ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1976, p. 817-823

Vol. 9, No. 5
Printed in U.S.A.

Altered Ribosomes in Antibiotic-Resistant Mutants of *Mycobacterium smegmatis*

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Received for publication 11 February 1976

Two alleles for viomycin-capreomycin resistance (*vic*) in *Mycobacterium smegmatis* affect ribosome structures. One (*vicA*) affects a component of 50S subunits and the other (*vicB*) affects a component of 30S subunits. The locus for neomycin-kanamycin resistance (*nek*), which is linked to *vicA* and *vicB*, affects a component of 30S subunits. Although the erythromycin resistance locus (*ery*) is linked to *vic* and *nek*, no ribosomal alterations could be detected. Mutations at the streptomycin locus (*str*) not linked to *vic* and *nek* caused alterations of 30S subunits.

In our previous paper (34), we reported that viomycin (VM)-resistant mutants isolated from *Mycobacterium smegmatis* ATCC 14468 had altered ribosomes. One of these mutants had altered 50S subunits. By contrast, others had altered 30S subunits. Thus, it was concluded that VM action could be counteracted to some extent by changes in both 50S and 30S subunits. These altered ribosomes demonstrated pleiotropic resistance to kanamycin (KM) and other antibiotics (33). The genetics of these mutants have not been reported.

Recent studies reveal that there is genetic recombination between two different strains of *M. smegmatis* (24, 31). Using compatible strains of *M. smegmatis* Rabinowitchi and PM5, we found close linkage between the loci conferring resistance to neomycin-kanamycin (*nek*), viomycin-capreomycin (*vic*), and erythromycin (*ery*). These three genes were closely linked to the genes for arginine biosynthesis (*argA*, *argB*). No linkage was observed between *str* and *nek* or *vic*. By the complementation tests, we also noticed that *vic* resistance was determined by mutations in two cistrons (23).

As a continuation of this work, the localization of drug resistance on ribosomal subunits of these genetically characterized mutants has been tested. Based on the results obtained in this study and observations of the previous publications (23, 28, 33, 34), genetic and biochemical aspects of drug resistance of *M. smegmatis* will be discussed.

MATERIALS AND METHODS

Antibiotics and reagents. VM was a product of Pfizer Ltd. (Japan), and streptomycin (SM) sulfate was a product of Kaken Co., Ltd. (Japan). KM sulfate was obtained from Takeda Chemical Industries, Ltd. (Japan) and capreomycin (CPRM) from Shionogi Co., Ltd. (Japan). Neomycin (NM) was purchased from Nippon Kayaku Co., Ltd. (Japan). Erythromycin (EM) lactobionate was a product of Abbott Laboratories. Transfer ribonucleic acid (*Escherichia coli* B) and polyuridylic acid [poly(U)] were obtained from Miles Laboratories, Inc., and pyruvate kinase and phosphoenolpyruvate monopotassium salt were from Sigma Chemical Co. [^{14}C]phenylalanine was purchased from Daiichi Pure Chemical Co., Ltd. (Japan).

Strains and culture media. *M. smegmatis* strain Rabinowitchi (R) and *E. coli* A19 (ribonuclease I$^-$) were used. Methods for isolation of antibiotic-resistant mutants were reported previously (23, 28). In brief, heavy bacterial suspensions, which had been treated with ultraviolet light, were inoculated onto complete agar plates that contained either 100 μg of SM, 10 μg of KM, 20 μg of VM, or 500 μg of EM per ml. Colonies grown on these plates were picked up, purified, and tested for susceptibility to these antibiotics. The mutant substrains of *M. smegmatis* Rabinowitchi used in this study are listed in Table 1. Since a *vicA* mutant could not be isolated from parental strain R-15, we used the *vicA* mutant R-31 isolated from SM-resistant mutant R-16. All culture media have been described previously (34).

Minimal inhibitory concentration. The methods for the determination of the minimal inhibitory concentration were described previously (28).

Ribosomes and supernatant. Ribosomal subunits and supernatant fluid were prepared as described previously (34).

