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Inactivation of Dihydrostreptomycin and Spectinomycin by *Staphylococcus aureus*

HARUHIDE KAWABE, MATSUHISA INOUE, AND SUSUMU MITSUHASHI

Department of Microbiology, School of Medicine, Gunma University, Maebashi, Japan

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Three types of *Staphylococcus aureus* strains were isolated with respect to streptomycin (SM) and spectinomycin (SPC) resistance, namely, SM^rSPC^r, SM^rSPC^s, and SM^sSPC^r (r, resistant; s, sensitive). Curing experiments and transduction analysis of strain MS7990 ($SM^r.SPC^r.EM^r$) (EM, erythromycin) disclosed that the loci governing SM and SPC resistance are different and exist on different nontransferable plasmids (*r* factor), one plasmid carrying the genes governing SM resistance and another possessing the genes governing resistance to both SPC and EM. Strain MS7990 ($SM^r.SPC^r$) inactivated both drugs by adenylylation. Similarly, the SM^rSPC^s and SM^sSPC^r strains inactivated SM and SPC, respectively, by adenylylation, although the adenylylated positions of both drugs have not been established as yet. The adenylylated SM in staphylococci was shown to be different from 3''-adenylyl-SM, indicating the possibility of the existence of a different enzyme from SM3''-adenylyl transferase demonstrated in *Escherichia coli* strains.

It was reported that streptomycin (SM) was inactivated by cell-free extracts from SM-resistant strains of *Escherichia coli* (1, 3, 9, 13, 14, 16) and *Pseudomonas aeruginosa* (2, 5, 7) in the presence of adenosine 5'-triphosphate (ATP) by either adenylylation or phosphorylation of the drug. A SM-resistant but spectinomycin (SPC)-sensitive strain of *E. coli* carrying an R factor could inactivate SM by phosphorylation (9). However, the cell-free extract from an SM- and SPC-resistant *E. coli* strain carrying a different R factor inactivated both SM and SPC by adenylylation, indicating the presence of an enzyme capable of adenylylating both SM and SPC (1, 11). It is known that in staphylococcal strains SM resistance can be classified into two groups, i.e., intermediate (50 to 400 μg/ml) and high (1,600 μg/ml or more) resistance. *Staphylococcus aureus* carrying intermediate SM resistance could inactivate the drug by adenylylation (6). By contrast, *S. aureus* possessing high SM resistance could not inactivate the drug under the same conditions; this inactivation mechanism is presently unknown (6). Recently, we isolated three types of *S. aureus* strains with respect to SM and SPC resistance, namely, SM^rSPC^r, SM^rSPC^s and SM^sSPC^r. This paper deals with their inactivation mechanisms and a comparison of the SM-adenylylating enzyme in *E. coli* strains.

MATERIALS AND METHODS

Bacterial strains. Strains of *S. aureus* were all isolated from clinical specimens. Fifty-five strains were selected from our stock cultures and, among them, 44 strains were SPC^r. Fifty-five strains were SM sensitive, and 20 strains among them were SPC^r. The relationship between SM resistance and SPC resistance in these strains is shown in Table 1. *S. aureus* strains MS27 ($SM^r.SPC^s$), MS7990 ($SM^r.SPC^r$), and MS9263 ($SM^s.SPC^r$) were selected from these strains for the present study. *S. aureus* MS7990-5 ($SM^s.SPC^r$) was obtained by spontaneous loss of SM resistance from strain MS7990 ($SM^r.SPC^r$) after cultivation at 42 C. A *rec*$^-$ derivative of strain MS3937 was used as a recipient in transduction to examine whether a gene is located on the plasmid (4). The strains used are shown in Table 2.

Media. Medium B, used for liquid culture, consisted of the following: Na_2HPO_4, 7.0 g; KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.2 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; glucose, 2.0 g; peptone (Eiken), 10 g; yeast extract (Difco), 1.0 g; and 1,000 ml of deionized water.

Determination of drug resistance. One loopful of overnight culture of each strain was spotted on HI agar plates containing serial twofold dilutions of each antibiotic. The minimal inhibitory concentration of each drug was scored after 18 h of incubation at 37 C.

