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Streptomycin and Spectinomycin Resistance Mediated by Plasmids

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Resistance to tetracycline (Tc), chloramphenicol (Cm), streptomycin (Sm), and sulfanilamide (Sa) was surveyed in clinical isolates of *Escherichia coli* and *Shigella* strains. Among the Sm Sa-resistant strains, the frequency of nonconjugative r (Sm Sa) plasmids was much higher than that of conjugative R plasmids encoding double resistance. The biochemical mechanism of Sm resistance mediated by the conjugative plasmids R(Tc Cm Sm Sa) and R(Cm Sm Sa) and about half the numbers of conjugative R(Tc Sm Sa) and R(Sm Sa) plasmids tested were found to be due to adenylylation of the drug. The remaining conjugative R(Tc Sm Sa) and R(Sm Sa) plasmids and all nonconjugative (Sm Sa) plasmids tested inactivated Sm by phosphorylation.

Sulfanilamides (Sa) and many antibiotics have caused dramatic changes in practical medicine by decreasing the death rate caused by infectious diseases. Following the introduction of chemotherapeutic agents, however, bacterial strains emerged which were resistant to the drugs, developing from single to multiple resistance. Conjugative resistance (R) plasmids have probably played the more important role in the rapid and wide spread of drug resistance in clinical isolates, causing the emergence of multiply resistant strains seen today in hospitals (6).

In a previous paper (7), we reported nonconjugative (r) Sa plasmids from singly Sa-resistant strains. This paper deals with nonconjugative plasmids encoding streptomycin (Sm) and Sa resistance and compares the biochemical mechanisms of Sm resistance encoded by conjugative (R) plasmids with those of nonconjugative plasmids.

MATERIALS AND METHODS

Bacterial strains. The strains used are stock cultures of this laboratory and were isolated from clinical specimens. *Escherichia coli* ML1410 (F^- *met*$^-$ *nal*^{r}, nalidixic acid resistant) and *E. coli* W3630 (F^- *mal*$^-$) were used as recipients of Sm Sa resistance in conjugation; they are substrains of *E. coli* K-12. *E. coli* C Rif^r (resistant to rifampin) was used as a recipient of Sm Sa resistance in transformation.

Media. Brain heart infusion (BHI) broth (Difco) was used for liquid culture of bacterial strains and for transfer experiments. For the assay of Sa resistance or the selection of Sa-resistant transconjugants, a semisynthetic medium (S) was used that consisted of 1,000 ml of medium A (4), 2 g of Casamino Acids (Difco), 10 mg of tryptophan, 1 mg of nicotinic acid, 10 mg of thiamine HCl, and 2 g of glucose. For the determination of other drug resistances, heart infusion agar (Eiken Chemical Co., Tokyo) and peptone water were used. Peptone water consisted of 10 g of peptone, 5 g of sodium chloride, and 1,000 ml of distilled water. Bromothymol blue (BTB) lactose agar, each containing selective drugs and nalidixic acid (Na, 25 μg/ml), was used as the selective medium for ML1410 R^+ or r^+ transconjugants.

Drugs. The antibiotics were provided by the following laboratories: Sa, Dai Nippon Seiyaku; tetracycline (Tc), Pfizer Taito, Sm, Meiji; chloramphenicol (Cm), Sankyo; Na and rifampin, Daiichi Seiyaku.

Drug resistance. Drug resistance was determined by the agar dilution method, and the level of resistance was expressed as the minimum inhibitory concentration of the drug. An overnight culture in peptone water was 100-fold diluted with peptone water, and one loopful of the diluted culture was spotted on a series of agar plates containing serial twofold dilutions of the drug. After 18 h of incubation at 37°C, bacterial growth was scored.

Elimination of drug resistance. Each bacterial strain to be tested was inoculated in cooked meat medium (Eiken) at 37°C. After 18 h of incubation, the culture was placed in a cold room at 4°C for at least 4 weeks. The culture was then spread on BHI agar plate, and the loss of Sm resistance was examined by the replica plating method. The loss of Sa resistance was examined by inoculating the culture on S agar plate by the replica plating technique. Artificial elimination of resistance was examined by inoculating each bacterial strain in peptone water containing various concentrations of acriflavine. After incubation at 37°C for 18 h, a turbid culture that contained the maximum concentration of the drug was spread on S agar or BHI agar plate, and the loss of Sm Sa resistance was examined by the replica plating method.