Cell-free system. Experimental conditions for poly(U)-directed polyphenylalanine synthesis were as described previously (33), with a few modifications. 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 phosphenolpyruvate monopotas-

TABLE 1. *Description of substrains of M. smegmatis strain Rabinowitchi*

Substrain	Genotype[a]
R-15	*argA-6, met-5*
R-16	*argA-6, met-5, str-15*
R-30	*argA-6, met-5, nek-3*
R-31[b]	*argA-6, met-5, vicA-1, str-15*
R-33	*argA-6, met-5, vicB-3*
R-46[c]	*met-5, ery-1*

[a] Markers are designated by gene letter or allele number. Abbreviations used for nutritional requirements are: *arg*, arginine; *met*, methionine. For resistance markers they are: *str*, streptomycin; *nek*, neomycin-kanamycin; *vic*, viomycin-capreomycin; *ery*, erythromycin.

[b] Isolated from R-16 by one-step selection.

[c] Isolated from the *argA-6*$^+$ revertant of R-15 by one-step selection.

sium salt; 0.9 mM adenosine triphosphate; 0.028 mM guanosine triphosphate; 0.1 mM [^{14}C]phenylalanine (specific activity, 100 μCi/5.5 μmol); 48 mM NH_4Cl; 1.5 μg of phosphoenolpyruvate kinase; 10 μl of supernatant fluid of *E. coli* A19 (absorbance at 280 nm, 25); 60 μg of 30S ribosomal subunits; 120 μg of 50S ribosomal subunits; 20 μg of poly(U); 50 μg of *E. coli* transfer ribonucleic acid; 13 mM $MgCl_2$. 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 glass-fiber papers (Whatman GF83). Radioactivity was counted in a liquid scintillation spectrometer.

RESULTS

Characteristics of the antibiotic-resistant mutants. As reported (23, 28), in the crosses between strains Rabinowitchi and PM5, a particular selection produced stable heterogenotes in the *vic* or *nek* allele at high frequencies. This discovery made possible the complementation tests between different mutants, since *vic* resistance or *nek* resistance is recessive to *vic*-susceptible and *nek*-susceptible alleles. The results showed that *vic* mutants are classified into two groups (*vicA*, *vicB*), whereas *nek* mutants are classified into one.

The *vicA* mutant R-31 (*argA-6, met-5, vicA-1*) was resistant to 300 μg of VM, 1,000 μg of CPRM, and 1,000 μg of SM per ml and was susceptible to KM and NM (Table 2). On the other hand, the *vicB* mutant R-33 (*argA-6, met-5, vicB-3*) showed a slightly lower level of resistance to VM (100 μg/ml) and CPRM (500 μg/ml) than did *vicA* and an elevated level of resistance to KM and NM. The KM-resistant mutant R-30 (*argA-6, met-5, nek-3*) was resistant to 1,000 μg of KM and 500 μg of NM per ml. R-30 showed an elevated level of resistance to VM and CPRM. SM-resistant mutant R-16 and EM-resistant mutant R-46 did not show cross-resistance to any other antibiotics.

Analysis of VM-resistant mutants (*vic*). A cell-free system containing ribosomes and supernatant fluid from *vic* mutants was made to test for VM susceptibility at various concentrations of the antibiotic. No significant inhibition of poly(U)-directed polyphenylalanine synthesis was observed at a concentration of 0.5 or 1 μg of VM per ml, whereas a similar system from the parental strain was inactive in the presence of the same concentration of VM (data not shown). Ribosomes from the parental and these resistant strains were combined with the supernatant fractions from both strains and tested for inhibition of polyphenylalanine synthesis by VM in a cell-free system. The results showed that the drug resistance of these mutants was due to the altered ribosomes (data not shown).

To determine subunit localization of drug resistance in *vic* mutants, the following experiments were carried out. Ribosomal subunits isolated from parental and resistant strains were reconstructed in vitro to give normal and hybrid ribosomes, and the antibiotic susceptibility of these were tested in a cell-free system. The ribosomes from *vicA* mutant R-31 were resistant to VM because of altered 50S subunits (Table 3). Since *vicA* mutant R-31 was co-resistant to CPRM in culture (Table 2), susceptibility of altered 50S subunits to CPRM was tested in a cell-free system. Co-resistance to CPRM was localized on 50S subunits (Table 3).