Elimination of drug resistance. One loopful of overnight broth culture of a tested strain was inoculated in fresh broth containing various concentrations of rifampin or ethidium bromide. After incubation at 37 C for 18 h, the culture that contained the highest concentration of each drug and still showed visible growth was selected, and an appropriate dilution was spread on an agar plate. After 18 h of incubation at 37 C, the loss of resistance in each colony was tested by replica-plating. The loss of resistance was confirmed further by assay of resistance after purification

by single-colony isolation. Elimination of resistance was also carried out by cultivating a tested strain at elevated temperature. Overnight broth cultures of tested strains were diluted 1,000-fold with fresh broth and incubated at 42 C. After 7 h of incubation, an appropriate dilution of the culture was spread on an agar plate, and the loss of resistance was examined as described above.

Preparation of cell-free extracts. Cell-free extracts were prepared as described previously (6).

Inactivation reaction of antibiotics. The SPC inactivation was carried out as described previously (6), with the antibacterial activity remaining in the reaction mixture determined by bioassay. The concentration of SPC used was 50 μg/ml. The incorporation of isotope from labeled ATP into the drug was investigated by the method of Ozanne et al. (9). The reaction mixture contained: S-30 fraction (0.5 mg of protein/40 μliters), 0.01 μmol of [^{14}C]ATP (53 mCi/mmol) or γ-[^{32}P]ATP (515 mCi/mmol), 0.25 mol of ATP, 5 nmol of each drug, 2.5 μmol of tris(hydroxymethyl)aminomethane buffer (pH 8.1) in the case of MS27 or acetate buffer (pH 6.0) in the case of strains MS7990, MS7990-5, and MS9263, 0.1 μmol of $MgCl_2$, 0.6 μmol of KCl, and 0.06 μmol of 2-mercaptoethanol, in a total volume of 40 μliters. The reaction procedure was as described previously (6).

Electrophoresis of inactivated products. The reaction mixture was pipetted onto a disk of phosphocellulose paper and washed with 100 ml of distilled water. The inactivated product of SM absorbed on the disk was extracted with 15 ml of 0.5 N HCl and lyophilized. The lyophilized sample was dissolved in 20 μliters of distilled water, and all of the sample was spotted on a strip (6 by 22 cm) of filter paper (no. 51 A; Toyo Roshi Co., Tokyo). Electrophoresis was carried out in 0.05 M acetate buffer (pH 5.0) for 150 min at 200 V per strip. The strip was cut in 0.5-cm lengths, and the radioactivity of each sample was counted in a scintillation counter. Authentic samples of SM and 3''-adenylyl-SM were used as controls and monitored by the Sakaguchi reaction (10).

Antibiotics and chemicals. A sample of 3''-adenylyl-SM was kindly supplied by H. Umezawa, Institute of Microbiol Chemistry, Tokyo. SM and dihydrostreptomycin were supplied by the Meiji Seika Co., Tokyo. SPC and erythromycin (EM) were kindly supplied by the Upjohn Co., Kalamazoo, Mich. Disodium ATP was purchased from the Sigma Chemical Co., St. Louis, Mo. Mitomycin C was obtained from the Kyowa Hakko Co., Tokyo. The isotope-labeled preparations of ATP, i.e., γ-[^{32}P]ATP (515 mCi/mmol) and [8-^{14}C]ATP (53 mCi/mmol), were purchased from the Radiochemical Centre, Amersham, England.

Preparation of phage lysates. Overnight cultures of the donor strains were diluted 100-fold with fresh nutrient broth and shaken at 37 C. After incubation for 4 h, mitomycin C was added (final concentration, 1 μg/ml), and further incubation with shaking was carried out. After incubation for 50 min, 10 ml of the cell culture was centrifuged, and the sedimented cells were suspended in 20 ml of fresh nutrient broth. After 2 h of incubation at 37 C with shaking, the phage lysate was filtered through a membrane disk (type HA; Millipore Corp., Bedford, Mass.). The titer of the lysate thus obtained was about 10^9 plaque-forming units per ml. In some experiments, a phage lysate from strain MS3878 was propagated on strain MS7990 by the soft-agar technique of Swanstrom and Adams (12).