Isolation of plasmid DNA for electron microscopy. Isolation of the Sm Sa plasmid deoxyribonucleic acid (DNA) was followed by the method described by Clowes (2). Briefly, bacterial cells were harvested in a late exponential phase and were lysed by ethylenediaminetetraacetic acid, lysozyme, and sodium dodecyl sulfate. Closed circular form DNA was obtained by ethidium-CsCl density gradient centrifugation. A dialyzed DNA was mixed with 0.01% cytochrome *c*, spread as a monolayer, dried, and shadowed by platinum-palladium.

Transformation of Sm Sa resistance. The transformation procedure is essentially as described by Cohen et al. (3). *E. coli* C Rif^r was used as a recipient for Sm Sa resistance. Bacteria were cultured in BHI broth (250 ml) and shaken at 37°C. Cells in the late logarithmic growth phase were harvested and used for DNA preparation. The DNA preparation thus obtained was kept in 20 mM tris(hydroxymethyl)aminomethane buffer (pH 8.0). To 0.3 ml of *E. coli* C Rif^r in 50 mM $CaCl_2$, 0.1 ml of DNA solution was added, and the mixture was allowed to stand in ice for 1 h, placed in a 42°C water bath for 2 min, and then placed in ice. This mixture was diluted into BHI broth, incubated at 37°C for 1 h, and then plated on bromothymol blue lactose agar plate containing Sm (6.3 μg/ml).

Plasmid transfer. Overnight BHI broth cultures of donor and recipient were diluted 50-fold with fresh broth and grown for 4 h with shaking. Two milliliters of recipient and 0.5 ml of donor cultures were mixed and gently shaken at 37°C. After 1 h of incubation, the appropriate dilutions of the mixture were spread on selective plates. S medium agar containing both Sa (150 μg/ml) and Na (25 μg/ml) or bromothymol blue lactose agar containing both 25 μg of Na per ml and one of the drugs to select drug-resistant markers on the R factor were used for the selection plate for R factor transfer from donor strains to ML1410. The concentrations of selective drugs used for plasmid transfer were Tc (25 μg/ml), Cm (25 μg/ml), and Sm (12.5 μg/ml). Rms306, a conjugative Tc-resistant plasmid, was carried by *E. coli* ML1410 Nal^r and was used to mobilize the nonconjugative Sm Sa plasmids. Rms306 was conjugally transferred from *E. coli* ML1410 Rms306$^+$ to *E. coli* or *Shigella* strains carrying nonconjugative Sm Sa-resistant plasmids. These strains were then used for the donors of resistance, and W3630 was used as a recipient to mobilize the nontransmitted Sm Sa resistance.

Preparation of cell-free extracts and inactivation reaction of Sm. Cell-free extracts were prepared as described previously (5). The reaction mixture consisted of 0.2 ml of 0.2 M acetate buffer (pH 6.0) or 0.2 M tris(hydroxymethyl)aminomethane buffer (pH 8.0), 0.05 ml of 20 mM disodium adenosine triphosphate (ATP), 0.05 ml of 0.02 M magnesium acetate, 0.05 ml of 0.5 mM Sm, and 0.15 ml of an S-30 fraction (10 mg of protein per ml). After incubation at 37°C for 60 min, the reaction was stopped by heating in boiling water for 3 min. Residual antibiotic activity in the reaction mixture was determined by bioassay using *Bacillus subtilis* ATCC 6633.

Incorporation of isotope-labeled ATP into Sm. The incorporation of isotope-labeled ATP into Sm was carried out in the following reaction mixtures, which consisted of 30 μl of 0.2 M acetate buffer (pH 6.0) or 0.2 M tris(hydroxymethyl)amonimethane (pH 8.0), 10 μl of 0.5 mM of Sm, 10 μl of 0.02 M magnesium acetate, 40 μl of an S-30 fraction of the strain to be tested (10 mg of protein per ml), and 0.5 μCi of [8-^{14}C]ATP (53 mCi/mmol) or [γ-^{32}P]ATP (515 mCi/mmol). After 60 min of incubation at 37°C, 20 μl of the reaction mixture was pipetted on a phosphocellulose paper (about 2 cm^2; Whatman P-81), washed with distilled water, and dried. Radioactivity on the paper was counted in a toluene-based scintillation counter (Packard Instrument).