The subunits' localization of drug resistance of *vicB* mutant R-33 was also determined in the cell-free system. It turned out that VM resistance of *vicB* mutant R-33 was due to altered 30S subunits in contrast to the *vicA* mutant (Table 4). These altered 30S subunits were co-resistant to CPRM (Table 4). Since the *vicB* mutant R-33

TABLE 2. *Minimal antibiotic inhibitory concentrations for mutants of M. smegmatis strain Rabinowitchi*

Strain	Minimal inhibitory concn (μg/ml)[a]					
	SM	KM	NM	VM	CPRM	EM
R-15	1.25	2.5	1.25	5	20	200
R-16	**>1,000**	2.5	1.25	5	20	200
R-30	1.25	**1,000**	**500**	*20*	*100*	200
R-31[b]	**>1,000**	2.5	1.25	**300**	**1,000**	200
R-33	1.25	10	10	**100**	**500**	200
R-46	1.25	2.5	1.25	5	20	*1,000*

[a] Italicized numbers represent a change in minimal inhibitory concentration from the parent strain of a factor of 8 or less. Numbers in boldface represent a change in resistance greater than 20-fold from the parental strain.

[b] Isolated from strain R-16.

TABLE 3. *Analysis of hybrid ribosomes between susceptible and vicA mutants*[a]

Constitution of hybrids[b]		Concn of antibiotic[c] (μg/ml)	Phenylalanine incorporated			
			VM		CPRM	
50S	30S		Counts/min[d]	%	Counts/min[e]	%
S	S	0	3,616	100	1,641	100
S	S	0.5	1,346	37	381	23
S	S	1	519	14	99	6
R	R	0	5,422	100	2,099	100
R	R	0.5	5,273	97	2,374	110
R	R	1	4,948	91	1,623	77
R	S	0	4,261	100	1,975	100
R	S	0.5	4,421	103	1,646	83
R	S	1	4,144	97	1,777	90
S	R	0	3,825	100	1,257	100
S	R	0.5	548	14	88	7
S	R	1	488	13	103	8

[a] Experimental procedures as described in the text. *M. smegmatis* R-15 was used as a susceptible strain and R-31 was used as a resistant strain.

[b] Constitution of hybrid and reconstituted ribosomes: hybrid and reconstituted ribosomes were composed of subunits derived from susceptible (S) or resistant (R) strains.

[c] Antibiotic was added at the onset of incubation.

[d] The radioactivity (200 to 500 counts/min) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. Incorporation on susceptible reconstituted particles in the presence of 1 μg of VM per ml ranged between 6 to 24% of control with 19 independent preparations.

[e] The radioactivity (100 to 200 counts/min) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. Incorporation on susceptible reconstituted particles in the presence of 1 μg of CPRM per ml ranged between 6 to 33% of control with 11 independent preparations.

was also co-resistant to KM in culture (Table 2), the susceptibility of altered 30S subunits to KM was tested. The property of co-resistance to KM was observed on 30S subunits derived from this mutant (Table 5).

Analysis of the NM-KM-resistant mutant (*nek*). As reported, all of the *nek* mutants except one were resistant to 1,000 μg of KM and 500 μg of NM per ml and were co-resistant to VM at a low level (24). The levels of resistance of one of these mutants (designated R-30) to the antibiotic are shown in Table 2. This mutation occurred at a chromosomal site very closely linked to *vicB* (Fig. 1). Ribosomes from R-30 were tested for drug susceptibility in a cell-free protein-synthesizing system and were found to be resistant to NM and KM. From studies with hybrid ribosomes, it was evident that resistance was due to change with 30S subunits (Table 6).

