on the *M. smegmatis* chromosome (Mizuguchi et al., submitted for publication). The details of genetic compositions of each mutant will be published later.

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