Transduction. Recipient cultures and phage lysates were mixed at a multiplicity of infection of 0.2 and incubated at 30 C. After incubation for 40 min, the mixture was washed three times to remove free phage particles. The cell pellet was suspended in nutrient broth and spread onto HI agar plates containing either SM (25 μg/ml), SPC (200 μg/ml), or EM (0.8 μg/ml). The colonies which developed on selective plates after incubation for 48 h were picked. After three successive single-colony isolations, their drug resistance and phage type were determined. As controls, sterility tests of the phage lysates and mutation of the recipient organisms without phage lysate were carried out.

TABLE 1. *Relation between SM and SPC resistance in S. aureus strains used*

Bacterial strains	Resistance pattern[a]	No. of strains
SM resistant	SM^r SPC^s	11
	SM^r SPC^r	44
SM sensitive	SM^s SPC^s	35
	SM^s SPC^r	20

[a] Level of resistance: SM resistance, 25 μg/ml or more; SPC resistance, 200 μg/ml or more. r, Resistant; s, sensitive.

TABLE 2. *Drug resistance and origin of the S. aureus strains used*

Strain	Origin	Resistance pattern[a]	Plasmid that exists[b] in a bacterial cell
MS27	Clinical source	SM^r SPC^s EM^s	
MS7990	Clinical source	SM^r SPC^r EM^r	r_{ms10}(SM), r_{ms10}(SPC.EM)
MS7990-5	Mutant from MS7990	SM^s SPC^r EM^r	
MS9263	Clinical source	SM^s SPC^r EM^r	
MS3878	Clinical source	SM^r SPC^r EM^r	r_{ms11}(SM), r_{ms11}(SPC.EM)
MS353	Clinical source	SM^s SPC^s EM^s	

[a] See the footnote of Table 1. Level of resistance: EM resistance, 6.3 μg/ml or more.
[b] *r*, Nontransmissible plasmid that carries drug-resistance determinant.

RESULTS

SM- and SPC-resistance patterns in S. aureus. We selected from our stock cultures four types of *S. aureus* strains with respect to SM and SPC resistance, i.e., SM^rSPC^r, SM^rSPC^s, SM^sSPC^r, and SM^sSPC^s (Table 1). We reported previously that staphylococcal strains possessing intermediate SM resistance could inactivate the drug by adenylylation, but did not report on those carrying high-level SM resistance (6). Strain MS7990, carrying intermediate SM resistance, was selected for investigation of the genetics of the SM resistance and comparison to that governing SPC resistance. Strain MS7990 ($SM^r.SPC^r.EM^r$) was harvested in broth at 37 C, and the loss of resistance was examined by treatment with drugs or after cultivation at elevated temperature. Strains were obtained which were SM sensitive but SPC resistant and, therefore, had lost only the SM resistance (Table 3). These results indicate that the loci governing intermediate SM resistance and SPC resistance are different and exist on different genetic elements, probably on different nontransferable plasmids (*r* factor).

Transduction of resistance to SM, SPC, and EM. Strains MS7990 ($SM^r.SPC^r.EM^r$) and MS3878 ($SM^r.SPC^r.EM^r$) were used as the donors of resistance (Table 4). When selected for SM resistance, transductants resistant only to SM were obtained. We could not obtain the doubly SM^rSPC^r transductants. When selected for either SPC or EM resistance, the doubly $SPC^r.EM^r$ transductants were obtained but neither of the singly SPC- or EM-resistant transductants. In this case, it was not possible to obtain the doubly $SM^r.SPC^r$ transductant. From these results, it is concluded that the loci governing intermediate SM resistance and SPC resistance are different. Next, we used the transductant MS353 ($SPC^r.EM^r$) as the donor of resistance, which had acquired (SPC.EM) resistance from either strains MS7990 or MS3878. The transductants were all resistant to both SPC and EM. These results indicate that the genes governing resistance to EM and SPC are very closely linked. Therefore, the genes governing SPC resistance and intermediate SM resistance are concluded to be different. To

TABLE 3. *Artificial elimination of SM resistance*[a]

Strain	Treatment with	No. of colonies tested	No. of colonies that lost resistance to:				
			SM	SM.SPC	SPC	SPC.EM	EM
MS7990 ($SM^r.SPC^r.EM^r$)	—[b]	600	2	0	0	0	0
	43 C	360	1	0	0	0	0
	RF	390	0	0	0	0	0
	EB	376	0	0	0	0	0

[a] Abbreviations: RF, rifampin; EB, ethidium bromide.
[b] Overnight broth culture without any treatment was plated.