Analysis of SM-resistant (*str*) and EM-resistant (*ery*) mutants. Since the locus for SM resistance was not linked to the *vic-nek* region (Fig. 1), it was of interest to check ribosomal alteration of the mutant. One of the SM-resistant mutants, R-15, was taken for isolating ribosomes. Poly(U)-directed polyphenylalanine synthesis on the isolated ribosomes was examined in the presence or absence of SM in the cell-free system. The incorporation of [^{14}C]phenylalanine into the acid-insoluble fraction on the mutant ribosomes was not inhibited by adding 5 μg of SM per ml, whereas significant inhibition of polypeptide synthesis was observed by adding the same concentration of drug to parental ribosomes in the cell-free system (data not shown). Ribosomal subunits were isolated from mutant and parental strains. They were exchanged to make normal and hy-

TABLE 4. *Analysis of hybrid ribosomes between susceptible and vicB mutants*[a]

Constitution of hybrids[b]		Concn of antibiotic[c] (μg/ml)	Phenylalanine incorporated[d]			
			VM		CPRM	
50S	30S		Counts/min	%	Counts/min	%
S	S	0	1,466	100	1,691	100
S	S	0.3	680	46	726	43
S	S	0.5	145	10	208	12
R	R	0	3,064	100	2,343	100
R	R	0.3	3,164	100	2,665	114
R	R	0.5	2,661	87	2,388	100
R	S	0	1,850	100	1,568	100
R	S	0.3	1,231	67	437	28
R	S	0.5	278	14	418	27
S	R	0	1,956	100	2,415	100
S	R	0.3	1,548	79	3,038	125
S	R	0.5	1,532	78	2,311	96

[a] Experimental procedures as described in the text. *M. smegmatis* R-15 was used as a susceptible strain and R-33 was used as a resistant strain.

[b] Constitution of hybrid and reconstituted ribosomes: hybrid and reconstituted ribosomes were composed of subunits derived from susceptible (S) or resistant (R) strains.

[c] Antibiotic was added at the onset of incubation.

[d] The radioactivity (100 to 200 counts/min) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. Incorporation on susceptible reconstituted particles in the presence of 0.5 μg of VM per ml and 1 μg of CPRM per ml ranged between 7 to 37 and 10 to 27% of control, respectively, with 10 independent preparations.

TABLE 5. *Analysis of hybrid ribosomes between susceptible and vicB mutants*[a]

Constitution of hybrids[b]		KM[c] (μg/ml)	Phenylalanine incorporated[d]	
50S	30S		Counts/min	%
S	S	0	2,682	100
S	S	1	1,019	38
S	S	5	413	15
R	R	0	2,616	100
R	R	1	2,109	80
R	R	5	906	34
R	S	0	2,457	100
R	S	1	1,090	44
R	S	5	220	9
S	R	0	3,447	100
S	R	1	2,126	62
S	R	5	854	25

[a] Experimental procedures as described in the text. *M. smegmatis* R-15 was used as a susceptible strain and R-33 was used as a resistant strain.

[b] Constitution of hybrid and reconstituted ribosomes: hybrid and reconstituted ribosomes were composed of subunits derived from susceptible (S) or resistant (R) strains.

[c] Antibiotic was added at the onset of incubation.

[d] The radioactivity (100 to 200 counts/min) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. Incorporation on susceptible reconstituted particles in the presence of 1 μg of KM per ml ranged between 30 to 38% of control with three independent preparations.

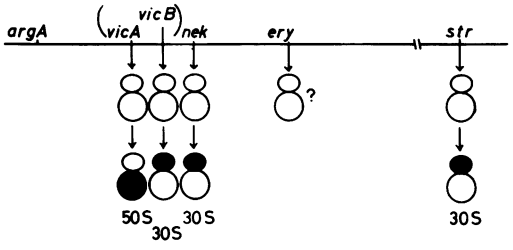

FIG. 1. *Diagrammatic presentation of genetic mapping of M. smegmatis. The order of vicA and vicB has not yet been determined. The ribosomal alteration is indicated for each antibiotic by shading. The figure is a composite of Tables 1 through 7 and previously described publications (23, 28).*

brid ribosomes, and drug susceptibility of these were tested in the cell-free system. The results in Table 7 show that the resistance to SM was localized on the 30S subunits. Thus, it became clear that the SM locus that conferred a component of 30S subunits was not linked to *vic-nek* regions.