RESULTS

Resistance patterns. Resistance patterns of *Shigella* and *E. coli* strains to Tc, Cm, Sm, and Sa are shown in Table 1. Quadruply resistant strains were isolated most frequently, followed by those carrying single resistance. Triple (or double) resistance was found at the lowest frequency. The frequency of conjugative resistance (R) plasmids was shown to be high in triply and quadruply resistant strains, but was lower in doubly and singly resistant strains. Among the doubly resistant strains, those carrying Sm Sa resistance were most frequently isolated, followed by those possessing Tc Sa resistance, and strains with other combinations of double resistance were infrequently isolated. The frequency of conjugative (R) plasmids in doubly resistant strains was low, except those strains carrying Tc Sa resistance (Table 1).

Nonconjugative Sm Sa plasmids. We examined for the presence of nonconjugative resistance (r) plasmids in those encoding Sm Sa resistance. We randomly selected 30 *E. coli* and 30 *Shigella* strains possessing nontransmitted Sm Sa resistance in Table 1 and checked for the loss of Sm Sa resistance. As shown in Table 2, about 70 to 80% of strains lost both Sm and Sa resistance during storage or by treatment with acriflavine, indicating that the determinants governing nontransmissible Sm Sa resistance are usually located on a single genetic element, i.e., a nonconjugative resistance (r) plasmid. All strains whose Sm Sa resistance was cured could inactivate Sm under the conditions used. All of a number of determinants governing Sm Sa resistance in *Shigella* and *E. coli* strains that were tested could be mobilized to W3630 at frequencies of 7×10^{-5} to 6×10^{-7} by the concomitant presence of Rms306 plasmid in the donor strain.

Transduction of Sm Sa resistance. Using bacteriophage P1, Sm Sa resistance in W3630 (Tc Sm Sa)-resistant transconjugants obtained in Table 3 was cotransduced at a frequency of 2×10^{-8} to 5×10^{-8} when selected for Sm resistance. According to these results, we can conclude that the determinants governing nonconjugative

Sm Sa resistance in *Shigella* and *E. coli* isolates are located on a single genetic element, i.e., a nonconjugative plasmid (Table 4).

Contour length measurements. The nonconjugative Sm Sa plasmids in *Shigella* and *E. coli* strains were transformed into *E. coli* C Rif^r. Plasmid DNA was prepared for the purified transformants for electron microscopy, and micrographs of open circular molecules were enlarged, traced, and measured. Mean contour lengths of six of the nonconjugative Sm Sa plasmids were 2.5 ± 0.2 μm, corresponding to molecular weights of 4.9 to 5.6 megadaltons.

Inactivation of Sm by nonconjugative plasmids. According to the finding that Sm was

TABLE 1. *Frequency of resistant strains and conjugative R plasmids*[a]

Resistant strains[b]	*Shigella*		*E. coli*	
	%	R$^+$ strains (%)[c]	%	R$^+$ strains (%)[c]
Resistance type				
Quadruple	75.7	75.5	69.6	70.2
Triple	4.7	86.7	5.7	83.2
Double	2.8	35.1	3.5	22.3
Single	16.8	9.6	21.2	7.8
Double resistance[d] patterns				
Sm Sa	73.2	18.0	75.2	19.2
Tc Sa	21.2	88.9	20.3	86.3
Cm Sa	2.5	27.3 (3/11)[e]	2.5	14.3 (1/7)[e]
Tc Cm	1.9	50.0 (4/8)	1.0	33.3 (1/3)
Tc Sm	0.9	25.0 (1/4)	0.8	50.0 (1/2)
Cm Sm	0.3	0.0 (0/1)	0.2	0.0 (0/1)

[a] Results based on surveys from 15,903 strains including *Shigella* (12,453) and *E. coli* (3,450).

[b] Survey for resistance to Tc, Cm, Sm, and Sa. Quadruple resistance, Tc Cm Sm Sa; triple resistance, Tc Sm Sa, Cm Sm Sa, Tc Cm Sa, and Tc Cm Sm; double resistance, Cm Sa, Tc Sa, Tc Cm, Tc Sm, and Cm Sm.

[c] Percentage of conjugative plasmids was computed among the strains carrying the indicated resistance type.

[d] Results based on surveys of doubly resistant 445 *Shigella* and 307 *E. coli* strains.

[e] Numbers in parentheses indicate number of R$^+$ strains per number of strains tested.