TABLE 4. *Transduction of resistance to SM, SPC, and EM*[a]

Donor	Selective drug	Transduction frequency ($\times 10^{-6}$)	No. of transductants tested	No. of transductants resistant to:				
				SM	SM.SPC	SPC	SPC.EM	EM
MS7990 ($SM^r.SPC^r.EM^r$)	SM	1.2	166	154	0	0	0	0
	SPC	2.4	164	0	0	0	164	0
	EM	1.3	105	0	0	0	105	0
MS3878 ($SM^r.SPC^r.EM^r$)	SM	0.8	114	114	0	0	0	0
	SPC	3.7	240	0	0	0	240	0
	EM	2.6	221	0	0	0	221	0
MS353[b] ($SPC^r.EM^r$)	SPC	1.9	124			0	124	0
	EM	3.5	264			0	264	0
MS353[c] ($SPC^r.EM^r$)	SPC	10	165			0	165	0
	EM	13	237			0	237	0

[a] Strain MS353 was used as a recipient. Multiplicity of infection was 0.2.
[b] Transductant from strain MS3878, which had acquired resistance to both SPC and EM.
[c] Transductant from strain MS7990, which had acquired resistance to both SPC and EM.

determine whether the genes governing resistance to SM and (SPC.EM) are located on a nontransferable plasmid (r factor), transduction of SM and (SPC.EM) resistance to strain MS3937 (*rec*$^-$) was carried out. The ratio of the transduction frequency with strain MS3937 to that with MS3937 (*rec*$^-$) indicates that the genes governing SM and (SPC.EM) resistance are located separately and on different plasmids (Table 5). Furthermore, the SM and (SPC.EM) resistance were found to be cured from the transductants MS3937 (*rec*$^-$) (SM^r) and MS3937 (*rec*$^-$) (SPC^r.EM^r) by treatment with ethidium bromide, indicating the extrachromosomal existence of the resistance determinants. The resultant SM-sensitive and (SPC.EM)-sensitive strains of MS3937 were found to be still *rec*$^-$ by ultraviolet light sensitivity and by transduction of the penicillin-resistance determinant of PS80 which exists on a chromosome.

Biochemical mechanisms of SM and SPC resistance. We have investigated the biochemical mechanisms of SM and SPC resistance by using cell-free extracts from the resistant strains of *S. aureus*. Strain MS7990 (SM^r.SPC^r) inactivated both SM and SPC by adenylylation of the drugs (Table 6). Singly SM-resistant strain MS27 (SM^r.SPC^s) inactivated only SM by adenylylation without inactivation of SPC. To the contrary, the SPC.EM-resistant strains MS7990-5 (SM^s.SPC^r), MS353 (SM^s.SPC^r), and MS9263 (SM^s.SPC^r) could inactivate SPC by adenylylation without inactivation of SM. To confirm the inactivation of SM by adenylylation, electrophoretic analysis of the inactivated products was carried out with an authentic sample of 3″-adenylyl-SM as control. SM and 3″-adenylyl-SM migrate toward the cathode; however, the radioactivity of the inactivated product of SM did not coincide with the authentic sample of 3″-adenylyl-SM, and its mobility was lower than that of 3″-adenylyl-SM (Fig. 1).

TABLE 5. *Comparison of the transduction frequencies between strains MS3937 and MS3937 (rec*$^-$*)*[a]

Donor	Ratio of transduction frequency[b]	
	SM	SPC.EM
MS7990 (SM^r.SPC^r.EM^r)	0.4	2.8

[a] Multiplicity of infection was 0.2.

[b] Ratio: transduction frequency of strain MS3937 to MS3937 (*rec*$^-$).