EM resistance was shown to be linked to *vic-nek* regions (Fig. 1). Therefore, it was expected that EM-resistant mutants might have altered ribosomes. Ribosomes were purified from an EM-resistant mutant (designated R-46) and compared to parental ribosomes for drug susceptibility in a cell-free protein-synthesizing system. Poly(UG) was used as messenger ribonucleic acid in the cell-free system, since poly(U)-directed polyphenylalanine synthesis was not susceptible to EM for parental ribosomes. An amino acids mixture, except valine and [^{14}C]valine (specific activity, 100 μCi/3 μmol), was added at 0.186 and 0.1 mM final

TABLE 6. *Analysis of hybrid ribosomes between susceptible and nek mutants*[a]

Constitution of hybrids[b]		Antibiotic[c] (μg/ml)	Phenylalanine incorporated[d]	
50S	30S		Counts/min	%
S	S		3,327	100
S	S	NM(1)	1,863	56
S	S	NM(5)	1,930	58
S	S	KM(1)	1,164	35
S	S	KM(5)	532	16
R	R		3,060	100
R	R	NM(1)	2,907	95
R	R	NM(5)	2,601	85
R	R	KM(1)	2,938	96
R	R	KM(5)	2,448	80
R	S		3,627	100
R	S	NM(1)	870	24
R	S	NM(5)	1,015	28
R	S	KM(1)	1,456	40
R	S	KM(5)	1,088	30
S	R		3,838	100
S	R	NM(1)	3,454	90
S	R	NM(5)	3,339	87
S	R	KM(1)	3,416	89
S	R	KM(5)	3,377	88

[a] Experimental procedures as described in the text. *M. smegmatis* R-15 was used as a susceptible strain and R-30 was used as a resistant strain.

[b] Constitution of hybrid and reconstituted ribosomes: hybrid and reconstituted ribosomes were composed of subunits derived from susceptible (S) or resistant (R) strains.

[c] Antibiotics were added at the onset of incubation.

[d] The radioactivity (200 to 300 counts/min) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. Incorporation on susceptible reconstituted particles in the presence of 1 μg of NM per ml and 1 μg of KM per ml ranged 49 to 59 and 30 to 38% of control, respectively, with three independent preparations.

TABLE 7. *Analysis of hybrid ribosomes between susceptible and str mutants*[a]

Constitution of hybrids[b]		SM[c] (μg/ml)	Phenylalanine incorporated[d]	
50S	30S		Counts/min	%
S	S	0	1,782	100
S	S	5	677	38
S	S	10	579	32
R	R	0	1,018	100
R	R	10	1,019	100
R	S	0	1,224	100
R	S	5	664	54
R	S	10	672	55
S	R	0	1,227	100
S	R	5	1,287	105
S	R	10	1,011	82

[a] Experimental procedures as described in the text. *M. smegmatis* R-15 was used as a susceptible strain and R-16 was used as a resistant strain.

[b] Constitution of hybrid and reconstituted ribosomes: hybrid and reconstituted ribosomes were composed of subunits derived from susceptible (S) or resistant (R) strains.

[c] Antibiotic was added at the onset of incubation.

[d] The radioactivity (100 to 150 counts/min) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. Incorporation on susceptible reconstituted particles in the presence of 10 μg of SM per ml ranged between 32 to 69% of control with 10 independent preparations.

concentrations, respectively. The results, however, showed no significant difference in drug susceptibility between mutant and parental strains (unpublished data).

DISCUSSION

Genetic mapping of *str, ery, nek,* and *vic* mutants of *M. smegmatis* was first reported in crosses between strains Rabinowitchi and PM5 (23, 28). Since these markers were presumed to define genes for ribosomal components, it was anticipated that these genes would form a cluster in the region of *str* locus, as described for *E. coli* (29) and *Bacillus subtilis* (13, 16, 17). The results showed that *vic, nek,* and *ery* loci were closely linked to *argA* and *argB* but were not linked with the *str* locus (28). Recent studies on ribosomal protein genes in *E. coli* have revealed that not all the ribosomal protein genes map at the *str-spc* region, but the structural genes for at least six 50S ribosomal proteins map near the gene *rif* (14, 15, 21, 32). The structural gene for S18 is outside the *str-spc* region (8). The kasugamycin resistance locus (*ksgC*), which affects ribosomal protein S2, is separated from these clusters (35). The *vic* genes in *M. smegmatis* consisted of two groups (*vicA* and *vicB*), as reported previously (23). From the results described in this paper, it is clear that *vicA* conferred a component of 50S subunits and *vicB* conferred a component of 30S subunits. These conclusions are consistent with the results obtained in a different strain, ATCC 14468, of *M. smegmatis* (34). The fact that VM action can be counteracted by changes of both 30S and 50S subunits was previously reported (34). However, these mutants had been isolated by multiple-step selection in increasing concentrations of VM. Therefore, the genetic composition was presumed to be complicated multiple mutations in those mutants. In the present study, we have clearly demonstrated, by the use of genetically defined strains, that resistance to VM can occur through alterations to either or both ribosomal subunits.