TABLE 2. *Properties of E. coli and Shigella strains possessing nonconjugative Sm Sa resistance*

No. of strains tested	Minimal inhibitory concn (μg/ml)		Loss of Sm Sa resistance[a]	Inactivation of Sm[b]
	Sm	Sa		
E. coli, 30	100–800	800–3,200	+ (21/30)	+ (21/30)
Shigella, 30	100–800	800–3,200	+ (25/30)	+ (25/30)

[a] Number of strains whose Sm Sa resistance was eliminated per number of strains which inactivated Sm.

[b] Number of strains which inactivated Sm per number of total strains examined from which Sm Sa resistance was cured.

TABLE 3. *Mobilization of nonconjugative Sm Sa resistance by the conjugative Rms306 plasmid*[a]

Donor[b]	Selection	Transfer frequency of Sm Sa resistance
E. coli (Sm Sa)^r (Rms306)$^+$	Sm + Tc	7×10^{-5} to 6×10^{-7}
Shigella (Sm Sa)^r (Rms306)$^+$	Sm + Tc	3×10^{-5} to 8×10^{-7}

[a] Sm (12.5 μg/ml) and Tc (25 μg/ml) were used as selective drugs. W3630 was used as a recipient.

[b] Six *E. coli* and six *Shigella* strains whose Sm Sa resistance was eliminated in the experiments shown in Table 2 were randomly selected. Donor strains were obtained by conjugal transmission of Rms306 plasmid into these strains.

TABLE 4. *Transduction of the mobilized Sm Sa resistance in W3630*[a]

Original host	Donor of transduction[b]	Selection	Transduction frequency	No. of transductants examined	No. of transductants resistant to:		
					Tc	Sm	Sa
E. coli							
GN3475	W3630(Tc Sm Sa)^r	Sm	3×10^{-8}	30	0	30	30
GN3702	W3630(Tc Sm Sa)^r	Tc	2×10^{-7}	30	30	0	0
GN3697	W3630(Tc Sm Sa)^r	Sm	5×10^{-8}	30	0	30	30
		Tc	6×10^{-7}	30	30	0	0
		Sm	2×10^{-8}	20	0	20	20
		Tc	1×10^{-7}	30	30	0	0
Shigella							
JS12535	W3630(Tc Sm Sa)^r	Sm	4×10^{-8}	30	0	30	30
JS12610	W3630(Tc Sm Sa)^r	Tc	7×10^{-7}	30	30	0	0
JS13792	W3630(Tc Sm Sa)^r	Sm	2×10^{-8}	20	0	20	20
		Tc	6×10^{-7}	30	30	0	0
		Sm	5×10^{-8}	30	0	30	30
		Tc	8×10^{-7}	30	30	0	0

[a] ML1410 was used as a recipient.

[b] The donor strains were obtained from the experiments shown in Table 3 by the transfer of the conjugative plasmid Rms306 into the wild-type Sm Sa-resistant strains.

inactivated by nonconjugative plasmids encoding Sm Sa resistance, we investigated the biochemical mechanism of Sm resistance conferred by various plasmids and also tested the plasmids for spectinomycin (Sp) resistance. We selected various plasmids from *Shigella* and *E. coli* strains.

To know whether inactivation occurred by adenylylation or phosphorylation, we examined the incorporation of γ-^{32}P or 8-^{14}C from isotope-labeled ATP into Sm. As shown in Table 5, all nonconjugative Sm Sa plasmids inactivated Sm by phosphorylation, whether or not they determined Sp resistance. About half the numbers of conjugative R(Sm Sa) and R(Tc Sm Sa) plasmids carried the gene(s) governing Sp resistance and inactivated Sm by adenylylation, whereas the remaining conjugative R(Sm Sa) and R(Tc Sm Sa) plasmids without Sp resistance inactivated Sm by phosphorylation. All the conjugative R(Tc Cm Sm Sa) and R(Cm Sm Sa) plasmids so far isolated also determined Sp resistance, and they were all found to inactivate Sm by adenylylation.

DISCUSSION

Our surveys of resistance to Tc, Cm, Sm and Sa in clinical isolates of *Shigella* and *E. coli* strains have disclosed the following: (i) a large proportion of the strains are quadruply resistant, (ii) Cm Sm Sa or Tc Sm Sa resistance is prevalent among triply resistant strains, (iii) a high frequency of doubly resistant strains are Sm Sa or Tc Sa resistant, and (iv) Sa resistance is prevalent among singly resistant strains. Moreover, the proportion of singly Cm- or Sm-resist-

TABLE 5. *Inactivation of Sm by nonconjugative or conjugative plasmids encoding Sm resistance*