DISCUSSION

It was reported that the 3-hydroxy group of the *N*-methyl-L-glucosamine moiety of streptomycin was adenylylated by *E. coli* strains carrying an R factor (13, 16). It was also found that a SM- and SPC-resistant strain of *E. coli*

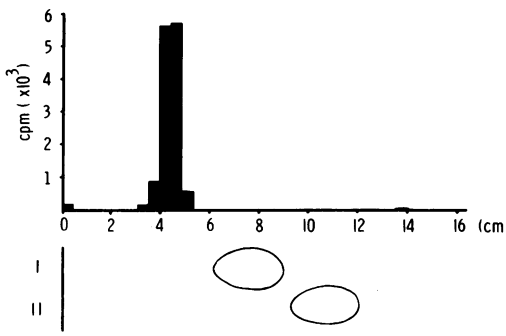

FIG. 1. *Electrophoresis of the inactivated SM by cell-free extract from strain MS27 (SM^r.SPC^s). I, 3″-adenylated SM; II, SM.*

TABLE 6. *Inactivation of DHSM and SPC by cell-free extracts from resistant strains of S. aureus*[a]

Strains[b]	MIC[c] (μg/ml)		Inactivation[b] (%)		Incorporation of labeled ATP into drug (counts/min)[b]			
					^{14}C		γ-^{32}P	
	DHSM	SPC	DHSM	SPC	DHSM	SPC	DHSM	SPC
MS27 (SM^r.SPC^s)	400	25	100	0	917	0	0	0
MS7990 (SM^r.SPC^r)	100	1,600	75	95	353	1,298	0	0
MS7990-5 (SM^s.SPC^r)	1.5	1,600	0	92	0	1,591	0	0
MS353 (SM^s.SPC^r)	1.5	1,600	0	90	0	989	0	0
MS9263 (SM^s.SPC^r)	1.5	1,600	0	98	0	2,136	0	0

[a] Streptomycin resistance in strains MS27 and MS7990 was intermediate. Strain MS353 (SM^s.SPC^r) was one of the transductants of MS7990 to MS353, which were obtained from the experiments shown in Table 4. Strain MS7990-5 (SM^s.SPC^r) was a mutant of MS7990, from which SM resistance had been lost after cultivation at 42 C.

[b] See Materials and Methods.

[c] Minimal inhibitory concentration.

carrying the R factor inactivated both SM and SPC by adenylylation, indicating the presence of an enzyme capable of adenylylating both SM and SPC (1, 11). According to epidemiological surveys, we noticed that there are three types of SM and SPC resistance in staphylococci, i.e., SM^rSPC^r, SM^rSPC^s, and SM^sSPC^r. These results strongly suggest that the loci governing SM and SPC resistance are different. As shown in this paper, the SM^rSPC^r strains of *S. aureus* could inactivate both SM and SPC by adenylylation, but the SM^rSPC^s and SM^sSPC^r strains inactivated SM and SPC by adenylylation, respectively. These results indicate that in staphylococcal strains two enzymes are present, with their specificity limited to adenylylation of only one of the antibiotics.

Curing experiments and transduction analysis of MS7990 ($SM^r.SPC^r$) indicate that the loci governing SM and SPC resistance exist separately and on different plasmids (for a review, see reference 8), one plasmid carrying the gene governing SM resistance and another one possessing the genes governing both SPC and EM resistance. Biochemical analysis strongly suggests that SM and SPC are inactivated by adenylylation of the drugs, although the adenylylated positions of both drugs are not known at present. Electrophoresis of the inactivated product has shown that the adenylylation of SM by strain MS27 ran toward the cathode but did not coincide with an authentic sample of 3''-adenylyl-SM, indicating the existence of an enzyme in staphylococci that adenylylates SM but that is different from the enzyme demonstrated in *E. coli* strains.

Walker and Skorvaga (15) reported that three enzymes, i.e., streptomycin 6-kinase, dihydrostreptomycin 3'-α-kinase, and streptomycin 3''-kinase, were demonstrated in *Streptomyces*. This fact also suggests the possibility that the enzymes capable of diadenylylating SM exist in staphylococci.

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