It is not known whether VM binds to 30S or 50S subunits. However, our results on the analysis of resistant mutants can be best explained by the fact that a mutation in one subunit can change the response to VM on another ribosomal subunit. Thus, alterations in the 30S subunit conferred by *vicB* and alterations in the 50S subunit conferred by *vicA* must interact in response to VM. There have been a number of reports that show functional relationships between different components of ribosomes. Reversion from SM dependence to independence by a second ribosomal alteration has been extensively studied (2, 6, 9, 10, 12, 18–20, 22, 27). The interaction between the *nek* mutation and *spc* or *str* mutation has been reported (3, 4). Mutations to SM resistance in *E. coli,* when transferred to a C strain, conferred dependence on SM. These alternatives in expression of the allele were explained as a result of interaction between two ribosomal proteins (22). Pleiotropic drug resistance has been reported in VM-resistant ribosomes in our previous publication (33). It is also possible that mutational alteration of one subunit can change the response of an antibiotic that exerts its primary effect through the other ribosomal subunit (11, 26). Recently, a strong interaction between 50S and 30S components was demonstrated (1). Expression of resistance to EM in *E. coli,* casued by an altered L4 protein in the 50S ribosomal subunits, can be phenotypically lost when two additional ribosomal mutations causing resistance to spectinomycin and SM, alterations in the 30S proteins S5 and S12, are introduced into the subunit (1). The observation was shown that some spectinomycin-resistant mutants were cold sensitive because of blocks in the

assembly of both ribosomal subunits. It was concluded that the assembly of 50S subunits was dependent on simultaneous assembly of 30S subunits in vivo (25). In addition, the binding of dihydrostreptomycin to the 30S ribosomal subunit from *E. coli* was stimulated by the concomitant addition of a 50S subunit, which has no ability to bind the antibiotic (30). We were unable to isolate *vicA* mutants from drug-susceptible *M. smegmatis* Rabinowitchi R-15. The *vicA* mutant used in this study was isolated from the SM-resistant mutant R-16. This also might be due to subunit interactions of the type discussed above. On the contrary, *vicB* mutants were isolated from both SM-susceptible and -resistant strains at the same frequencies.

Mutant strains resistant to NM and KM were first isolated from *E. coli* (3), and the *nek* locus was found to be linked to the *str* locus. Alterations of the ribosomes in mutant strains were shown (3). However, it is not known if *nek* mutations affect the 30S or 50S subunits, and no reconstitution experiments have been reported (7). Unlike in *E. coli*, the *nek* locus was not linked to the *str* locus in *M. smegmatis* (28). In this study, we report that the ribosomes from *nek* mutants of *M. smegmatis* have altered 30S subunits (Table 6). These mutants were also resistant to VM at a low level (Table 2). It is interesting to note that the 30S mutant *vicB*, closely linked to *nek*, also showed co-resistance to KM and NM at a low level. Unlike *E. coli* (3) and a VM-resistant mutant of *M. smegmatis* ATCC 14468 (33), however, the *nek* and *vic* mutants of strain Rabinowitchi did not show co-resistance to SM.

We could not get clear-cut results to show that the EM resistance locus was a ribosomal gene. However, the gene *ery* is located in the region of the *M. smegmatis* chromosome that contains the ribosomal gene. Therefore, we cannot rule out that EM resistance is due to a mutational alteration in the protein-synthesizing machinery that cannot be detected in our cell-free system.

The results obtained are summarized in Fig. 1; further studies on the genetics and biochemistry of drug resistance in *M. smegmatis* are in progress.

ACKNOWLEDGMENTS

We thank J. Davies for a critical reading of the manuscript. We also thank N. Okada for skillful and dedicated assistance.

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