Original host	S-30 fraction from ML1410 carrying:	Resistance pattern	Inactivation of Sm	Incorporation of ^{32}P or ^{14}C (CPM)	
				Phosphorylation	Adenylylation
E. coli	Nonconjugative plasmids				
	Rms26	Sm Sa	+	11,605	67
	Rms28	Sm Sa	+	13,303	153
	Rms25	Sm Sa Sp	+	4,255	52
	Rms30	Sm Sa Sp	+	3,505	48
Shigella					
	Rms44	Sm Sa	+	10,790	120
	Rms45	Sm Sa	+	11,306	112
	Rms46	Sm Sa Sp	+	2,206	74
	Rms47	Sm Sa Sp	+	2,902	59
E. coli and *Shigella*	Conjugative plasmids				
	Rms302[a]	Sm Sa	+	9,852	72
	Rms303	Sm Sa	+	33,985	55
	Rms15	Sm Sa Sp	+	65	8,076
	Rms397	Sm Sa Sp	+	56	4,128
	Rms300[a]	Tc Sm Sa	+	37,213	214
	Rms398	Tc Sm Sa	+	11,718	98
	Rms400	Tc Sm Sa Sp	+	72	12,716
	Rms401	Tc Sm Sa Sp	+	91	9,618
	Rms262[a]	Cm Sm Sa Sp	+	75	6,740
	Rms298	Cm Sm Sa Sp	+	80	5,940
	Rms299	Cm Sm Sa Sp	+	98	13,381
	Rms11[a]	Tc Cm Sm Sa Sp	+	65	9,358
	Rms304	Tc Cm Sm Sa Sp	+	91	7,897
	Rms305	Tc Cm Sm Sa Sp	+	120	8,355
	ML1410			86	108
	E. coli C			128	130

[a] R plasmids were randomly selected from our stock cultures.

ant strains is rather low (6), and although plasmids encoding single Tc resistance are frequently isolated, those encoding linked Tc and Sa resistance are very infrequent in doubly Tc Sa-resistant strains (unpublished observation). Sm-phosphorylating enzyme was found from nonconjugative (r) (Sm Sa) and conjugative (R) (Sm Sa) and R(Tc, Sm Sa) plasmids. Sm-adenylylating enzyme was found from r(Sm/Sp Sa), R(Sm/Sp Sa), R(Tc Sm/Sp Sa), R(Cm Sm/Sp Sa), and R(Tc Sm/Sp Sa) plasmids. From these results, the formation of a multiple resistance plasmid could be explained by two assumptions: (i) r(Sa) → r(Sm Sa) → R(Sm Sa) → R(Tc Sm SA), and (ii) r(Sa) → r(Sm/Sp Sa) → R(Sm/Sp Sa) → R(Tc Sm/Sp Sa) or R(Cm Sm/Sp Sa) → R(Tc Cm Sm/Sp Sa).

It has been reported that conjugative R plasmids which are Sm resistant and Sp sensitive inactivate Sm by phosphorylation (8), and one conjugative plasmid-containing strain of *E. coli* which is known to inactivate Sm by adenylylation has been shown to be Sp resistant (1). These results indicate that there are two kinds of plasmids encoding resistance to Sm, those which determine Sp resistance and those which do not.

As reported previously (6) and in this article, conjugative R(Tc Cm Sm Sa) and R(Cm Sm Sa) plasmids are isolated at high frequencies from *E. coli* and *Shigella* strains, and all of them also carry the gene(s) governing Sp resistance, the biochemical mechanism of Sm and Sp resistance mediated by these plasmids being due to adenylylation of the drugs. About half the number of conjugative R(Sm Sa) and R(Tc Sm Sa) plasmids determined Sp resistance and could also inactivate both antibiotics by adenylylation, whereas the remaining plasmids did not determine Sp resistance; they all inactivated Sm by phosphorylation. However, the nonconjugative Sm Sa plasmids inactivated Sm by phosphorylation regardless of the presence or absence of the gene(s) governing Sp resistance, although the biochemical mechanism of Sp resistance has not so far been reported.

We could thus classify resistance plasmids into three groups, i.e., a group 1 (Sm-adenylylating plasmid (Sm-r, Sp-r), a group 2 (Sm-phosphorylating plasmid Sm-r, Sp-s), and a group 3 (Sm-phosphorylating plasmid Sm-r, Sp-r). Conjugative plasmids have the characteristics of group 1 or 2, although most of them belong to group 1. Nonconjugative plasmids belong to group 2 or 3.

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